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Research on Captive Broodstock Programs for Pacific Salmon

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ASSESSMENT OF CAPTIVE BROODSTOCK TECHNOLOGIES

ANNUAL REPORT
(PERFORMANCE PERIOD: 1 JUNE 2003 THROUGH 31 MAY 2004)

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ABSTRACT

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

We were able to develop an analytical method for optimizing the detection of spawning events in Chinook salmon using EMG signals. The method developed essentially captured the consistently greater frequency of higher EMG values associated with females cover digging immediately following spawning. However, females implanted with EMG tags retained the majority of their eggs, which significantly reduced their reproductive success compared to non-tagged females. Future work will include increased sample sizes, and modified tagging methods to reduce negative effects on reproductive success.

Upper Columbia River sockeye salmon exposed to the odorants PEA, L-threonine, L-arginine and L-glutamate were able to learn and remember these odorants as maturing adults up to 2.5 years after exposure. These results suggest that the alevin and smolt stages are both important developmental periods for successful olfactory imprinting. Furthermore, the period of time that fish are exposed to imprinting odors may be important for successful imprinting. Experimental fish exposed to imprinting odors as smolts for six or one weeks successfully imprinted to these odors but imprinting could not be demonstrated in smolts exposed to odors for only one day.

A 2 – 3 ºC reduction in seawater rearing temperature during the fall and winter prior to final maturation had little effect on reproductive development of spring Chinook salmon. Body size at spawning and total ovary mass were similar between temperature treatments. The percentage of fertilized eggs was significantly higher for females exposed to the ambient temperature compared to those exposed to the chilled temperature. However, the percentage of embryos surviving to the eye-stage, total fecundity, and mean egg mass did not differ between treatments. This work is being continued with larger samples sizes and increased duration of temperature exposure.

Exercise during the months prior to final maturation had no detectable effects on fertilization success or embryo viability in Redfish Lake Sockeye. Problems with highly variable or low eyed-embryo survival are most likely due to problems with fertilization. Synchronizing spawn timing between males and females may improve gamete fertility, perhaps by making oocyte maturation and ovulation more readily detectable and synchronous within the individual. Improvements in milt production (using GnRHa) and fertilization protocols have apparently increased fertilization success in Redfish Lake sockeye over previous years.

Broodstock treatment with azithromycin immediately prior to spawning can protect against acute challenge with *R. salmoninarum*. Among fish challenged with 10,000 virulent *R.*

salmoninarum cells per fish, progeny of broodstock treated with azithromycin exhibited significantly greater survival than progeny of sham-treated broodstock. Work on the efficacy of antibiotic treatment and vaccination against BKD before and after smoltification in offspring chinook salmon captive broodstocks is ongoing.

To date, the long-term study of inbreeding indicates that the potential for anadromous Chinook salmon to respond rapidly to close inbreeding, with adverse consequences for marine survival and, possibly, growth. The effects of inbreeding expressed during early life history do not reveal significant effects. Overall, the results would support recommendations for initiating artificially propagated populations with sufficient, outbred broodstock and implementing carefully monitored breeding practices to minimize rates of inbreeding during a program's duration.

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OBJECTIVE 1 - IMPROVE REINTRODUCTION SUCCESS

TASK 1a. QUANTIFY REPRODUCTIVE BEHAVIOR AND SUCCESS OF CHINOOK SALMON REARED IN EXPERIMENTAL TREATMENTS IN STREAM CHANNELS

By

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

Introduction

As with other vertebrates, the difficulty of reintroduction to the natural environment is a major impediment to the success of captive broodstock programs for Pacific salmon. Empirical studies of Chinook salmon reared to adult and released for natural spawning indicate that, while they are capable of producing juvenile offspring, reproductive deficiencies include delayed spawn timing, mate selection against captive males, and poor egg deposition.

Captive broodstocks for recovery of threatened or endangered Pacific salmon and steelhead populations are derived from local wild populations, and salmon released as adults for natural spawning may be held in captivity for less than one full generation. In such cases where eggs or parr are collected from the natural environment, reared to adulthood, and released for natural spawning (i.e., the 'captive rearing' strategy), rearing environment effects on reproductive success supercede genetic concerns (such as domestication selection) associated with multiple generations of captive culture. Sea-ranched coho salmon populations and escaped farmed Atlantic salmon have exhibited reduced reproductive success relative to wild populations (Fleming and Gross 1992, 1993, Fleming et al. 1996, 2000). The confounded genetic effects of multiple generations of captive culture and environmental rearing effects on reproductive success reduce the relevance of such findings to captive rearing programs for recovery of ESA-listed Pacific salmon populations that are initiated with locally adapted, naturally produced fish. Nevertheless, studies conducted between 1995 and 2001 also under Project #199305600 have demonstrated that the rearing environments associated with full-term culture, independent of genetic factors, affect reproductive behavior and reduce breeding success of both coho and Chinook salmon relative to wild cohorts.

Reproductive success in salmon, defined as the ability to produce reproductive offspring, may be largely determined by breeding behavior characteristics and their underlying mechanisms. For example, the dramatic differences in lifetime reproductive success (LRS) documented between farmed and wild Atlantic salmon were explained almost entirely by the ability of adults to spawn and produce age-0 parr (Fleming et al. 2000). Farmed fish achieved only 19% relative adult-to-parr reproductive success and 16%

relative LRS of wild salmon, which means survival from parr to adult did not differ between progeny from the two populations. Therefore, adult-to-parr reproductive success, which is largely explained by variation in breeding behavior (Foote et al. 1997, Mjolnerod et al. 1998, Fleming et al. 2000), may provide a strong indicator of LRS, particularly as it relates to captively-reared (hereafter referred to as 'captive') adult salmon. In addition, controlled breeding behavior and success studies conducted in artificial arenas produce results similar to those in natural streams (Fleming et al. 2000, Berejikian et al. 1997, Berejikian et al. 2001a) and provide an effective tool for evaluating reproductive success.

Studies of captive coho salmon (*O. kisutch*) show them to be competitively inferior to their wild counterparts. Captive males participate in fewer oviposition events than wild males (Berejikian 2001a), and captive females are out-competed for nest sites (Berejikian et al. 1997). Secondary sex characteristic development and nuptial coloration are muted, overall body shape is altered from the wild state in captive fish (Hard et al. 2000a), and morphological characteristics have been correlated with behavioral measures of breeding success (Quinn and Foote 1994, Berejikian et al. 2001b).

Captive Chinook salmon studied thus far exhibit maladaptive behavioral patterns not documented in wild Chinook. For example, captive females often abandon their nests without spawning and as a result fail to deposit all of their eggs, and males often exhibit little courtship response to nearby nest-digging females (Berejikian et al. 2001b). In addition, eggs of adult captive Chinook salmon spawning in natural streams suffer high mortality. Fertilization rates are lower and post-fertilization mortality is higher than exhibited by either naturally spawning wild fish or captive fish spawned artificially (P. Kline, Idaho Department of Fish and Game (IDFG), personal communication). Thus, poor fertilization during natural spawning may reflect deficits in behavioral communication during spawning, and high post-fertilization mortality may reflect inability to construct a suitable incubation environment (a 'redd').

Captive Chinook salmon also exhibit delayed final maturation and spawn timing in both natural streams and experimental arenas (Berejikian et al. in press). Delayed spawn timing reduces chances for interbreeding with wild salmon (a main objective of the Idaho captive Chinook program, IDFG 1996), and may negatively affect offspring fitness. Salmon populations have locally adapted spawn timing, which increases the chances that offspring will emerge from the gravel under favorable environmental conditions. For example, Einum and Fleming (2000) demonstrated selective mortality on Atlantic salmon parr produced from eggs in which fertilization was artificially delayed by 10 – 12 d. The range of accumulated incubation water temperature units (d x average °C/d) required for embryonic development is narrow within populations of Chinook salmon (Billard and Jensen 1996). Thus, delayed spawning of captive Chinook salmon, released as adults during late summer when temperatures are declining, further delays emergence, may affect the condition of emerging parr (Beer and Anderson 2001), and magnifies the disparity between emergence timing of their offspring and that to which the population is locally adapted.

Project #199305600 aims to conduct scientific research that can be applied to improving captive broodstock methodologies to the greatest extent possible. It addresses three critical questions regarding the release of captive adult salmon, viz:
(i) What specific environmental parameters are responsible for the documented reproductive deficiencies?
(ii) What mechanisms (i.e., behavioral, physiological, or morphological), either alone or in combination, are causing reduced reproductive performance?
(iii) How might culture environments be modified to improve reproductive performance?

Several conditions encountered by captive salmon differ from probable experiences in the natural environment. The water temperature during the period of gonadal development is substantially different for captive fish, and potentially contributes to reproductive deficiencies. The proposed experimental approach is to manipulate each variable independently, and determine the effects on breeding behavior and adult-to-parr reproductive success under both experimental and natural conditions.

In addition to documented delays in final maturation timing and spawn timing, recent studies have identified ovarian development is delayed in captive Chinook salmon prior to transfer to freshwater (Swanson et al., unpublished data). Thus, delayed final maturation may be partially caused by environmental conditions during seawater rearing. Seawater temperatures at the Manchester Research Station, where six ESA-listed Chinook salmon populations are reared, range between an average daily temperature of 9.0°C in winter to 13.5°C in summer. In Chinook salmon, gonadal development begins nearly a year before final maturation (Shearer et al. 2000), thus maturing fish experience a wide range of seasonal temperature fluctuations before re-entry to freshwater. Little is known about the actual temperature regimes experienced by salmon in the open ocean. However, recent information from data storage tags on other species of Pacific salmon in the Gulf of Alaska and the Bering Sea indicates that fish make vertical migrations during daylight hours and experience temperatures typically less than 11°C (Walker et al. 2000). In the spring, the abundance of several salmon species declines dramatically in areas where sea surface (i.e., warmest) temperatures exceed 9°C (Welch et al. 1995), and in the winter the thermal maximum is approximately 6°C for sockeye salmon (Welch et al. 1998).

Available data, although general and limited, suggest that rearing temperatures for captive Chinook salmon may be several degrees higher than they would experience in the ocean, and may be partly responsible for the documented reproductive dysfunctions. The negative effects of elevated rearing temperature on reproductive characteristics of captive salmonid populations are described in detail under Objective 3, below. The present work approach was to rear fish at ambient temperatures experienced by ESA-listed captive broodstocks, and at temperatures continuously 3°C below ambient for 11 months prior to final maturation. Temperature effects on spawning behavior, spawn timing, reproductive behavior, and adult-to-parr reproductive success was evaluated under experimental conditions.

Materials, Methods, and Description of Study Area

Captive Chinook salmon from the Carson National Fish Hatchery (BY1999) were reared at the Manchester Research Station from the eyed egg stage of embryonic development. The 449 salmon, which exhibit a stream-type life history, were transferred to sand-filtered, UV-sterilized seawater as yearling smolts. On 4 October 2002, the fish were weighed, measured, PIT tagged, and divided equally into four rearing vessels (4 m diameter). Two tanks received non-chilled water similar in temperature to that experienced by six populations of ESA-listed Chinook salmon (i.e., 'standard' temperature), and the other two were maintained at 3°C below standard temperature (Fig. 1). Feeding followed manufacturer (Moore-Clarke) guidelines. Beginning on 15 May 2003, all fish were transferred to back to freshwater. For more details also see Task 3c.

On 18 August 2003, age-4 maturing adults were allocated to the following reproductive behavior and success experiment. A total of 32 females (8 from each tank) and 12 males were used in the experiment. Fish were anaesthetized, weighed, measured for fork length, postorbital−hypural (POH) length, and a numbered 2.5-cm Peterson Disk tag (Floy Tag Co., Seattle, WA) was attached to each fish for external individual identification (Berejikian, 2001b). Mean (± s.d.) female adult weight averaged 1.8 kg (± 0.7 kg) and 51.2 cm (± 5.2 cm) in forklength. Because too few males survived to age-4, maturing spring Chinook males were obtained from the Cowlitz Salmon Hatchery. The males averaged 6.3 kg (± 1.8 kg) and 81.2 cm (± 8.1 cm). In a recently conducted study of captive Chinook reproductive behavior, female sample sizes of 16 per treatment were sufficient to detect relative behavioral frequency, duration, and status differences of approximately 25% (Berejikian et al. 2003).

The spawning experiment was conducted in a stream channel (40 m x 6 m) located at the Manchester Research Station (Berejikian et al. 2001b). A constant flow of 40 $L \cdot min^{-1}$ of fresh well water was continuously added to the channel, and water in the channel was recirculated at a rate of 5,821 $L \cdot min^{-1}$. Water depth (10 - 35 cm), current velocities at nest sites (0.1 - 0.6 $m \cdot sec^{-1}$), water temperatures (controlled by a chiller), and gravel size (approximately 3 - 12 cm diameter) in the channel was within the range of spawning habitat used by several Columbia River Basin Chinook salmon populations in Washington State (Burner 1951). A centerboard separated the channel into two replicate 40-m by 3-m wide sections. Sixteen females (4 from each tank) and 6 males were stocked into each section.

Four flood lights positioned horizontally 4.5 m above the channel illuminated the water surface to provide enough light (mean $\pm$ s.d. = 4.8 $\pm$ 0.9 lux) for night-time video recording. Four cameras (Watec, model 902HS; 00015 lux sensitivity @ F1.4; 26 mm lens) were positioned approximately 3.8 m above the stream channel, so that each camera captured images from 25% of the channel. Video values were recorded on time-lapse recorders (Gyyr, model TLC 2124-GY) at approximately 5 frames/sec between 1700 hrs and 0730 hrs. All nocturnal spawning events were recorded and observed upon

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

In addition to the overhead video cameras connected to time-lapse recorders, and underwater video recording, reproductive behavior of fish in the stream channel were recorded by direct observation 'scan' sampling. Reproductive behaviors (described in Table 1) were quantified and categorized in three classes: nesting behavior, aggression, and spawning duration. Each female in the channel was observed for 3 min daily between 0700 and 1900 hrs from behind an observation blind.

Data Management Activities

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

Results and Discussion

Within a week of stocking the maturing Chinook salmon into the stream channel, females began to develop lesions and fungus. The first female was found dead on 25 August 2003, exactly 7 d after stocking. Eight more of the original 32 female Chinook salmon stocked died on 27 August 2003. Losses continued at a rate of about three females daily until 5 September 2003. Post-mortem dissections did not indicate any definitive cause of the accelerated mortality.

There was only one female that was definitely observed spawning prior to death. Ten of the 32 female Chinook salmon were observed digging. Behavioral data and reproductive lifespan are shown in Table 2. No further analyses were conducted on these data because of the premature mortality.

Because of the premature mortality, we did not quantify embryo viability. However, the single female we documented as having spawned produced a total of 76 fry, which were captured between 21 December 2003 and 30 December 2003. Fecundity of chilled and non-chilled females is reported under Objective 3.

The planned DNA pedigree analysis was not performed on this experiment, and was applied instead to Task 1b. This decision was made following consultation with the

Snake River Chinook Salmon Captive Broodstock Technical Oversight Committee and the BPA COTR.

Table 1. Definitions of female reproductive behaviors and states for Chinook salmon placed into the stream channel.

Class	Behavior	Definition
Nesting behavior		
	Nest dig	Excavating dig performed during nest construction
	Cover dig	Dig which either covers recently deposited eggs or covering a previously constructed nest
	Red Holding	Female holds over excavated area without digging or courtship
Activity level		
	Inactive	Pre-spawn
	Inactive	Post-spawn
Aggression		
	Nest guarding	Female is on or near previously spawned nest or redd and exhibits aggression against any other fish

Table 2. Body length, weight, longevity, and behavioral information for Carson NFH Spring Chinook females reared in chilled (C) and non-chilled (NC) vessels.

					Proportion of time spent in status						
		Length (cm)	Weight (kg)	Longevity d:hrs	Inactive pre-spawn	Inactive post-spawn	Redd holding	Nest digging	Cover digging	Nest guarding	Courted by male
Chilled	Mean	51.8	2.45	11:16	1.00	0.00	0.00	0.00	0.00	0.00	0.03
	s.d.	5.34	1.24	3:55	0.00	0.00	0.00	0.00	0.00	0.00	0.09
Non-Chilled	Mean	50.6	1.85	11:04	0.93	0.05	0.02	0.03	0.02	0.02	0.00
	s.d.	5.23	0.68	3:10	0.25	0.19	0.06	0.08	0.06	0.06	0.00

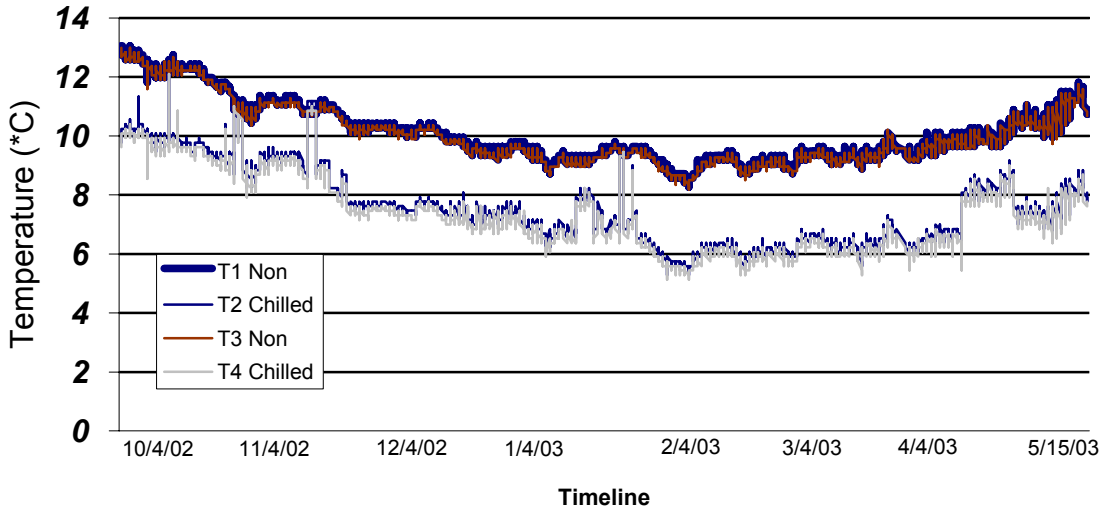


Figure 1. Water temperatures in four Chinook salmon rearing tanks (two chilled and two non-chilled) from 4 October 2002 through 15 May 2003.

TASK 1b. QUANTIFY THE REPRODUCTIVE BEHAVIOR OF ESA-LISTED CHINOOK SALMON IN IDAHO STREAMS

By

Barry Berejikian, Don VanDoornik, Anita LaRae, Rob Endicott, Rich Brown, Skip Tezak

Introduction

Release of captive adult Chinook salmon is the main reintroduction strategy for three populations of endangered Chinook salmon in Idaho. Monitoring of adult releases in 1998 - 2002 has revealed that captive Chinook salmon undergo final maturation several weeks to more than a month later than wild fish, egg deposition rates are less than in wild fish, and nesting locations differ from those utilized by wild fish (Venditti et al. 2003). The success of the adult release strategy depends on natural maturation timing, nesting location and behavior to allow for intermixing of captive and wild Chinook salmon, natural emergence timing of the offspring, and optimum survival.

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 NOAA Fisheries (e.g., Berejikian et al. 2000a, 2000b) as a result of this project. The expertise of NOAA Fisheries scientists in evaluating breeding behavior and success of spawning salmon and invited participation has substantially improved the effectiveness of reintroduction monitoring efforts in Idaho streams, as carried out between 1998 and 2002.

In past years, breeding success evaluations have included behavioral measures on individually marked Chinook salmon released for natural spawning in their natal streams. This work provides important and revealing information on spawn timing and location, courtship and nest construction behavior, inter- and intra-specific interactions, and egg deposition rates. In the past, observers have relied on direct observations of spawning events to determine rearing treatment effects on reproductive performance. Witnessing the act of spawning is rare, because of the large area that needs to be covered and the brief nature of salmon spawning, which lasts only about 5 to 10 sec per event. Therefore, methods need to be developed to predict when and where spawning is soon to occur and to document spawning of individuals without direct observation. This work was design to continue to assist IDFG in evaluating the reproductive performance of captively reared Chinook salmon by developing data collection methods that will allow biologists to determine where and when individual salmon have spawned.

Chinook, like other salmon exhibit stereotypic nest digging and courtship behaviors. The nature of these activities involves muscle activity that is different from that during basal swimming activity. For example, females digging motions performed to excavate nests and cover deposited eggs involve strong, rapid trunk muscle undulations (more than 10

body flexures may be involved in a single dig). The frequency of female digging activity is fairly constant within an hour of spawning (about one dig per 2 min), but increases dramatically during the 5 min following spawning (about 6 digs per minute) as the female covers eggs in the nest pocket (Berejikian et al. 1997, 2001b). High frequency head-to-tail vibrations characterize male 'quivering', which is the principle courtship behavior. The frequency of quivering increases as spawning nears, extended quivering (5 to 10 sec) occurs during spawning, and quivering frequencies are dramatically reduced following spawning (Berejikian et al. 1997, 2001b).

The recent development of electromyogram (EMG) tags that read and transmit electrical signals associated with muscle activity provide the potential opportunity to record swimming activity and energy use in fish (see Geist et al. 2002). The present study was conducted to develop quantified relationships between values emitted from EMG tags and spawning activity. We hypothesized that spikes in the EMG values would occur just prior to, during, and just after spawning for both males and females in patterns sufficiently predictable to unequivicolly detect spawning events. To determine whether implantation of EMG tags interfere with natural reproduction, adult-to-fry reproductive success of tagged and non-tagged fish was quantified by applying DNA microsatellite pedigree analyses. In the future, the intent is to utilize EMG tags to monitor reproductive performance of ESA-listed Chinook salmon being released for natural spawning in Idaho streams.

Materials, Methods, and Description of Study Area

Maturing fall Chinook salmon were obtained in September 2003 from the University of Washington's Big Beef Creek Research Station, which is located at the Big Beef Creek estuary in Hood Canal (Kitsap Co., WA). Sixteen adults were transported to the NMFS Manchester Research Station, Manchester, WA, on 24 September 2003. All 16 fish under study were anaesthetized, weighed, and measured. For external individual identification, a numbered 2.5-cm Peterson Disk tag (Floy Tag Co., Seattle, WA) was attached to each fish slightly posterior and ventral to the dorsal fin. The females weighed an average of (± s.d.) 4.8 kg (± 1.3 kg) and measured 72.2 cm (± 6.6 cm) in fork length. The males weighed an average of 3.3 kg (± 0.7 kg) and measured 60.8 cm (± 8.7 cm) in fork length.

The spawning experiment was conducted in a stream channel (40 x 6 m) located at the Manchester Research Station (Berejikian et al. 2001b). See Task 1a for a detailed description of the stream channel configuration. All 16 fish (8 EMG-tagged and 8 non-tagged)were simultaneously stocked into one side of the stream channel on 24 September 2003. The channel was modified to produce a recirculating flow of 3,842 $L \cdot min^{-1}$ through the side of the channel occupied by the fish.

EMG Tagging Procedures

Four female and four male Chinook salmon were surgically implanted with electromyogram (EMG) radio transmitter tags (Lotek Engineering, Ontario, Canada). The transmitters were 5 cm long, 1.5 cm in diameter, and weighed 20 g in air. A 3 to 4-cm incision was made in the belly anterior to the pelvic fins. Two gold-tipped steel-wire electrodes were secured in the red musculature just under the surface of the skin beneath the lateral line on the left side of the fish with the antenna extending posteriorly through the body wall (Geist et al. 2003). The tag itself was placed in the coelomic cavity. The incision was closed with four silk sutures and an antibiotic applied to the surface of the closed incision to prevent infection. The method used is described in detail in Hinch et al. (1996).

EMG and video data collection

Values from the EMG tags were transmitted to microprocessor controlled receivers (SRX_400, Lotek, Ontario, Canada). A total of four receivers were used in the study. Two of the four receivers were each set to record EMG values from one fish at a time. A single EMG value was transmitted to the receiver every 3.2 sec. We hereafter refer to this as "focal sampling". Whenever possible, focal receivers were set to record values from females that were constructing nests and appeared to be nearing spawning. This allowed us to compare EMG values with behavioral data.

The third receiver was configured to 'scan sample' the four EMG-tagged females. In this configuration, values from an individual female were transmitted to the receiver every 3.2 sec for a total of 48 sec. These data were then automatically downloaded (~16 sec) to the receiver's internal memory, and the receiver automatically switched to record data from the next female. This cycle was repeated from the time the females were introduced until the death of the last female. The fourth receiver was configured in the same manner to receive and record values from the four males. Data from each of the four receivers were downloaded to a PC laptop computer each day and converted into Microsoft Excel format. Each row of data contained the date, time (to the nearest second) and the EMG value.

Four flood lights positioned horizontally 4.5 m above the channel illuminated the water surface to provide enough light (mean $\pm$ s.d. = 4.8 $\pm$ 0.9 lux) for night-time video recording. Four cameras (Watec, model 902HS; 00015 lux sensitivity @ F1.4; 26 mm lens) were positioned approximately 3.8 m above the stream channel, so that each camera captured images from 25% of the channel. Video values were recorded on time-lapse recorders (Gyyr, model TLC 2124-GY) at approximately 5 frames/sec between 1700 hrs and 0730 hrs. All nocturnal spawning events captured on video tapes were observed and documented upon reviewing the videotapes between 0800 and 1200 hrs the following day.

During daylight, three remote underwater cameras were used to record the behavior of females before, during and after individual spawning events. Females that were actively constructing a nest while being attended by a courting male indicated that spawning was imminent. An underwater camera was positioned nearby nesting females, and the video

recorded in real time (JVC Super VHS, model HR S-7300-U). By a combination of direct observation, daytime underwater video, and night-time overhead video we were able to document the time, location, and female participant for every spawning event that occurred during the study.

For each female, we reviewed the day and night-time video recordings and entered the nest construction digs and nest covering digs alongside the actual time (to the nearest second) that the behavior occurred. Digging events were recorded for the period 60 min prior to 30 min after each documented spawning event. Each digging event was entered into the Excel spreadsheet alongside the corresponding EMG value.

At least three spawning events by each of the four females were recorded simultaneously by video and EMG receivers. We enumerated nest covering digs during the first 5 min after each spawning event captured on videotape. The ratio of EMG values associated with covering digs to the average of all scan sampling EMG values was calculated to determine whether, as hypothesized, covering digs produce an elevated EMG value.

We gathered far less focal information on males than females because observations made during the study indicated that detecting spawning in males using EMG values would not be possible. Focal recordings on females were, therefore, emphasized. For males, reproductive behaviors (described in Table 1) were directly observed in real-time for a total of 45 to 60 min (three or four 15- min 'scans'). One focal EMG receiver was set to simultaneously record EMG values from the fish under observation. The frequency of male courtship behaviors, quivers and crossovers, and two types of aggressive behaviors, attacks and threat displays, were recorded. Also recorded with the attacks and threat displays were whether the aggressive behaviors were initiated by the fish being observed or received from another fish. Cooresponding focal sampling EMG values for each behavior were averaged across the 15-minute period.

Detecting spawning events with EMG values

To determine relationships between the frequency and amplitude of EMG values and known spawning events, we compiled all of the focal data for each female. We iteratively processed the data to optimize the number of spawning events detected by EMG values with a minimum of false detections. The following four steps describe the process we used for each female:

Step 1: We compiled a program in Visual Basic to create a frequency distribution of individual EMG values for each female. The program then calculated a percentile rank for each recorded value in the distribution.

Step 2: We created a separate spreadsheet for each female to filter large blocks of EMG values (because not all data would fit in a single worksheet). All EMG values above the 95^{th} percentile were considered "high" values, and the frequency of high values was calculated for each i) moving 5-min period and ii) each moving 60-min period.

Step 3: We calculated the ratio between each 5-min high value frequency and the subsequent 60 min high value frequency for the entire data set. All data for which the 5-min/60-min ratio was > 0.35 were removed from the data set. This had the effect of removing clusters of high EMG values that were not associated with a spawning event.

Step 4: To more precisely deterimine the time of spawning and further reduce the occurance of false detections, we further filtered the remaining 5-min high value frequency intervals. We took the average of two consecutive 5-min high value frequency intervals, and averages that exceeded 10.5 were considered as detected spawning events and those below were not.

These criteria were applied in the exact same manner to each of the four females to generate the association between actual (observed) spawning events and 'detected' spawning events from the EMG analysis (Fig. 2).

Fry collections

Alevins that emerged from the gravel with yolk sacs exposed were collected from the downstream screen on a daily basis. Mortalities were collected in a container and preserved in 100% non-denatured ethanol. Live fry were released back into the upstream end of the channel. On 9 January 2004 the downstream-end of the stream channel section was fitted with a trap to collect fry that emerged from the gravel and emigrated. The fry trap was checked daily and all fry removed. Live fry were released back into the channel and mortalities were preserved (Table 3). All non-emigrating fry were removed from the stream channel between 2 and 5 February 2004, approximately 1,040 to1,088 temperature units from the last spawning event.

DNA Pedigree analyses

A small portion of caudal fin tissue was removed from all adults before they were released into the experimental stream channel and preserved in 100% non-denatured ethanol. Likewise, a subset of 1,000 fry, selected in such a manner to proportionally represent the entire sample, was preserved in 100% non-denatured ethanol.

Genomic DNA was isolated from the adult and fry fin tissue samples using Wizard genomic DNA purification kits (Promega Corp.) following the manufacturer's instructions. The isolated genomic DNA was used in polymerase chain reactions (PCR) to amplify seven microsatellite loci. The resulting PCR products were analyzed using an Applied Biosystems 310 capillary electrophoresis system. Results of the electrophoretic runs were analyzed using Genescan and Genotyper software (Applied Biosystems) to determine the genotypes of every individual for each locus. The genotypes of the fry were compared to those of the adults using the computer program CERVUS (Marshall et al. 1998) to determine the parentage of each fry.

Results and Discussion

Female behavior and EMG values

The average number of spawning events by EMG-tagged females was 6.5 compared to 3.3 by non-tagged females. However, EMG-tagged females retained more eggs than the females without EMG tags (Table 2). Thus, problems with egg retention appear to be caused by the tagging procedure or the tag itself. There was no significant difference in longevity between tagged and non-tagged females.

Females varied widely in their mean EMG output over the course of the study (Table 4). The EMG tag implanted into female W51 provided higher mean outputs than the other tags. As hypothesized, nest covering digs occurred at a high frequency during the first 5 min following spawning (Table 4). EMG values associated with nest covering digs were about 2.5 times greater than the average EMG value during the reproductive lifespan, and variation among females and among spawning events within females was surprisingly low (Table 4).

Females surgically implanted with EMG tag spawned a combined 26 times during the study. Focal EMG receivers were set to record EMG values during 15 of the 26 spawning events. Focal EMG data were not collected during the other 11 spawning events because only two recorders were dedicated to focal sampling, and therefore only two fish could be recorded at once. The four step process for detecting spawning events by each of the four females was successful in detecting 14 of 15 spawning events for which we had both observational and EMG data. The spawning detection was based on a single set of criteria applied to all females (Fig. 2).

The EMG spawning detection procedure falsely detected spawning events for two of the four females, including one false detection for one female and four false detections for the other. However, three of the four false detections could be eliminated post-hoc because they occurred too closely together. For example, there were four false detections for one female (tag # W-57, Table 7), all four of which occurred between two spawning events (19:09 hrs and 22:51 hrs) that were both observed and detected by EMG analysis. Three of the detections occurring within this period could be eliminated because spawning at a rate of more than three times in a 3.5 hr span was never documented in this study or other studies of Chinooks salmon reproductive behavior (Berejikian et al. 2000, 2001, 2003).

Male behavior and EMG values

Observational data was focused on female behavior during actual spawning events; therefore, few courtship behaviors were observed during the 15-minute scans of the EMG tagged males. The ratio between inactive EMG averages and active EMG average output suggest only minimal increase in EMG values during male quivers and crossovers (Table 5). A more dramatic increase in EMG values was evident during periods when males

were engaged in aggressive contests (Table 6). There doesn't appear to be any significant pattern in value output across aggressive behaviors.

DNA Pedigree Analysis

A total of 789 emigrant and 8,565 non-emigrant fry were subsampled from the stream channel for DNA analysis. Both emigrants and non-emigrants were sampled at the same rate of 10.6% (Table 3). A total of 999 were analyzed to determine their parentage. A total of 989 fry were genotyped for at least 4 loci and assigned to a single parental pair. Non-tagged females produced a significantly greater number of fry (689) than tagged females (300; $P<0.05$). Likewise, non-tagged males produced a significantly greater number of fry (622) than tagged males (367; $P<0.05$). Female egg retention accounted for approximately 75% of the variability in offspring production (Fig. 1).

Data Management Activities

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

Summary and Conclusions

We were able to develop an analytical method for optimizing the detection of spawning events in Chinook salmon using EMG signals. The method developed essentially captured the consistently greater frequency of higher EMG values associated with females cover digging immediately following spawning. High EMG values were observed at various other times during the reproductive lifespan of the four females, but they did not occur with the predictable regularity associated with covering digs.

Several uncertainties remain before this approach can be reliably applied to detecting spawning of fish released into natural streams. First, we monitored a small number ($n = 4$) of females, so it is unclear whether a single set of EMG analysis criteria could be applied to a larger population and still achieve similar spawning detection rates. Sample sizes will be increased in subsequent studies. Second, all females had ovulated prior to their introduction to the stream channel. Thus, we did not collect EMG data for the period prior to spawning when the females would be expected to be far less active. Collecting data during an extended prespawning period should allow us to detect the onset of spawning activity for individual females, and perhaps more clearly identify spawning events. Third, spawning could only be detected through analysis of the focal

sampling data, which means that one receiver had to be dedicated to each fish. In our study, we switched frequencies based on fish behavior (mainly proximity to spawning) to maximize the number of spawning events recorded both by video and EMG receivers. For applications in natural streams this will not be possible. The high cost of receivers makes it impractical to have one receiver per fish. Thus, in the upcoming year, we will be testing whether similar spawning detection rates can be achieved by placing three tags on the same frequency. That would allow us to continuously monitor 12 females with 4 receivers.

Females implanted with EMG tags retained the majority of their eggs, which significantly reduced their reproductive success compared to non-tagged females. Post-mortem dissections did not reveal any apparent blockage caused by the tag itself. We suspect that the high egg retention was caused by the loss of ovarian fluid during the surgery. All females used in this study were implanted after having ovulated, and ovarian fluid was observed draining from the incision during surgery. The role of ovarian fluid in facilitating egg deposition is unclear, but ovarian fluid may serve as a lubricant or to maintain hydrostatic pressure in the body cavity. In work planned in 2004 we will attempt to obtain pre-ovulated females and possibly reduce the size of the incision by implanting the tag subcutaneously (see Healey et al. 2004). However, a small incision will still be required to implant the electrodes in the red muscualature.

Predictable increases in digging frequencies that occur immediately following spawning occurs for all species of Pacific salmon. Use of EMG technology may prove useful in field-detection of spawning for other species as well. However, criteria for detection will likely need to be developed for each species independently before they can be applied with any certainty to studies of spawning salmon in natural streams.

This work is designed to assist captive broodstock programs that opt to release adults for natural spawning. Such an approach has been adopted for steelhead in Washington, Atlantic salmon in Maine and Snake River sockeye and Chinook salmon in Idaho and Oregon (Berejikian et al. in press). Documenting successful spawning by captively reared females is very difficult by direct observation alone. We believe that application of the EMG procedures described here will greatly improve the ability of researchers to document spawning success or failure of released captively reared salmon in natural streams.

Table 1. Definitions of behavioral frequencies observed in male Chinook salmon placed into the stream channel.

Class	Behavior	Definition
Courtship behavior		
	Quiver	High frequency head-to-tail vibrations along side or against the body of a female.
	Crossover	Swims over the back or caudal peduncle of a female
	Nest Entry	'Sneaking' into the nest of a courting pair
Aggression		
	Attack give (+)	Attack (nip, chase, or charge) initiated by male
	Attack received (-)	Attack (nip, chase, or charge) received by another fish
	Lateral (+)	Parallel body presentation where the anal fin is lowered, the pelvic and pectoral fins are spread and dorsal fin is erect. Display initiated by male
	Lateral (-)	Parallel body presentation where the anal fin is lowered, the pelvic and pectoral fins are spread and dorsal fin is erect. Display received by another fish.

Frequencies were calculated for each minute of the 15-min scan.

Table 2. The number of nests constructed, eggs retained, and longevity for EMG-tagged (N=4) and non-tagged (N=3) BBC Fall Chinook females. One non-tagged female died prematurely from and apparent abdominal injury and did not spawn. That female was removed from the summary below. An asterisk indicates significance of probability (P) values at $\alpha = 0.05$.

	EMG-tagged (N=4)		No-tag (N=3)		t-test	df	P
	Mean	s.d.	Mean	s.d.			
# of nests	6.5	0.58	3.33	0.58	-7.18	4.5	0.001*
Egg retention	3011	896.9	921	790.39	-3.27	4.8	0.024*
Longevity	4.59	1.41	4.92	0.58	0.426	4.2	0.691

Table 3. Total number of Chinook fry removed from spawning channel. Live fry collected in the traps were reintroduced to the stream channel. Dead fry (caused by impingement on screens) were collected and preserved in separate weekly containers. A sampling rate of 10.6% was applied to both emigrant fry and those removed from the spawning channel between 2 and 5 February 2004.

Collection dates	Live	Dead	Total	Proportion of weekly DNA sample
11/24/03 - 11/26/03	5	37	42	0.05
12/01/03 - 12/05/03	6	27	33	0.03
12/09/03 - 12/13/03	4	25	29	0.03
12/15/03 - 1219/03	3	102	105	0.13
12/22/03 - 12/26/03	5	149	154	0.19
12/29/03 - 01/02/04	1	156	157	0.20
01/05/04 - 01/09/04*	1	144	145	0.18
01/12/04 - 01/16/04	0	12	12	0.02
01/19/04 - 01/23/04	0	31	31	0.04
01/26/04 - 01/30/04	0	118	118	0.15
Total emigrants collected	**25**	**801**	**826**	**0.08**
Total non-emigrants collected	**8,414**	**151**	**8,425**	**0.92**

Table 4. EMG data from four Big Beef Creek salmon females. The overall average was taken from 'scan' sampling, and cover dig averages were taken from 'focal' sampling (see methods). The cover dig ratio is the cover dig average divided by the overall average, and indicates that EMG values during 3.2 sec pulse intervals when covering digs occur are 1.83 to 4.04 times greater than the average EMG values for a female over her reproductive lifespan.

Female	N [a]	Overall average[b]	s.d.	Female nest number	n [c]	Cover dig average (1st 5 min) [d]	s.d.	Cover dig ratio (1st 5 min)	s.d.	CV
W51	11,538	11.25	4.33	1	13	28.85	6.26	2.56	0.56	.21
				2	15	24.80	6.75	2.20	0.60	.27
				7	11	24.73	5.98	2.19	0.53	.24
W55	15,308	3.04	2.26	1	15	12.27	3.56	4.04	1.17	.28
				2	13	11.92	2.87	3.92	0.94	.24
				4	18	5.56	2.62	1.83	0.86	.45
				5	9	7.11	2.37	2.34	0.78	.33
W57	14,521	6.05	4.05	1	7	15.43	2.15	2.55	0.36	.14
				2	19	12.73	3.98	2.10	0.66	.31
				3	13	14.31	2.06	2.36	0.34	.14
				4	16[e]	13.36	2.16	2.21	0.36	.16
W70	15,258	5.00	2.69	4	20	13.18	2.60	2.64	0.52	.19
				5	19	10.63	3.25	2.13	0.65	.30
				6	19	11.47	2.41	2.29	0.48	.21
				7	18	11.39	1.97	2.28	0.39	.17
All females and all nests combined					228			2.51	0.61	.24

[a] The total number of EMG values recorded per female over the entire course of the study
[b] The average EMG values for all n
[c] The number of covering digs observed during the 1st 5 min following spawning
[d] The average EMG values corresponding to the n cover digs during the first 5 min following spawning
[e] From time 15:47:54 to 15:50:18, 8 cover digs not observed because of poor visibility were included in this data set as the frequency and intensity of the EMG data indicated cover dig events had occurred.

Table 5. EMG data from four Big Beef Creek Chinook salmon males during periods of inactivity and courtship.

		Total				Quiver			Crossover		
Male	Total Min Scanned	Min inactive	Ave EMG inactive[a]	s.d.	Scan	Min (n)[b]	Ave[c]	Ratio[d]	Min (n)[2]	Ave	Ratio
Y11	45	20	8.48	0.85	1	2 (2)	9.76	1.15	0	0.00	0
					2	0	0.00	0	0	0.00	0
					3	0	0.00	0	0	0.00	0
Y15	60	44	4.87	1.30	1	0	0.00	0	0	0.00	0
					2	0	0.00	0	1 (1)	5.16	1.06
					3	0	0.00	0	0	0.00	0
					4	4 (4)	5.22	1.07	0	0.00	0
Y22	60	35	4.06	1.83	1	0	0.00	0	0	0.00	0
					2	0	0.00	0	0	0.00	0
					3	0	0.00	0	0	0.00	0
					4	0	0.00	0	0	0.00	0
Y42	60	48	8.51	2.09	1	0	0.00	0	0	0.00	0
					2	0	0.00	0	0	0.00	0
					3	1 (2)	12.06	1.42	0	0.00	0
					4	0	0.00	0	0	0.00	0

[a] The average EMG values for 1-min intervals during which the fish was inactive (not engaged in any aggressive or courtship behaviors).
[b] The number of 1-min intervals in which the behavior was observed. The total frequency of the behavior is shown in parenthases.
[c] The average EMG values for the intervals in which the behavior was observed.
[d] The average EMG values during intervals when the fish was active divided by the 'inactive' average.

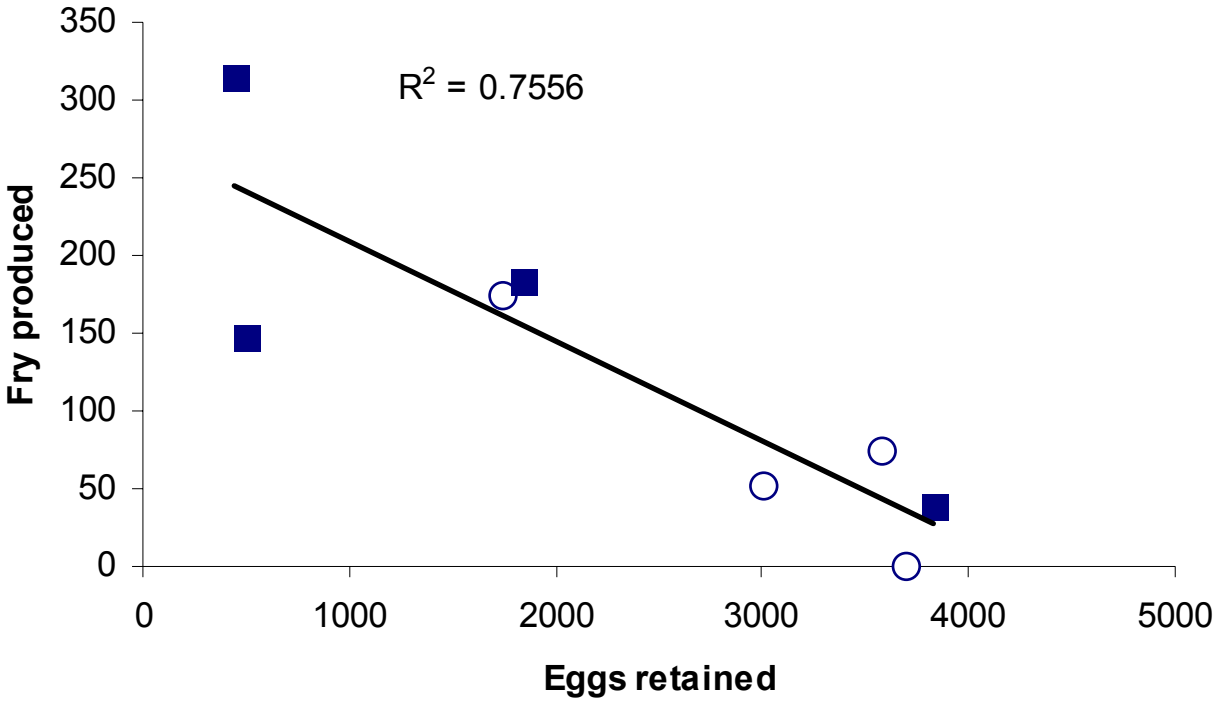
Table 6. EMG data from 15-min scans four each of four Big Beef Creek Chinook salmon males. Four aggressive behaviors were recorded: attacks given (attack +), attacks received (attack -), displays given (display +) and displays received (display -).

			Attack +			Attack -			Display +			Display -		
Male	Ave EMG inactive[a]	Scan	Min (n)[b]	Ave[c]	Ratio[d]	Min (n)[1]	Ave	Ratio	Min (n)[1]	Ave	Ratio	Min (n)[1]	Ave	Ratio
Y11	8.48	1	3 (3)	10.01	1.18	2 (2)	10.83	1.28	1 (1)	13.37	1.34	0	0.00	0
		2	0	0.00	0	4 (15)	12.73	1.50	3 (4)	13.30	1.57	0	0.00	0
		3	0	0.00	0	0	0.00	0	0	0.00	0	0	0.00	0
Y15	4.87	1	0	0.00	0	4 (9)	8.05	0	0	0.00	0	0	0.00	0
		2	2 (2)	4.13	0.85	4 (8)	4.99	1.02	0	0.00	0	0	0.00	0
		3	0	0.00	0	2 (3)	6.97	1.43	0	0.00	0	1 (1)	6.05	1.24
		4	1 (1)	6.26	1.29	2 (2)	5.32	1.09	0	0.00	0	0	0.00	0
Y22	4.06	1	0	0.00	0	0	0.00	0	0	0.00	0	0	0.00	0
		2	3 (8)	7.62	1.88	2 (4)	6.29	1.55	2 (2)	8.34	2.06	0	0.00	0
		3	7 (11)	7.36	1.81	9 (15)	8.57	2.11	1 (1)	10.89	2.61	0	0.00	0
		4	5 (20)	7.46	1.84	6 (32)	7.87	1.94	5 (18)	7.53	1.86	4 (12)	7.64	1.88
Y42	8.51	1	0	0.00	0	2 (5)	16.39	1.93	0	0.00	0	0	0.00	0
		2	0	0.00	0	0	0.00	0	1 (1)	7.68	0.90	0	0.00	0
		3	3 (4)	12.41	1.46	2 (3)	12.35	1.45	1 (1)	11.84	1.39	3 (3)	12.04	1.41
		4	3 (3)	7.91	0.93	1 (1)	9.26	1.09	0	0.00	0	1 (1)	10.00	1.17

[a] The average EMG values for 1-min intervals during which the fish was inactive (not engaged in any aggressive or courtship behaviors).
[b] The number of 1-min intervals in which the behavior was observed. The total frequency of the behavior is shown in parenthases.
[c] The average EMG values for the intervals in which the behavior was observed.
[d] The average EMG values during intervals when the fish was active divided by the 'inactive' average.

Table 7. Date and time of spawning events observed (directly or by video) and detected by analysis of EMG data. Each of the four females are shown separately. Of the four false detections for female W-57, three could be eliminated because spawning at a rate of more than three times in a 4-hour period (i.e., between 18:51 hrs and 22:51 hrs) was never documented in this study or other studies of Chinooks salmon reproductive behavior (Berejikian et al. 2000, 2001, 2003).

Female W-51		Female W-55		Female W-57		Female W-70	
Observed	Detected	Observed	Detected	Observed	Detected	Observed	Detected
9/24/03 15:16	9/24/03 15:15	9/24/03 18:19	9/24/03 18:19	9/24/03 18:51	9/24/03 19:09	9/25/03 7:00	9/25/03 7:27
Not observed	9/24/03 16:40	9/24/03 21:17	9/24/03 21:16	Not observed	9/24/03 19:25	9/25/03 17:16	9/25/03 17:15
9/24/03 17:51	9/24/03 17:47	9/25/03 1:15	9/25/03 1:14	Not observed	9/24/03 20:07	9/25/03 21:24	9/25/03 21:29
9/27/03 0:50	9/27/03 0:49	9/25/03 22:07	Not detected	Not observed	9/24/03 20:42	9/26/03 3:33	9/26/03 3:37
				Not observed	9/24/03 21:09		
				9/24/03 22:51	9/24/03 22:51		
				9/25/03 3:13	9/25/03 3:11		
				9/25/03 15:47	9/25/03 15:47		


350
300
250
200
150
100
50
0
Fry produced
R2 = 0.7556
0
1000
2000
3000
4000
5000
Eggs retained

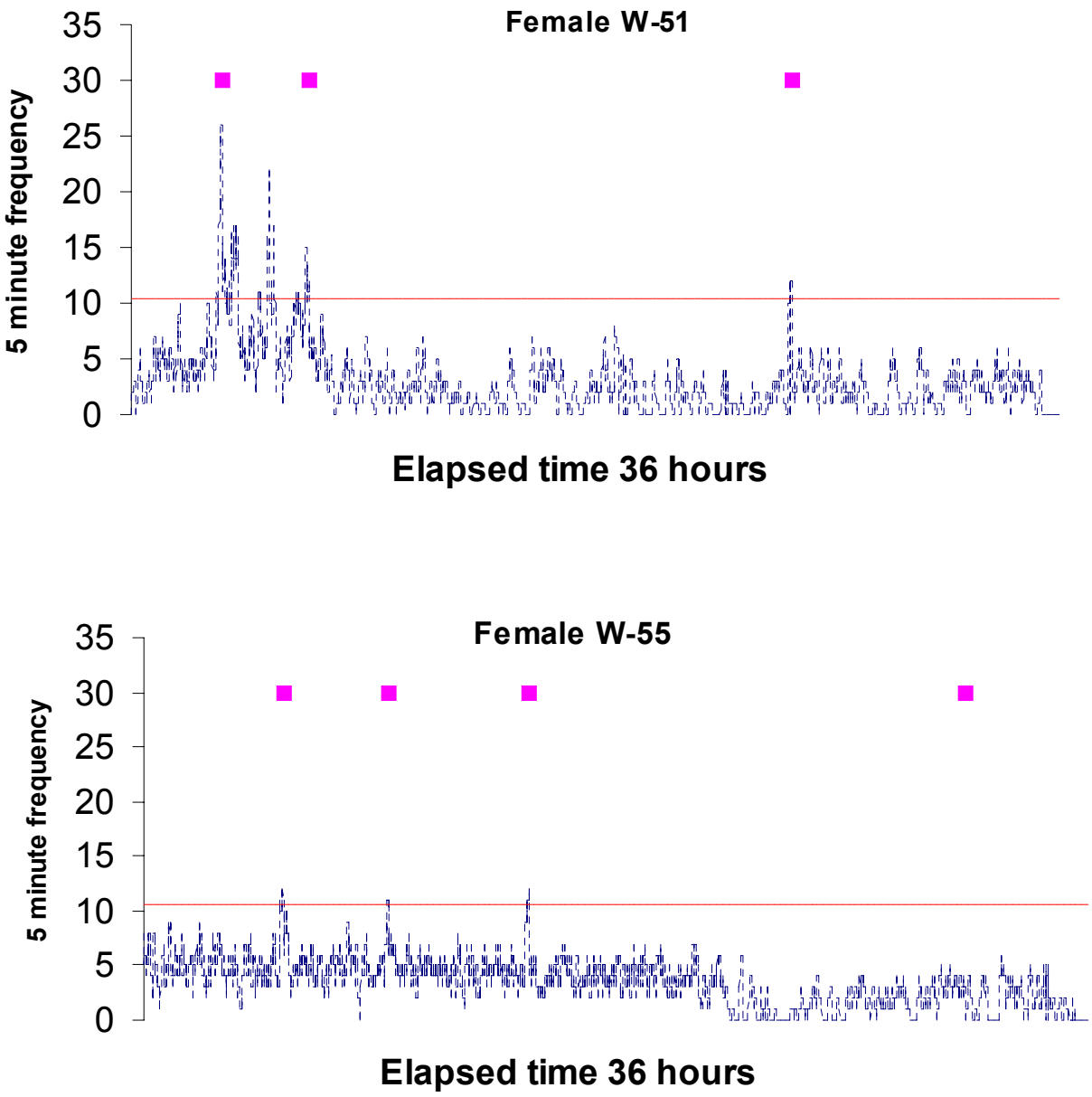
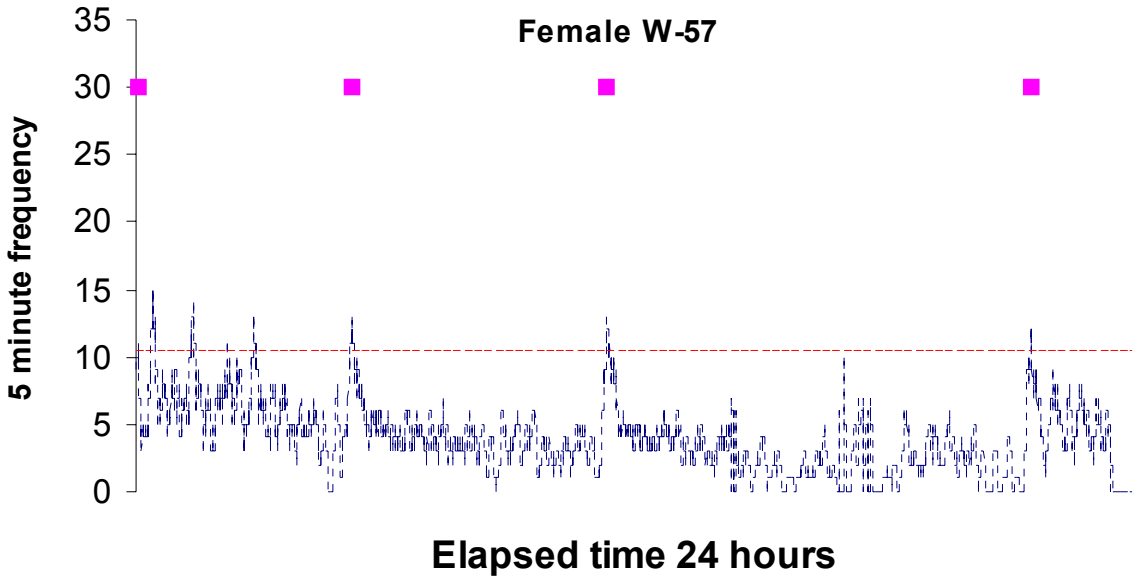


Figure 2. Moving frequency per 5 min of the number of EMG values that exceeded the 95th percentile of all EMG values for an individual female. Spawning events observed directly or by video analysis are indicated by the squares. Spikes in EMG frequencies above the horizontal line indicate EMG-detected spawning events (see also Table 7).



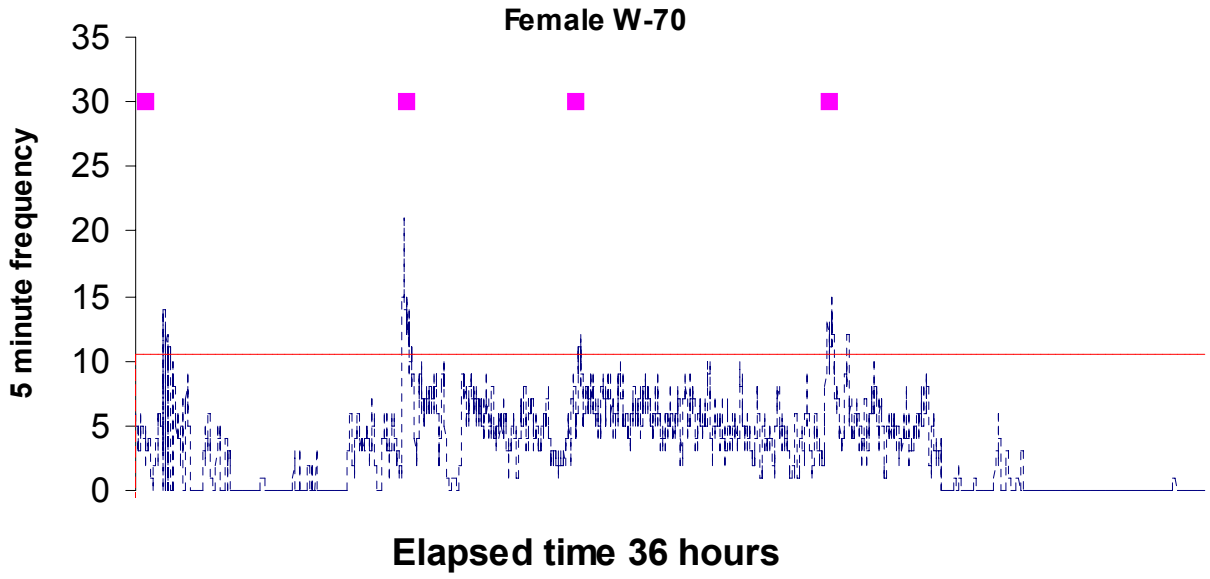


Figure 2. Continued.

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OBJECTIVE 2 - IMPROVE OLFACTORY IMPRINTING

TASK 2. DETERMINE CRITICAL IMPRINTING PERIODS FOR SOCKEYE SALMON

By

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Introduction

Over the last several decades, many of the distinct salmon populations in the Columbia River have experienced a steady decline due to habitat loss, dams, and over fishing (NRC 1996, NMFS 2000a, 2000b). In response to these declines, a number of captive propagation and conservation hatchery programs have been initiated to preserve the genetic resources associated with these population, and to re-introduce and restore these populations as environmental conditions associated with the original declines are mitigated (NWPPC 1999). Several Columbia River salmon populations and ESUs have reached critically low levels, and NOAA Fisheries (NMFS 2000a), the Northwest Power Planning Council's Columbia River Basin Fish and Wildlife Program (NWPPC 2000), and several state and tribal agencies, have endorsed and implemented captive broodstock programs as a safety net for endangered populations.

The initial focus of most of these programs has been to capture wild fish from imperiled populations to rear them in captivity, thereby increasing the juvenile-to-adult survival. Ultimately, as their numbers increase, these fish or their progeny are reintroduced into their natal environment. While captive rearing may be necessary to preserve these populations, there are several potential problems that must be addressed in developing successful captive broodstock programs. For example, artificial propagation removes salmon from their natural environment and hatchery rearing and release practices can have profound effects on the development, physiology, behavior and ecological interactions of fish when they are released back into their native environment. One area of particular concern is the effect of artificial propagation and inappropriate reintroduction strategies on the subsequent homing ability of released fish (Grant 1997, NWPPC 1999). Concerns about excessive straying of hatchery-reared fish have led to a call for the re-evaluation of hatchery programs to "avoid unnatural patterns of straying by adult returns" (NRC 1996) and for further research on the causes and consequences of homing and straying of hatchery-reared fish (Grant 1997, Flagg and Nash 1999). The recent draft, Mainstem/Systemwide Province Artificial Production Program Summary, has indicated that studies on the effect of hatchery rearing "on straying (homing and imprinting) are needed to manage straying rates of hatchery adults," and such studies were identified as actions toward RPA 107 and 184 "needing immediate implementation" under both BIOP and FWP (NWPPC 2002).

The tendency to home to the natal stream to spawn is fundamental to the unique biology and management of Pacific salmon. Homing results in genetic isolation of populations of salmon uniquely adapted for conditions in their natal streams (Ricker 1972, Taylor 1991). The final freshwater stages of these homing migrations are governed by the olfactory discrimination of home-stream water. Prior to their seaward migration, juvenile salmon learn (imprint on) site-specific odors associated with their home stream, and later use these retained odor memories to guide the final phases of their homing migration (Hasler and Scholz 1983). This imprinting process is critical for the 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 (Quinn 1993). While low levels of straying from the natal site are normal in the wild, inappropriate hatchery rearing conditions and juvenile release procedures can dramatically increase the level of straying by adult fish (Grant 1997, Pascual and Quinn 1994, Pascual et al.1995). Reintroduction of captively-reared fish into the wild at inappropriate developmental periods, or after insufficient periods of exposure to appropriate olfactory cues, may result in elevated levels of straying (Quinn 1993, Nevitt and Dittman 1998). Straying by captively-reared salmon can jeopardize efforts to enhance endangered populations by either lowering the effective number of spawning adults in a captively-reared target population (USFWS1996), or via competition and interbreeding of hatchery salmon with endangered wild populations (Crateau 1997, Carmichel 1997).

Hatchery rearing does not necessarily result in increased levels of straying (Labelle 1992, Quinn et al. 1989, Hard and Heard 1999). However, rearing wild fish for even short periods in a hatchery can increase straying (McIsaac 1990), and certain hatchery practices do clearly increase stray rates (Quinn 1993). For example, while salmon typically return to the site from which they were released (Donaldson and Allen 1958, Ricker 1972), fish reared and released from a single site generally stray less than fish transported and released off-site (Reisenbichler 1988, Boydstun et al. 1992 reviewed in Quinn 1993). In general, the closer the rearing and release sites are to each other, the more likely adults will return to the rearing site (Lister et al. 1981, Johnson et al. 1990, Slaney et al. 1993). Another important factor that may influence homing fidelity in hatchery fish is the timing of releases and reintroductions into the wild. A number of studies have identified the parr-smolt transformation as an important period for olfactory imprinting for some species (Hasler and Scholz 1983, Dittman et al. 1996) and chinook salmon released just before or after smolting tended to stray more than fish released as smolts (Pascual et al. 1995, Unwin and Quinn 1993). Finally, the length of time fish are exposed (acclimated) to water at the release site prior to release may influence subsequent homing to that site (Johnson et al. 1990, Savitz et al. 1993, Kenaston et al. 2001).

Captive rearing programs for endangered wild salmon require special considerations and have unique constraints not normally required in production hatcheries. The primary goals of most programs are to, (i) increase the number of individuals within a population; and (ii) maintain the genetic and phenotypic integrity and complexity of the wild population until reintroduction (Flagg et al. 2000). The strategies typically employed to achieve one goal often have negative consequences for the other. The longer fish are

maintained in captivity the greater the immediate increases in survival, but also the greater the risk of genetic and phenotypic changes in the population (Waples and Do 1994). This is also true for reintroduction strategies. In general, the earlier in the life cycle a fish is released into its ancestral environment (or at least experiences its natal water for imprinting), the better the opportunity for proper imprinting and successful homing. However, re-introduction at these early times carries with it the risk of lower survival. These two competing concerns force managers of captive rearing programs to weigh the likely tradeoffs and benefits of different release strategies to maximize survival but minimize straying. These problems are further compounded by the need for large and expensive culture facilities to maintain captive broodstocks through all phases of their life cycle. In most cases, these appropriate rearing facilities are not located in the ancestral watershed, and *in situ* rearing of endangered populations is not practical. Fish are typically reared offsite in non-homestream water and then transferred back for reintroduction (Kline and Heindel 1999).

One example that illustrates some of these challenges for a captive broodstock conservation hatchery program is the Redfish Lake sockeye salmon captive rearing program (BPA Projects #199107200 and #199204000). Snake River sockeye salmon were listed as endangered by NOAA Fisheries in 1991, and in that same year IDFG initiated a captive broodstock program with the ultimate goal of re-establishing sustainable sockeye runs to Stanley Basin waters (Kline and Heindel 1999). During the program's initial years all returning wild anadromous adults (16), residual sockeye adults and wild juvenile out-migrants were captured to establish a captive broodstock. Captive rearing dramatically increased juvenile-to-adult survival and the population numbers have increased to the point that since 1993 captively-reared fish have been re-introduced annually into the Stanley Basin. To avoid unanticipated negative consequences of any one reintroduction approach, the IDFG, in conjunction with the Stanley Basin Sockeye Technical Oversight Committee (SBSTOC), has adopted a "spread-the-risk " strategy for reintroducing sockeye back into the wild that includes planting of eyed eggs, net pen and direct lake releases of pre-smolts, smolt releases, and releasing captively-reared adults to spawn naturally (Kline and Heindel 1999).

Despite successful out-migrations of smolts from all the different release strategies, prior to 1999 none of these strategies successfully produced adult sockeye salmon back to Idaho. Fish for these releases were reared at several out-of-basin facilities (NOAA Fisheries hatchery at Big Beef Creek, Washington; IDFG hatchery at Eagle, Idaho, ODFW Bonneville hatchery) because there were no appropriate Stanley Basin facilities, and to avoid the risk of cataclysmic events at a single facility. The wisdom of this approach, and the need to evaluate all reintroduction strategies for imprinting success, was illustrated by the recent IHNV outbreak that destroyed the Stanley Basin sockeye smolt release group being reared at the Bonneville hatchery. 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. Earlier studies with coho salmon have indicated that fish released as smolts tended to stray more than fish released as fingerlings a year prior to smolting (McHenry 1981).

Concerned that the lack of adults returning to Stanley Basin may be due to unsuccessful imprinting and straying, the sponsors of the captive broodstock Projects #199107200 and #199204000, and the SBSTOC recommended that Project #9305600 initiate research on the timing of imprinting and environmental factors that influence imprinting, especially in sockeye salmon. Research to examine the timing of imprinting in Columbia Basin sockeye salmon was initiated in 2000. In 1999, the first hatchery-produced adults returned to the Stanley Basin and as of fall 2001, 290 adults had returned to the Stanley Basin. The majority of adult fish recovered to date have come from the smolt-release group of fish that only briefly experienced Stanley Basin water before emigration. These results appear to indicate that sockeye salmon are capable of imprinting as smolts, but the numbers of returning fish relative to other release groups may be more a reflection of the number of fish released and smolt-to-adult survival than homing success. The stray rates of these smolt release groups are unknown. However, radio telemetry and Snake River dam passage data indicated that a high percentage of fish that migrated past Lower Granite Dam do not reach the Stanley Basin watershed (Paul Kline, IDFG, personal communication), suggesting that they are either straying or suffering pre-spawn mortality within the Snake River.

The spread-the-risk strategy for reintroduction of Stanley Basin Sockeye is necessitated in part by the lack of knowledge about the physiological and developmental processes underlying olfactory imprinting and the ecological factors, which facilitate successful homing. For sockeye salmon reintroductions to be successful in the Stanley Basin (and throughout the Columbia Basin (e.g., BPA Project # 200001300; 29016) 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.

To date, research on the timing of olfactory imprinting has focused on coho salmon because of their relatively simple life histories. Juvenile coho salmon generally rear in their natal stream until they smolt and migrate to sea during the second or third spring after hatching. Experiments with hatchery-reared coho salmon indicated that this smolting period is the critical period for olfactory imprinting (Hasler and Scholz 1983, Dittman et al. 1996). Unfortunately, the understanding of imprinting inferred from studies of hatchery-reared coho salmon underestimates the complexity and temporal plasticity of the imprinting process in the wild and in other salmonid species (Dittman and Quinn 1996, Nevitt and Dittman 1998). For example, sockeye salmon fry typically emerge from their natal gravel and immediately migrate to rearing areas within a lake where they live for 1 or 2 years before smolting and migrating to sea. During their homing migration, adults migrate past the outlet stream and lake where they smolted and return to their natal area to spawn, suggesting that olfactory imprinting must also occur

prior to or during emergence from the gravel. Studies to determine the critical period(s) for imprinting for sockeye salmon were initiated in fall 2000 and the outcome of these experiments will help captive broodstock biologists develop and prioritize future rearing and release plans to minimize straying.

The overall goal of the research conducted under this objective is to identify hatchery practices that influence olfactory imprinting and thereby develop strategies to minimize straying of artificially produced salmonids. 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. Reintroduction of captively-reared fish into the wild at inappropriate developmental periods or insufficient periods of exposure to appropriate olfactory cues may result in elevated levels of straying. Results from these studies will help develop captive broodstock reintroduction and hatchery release strategies that will minimize straying.

Materials, Methods, and Description of Study Area

Odor exposures 2000-2002

Experiments to determine the critical period(s) for olfactory imprinting by sockeye salmon were initiated in fall 2000. After emergence from their natal gravel, sockeye salmon migrate to or remain in a lake where they reside for 1 or 2 years before smolting and migrating to sea. To determine the relative importance of odorant exposure during these key developmental periods, sockeye salmon were 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).

Assessments of imprinting were conducted in fall 2003 by measuring olfactory sensitivity to exposure odorants using behavioral assays and electro-olfactograms (EOG), a relatively simple electrophysiological technique used extensively in fishes to measure the sensitivity of the olfactory epithelium to specific odorants (Hara 1992, Sorensen and Caprio 1997). EOG responses reflect the summated responses of many receptor neurons in the olfactory epithelium (Ottoson 1971). Because the olfactory epithelium is apparently sensitized to specific odors during imprinting (Nevitt et al. 1994, Dittman et al. 1997), the EOG may provide a rapid, sensitive, and cost-effective method for assessing sensitization to imprinted odorants.

Experimental animals and treatments

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

Embryos were transferred to the NMFS Northwest Fisheries Science Center and reared in chilled dechlorinated Seattle City water. Subsequently, they were divided into three treatment groups, (i) alevins/emergent fry exposure, (ii) smolt exposure, and (iii) control. The smolt exposure group was further divided into 3 groups with different exposure lengths. The alevins/emergent fry were continually exposed to a mixture of imprinting odorants (phenylethyl alcohol (PEA), L - arginine, L - threonine, and L – glutamate) at a final concentration of 100 nM each from 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 Big Beef Creek (BBC) in August 2001 and reared exclusively in well water (constant 10° C) throughout the remainder of the experiment. Beginning in 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. The experimental fish demonstrated a significant increase in gill Na^+/K^+ ATPase activity, suggesting that these fish successfully smolted during the imprinting period (Dittman 2003). 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); releases of smolts into outlet streams (1 day). All groups were maintained separately until after the parr-smolt transformation (31 May 2002), and then marked by treatment. Behavioral and physiological assessments of imprinting in sockeye were initiated in Fall 2003. Of the 518 experimental fish remaining, 356 fish (68.7%) matured in Fall 2003. Approximately equal numbers of males (53.09%) and females (46.9%) matured in 2003.

Behavioral testing

Behavioral responses of maturing sockeye salmon to imprinting odorants were tested in three 2-choice mazes constructed at the BBC facility. Each maze consisted of a 3.05 m x 1.22 m x 1.22 m fiberglass raceway connected to a 0.91 m x 0.61 m x 0.91 m tank and a 0.91 m x 0.61 m circular polyethylene tank on both sides (Fig. 1). A false bottom was placed in each raceway to create a water depth of 36.0 cm. The uppermost 0.76 m of the raceway was divided to generate the two choice areas. Big Beef Creek water was pumped from the BBC spawning channel into each circular tank, which then flowed into the intermediate rectangular tank and finally into the main raceway (Fig. 1). The flow rate into each arm of the maze was 150 L/min. Fish were tested in BBC water because an unfamiliar background water may be important for experimentally determining that salmon have imprinted to the test odorants (Hasler and Scholz 1983, Dittman et al. 1986).

Because the temperature of the well water used for rearing and BBC water differed by as much as 5.0 ° C over the course of the experiment, salmon were acclimated to BBC water for 1-2 d prior to testing.

To start a trial, one mature fish was randomly selected from the holding tank, placed in the downstream section of a raceway and allowed to acclimate for 30 min. During the acclimation period, a screen placed at the downstream end of the divider prevented fish from entering either arm. During the final five min of the acclimation and for the remainder of the trial, test odors (PEA, L - arginine, L - threonine, and L – glutamate) were pumped into the inlet of one arm to generate a final concentration of 100 nM of each odorant. After the acclimation period, the screen was lifted and the fish was allowed to swim freely throughout the tank for 40 min. First and last arm entered, number of entries and time spent in each arm was recorded for each fish. For the first 89 trials, data were collected by real-time observations, staggering trials to observe the first and last 10 min of a 40-minute trial for each maze. For the final 145 trials, underwater video cameras mounted at the extreme downstream end of each maze captured all 40 min of each trial for later review. At the end of each trial, the odor pump was turned off, fish were removed, scanned for PIT tags and fin clips to determine experimental group, and its reproductive status (spermiating, ovulating) was determined. Tanks were allowed to clear of test odors for approximately 5-10 min. between each trial. During any given day, the arm into which odors were pumped remained the same (typically 4-5 trials/maze/day). At the end of each day, the mazes were drained, scrubbed and the odor pump was switched to the opposite arm for the next day's trials. A total of 115 mature males (100% spermiating) and 119 mature females (96.6% ovulating) were tested for behavioral responses to the imprinting odors over 20 d between 18 November and 9 December 2003.

EOG testing

Imprinting success of the treatment groups was also assessed in fall 2003 by measuring olfactory sensitivity to exposure odorants using electro-olfactograms (EOG), a relatively simple electrophysiological technique used extensively in fishes to measure the sensitivity of the olfactory epithelium to specific odorants (Hara 1992, Sorensen and Caprio 1997). EOG responses reflect the summated responses of many receptor neurons in the olfactory epithelium (Ottoson 1971). Because the olfactory epithelium is apparently sensitized to specific odors during imprinting (Nevitt et al. 1994, Dittman et al. 1997, Dittman 2003), the EOG may provide a rapid, sensitive, and cost-effective method for assessing sensitization to imprinted odorants. In brief, the EOG technique involves placing a recording electrode near the medial surface of one lamellae within the olfactory rosette and monitoring electrical activity in response to perfusion of the rosette with odorants at different concentrations. Development and testing to ready the EOG apparatus for testing odor-exposed sockeye was initiated during 2001. Initial results using coho salmon, a species for which sensitive periods for imprinting have been identified, indicated that the EOG technique is effective for measuring olfactory sensitivity to exposure odorants (Dittman 2003). Perfusion of the olfactory epithelium with the amino acids (e.g. L-arginine (Fig. 2), bile acids (e.g. Taurocholic acid (Fig. 3),

and artificial odorants elicits concentration-dependent EOG responses. Furthermore, by Fall 2002, the coho salmon exposed to amino acid odorants during sensitive imprinting periods demonstrated a heightened sensitivity to these odorants relative to control fish (Dittman 2003).

In Fall 2003, six mature and two immature sockeye from each of the five experimental groups were transferred from the BBC facility to the NWFSC for EOG testing. Fish were held for a period of 2 to 14 d in dechlorinated Seattle city water (10° C) prior to testing. EOG analysis was performed as described by Baldwin et al. (2003) with minor modifications. Briefly, each fish was anaesthetized in MS-222 (50mg/L) for approximately 10 min, paralyzed by injection with gallamine triethiodide (0.3mg/100g of body mass) and placed in a V-shaped Plexiglas holder on a vibration isolation table. Gills were constantly perfused with chilled (11° C), aerated dechlorinated city water containing MS-222 (50mg/ml) at a flow of 400ml/min. A glass recording microelectrode was positioned along the midline of the rosette and the rosette was constantly perfused with chilled artificial freshwater. For odorant testing, odorants were delivered by a computer-activated pinch-valve manifold (Automate) with a pulse duration of 10 sec. Odor-evoked responses were measured as the peak negative amplitude (millivolts) relative to the pre-stimulus baseline. Odor pulses were separated by at least 2 min to ensure recovery of the rosette to prestimulus sensitivity. Data acquisition and preliminary analysis utilized Labview software (National Instruments). Sensitivity to imprinting odorants was assessed by measuring the EOG responses of each fish to L-Serine (10^{-4} M), a standard reference odorant, the imprinting amino acids odorants: L-threonine, L-glutamate and L arginine each at 10^{-5} M and an equimolar combination of these odorants plus PEA at 10^{-6}, 10^{-5} and 10^{-4} M. The standard (L - serine) was tested before and after each odorant dose-response profile.

Molecular testing

The initial events in odor (e.g., homestream water) recognition are mediated via binding of odorant molecules to specific receptor proteins expressed in the olfactory receptor neurons (ORNs) of the salmon olfactory rosette (for review see Hara 1992). Each of the millions of ORNs in the rosette expresses one of a family of approximately 100 distinct odorant receptors (Ngai et al. 1993). During imprinting, the number of olfactory neurons that are responsive to an imprinting odorant increases and the sensitivity of those neurons to that odorant is heightened (Nevitt et al. 1994, Nevitt and Dittman 1998). It is hypothesized that this heightened cellular sensitivity is due to increased numbers of cells expressing a given receptor protein and increased expression of the receptor within responsive neurons. In the FY 2004 statement-of-work (SOW), we proposed to exploit the up-regulation of odor receptor proteins during imprinting to develop a simple PCR-based assay for imprinting by examining the salmon basic amino acid odorant receptor (BAAR). Results from a separate study indicated that juvenile coho salmon exposed to arginine during smolting, a critical period for imprinting, demonstrated heightened sensitivity of the olfactory epithelium to this odorant, and initial molecular assessments of rosettes from these fish indicate that BAAR expression may change during different life stages and after odor exposure (Dittman unpublished). These results are encouraging

and preliminary experiments to test the feasibility of molecular assays for imprinting in sockeye salmon will be conducted under the FY 2004 SOW. Olfactory rosettes for conducting these studies were collected from 10 mature and 2 immature fish from each of the five experimental groups, immediately frozen in liquid nitrogen, and transferred to a –80°C freezer for storage until they are analyzed for receptor expression levels.

Data analysis

The first and last arm entered during a time period and the arm in which experimental fish spent the majority of their time in response to odorants were compared with the 50:50 distribution expected by chance and to responses of odorant-naïve control fish using χ^2 contingency tests. Differences in the proportion of time spent in the odor vs. the control arm of the maze were assessed by comparing responses of experimental groups to control fish using a two-tailed t test. Proportions were normalized by arcsine square root transformation (Zar 1984). Differences between number of entries into each arm were analyzed by a paired t test. Fish that did not enter an arm during the time period examined were excluded from the analysis. In all cases, departures from no preference were tested at a P=0.05 significance level.

Responses to each odorant concentration were either expressed directly in millivolts (mV) or expressed as a percentage of the response to the L-serine (10^{-5} M) standard and then averaged. Differences between treatment groups in EOG responsiveness to specific odorants was examined by analysis of variance (ANOVA) followed by Fisher's PLSD (Statview, 1992). Significance for all analyses was established at the $p<0.05$ level.

Odor exposures 2002-2004

In December 2002, a second experimental group of odorant-exposed fish was initiated for studying the timing of imprinting and the effects of artificial incubation and early rearing environments on imprinting. Particular emphasis was be placed on experimental treatments that parallel rearing and release strategies being tested as part of the Snake River Sockeye Salmon Captive Broodstock program (BPA Project # 199107200). Based on the current and projected release strategies for Stanley Basin sockeye the following odor exposure experiments were conducted: Stanley Basin sockeye salmon (eyed eggs) were transferred to the Big Beef Creek and reared in constant 10°C well water. In this experiment fish are being exposed to imprinting odorants for longer durations to parallel release strategies utilized in Stanley Basin. Specifically, fish were divided into six treatment groups: (i) eyed egg to smolt exposure (January 2002 to May 2004) - odor exposures history similar to eyed egg plants and naturally produced fish; (ii) fry to smolt exposure (April 2003 to May 2004) - similar to Sawtooth hatchery rearing and smolt release; (iii) brief smolt exposure (May 2004) - Bonneville/Eagle hatchery rearing and smolt release. From February-July 2004, 15 fish from each exposure group were sacrificed every three weeks for physiological sampling of gill Na^+/K^+ ATPase activity (McCormick 1993), plasma thyroxine (Dickhoff et al. 1982) and olfactory rosette collection. ATPase assays will be conducted in the 2004 work plan.

Long-term odor exposure experiments are utilizing a single amino acid odor because the expense of amino acid odorants precludes long term exposures to complex mixtures of these defined odorants. Arginine was chosen because concurrent electrophysiological and molecular studies of arginine odorant receptors (BAAR) are being conducted. Two additional smolt exposure groups were exposed to more complex odors (Redfish Lake water, Upper Salmon River water) to examine the importance of complex odor types (i.e., lake water versus river water) for imprinting and successful homing. After odor exposure, the fish will be marked and reared communally at Big Beef Creek. The majority of these fish will not mature until Fall 2005. EOG and molecular testing for imprinting will be conducted throughout 2004-2005. Behavioral testing of maturing fish will be conducted in fall 2005. During the 2004 work plan, olfactory rosettes for molecular analysis will be collected every three months (July, October, January, April) from 15 fish/treatment group and EOG analysis of odor sensitivity will be performed in July 2004 and April 2005 (8 fish/treatment).

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, as indicate above, the majority of the behavioral and EOG evaluations of olfactory imprinting will be conducted in fall 2005 when these fish are expected to mature. While a few early maturing males (W. McAuley, NMFS, personal communication) may be available in September-October 2004 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 remains a major obstacle for development of routine assays for imprinting. Recent studies suggest that the maturational hormone GnRH can stimulate migratory behavior in homing salmon (Dittman, unpublished), and heighten olfactory sensitivity (Eisthen et al. 2000). Using coho salmon exposed to imprinted odorants during smolting (March - May 2001), the known critical period for imprinting in hatchery-reared coho, the efficacy of GnRH analog implants was tested for inducing EOG olfactory sensitivity to imprinting odorants in odorant-exposed fish. EOG implants had no apparent effect on sensitivity to imprinted odorants but direct application of this hormone to the olfactory epithelium resulted in increased sensitivity to a number of odorants. Complete analysis of these results and follow-up studies to confirm this phenomenon and to eliminate potential instrumental artifacts will be reported in the next Annual Report.

Results and Discussion

Behavioral assays

To assess the efficacy and efficiency of the two-choice maze for examining olfactory imprinting and homing behavior, we compared the responses of fish over time during the 40-minute trial. Previous experiments utilizing two-choice arenas to study chemosensory-mediated behaviors in Pacific salmon have indicated that early responses after

acclimation yield the most robust behavioral responses for chemosensory discrimination between experimental groups (e.g. Courtenay 1989, Dittman and Quinn 1994, Dittman et al. 1998). To ensure this was also true for adult sockeye salmon, we subdivided the trials into three time periods (first 10 min, second 20 min and final 10 min) and analyzed each response variable (proportion of time in odor arm, number of entries, etc) for every time period and for the trial as a whole. Over the course of the trials, general activity, as measured by the number of entries into arms, increased for all treatments (ANOVA; $P<0.05$). The specific odor-related responses of individual fish changed dramatically over the course of the trial. For example, while the percentage of time that control fish spent in the odor-scented arm remained relatively constant, the percentage of time in the odor arm for the treatment groups declined over the three time periods (ANOVA; $P<0.05$) (Fig. 4). Indeed, with further validation, this decline in time spent in the odor arm may ultimately provide a metric for odor discrimination. As previously observed for other *Onchorhynchus* species and life stages, the most robust behavioral responses to odors were observed during the first 10 min of the trial and subsequent analysis was confined to this time period.

Thirty-one of the 234 mature fish tested in the two-choice maze did not enter either arm of the maze during the first 10 min of the trial and were excluded from the analysis. Of the remaining 203 fish, the behavioral responses of mature fish from 3 of the 4 experimental groups that had been exposed to the imprinting odorants (PEA, threonine, arginine, and glutamate) at least one year earlier as juveniles differed from odorant–naïve control fish. In particular, adult sockeye that had been exposed to odors for four weeks as alevins or for one or six weeks during smolting differed from control fish by at least one of several non-independent response variables (Tables 1-3; Fig. 5). However, the responses of salmon exposed to the odors for only one day during smolting did not differ from the control fish by any of the measures of odor recognition. Alevin-exposed and six-week smolt exposure fish spent significantly more time in the odor scented arm, 60.1 % ($P=0.038$) and 58.5 % ($P=0.043$) respectively, compared to control fish that tended to avoid the imprinting odors (45.4 %, see Fig. 5). Fish exposed to odors for one week or one day as smolts spent the majority of time in the odor arm (52.0% and 50.7%, respectively) but responses were not significantly different from controls ($P>0.05$; Fig. 5). Using a different measure of time preference, the majority of fish exposed to odors as alevins and smolts (1 and 6 week exposures) spent more time in the odor arm vs. the control arm during the first 10 min of the trial but these responses did not differ significantly from control fish ($P>0.050$; see Table 1). Similarly, the odor scented arm was final arm choice of the majority of the fish from the alevin (ns; $P=0.138$) , one week smolt ($P=0.010$) and six week smolt ($P=.009$) exposure groups while the final choice of the majority of the control fish was the unscented arm (Table 2). Control fish made equal number of entries into the odor (3.83 ± 0.65 s.e.) and control arms (3.83 ± 0.69) during the first 10 min of the trial while only the six week smolt exposure group made significantly more entries into the odor arm compared to the nonscented arm ($P=0.03$) (Table 3). Finally, unlike previous two-choice maze studies (Dittman and Quinn 1994, Dittman et al. 1998), there was no effect of treatment on the first arm entered (Table 4).

Taken together the data from this experiment indicated that upper Columbia River sockeye salmon exposed to the odorants PEA, L-threonine, L-arginine and L-glutamate were able to learn and remember these odorants as maturing adults up to 2.5 years after exposure. These results suggest that the alevin and smolt stages are both important developmental periods for successful olfactory imprinting. Furthermore, the period of time that fish are exposed to imprinting odors may be important for successful imprinting. Experimental fish exposed to imprinting odors as smolts for six or one weeks successfully imprinted to these odors but imprinting could not be demonstrated in smolts exposed to odors for only one day. The two-choice maze affords the ability to determine if odorants have been learned and elicit a behavioral response, but it is difficult to make inferences about the ecological relevance of responses observed in an artificial maze. With that caveat, responses to the imprinting odorants tended to be more robust the longer fish were exposed to the imprinting odorants (e.g., Fig. 5). Previous studies have indicated that imprinting for coho salmon may involve a very rapid learning process (Dittman et al. 1996, Dittman and Quinn 1996) and may require as little as 2 d exposure to a new water for successful imprinting (Jensen and Duncan 1971). The current results indicated that sockeye smolts may require longer odor exposure periods for imprinting. While managers of captive rearing programs must balance tradeoffs between post-release survival and appropriate periods of imprinting acclimation, these results suggest that earlier releases and longer acclimation may be beneficial for successful imprinting and homing. Experiments with the 2002-2004 exposure groups that were exposed for varying period times to imprinting odors should provide more insight about this question.

The current experiment utilized sockeye salmon that were fully mature (i.e., spermiating or ovulating). Mature coho and fall Chinook salmon still show strong or even heightened homing responses as they approach spawning (Quinn et al. 1988, Dittman unpublished). For most sockeye salmon, including the Okanogan River and Stanley Basin populations used for these studies, the homing migration involves two phases; migration from the ocean to their nursery lake in June-August and migration to specific sites for spawning in October-November (Gustafson et al. 1997, Kline and Heindel 1999). Fish in this study were tested in November when naturally spawning fish would be seeking their spawning grounds. Imprinting to the natal spawning site must occur prior to or during emergence from the natal gravel and therefore it may be that mature sockeye salmon seek odors they specifically learned as alevins. Mature fish also responded to odors learned as smolts, which may facilitate late migrating fish to locate their natal lake and spawning grounds. Future studies will examine the behavioral and EOG responses of maturing fish both in July (ocean to lake migration) and October (spawning site migration) to determine if odor mediated responses are dependent on the juvenile stage that imprinting odors were experienced.

EOG assays

Of the 30 mature sockeye utilized for EOG analysis of imprinting, only 14 fish produced reliable olfactory responses as measured by EOG. The mature fish that did produce an EOG response had dramatically lower sensitivity to all odors tested when compared to

immature fish. For example, the mean EOG responses (± s.e.) to the 10^{-4} M L-serine standard was 1.76 mV (±0.22) for immature fish (N=7) but only 0.80 mV (±.082) for mature fish (N=14). This dramatic decline in the olfactory sensitivity of mature fish at, or near, the time of spawning has been previously observed in other salmon species (Hasler and Scholz 1983, Dittman et al. 1997, Dittman unpublished). Given the small sample size of responsive fish and the reduced odor sensitivity of those that did respond, it is perhaps not surprising that we observed no effect of treatment (i.e. exposure to imprinting odors as alevins and smolts) on EOG sensitivity to the imprinting odor mixture or the individual component odors (L-arginine, L-glutamate, L-threonine) (Fig. 6). The timing of EOG testing was designed to occur just prior to spawning and overlap with the behavioral assessments of imprinting but approximately 25% of the fish were tested after the two-choice tests were completed. While the behavioral assays indicated that salmon were able to recognize and respond to odors in mid-late November, EOG testing may have occurred as a precipitous decline in sensitivity occurred. Future studies will examine the behavioral and EOG responses of these fish both in July (ocean to lake migration) and earlier in October (spawning site migration) to determine if odor mediated responses at different stages of maturation are dependent on the juvenile stage that imprinting odors were experienced.

Data Management Activities

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

Summary and Conclusions

Reintroduction of captively-reared fish into the wild at inappropriate developmental periods or insufficient periods of exposure to appropriate olfactory cues may result in elevated levels of straying. The overall goal of this research is to identify hatchery practices which influence olfactory imprinting, thereby develop strategies to minimize straying of artificially produced salmonids. By identifying developmental periods and acclimation periods that are important for olfactory imprinting, rearing and release strategies for each salmon species can be developed to lower stray rates in both production and recovery hatcheries. To determine the critical period(s) for imprinting for sockeye salmon, juvenile salmon were exposed to known odorants at key developmental stages. Behavioral assays of imprinting indicated that sockeye salmon learn imprinting odors at both the alevin and smolt stage. Furthermore, experimental fish exposed to imprinting odors as smolts for six or one weeks successfully imprinted to these odors but imprinting could not be demonstrated in smolts exposed to odors for only one day. Taken together, these results suggest that releasing captively-reared smolts so that they experience their natal lake for an extended period, may be helpful to facilitate proper odor learning and imprinting. In 2002-2003, the efficacy of EOG analysis for assessing

imprinting was demonstrated, and will be applied in these and other behavioral and molecular tools in the current work plan. Results of these experiments will be important to determine the critical periods for imprinting for the offspring of captively-reared fish destined for release into natal rivers or lakes.

Table 1. Behavioral responses of maturing sockeye salmon to imprinting odors. Data represent the final arm entered by individual fish during the first 10 min period. **Odor** indicates fish located in the arm scented with imprinting odorants, **Control** indicates fish located in the unscented arm and **No choice** indicates fish that were in neither arm at the end of this period. Significant differences from control responses were assess by $\chi 2$ contingency testing at a P=0.05 significance level.

	Final position (0-10 min)			
	Odor	**Control**	**No choice**	**$\chi 2$, p value**
Control	**22**	**32**	**7**	**-**
Alevin	**23**	**18**	**11**	$\chi 2$=2.2 P=0.138
Smolt – 6 weeks	**29**	**14**	**8**	**$\chi 2$=6.85 P=0.009**
Smolt – 1 week	**24**	**11**	**3**	**$\chi 2$=6.59 P=0.010**
Smolt – 1 day	**13**	**13**	**2**	$\chi 2$=0.61 P=0.433

Table 2. Behavioral responses of maturing sockeye salmon to imprinting odors. Data represent the arm preference of individual fish during the first 10 min. period. **Odor** indicates fish that spent more time in the arm scented with imprinting odorants, **Control** indicates fish that spent more time in the unscented arm. Fish that entered neither arm during this period were excluded from the analysis. Significant differences from control responses were assess by $\chi 2$ contingency testing at a P=0.05 significance level.

	Time Preference (0-10 min)		
	Odor	**Control**	**$\chi 2$, p value**
Control	**24**	**30**	-
Alevin	**27**	**15**	$\chi 2$=3.75 P=0.05
Smolt – 6 weeks	**26**	**18**	$\chi 2$=2.08 P=0.15
Smolt – 1 week	**18**	**17**	$\chi 2$=0.42 P=0.52
Smolt – 1 day	**12**	**14**	$\chi 2$=0.02 P=0.89

Table 3. Behavioral responses of maturing sockeye salmon to imprinting odors. Data represent the number of entries (mean ± standard error) during the first 10 min. period into the arm scented with imprinting odorants (**Odor**) and into the unscented arm **(Control).** Fish that entered neither arm during this period were excluded from the analysis. Significant differences between the number of odor and control entries for each treatment group were assessed by paired t tests at a P=0.05 significance level.

	Arm entries (0-10 min)		
	Odor	**Control**	**t value, P value**
Control	**3.83 (±0.65)**	**3.83 (±0.69)**	t=0.00
Alevin	**4.28 (±0.87)**	**3.52 (±0.74)**	t=1.25 P=0.22
Smolt – 6 weeks	**4.46 (±0.81)**	**3.04 (±0.46)**	**t=2.24** **P=0.03**
Smolt – 1 week	**3.35 (±0.55)**	**4.09 (±0.89)**	t=-0.81 P=0.42
Smolt – 1 day	**6.15 (±1.17)**	**4.10 (±0.85)**	t=1.89 P=0.07

Table 4. Behavioral responses of maturing sockeye salmon to imprinting odors. Data represent the number of fish that first entered the arm scented with imprinting odorants (**Odor**), first entered the unscented arm (Control) or entered neither arm during the entire 10 min. period (No choice). Significant differences from control responses were assess by $\chi 2$ contingency testing at a P=0.05 significance level. NS= not significant at P=0.05 level.

	First Arm Entered (0-10 min)			
	Odor	**Control**	**No choice**	**$\chi 2$, P value**
Control	**25**	**29**	**7**	**-**
Alevin	**19**	**22**	**11**	$\chi 2$=0.43 P=0.51
Smolt – 6 weeks	**25**	**19**	**8**	$\chi 2$=0.40 P=0.53
Smolt – 1 week	**14**	**21**	**3**	$\chi 2$=1.2 P=0.27
Smolt – 1 day	**14**	**12**	**2**	$\chi 2$=0.21 P=0.64

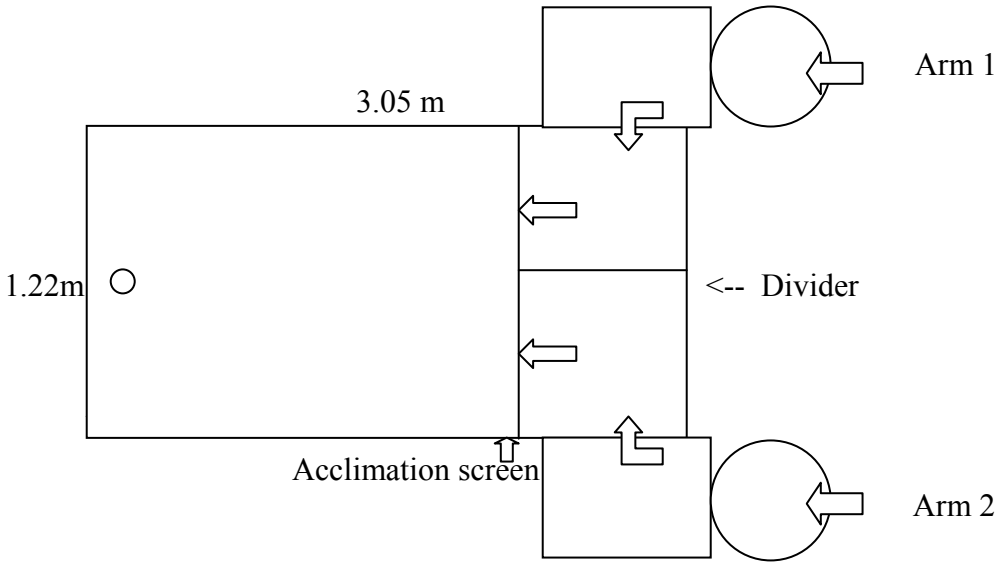


Figure 1. Schematic drawing of two-choice maze used for trials. Arrows indicate direction of water flow. There was an observer blind along the upstream end of the raceway to prevent disturbance of the trial.

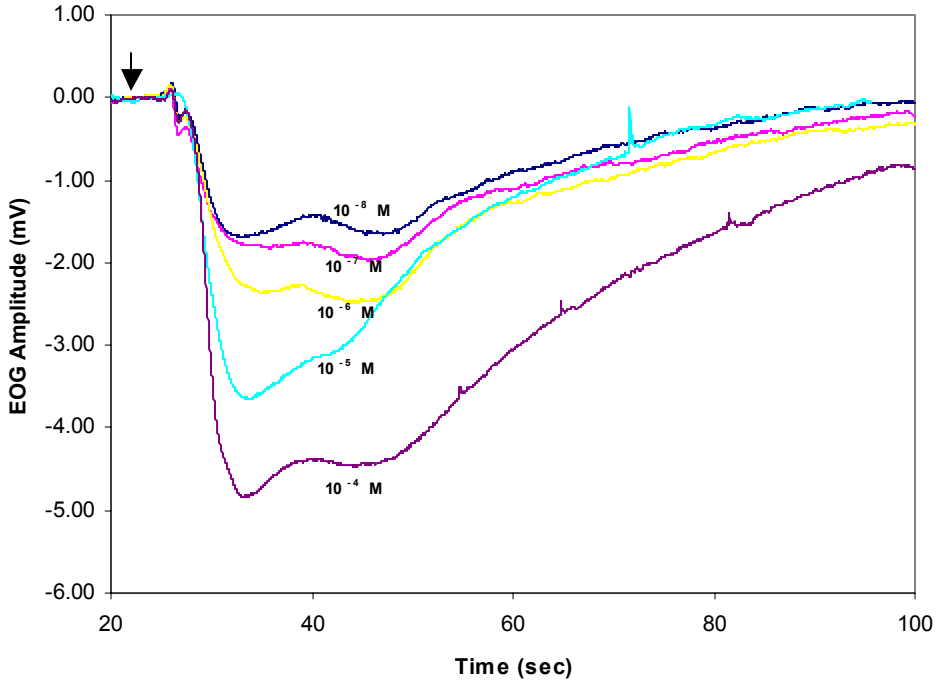


Figure 2. Representative L-arginine EOG dose-response from 2 year-old coho salmon.

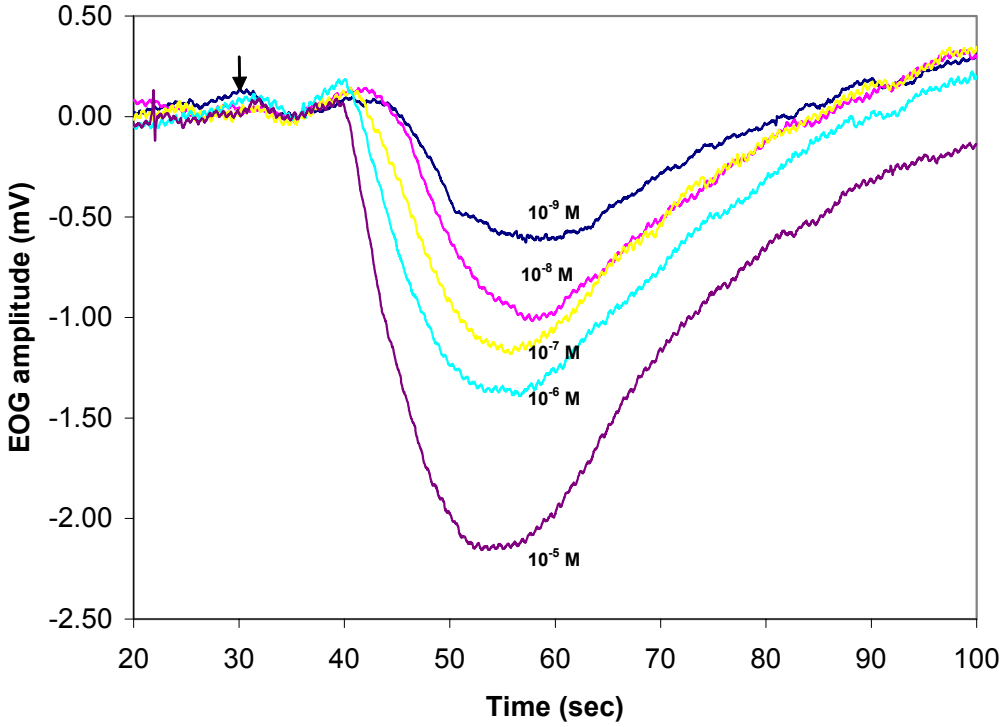


Figure 3. Representative Taurocholic Acid (TCA) EOG dose-response from 2-year-old coho salmon.

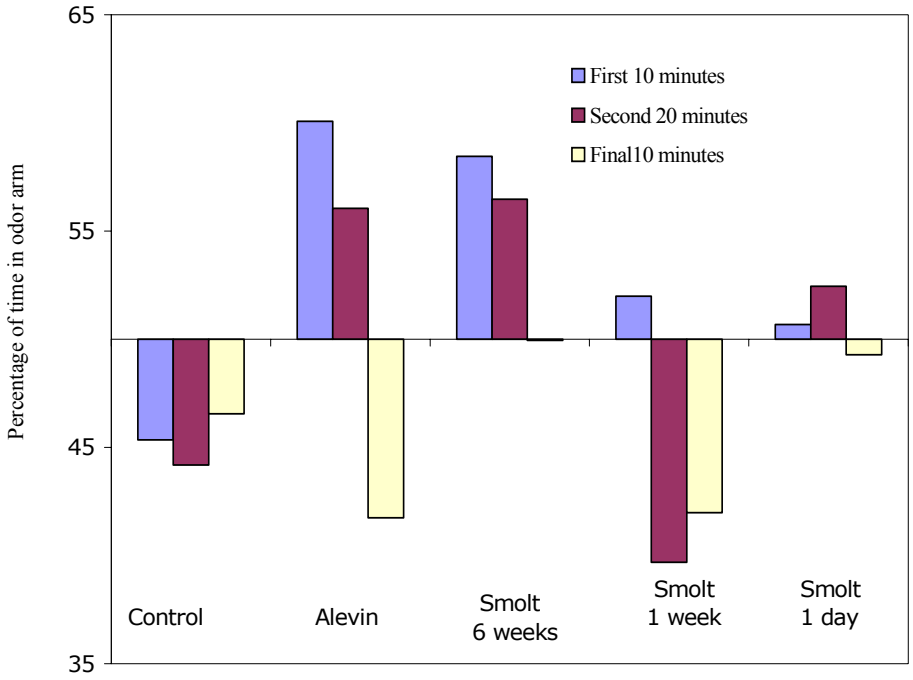


Figure 4. Behavioral responses of maturing sockeye salmon to imprinting odors. Data are mean percentage time spent in the arm scented with odorants relative to the total time spent in both arms during each time period. Fish that entered neither arm during this period were excluded from the analysis. Differences between the odor-exposed and control treatments were assessed by arcsine squareroot transformation of the data and two-way t-tests. Asterisks indicate a significant difference from controls at a P=0.05 significance level.

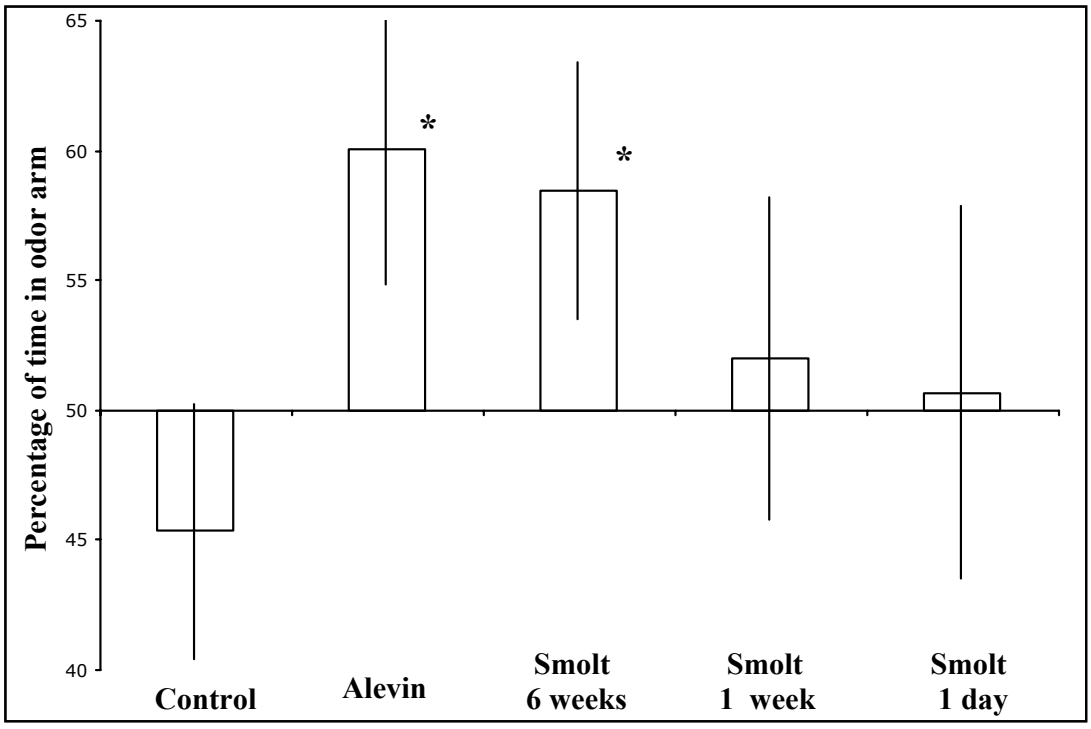
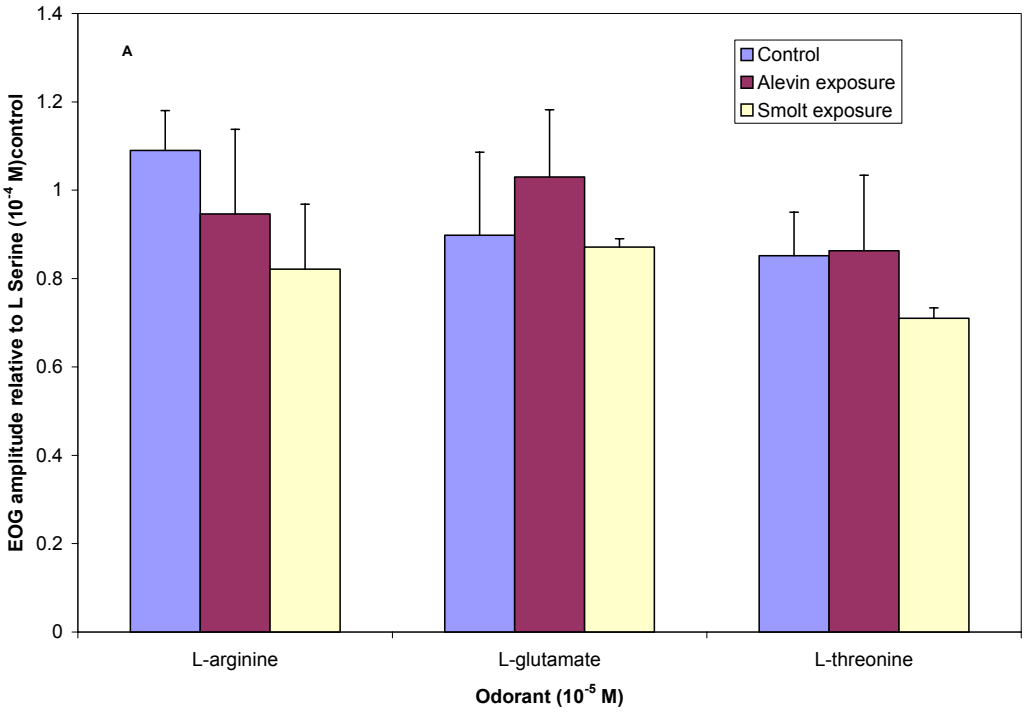


Figure 5. Behavioral responses of maturing sockeye salmon to imprinting odors. Data are mean percentage time spent in the arm scented with odorants relative to the total time spent in both arms during each time period. Fish that entered neither arm during this period were excluded from the analysis. Differences between the odor-exposed and control treatments were assessed by arcsine squareroot transformation of the data and two-way t tests. Asterisks indicate a significant difference from controls at a P=0.05 significance level.



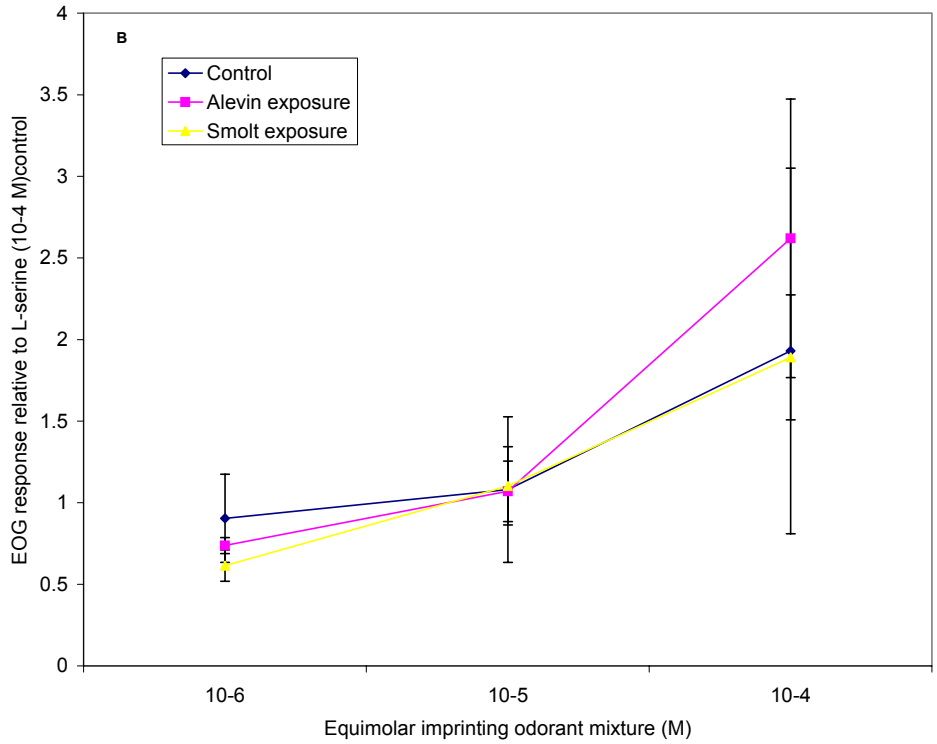


Figure 6. EOG sensitivity of mature sockeye to individual odorants (A); and the imprinting odor mixture (PEA, L-arginine, L-glutamate, L threonine) at increasing concentrations. Responses are expressed relative to peak responses elicited by a standard odorant (10^{-4} M L-Serine).

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OBJECTIVE 3 - IMPROVE PHYSIOLOGICAL DEVELOPMENT AND MATURATION

TASK 3a. THE EFFECTS OF GROWTH RATE/SIZE ON THE INCIDENCE OF EARLY MALE MATURITY AND ADULT QUALITY IN SPRING CHINOOK SALMON (*Oncorhynchus tshawytscha)*

by

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Introduction

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

Currently, early male maturity is being encountered in several Snake River Chinook salmon stocks presently in captive broodstock programs, however it is not unique to either Chinook salmon or captive broodstock programs. Recently, Larsen et al. 2004 documented greater than 40% age-2 maturation of Yakima River spring Chinook salmon males that were reared in a supplementation hatchery. The incidence of early male maturity has increased as the average size of smolts has increased in the Redfish Lake sockeye salmon captive broodstock program (W.T. Fairgrieve, PSMFC, and P. Kline, IDFG, personal communications); however, it appears to be a lesser problem than that of Chinook salmon programs. Therefore, methods to control age of maturity and minimize asynchronous maturation of males and females are needed for captive broodstock

programs for threatened spring Chinook and sockeye salmon, as well as for supplementation hatcheries.

The development of methods to control age of maturity in captive fish requires a fundamental understanding of mechanisms controlling onset of puberty. Extensive work in Atlantic and Chinook salmon, and rainbow trout indicate that both genetic and environmental factors control age of maturity. While water temperature and photoperiod clearly affect age and seasonal timing of spawning, both growth rate and body composition have also been shown to affect age of male maturity in salmonids (e.g. Rowe and Thorpe 1990a, 1990b, Rowe et al. 1991, Silverstein et al. 1998, Shearer and Swanson 2000). Because genetic selection should be minimized in a captive broodstock program for depleted stocks, rearing strategies that minimize expression of the trait are needed. Therefore the approach taken in the present research is to identify systematically factors that influence age of maturation and to determine seasonal periods when maturation is initiated. This information is then being used to develop diets and growth regimes that allow for better control of the age of maturity, provide sufficient stored energy for appropriate life-cycle transitions, support development of gametes in adult fish, and achieve target body size for release as adults.

Results from previously funded work demonstrated that both body fat and size affected the age of male maturity in male spring Chinook salmon (Silverstein et al. 1998, Shearer and Swanson 2000). But, body size or growth rate was the predominate factor. Body adiposity influenced the rate of maturation only in small fish. Subsequently, two studies were conducted to determine the threshold of size or growth rate that influenced the onset of male maturation. In the first study (Shearer et al. 2000), fish were fed high protein, low fat diets at graded ration levels. Fish size during the first autumn ranged from 50 - 100 g, and 65 - 90% of the males matured one year later (age 2). It was concluded that even though size or growth rate clearly influenced the rate of male maturation at age-2, the threshold had been exceeded in all groups. In a later study (Shearer et al. 2001, 2002), fish were reared on graded rations of a commercially available diet. The feeding regime targeted a much lower body size for the first year of rearing than the first study. The combined results are shown in Table 1. For Chinook salmon, the combined data suggest that a growth trajectory that produces a fish of 10 g body weight in the first year of rearing should be used to substantially reduce maturation in the subsequent year (age 2). Interestingly, this target size is similar to that of juvenile wild Yakima River spring Chinook salmon, which exhibit lower rates of early male maturation than hatchery-reared fish of the same stock (D. Larsen, NWFSC, personal communication).

Several questions remain to be addressed and were the subject of the present study. First, what are the critical periods when accelerated growth triggers the onset of maturation? In the our previous research with spring Chinook salmon, fish were reared on fairly constant growth rates throughout the two years of rearing. Thus, it is not known when growth can be accelerated without triggering maturation. As some captive rearing programs have been unable to achieve wild size adults, it is imperative to know when growth can be accelerated to increase body mass, and not trigger reproductive maturation. Second, are these data on the relationship between growth and male maturation more generally

applicable to other stocks and species of Pacific salmon? Willamette River spring Chinook salmon have been used for most of our research on growth and male maturation. It is possible that specific thresholds for size or growth rate may vary between stocks and species. Third, what are the effects of the growth manipulations on female maturation? If these growth manipulations are applied in captive broodstock programs or hatchery programs to reduced age-1 maturation, it is critical to determine the potential impacts on females. The present study was designed to address these questions and to develop a better understanding of when puberty is initiated in spring Chinook salmon.

Materials, Methods, and Description of Study Area

Experimental Design and Rearing Regime

This experiment was initiated in July 2002. The hypothesis tested was that increased growth after the spring equinox does not induce maturation in the subsequent autumn. It was speculated that a critical period exists during the autumn/winter when attainment of a threshold growth rate (or size) will initiate maturation for the following year. To determine if there are stock differences in the effects of growth on male maturation, the hypothesis was tested using Willamette River (WR) spring Chinook salmon together with two parallel treatment groups of Rapid River (RR) spring Chinook salmon. Hatchery facilities and numbers of RR fry available for this study were inadequate to compare maturation rates in all treatments. Thus, only two treatments were tested in the RR stock. At the end of the experiment in September 2003, the proportion of males maturing at age-2 was determined in all groups. Plasma, pituitary glands, and gonads were collected from subsamples of fish in all treatments at bimonthly intervals during the experiment to determine the progression of gametogenesis in relation to body growth. This allowed for testing a second null hypothesis; the rate of gonad growth and development is not altered by body growth.

In autumn 2001, eggs from WR and RR spring Chinook salmon were obtained and incubated at the Montlake Laboratory. In February 2002, fry were ponded into tanks containing re-circulated freshwater and fed a commercial feed (BioOregon starter). In June 2002, fish were split into treatment groups and fed a commercial grower diet (BioOregon) at one of eight growth regimes. Each growth regime consists of a combination of two growth rates, namely T10, with target body weight of 10g and T70 with target body weight of 70g in December 2002. The timing of the shift from T10 to T70 is varied by treatment group to encompass the hypothetical sensitive window (September-December) for initiation of maturation for the subsequent fall. Previous studies have indicated that the period when meiosis is evident throughout the testis of maturing fish occurs between April and May, indicating that the fish are committed to maturation the subsequent fall. It is speculated that increased growth during or after this spring period will not initiate maturation for the fall. The design of the growth manipulations during the first two years of rearing is represented in Table 2 and Figure 1.

WR fish were divided into 8 treatments (WRA-H; 2 tanks per treatment) and RR fish were divided into 2 treatments (RRA and RRH; 2 tanks per treatment). All fish were reared on the lowest growth rate from first feeding (February 2002) through June 2002. Using the bioenergetics model of Cho (1992), a daily ration was calculated to produce fish (WRH and RRH groups) of 10 g on 1 December 2002. The model requires inputs for: rearing temperature, the thermal growth coefficient (TGC of 44×10^{-5}) daily energy gain as a percent of the dietary energy fed (25%), heat increment of feeding (0.6) and energy digestibility (90%). These values were based on previous experience with this species and this diet at our facility. Two groups, WRH and RRH, were maintained on T10 ration until the end of the experiment in September 2003. Fish that were placed on an accelerated growth rate in July 2002 (WRA and RRA groups) were fed a ration that was based on a TGC of 126×10^{-5}. This was designed to produce fish with average body weight of 70g on 1 December. Subsequent accelerated groups were pair fed to the amount of feed given to this WRA fish when they were of a similar size. At two-month intervals, the ration of additional groups, WRB-WRG, was increased to produce a T70 growth rate. Ration adjustments up to T70 were done on 21 September (WRB), 21 November (WRC), 2002; 21 January (WRD), 21 March (WRE), 21 May (WRF), and 21 July (WRG), 2003.

Sampling of fish and tissues

From July 2002 onward, sample weights were collected bimonthly to monitor growth and adjust ration accordingly. During the two month intervals, samples of 20 WR fish per tank were collected for proximate composition, gonad histology, and plasma levels of 11-ketotestosterone (11KT) in males only, estradiol-17beta (E2) in females only, and plasma insulin-like growth factor I (IGF I) in all fish and follicle stimulating hormone (FSH) in pooled samples from fish of the same sex and stage fish. The intermediate sample collection was necessary to assess the progress of maturation, and determine the reproductive state on nonmaturing fish at the end of the experimental period. The reproductive status of fish that did not mature at age-2 will aid in determining proportions of fish that may mature at age-3. Novel data will be collected on the relationship between these endocrine parameters and ovarian development, and the effects of body growth on oocyte development at very early stages of oogenesis when egg number is likely being adjusted. See below for details on sample collection.

At the end of the experiment (September 2003), 160 fish per tank were examined for sex and maturity. Because of limited numbers of the RR fish, they were not sampled until the end of the first phase of the experiment in September 2003. Extra females in the WRA, RRA, WRH, and RRH groups are being reared for experiments (Task 3b) to examine the effects of growth on reproductive investment and success (behavior, adult size, fecundity, and egg size).

Plasma collection and tissue collection

Fish were anesthetized in buffered tricaine methanesulfonate (MS-222, 0.05%) and blood was collected into 5 cc heparinzed syringes from the caudal vein using 21 gauge needles. Blood was centrifugation at 800 x g for 15 min and plasma was stored at –70 ^{o}C. Plasma will be analyzed for insulin like growth factor I (IGF I) and sex steroids in the next funding period. Pituitaries and one ovary or testis were frozen directly in liquid nitrogen and stored at –70 ^{o}C for subsequent analysis of gene transcripts during the next funding period.

Gonad morphology and histology

Gonads were removed and one ovary or testis was fixed in Bouin's fixative for 48 h at 4 ^{o}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 hematoxylin and eosin. and photographed with a digital camera. From May 2003 onwards, gonads were weighed to the nearest 0.001 g. Gonadosomatic index (GSI) was calculated as (gonad weight/somatic weight) x100.

Body composition

Carcasses with gonads removed were collected and stored at –20 ^{o}C until analyzed for fat content. Carcasses were partially thawed, ground in a food processor, dried, then reground in a coffee grinder. A subsample of ground tissue was taken for fat analyses (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. Proximate composition values are expressed on a wet weight basis.

Statistical analyses

Treatment effects on final size, body composition were performed by one-way ANOVA using tanks as the experimental unit. Proportional data (percent maturation) was normalized by arcsin transformation, followed by one-way ANOVA. Multiple comparisons were made using Fishers PLSD test. Analyses were performed using Statview 512+ software and $P<0.05$ was considered significant.

Preliminary Results and Work to Be Completed

Fish Growth and Body Composition

The patterns of body growth in weight in immature males, immature females and maturing males in the various treatment groups over time are shown in Figures 2-4. The growth of the various treatment groups generally followed the intended growth regimes. The final body weight and length in all fish are shown in Figures 5 and 6 As expected, fish size declined with duration of the acclerated growth period. Body fat levels in the

sampled fish are shown in 7-9. The lowest growth group (WRH) had the lowest body fat levels, which fluctuated around 3%, with a major decline to 1% in spring 2003. During March-May 2003, when nonmaturing fish were smolting, body fat levels tended to decline in all treatment groups. Body fat levels in fish after the growth acceleration remained between 5-9%. Thus, 2 months after growth acceleration body fat levels appear to increase two to three-fold, and then remain relatively constant except for the period of smoltification when levels tended to decline.

Maturation at age-2

The rate of maturation of males at 2 years of age in September 2003 declined with the duration of the period of accelearated growth (Fig. 10). Growth acceleration after March did not significantly alter the rate of maturation in males.

Ovary Growth

Ovary weight appeared to be positively related to body weight (Fig. 11), but GSI was significantly higher in the fish with growth acceleration after March 2003 (Fig. 12). This suggests that ovarian growth is not strictly proportional to body growth; the rate of body growth appeared to exceed that of ovarian growth in fish with growth acceleration prior to March. A further analysis of the ovary size, mean oocyte size, and oocyte stage will be required to determine the relationship between body growth and oocyte development.

Testis Development

The progression of spermatogenesis in maturing and nonmaturing males in the highest (WRA) and lowest (WRH) growth groups are shown in Figures 13-16. Although the data have not been statistically analyzed at this point, the pattern of appearance of various stages of sperm development in the nonmaturing fish appears to be similar irrespective of growth regime. It is apparent that if meiosis is not initiated by March 2003 (as evident by appearance of late Type B spermatogonia and primary spermatocytes), spermatogenesis does not proceed during that year. No fish with late Type B spermatogonia or primary spermatocytes as the most advanced germ cell stage were evident after March. It appears that the general progression of spermatogenesis in maturing fish is similar in the high (WRA) and low growth (WRH) growth groups. However, it is notable that spermatozoa were first apparent in the testes of the WRA fish in May, while in the WRH group spermatozoa were not found until July. This suggests that either the rate of spermatogenesis is higher in the fast growing fish or that the process started earlier. Further statistical analyses are needed to determine if this is a significant effect of the treatment.

Work to Be Completed

During the next funding period, the focus will be on analyzing plasma hormone levels, pituitary gonadotropin gene transcripts and a variety of gonadal gene transcripts in maturing and nonmaturing fish from the WRA and WRH groups, and from the final samples in all treatment groups. A variety of gonadal gene transcripts will be measured: FSH and LH receptors; steroidogenic acute regulatory protein (StAR), which regulates the rate limiting step in steroid biosynthesis; IGF I and IGF II; growth hormone receptor; and growth factors that are known to regulate germ cell development in mammals. The histological analysis of ovarian stages and size of oocytes will be quantified using an image analysis system. Efforts will also be directed toward completion of statistical analyses of all data and drafting final reports and manuscripts.

Summary and Conclusions

Although this task has not been completed, the results so far suggest that the highest rates of maturation occur in the groups with the longest period of accelerated growth. A significant reduction in the rate of maturation occurred when growth acceleration was delayed, particularly from March onward of the second year of rearing.

Data Management Activities

Data are collected manually by NOAA, UW, and PSMFC researchers onto preformatted data sheets or directly into Power MacIntosh G4s. Data are entered and summarized on personal computers operated by researchers using Microsoft Excel, Statview 512+, Cricket Graph 1.3.2, and Prism. Data on histology of tissues is recorded using a digital camera and stored as either TIFF or JPG files on a Power MacIntosh G4. Backup copies of data are saved on an external Lacie hard-drive and on CD-ROMs. All data are checked for quality and accuracy before analysis. Analytical processes are described in the text of the annual report.

Table 1. Relationship between body size in December (age 1+) and maturation the following September (age 2) in Willamette River spring Chinook salmon.

Body Weight (Tank mean, n=2)	% Maturation (Tank mean, n=2)	Reference
10	12.0	Shearer et al. 2001, 2002
14	19.9	Shearer et al. 2001, 2002
18	29.9	Shearer et al. 2001, 2002
21	33.8	Shearer et al. 2001, 2002
24	34.9	Shearer et al. 2001, 2002
48	66.2	Shearer et al. 2000
60	75.8	Shearer et al. 2000
76	78.9	Shearer et al. 2000
76	88.0	Shearer et al. 2000
90	92.8	Shearer et al. 2000
95	87.5	Shearer et al. 2000
108	51.0	Shearer et al. 2001, 2002
110	81.9	Shearer et al. 2000

Table 2. Growth regimes for experimental groups. Each growth regime consists of two growth rates: T10 = target size of 10 g, and T70, target size of 70 g in December 2002. Changes in growth will be made in the middle of indicated months. Willamette River (WR) and Rapid River (RR) spring Chinook salmon will be used for the experiment. The hypothetical "sensitive" period when high growth influences the onset of maturation occurs from September-December.

Stock/ Treatment	Period of low growth rate (T10)	Period of high growth rate (T70)	Predicated rate maturation of age-2 males
WRA	02/2002-07/2002	07/2002-09/2003	High
RRA	02/2002-07/2002	07/2002-09/2003	High
WRB	02/2002-09/2002	09/2002-09/2003	High
WRC	02/2002-11/2002	11/2002-09/2003	Intermediate
WRD	02/2002-01/2003	01/2003-09/2003	Intermediate
WRE	02/2002-03/2003	03/2003-09/2003	Low
WRF	02/2002-05/2003	05/2003-09/2003	Low
WRG	02/2002-07/2003	07/2003-09/2003	Low
WRH	02/2002-09/2003	None	Low
RRH	02/2002-09/2003	None	Low

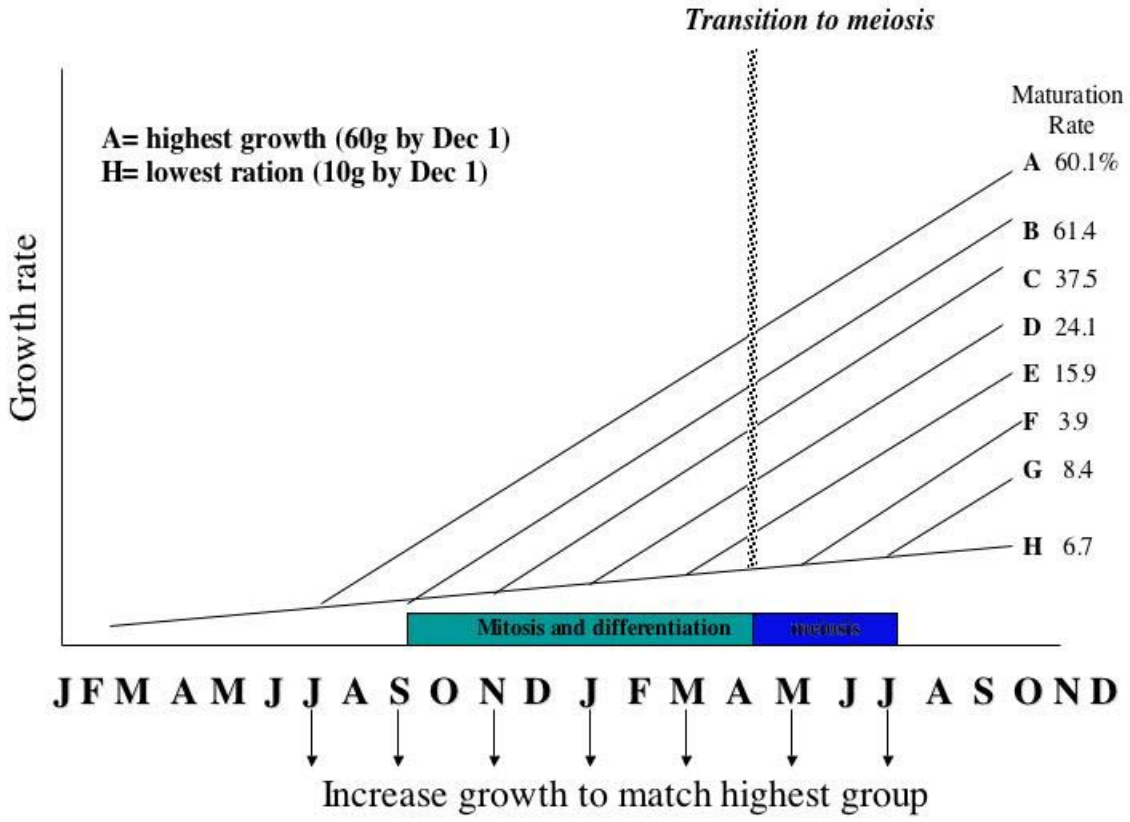

Figure 1. Schematic representation of intended growth regimes for various treatment groups. The timing of increased growth and sampling of fish is indicated by the arrow. The period when mitosis and differentiation of the spermatogonia and onset of meiosis n the testis is indicated by the green and blue bars. The transition from mitosis to meiosis is critical for the onset of puberty. The pattern of spermatogenesis was established from previously funded work on spring Chinook salmon. Maturation rates for males at the end of the experiment are indicated next to the treatment groups. These data are also shown in Figure 10.

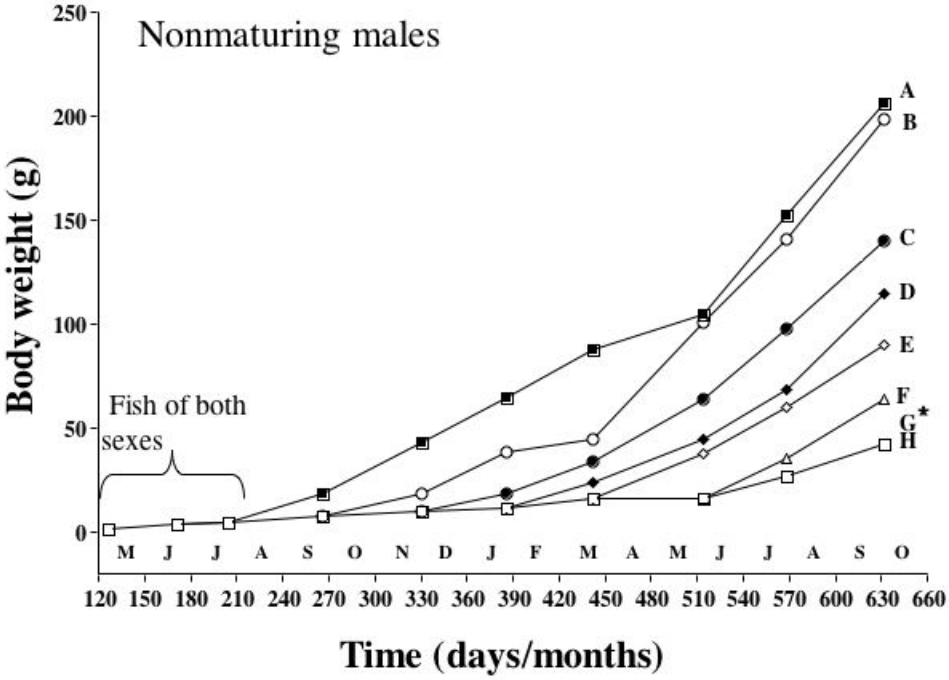

Figure 2. Body weight over time in Willamete River spring Chinook salmon nonmaturing males from various treatment groups. Data points are means of two replicate tanks. The first three data points are mean body weights for all fish, irrespective of sex.

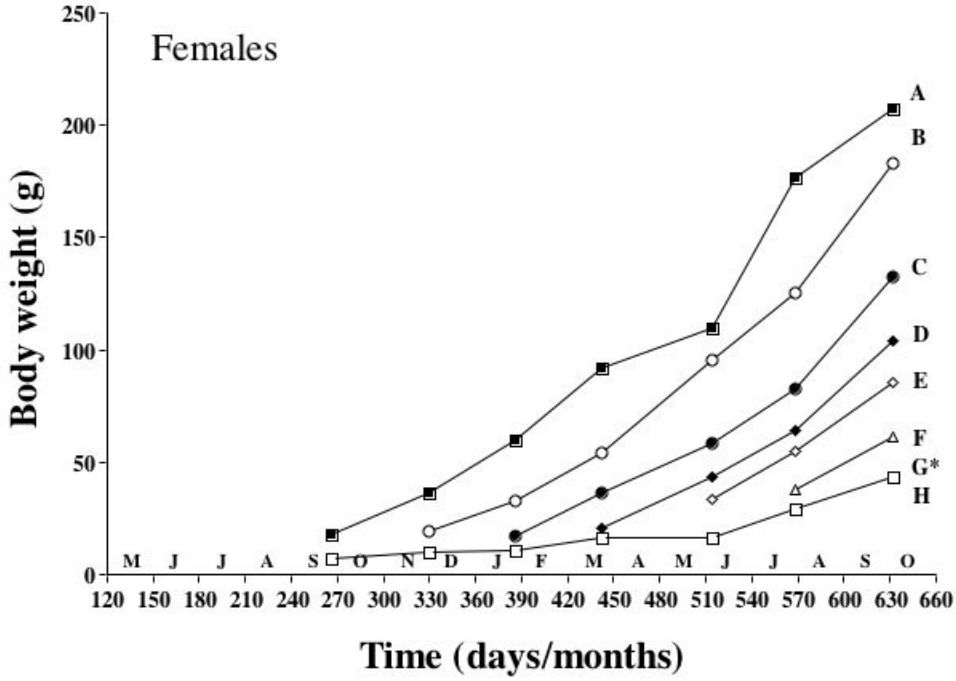

Figure 3. Body weights in Willamete River spring Chinook salmon females from various treatment groups. Data points are means of two replicate tanks.

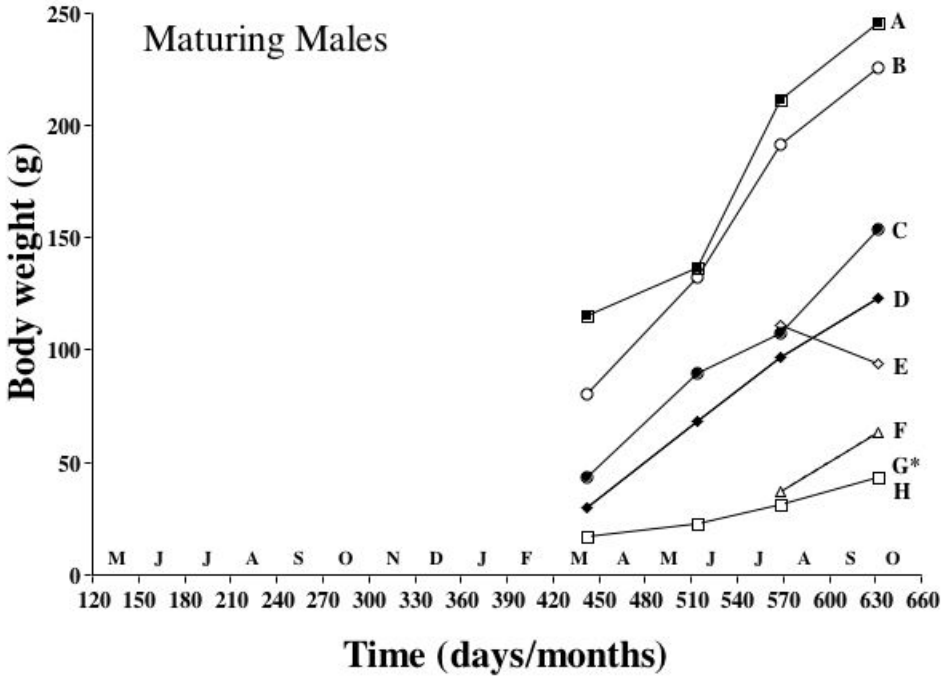

Figure 4. Body weights in Willamete River spring Chinook salmon maturing males from various treatment groups. Data are mean of two replicate tanks.

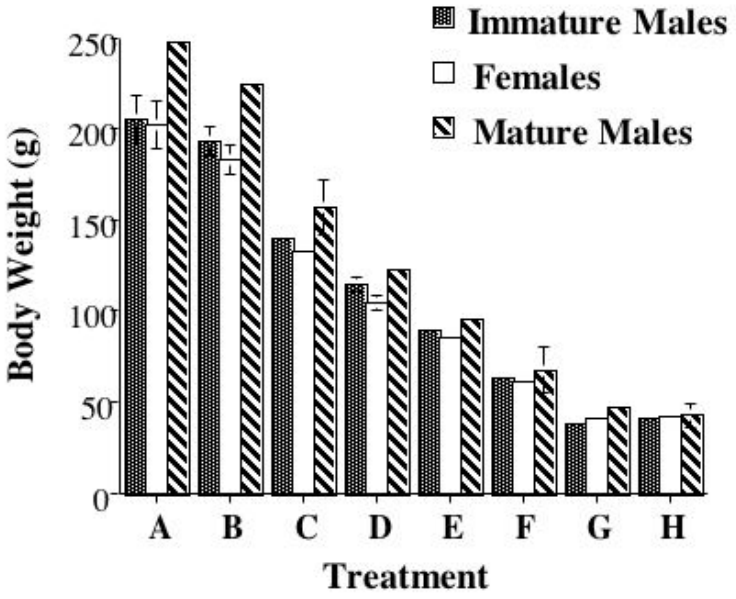

Figure 5. Final body weights of immature males, females and mature males in September 2003 when the experiment was terminated. Data are mean +/- standard error of two replicate tanks. A total of 20 fish per tank were sampled. Only data for Willamete River fish are shown.

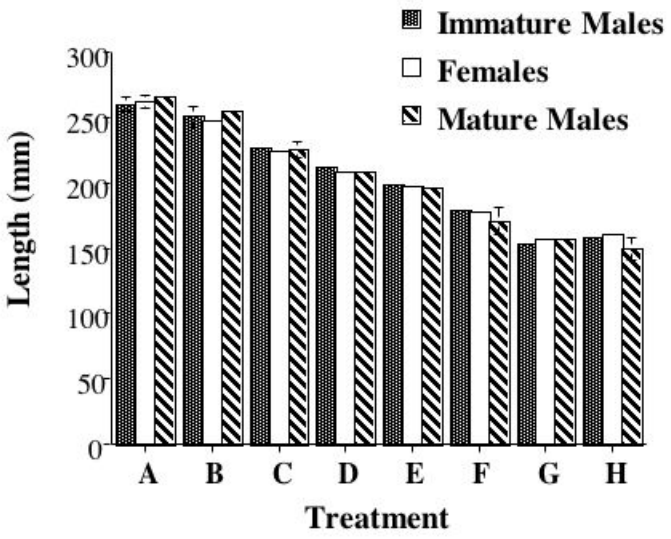

Figure 6. Final body lengths of immature males, females and mature males in September 2003 when the experiment was terminated. Data are mean +/- standard error of two replicate tanks. A total of 20 fish per tank were sampled. Only data for Willamete River fish are shown.

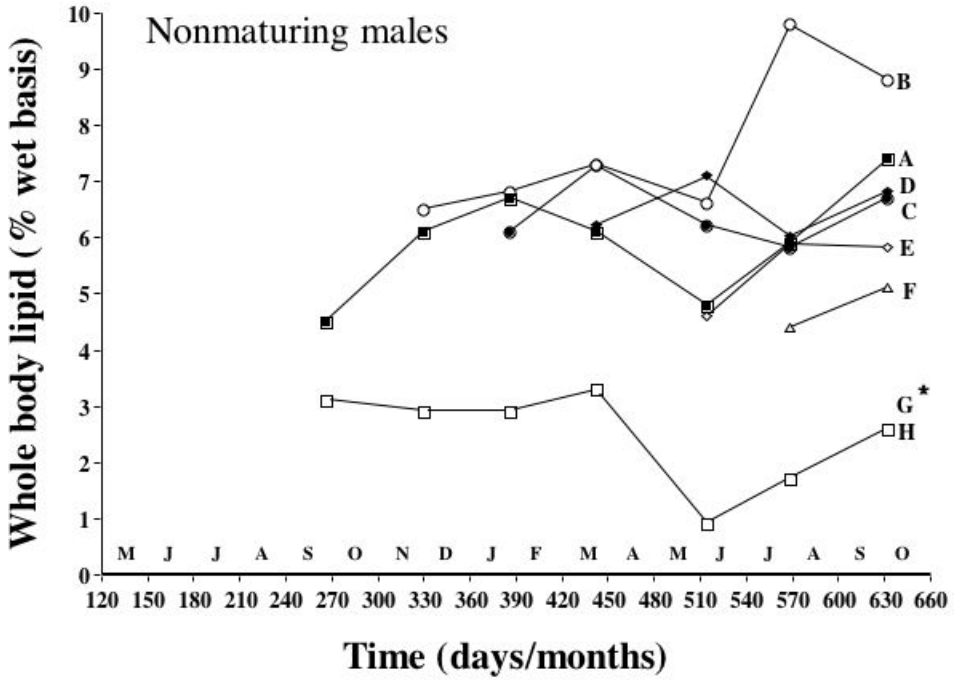

Figure 7. Whole body fat levels in Willamete River spring Chinook salmon nonmaturing males from various treatment groups. Data points are means of two replicate tanks.

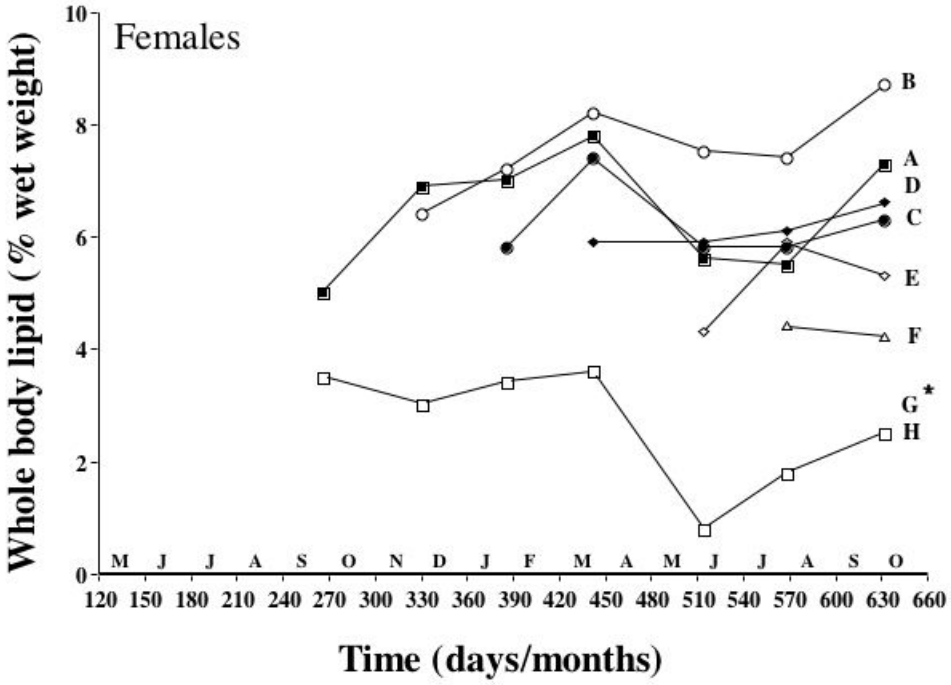

Figure 8. Whole body fat levels in Willamete River spring Chinook salmon females from various treatment groups. Data points are means of two replicate tanks.

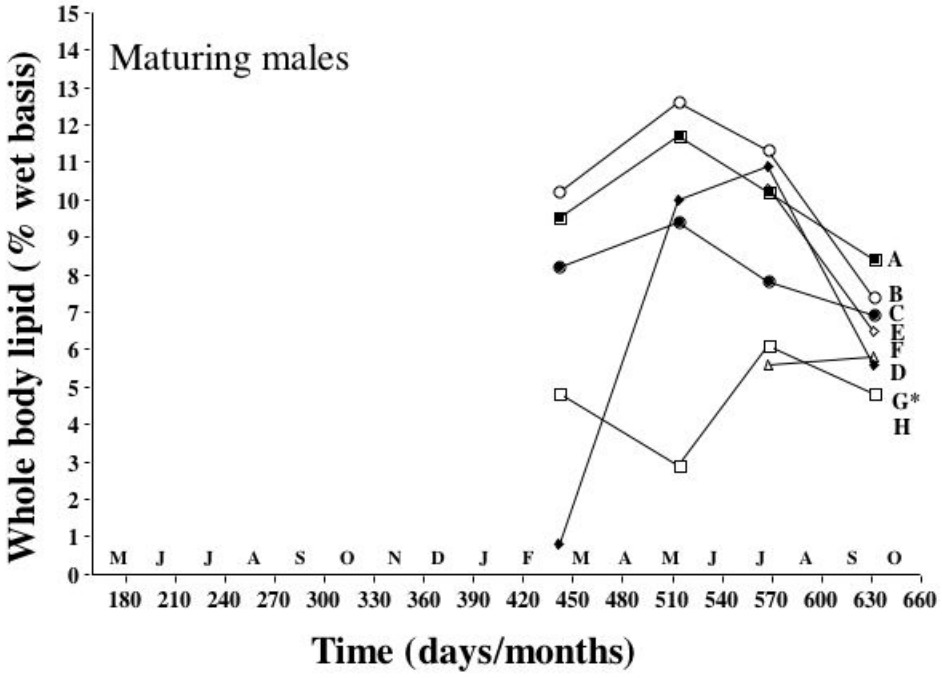

Figure 9. Whole body fat levels in Willamete River spring Chinook salmon maturing males from various treatment groups. Data are mean of two replicate tanks.

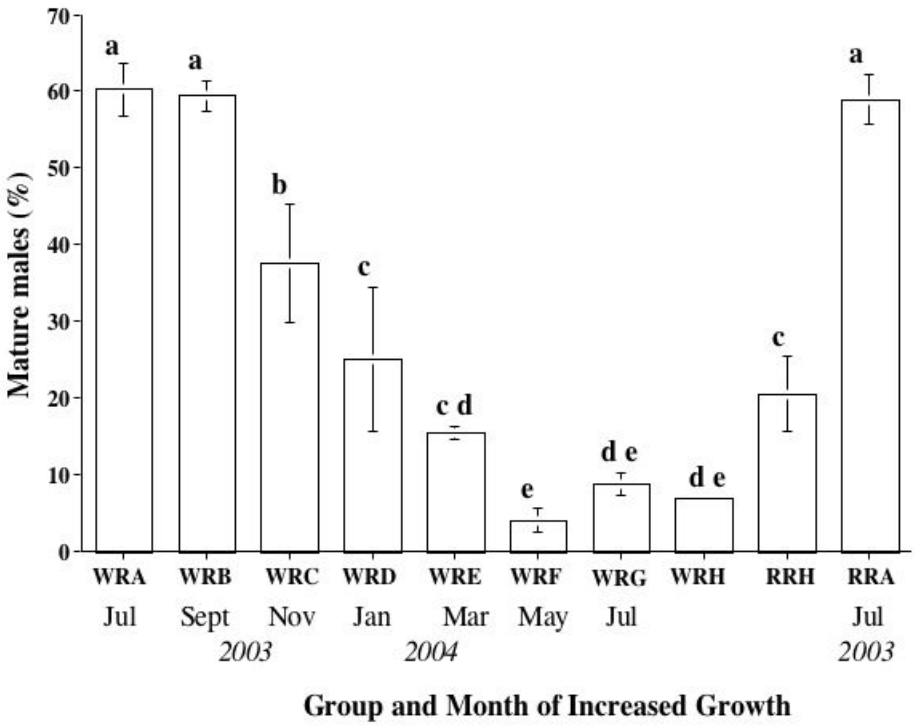

Figure 10. Percentage of 2-year old maturing males by treatment at the termination of the experiment in September 2003. WR (Willamete River), RR (Rapid River) spring Chinook salmon. Treatment groups and month of increased growth are indicated. Data are mean +/- sem of 2 replicate tanks. Significant differences ($P<0.05$) are indicated by different letters.

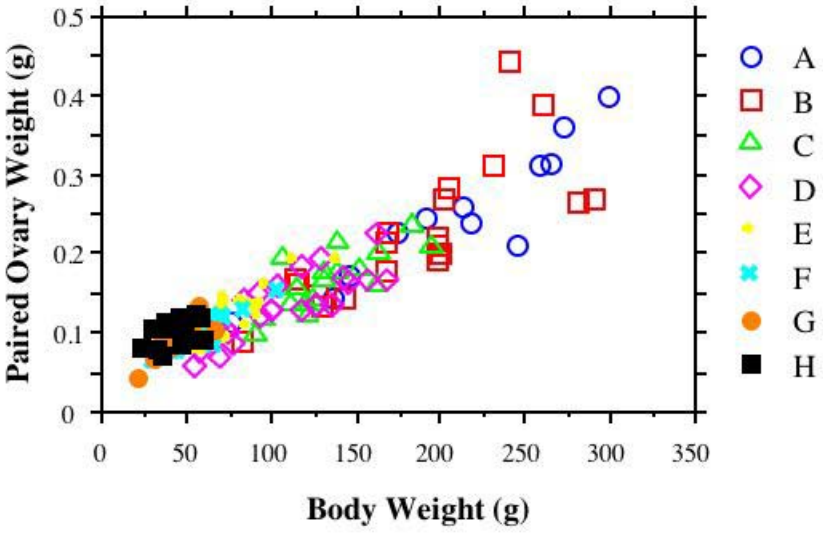

Figure 11. Ovarian and body weight of females at the termination of the experiment in September 2003.

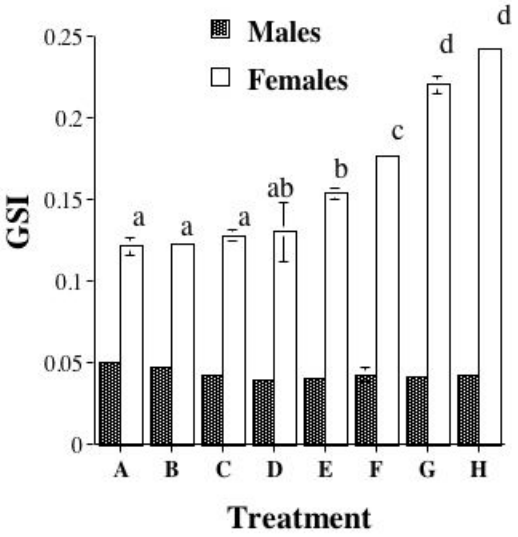

Figure 12. Gonadosomatic index(GSI) of immature males and females at the termination of the experiment in September 2003. Data are mean +/- SEM of N=2 tanks.

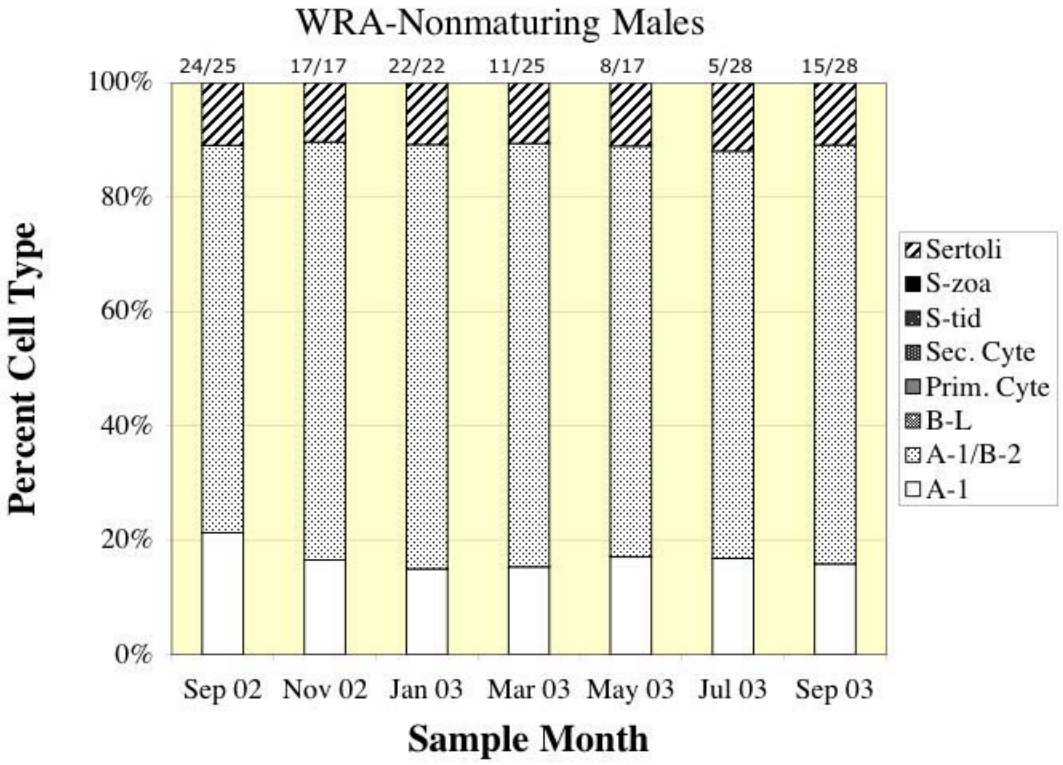

Figure 13. Percentage of various cell types within the testes of nonmaturing males from the WRA group (highest ration group) from September 2002 to September 2003. Sertoli, S-zoa (spermatozoa), S-tid (spermatid), Sec.Cyte (secondary spermatocyte), Prim. Cyte (primary spermatocyte), B-L (late B spermatogonia), A-1/B-2 (intermediate A and early B spermatogonia), A-1 (primary A spermatogonia). Numbers at the top of the columns indicate numbers of nonmaturing males out of total number of males examined at that month. Nonmaturing males were those without late B spermatogonia, which indicates the initiation of meiosis had not occurred in the germ cells within the testis.

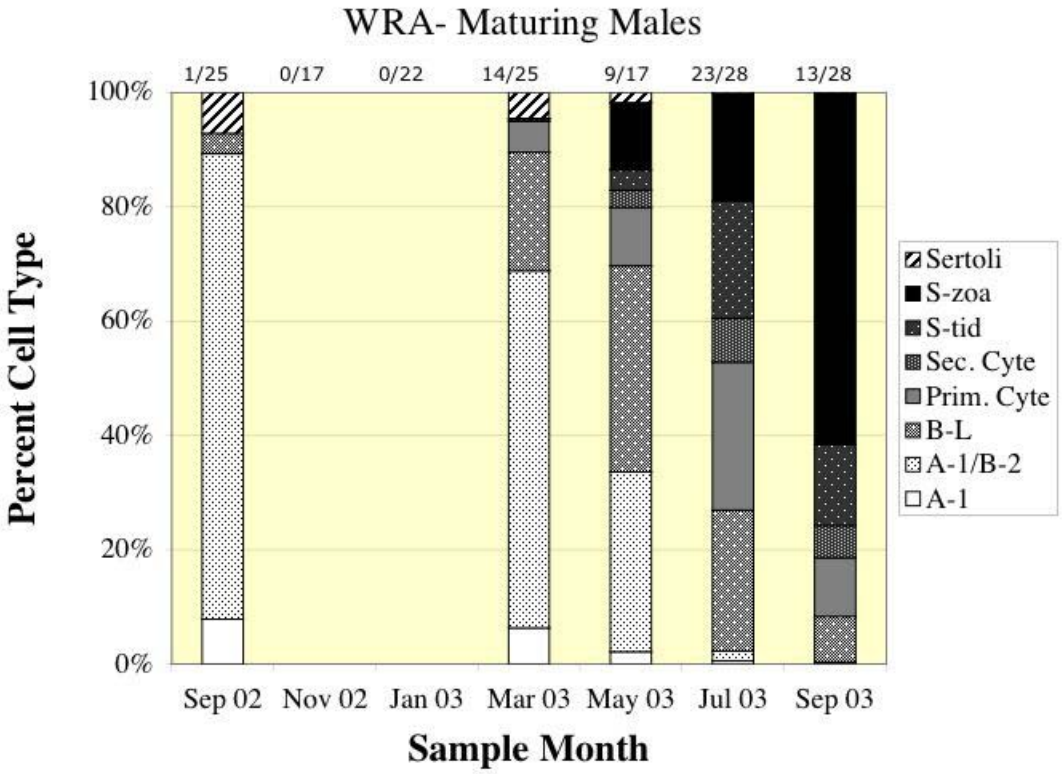

Figure 14. Percentage of various cell types within the testes of maturing males from the WRA group (highest ration group) from September 2002 to September 2003. Sertoli, S-zoa (spermatozoa), S-tid (spermatid), Sec.Cyte (secondary spermatocyte), Prim. Cyte (primary spermatocyte), B-L (late B spermatogonia), A-1/B-2 (intermediate A and early B spermatogonia), A-1 (primary A spermatogonia). Numbers at the top of the columns indicate numbers of maturing males out of total number of males examined at that month.

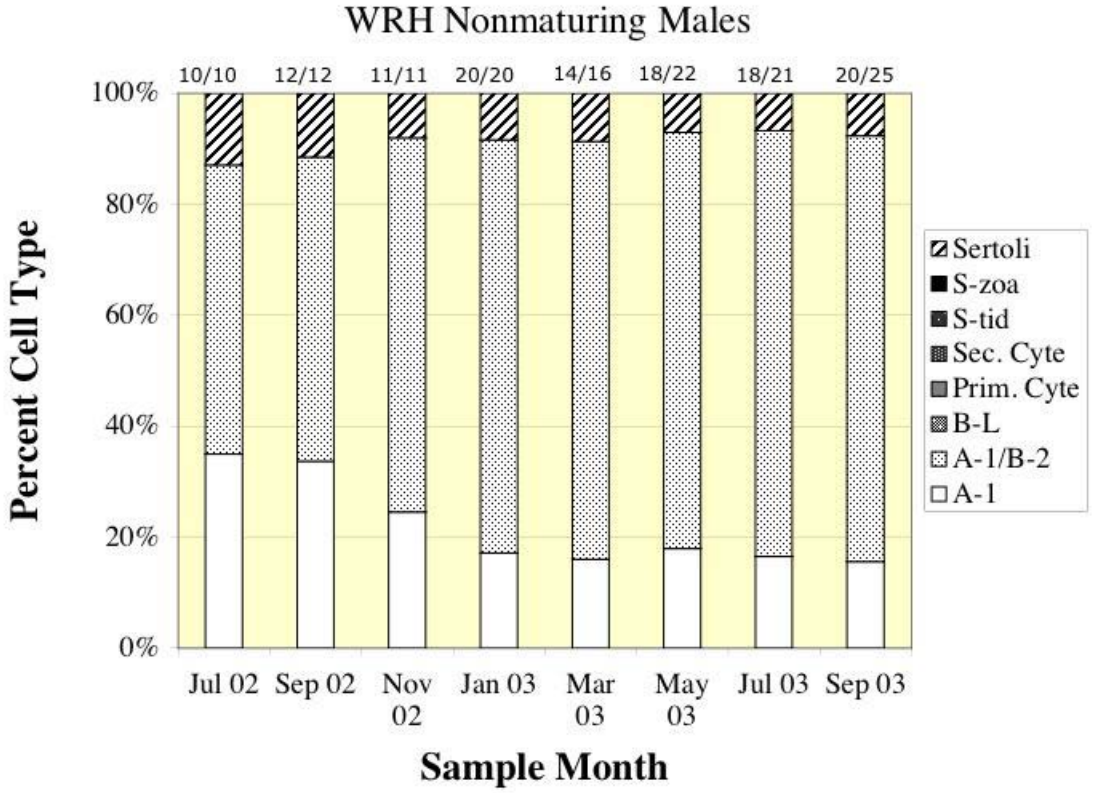

Figure 15. Percentage of various cell types within the testes of nonmaturing males from the WRH group (lowest ration group) from September 2002 to September 2003. Sertoli, S-zoa (spermatozoa), S-tid (spermatid), Sec.Cyte (secondary spermatocyte), Prim. Cyte (primary spermatocyte), B-L (late B spermatogonia), A-1/B-2 (intermediate A and early B spermatogonia), A-1 (primary A spermatogonia). Numbers at the top of the columns indicate numbers of nonmaturing males out of total number of males examined at that month. Nonmaturing males were those without late B spermatogonia, which indicates the initiation of meiosis had not occurred in the germ cells within the testis.

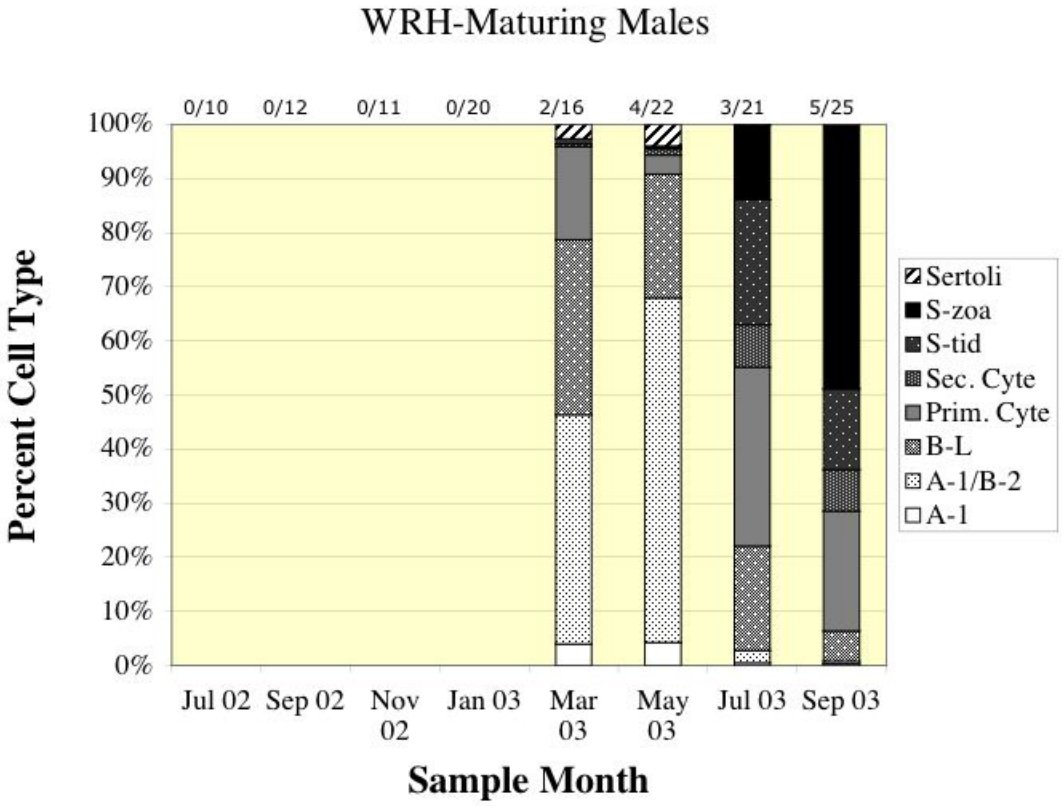

Figure 16. Percentage of various cell types within the testes of maturing males from the WRH group (lowest ration group) from September 2002 to September 2003. Sertoli, S-zoa (spermatozoa), S-tid (spermatid), Sec.Cyte (secondary spermatocyte), Prim. Cyte (primary spermatocyte), B-L (late B spermatogonia), A-1/B-2 (intermediate A and early B spermatogonia), A-1 (primary A spermatogonia). Numbers at the top of the columns indicate numbers of maturing males out of total number of males examined at that month.

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OBJECTIVE 3 - IMPROVE PHYSIOLOGICAL DEVELOPMENT AND MATURATION

TASK 3b. DETERMINE THE EFFECTS OF GROWTH ON MATURATION TIMING, OOCYTE NUMBER AND OOCYTE SIZE, IN SPRING CHINOOK SALMON

by

Briony Campbell, Nicholas Hodges, Jon Dickey, Brad Gadberry, and Penny Swanson

Introduction

Both the Redfish Lake sockeye salmon and Snake River spring Chinook salmon captive broodstock programs have encountered problems with highly variable egg production and quality, with females displaying a high rate of egg retention and abnormal ovarian development leading to reduced egg size and number. These problems could be a result of rearing practices that alter growth and development of ovaries, and cause abnormal timing of oocyte maturation. However, very little is presently known about the mechanisms whereby the systems within the animal controlling body growth also affect gonad growth.

In many stocks of salmon, individuals within a population can follow different developmental pathways, ultimately maturing at different sizes and ages with accompanying alterations in energy allocation to egg number and egg size (see Groot and Margolis 1991). There is accumulating evidence to suggest that growth during various life stages affects the partitioning of resources available for reproduction in the form of a trade-off between final egg size and egg number. 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, Lobon-Cervia et al. 1997, Jonsson et al. 1996, Morita et al. 1999, Tamate and Maekawa 2000). Therefore, growth history has a pivotal role in controlling the initiation and maintenance of maturation in salmonids.

In an artificial rearing environment it is possible that abnormal ovarian development may result from inappropriate growth during periods when adjustments in reproductive investment are being made. In general, there is an emphasis on obtaining a spawning size for captive fish that is equivalent to individuals within the wild population. Rearing regimes have focused on feeding fish high quality broodstock diets during the year of expected maturity. Far less consideration is given to the effect of rearing practices at earlier life history stages on the subsequent reproductive performance of adults 1-3 years later. In semelparous species, determining the seasonal timing and degree of growth that

influences the physiological commitment of oocytes to mature is crucial, as there is no second opportunity once this commitment is made.

In this task, one aim is to determine the effects of juvenile and adult growth on ovarian development. An important question to answer is whether fish can be grown on a restricted ration during juvenile rearing in fresh water to prevent males maturing early at 2 years of age without serious consequences to female maturation in subsequent years. In other words, if growth is accelerated in small smolts, does the ovary develop more rapidly than that of slow growing smolts and does ovarian development 'catch up' to that of the large smolts?. In this study, fish are reared on relatively constant water temperature and growth is manipulated by altering ration.

A second aim of this study is to gain more basic information on timing of ovarian growth during the spring Chinook salmon lifecycle to understand when critical stages of ovarian development (e.g. primary and early secondary oocyte growth) occur. Results from this study will aid in understanding how environmental conditions (either in the hatchery or ocean environment) may affect physiological processes that ultimately affect age of maturity, fecundity, and egg size.

Materials, Methods, and Description of Study Area

Experimental Design

Willamete River (WR) spring Chinook salmon smolts produced under Task 3a are being used for this study, specifically females from the WR-A (High) and WR-H (Low) groups. Fish from the WRA group have been individually tagged and sexed using a genetic marker for Y-chromosome (Du et al. 1993). Tagging of this group will allow for collection of data on growth history and ovary growth, oocyte size, and oocyte number. The WR-A group consists of large smolts on a high ration and the WR-H group consists of small smolts on a low ration that had average body sizes of 200 g and 43 g, respectively, in October 2003. Smolts from the WR-A and WR-H groups have been divided into either a High or Low ration group. Thus, in October 2003 we established four replicated treatments with differing growth conditions: Low to Low (LL), High to Low (HL), Low to High (LH), and High to High (HH), A graphical representation of the experimental design is shown in Figure 1. Fish are being reared in the recirculated freshwater system at the NWFSC Montlake hatchery and fed BioOregon BroodSelect.

It is anticipated that most fish will spawn during September 2005, and the sampling times in March 2004 and September 2004 will coincide with important stages of ovarian development. To date a total of 60 females (30 per tank) from each of 4 treatment groups have been sampled during March 2004. Fish have been sampled for body weight and length, and ovarian weight. Samples of blood plasma, pituitaries, ovary tissue and bodies have been collected for subsequent analysis detailed below. The last sampling of remaining fish will occur in September 2004.

Preliminary Results and Work to Be Completed

Body and ovary growth

The growth of fish in the experimental treatments up to March 2004 is shown in Figure 2. The different ration groups have separated as expected and are following the projected growth trajectories. The relationship between body size (as length) and ovary weight in all the females sampled in March 2004 is shown in Figure 3. It is clear that at an approximate body size of 300g many individuals have ovaries that are entering an accelerated growth period relative to general body growth as indicated by the change in slope of the ovary growth curve. Preliminary histological analyses from a few individuals suggest the ovaries in the growth-accelerated fish contain oocytes that are entering the cortical alveoli stage of development. This is the stage when the oocytes begin to accumulate mucopolysaccharides and glycoproteins in the ooplasm (Nagahama 1983). The origin and function of this material is not clear although some are apparently destined to form egg-shell proteins. Previous work in coho salmon has suggested that follicle-stimulating hormone (FSH) and steroids control this process (Campbell et al. 2003). We hope to confirm these findings in Chinook salmon and identify other factors that are crucial to this important stage in oocyte development.

Work to be completed

Estimation of oocyte number and size: We have developed a new method for assessing the number and size distribution of oocytes in the immature and maturing ovary. This technique uses an initial Trypsin treatment (1% for 4 h), followed by vigorous shaking and storage in 4% buffered formalin. This separates and preserves all the oocytes present including small immature oocytes that would be lost with digestion of tissue using Gilson's solution. Photographs of these dispersed oocytes will be taken and the number and size distribution of oocytes in the ovaries estimated by image analyses. This analyzes is based on that described for cod ovaries by Thorsen et al. 2001 and will be validated during the summer of 2004.

Oocyte development stage: Gonad pieces were fixed in Bouin's and stored in 70% ethanol. These will be processed in paraffin. During previous funding periods a technique has been developed which produces reliable results for staging early vitellogenic oocytes. Immunocytochemistry for proteins involved in the S-phase of DNA synthesis will determine if any proliferation of germ cells or somatic cells occurs, and is related to growth treatments.

Quantification of atresia: Pieces of ovarian tissue collected in March 2004 were fixed in paraformaldehyde and processed in paraffin. They will be stained for apoptotic cells by DNA fragmentation staining (Tunel method). This will allow for quantification of the

amount of atresia and its relationship to body growth. Similar samples will be collected from remaining fish in September 2004.

Measurement of reproductive hormones and steroidogenic enzymes: Plasma FSH, luteinizing hormone (LH), plasma 17ß-estradiol, testosterone, insulin-like growth factor I (IGF-I) will be measured to relate hormone levels to ovarian stage, growth rate, and body size. Levels of transcripts for steroidogenic enzymes, FSH receptor, IGF-I, and IGF-II will be measured in pieces of frozen tissue by real-time PCR and related to ovarian stage, body size, and growth regime (treatment).

Body composition: The effects of growth regime on body composition, and subsequent effects on ovarian growth will be determined. Carcass, ovary and vicera samples collected in March and September 2004 will be analyzed for fat content.

During FY04 the predominant activities will be rearing fish in their respective treatment groups, periodic measurements of body size, collection of the final set of terminal samples in September 2004, validation of methods, and initiation of sample analyses. Analyses of data and samples will continue through FY05.

Summary and Conclusions

Not applicable at this point of the study.

Data Management Activities

Data are collected manually by NOAA, UW, and PSMFC researchers onto preformatted data sheets or directly into a Sony Viao, IBM Thinkpad, or Power MacIntosh G4s. Data are entered and summarized on personal computers operated by researchers using Microsoft Excel, Statview 512+, Cricket Graph 1.3.2, and Prism. Data on histology of tissues is recorded using a digital camera and stored as either TIFF or JPG files on a Power MacIntosh G4. Backup copies of data are saved on an external Lacie hard-drive and on CD-ROMs. All data are checked for quality and accuracy before analysis. Analytical processes are described in the text of the annual report.

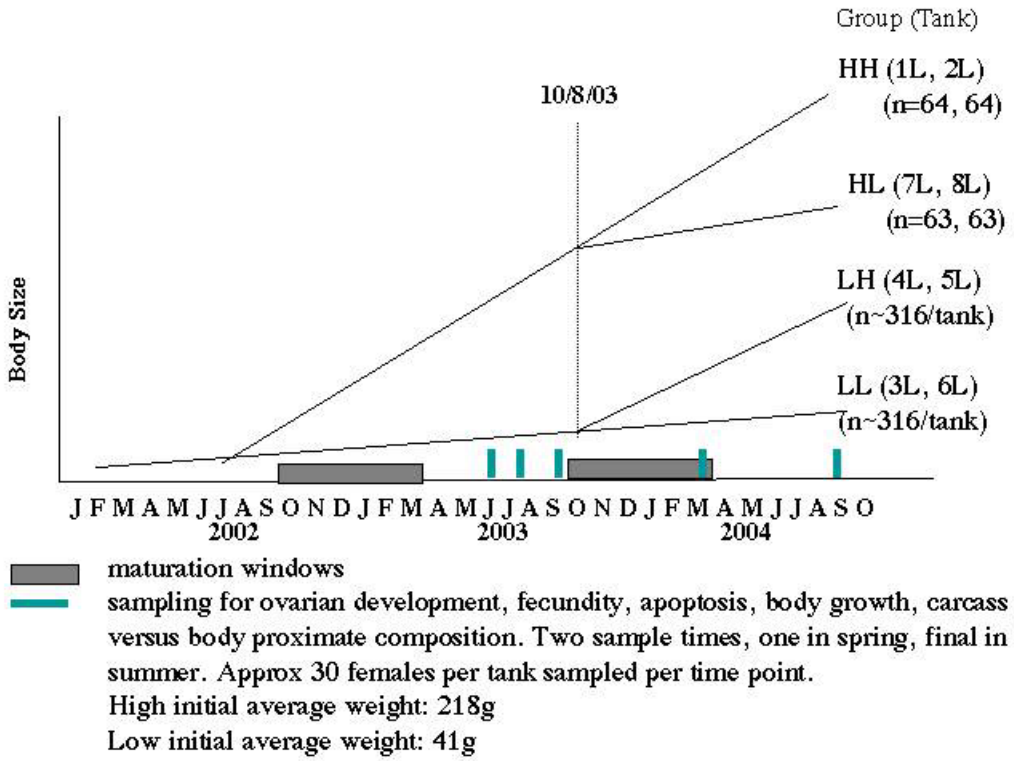

Figure 1. Graphic representation of experimental design and growth regimes for the growth experiment described in Task 3b using brood-year 2000 WR spring Chinook salmon. The lines represent theoretical growth patterns, and are shown to represent graphically the intended target sizes, and when growth will be altered in various groups. Vertical bars indicate the timing of sampling for ovarian stage.

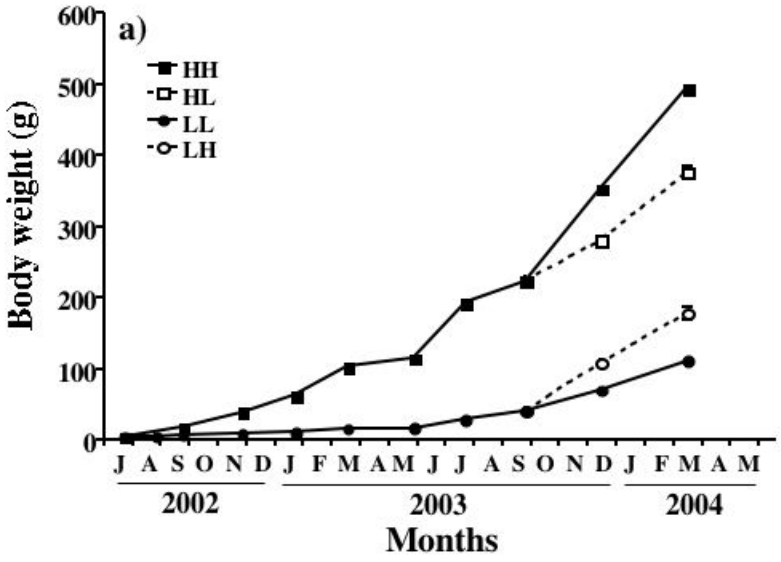

Figure 2. Change in body weight (g) for the four ration (growth) groups; high to high (HH), high to low (HL), low to low (LL) and low to high (LH). Data are means±sem (n=2 tanks).

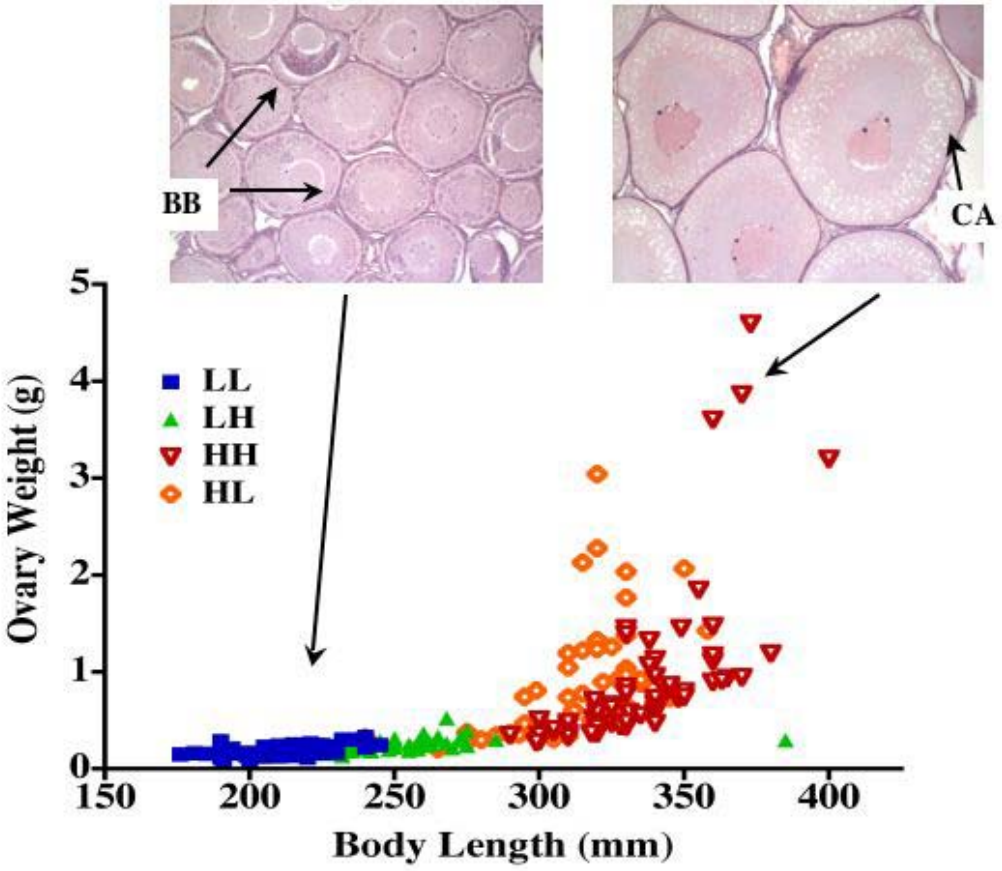

Figure 3. Plot of body length versus ovary weight for all the female Chinook salmon sampled in March 2004. The ration treatment groups are indicated by different symbols. Top-left photograph shows oocytes at the Balbiani Body (BB) stage of development seen in ovaries from the smaller females. Top-right photograph shows oocytes at the Cortical Alveoli (CA) stage of development seen in ovaries from the larger females.

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OBJECTIVE 3 - IMPROVE PHYSIOLOGICAL DEVELOPMENT AND MATURATION

TASK 3c. EFFECTS OF WATER TEMPERATURE DURING SEAWATER REARING ON TIMING OF SPAWNING AND EGG QUALITY IN SPRING CHINOOK SALMON

By

Briony Campbell, Nicholas Hodges, Jon Dickey, Rob Endicott, Barry Berejikian, and Penny Swanson

Introduction

In captive broodstock programs for Snake River spring Chinook salmon, there are substantial problems with delayed spawning time and poor egg quality relative to their wild counterparts (Venditti et al. 2003, Swanson et al. 2003). The degree of the delay in spawning time relative to wild fish ranges from 2-5 weeks depending on stock and year class. It is not clear whether the delay in spawning time and the reduction in egg fertility stem from the same physiological problem. However, the frequent occurrence of polarized over-ripe eggs in artificially stripped female spring Chinook salmon suggests that the processes controlling the timing of final oocyte maturation and ovulation are not functioning normally in some of these animals. The complete ovulation of fully mature eggs is essential for the efficient collection of good quality eggs timely (Springate et al. 1984). In addition, for broodstock programs that require mating of captive and wild fish, it is essential that the timing of spawning in captive adults coincide with that of the wild population. Therefore, improving the timing and completeness of ovulation in captive broodstock is important to increase the efficiency of the recovery programs.

The timing of spawning can be controlled with hormone treatments or by manipulating environmental factors. Implants containing hormone analogues, such as gonadotropin-releasing hormone (GnRH, Swanson 1995) are used in commercial aquaculture and captive broodstock programs to synchronize and advance spawning of fish in captivity on an 'as needed basis'. However, use of this drug is not permitted for released fish. Thus, for captive rearing programs that release fish to spawn in their native habitat, alternative methods for advancing spawning are needed to ensure that captive fish spawn at the same time as their wild counterparts.

It is well known that the seasonal timing of spawning in temperate zone fishes is primarily regulated by photoperiod and temperature (Bromage 1995, Bromage et al. 1993, Bromage et al. 2001). Manipulation of the prevailing photoperiod has been successfully used to alter the time of spawning in a number of salmon, including coho salmon (unpublished, B. Campbell, Univ. Washington, Seattle, WA) and Sacramento River winter run Chinook salmon (personal communication, K. Arkoosh, Bodega Marine Laboratory, University of California, Davis, CA). However, captive broodstock

programs often have year classes which are a mixture of maturing and non-maturing fish, and it is not clear how photoperiod regimes designed to advance the spawn time of the maturing fish would affect maturation of the non-maturing fish in following years. Once technologies, such as ultrasound, are developed to reliably separate maturing and non-maturing fish early in the year, photoperiod treatment will become a powerful tool for the synchronization and advancement of spawning in captive broodstock. However, alternative methods should be explored to provide broodstock program with options for controlling spawn timing and improve egg quality.

There is growing evidence that rearing temperatures can also alter the timing of spawning of salmonids and affect gamete quality (Henderson 1963, Goryczko 1972, Titarev 1975, Morrison and Smith 1986, Nakari et al. 1987 and 1988, Beacham and Murray 1988, Gillet 1991, Johnstone et al. 1992, Jobling et al. 1995, Pankhurst et al. 1996, Taranger and Hansen 1993, Pankhurst and Thomas 1998, Taranger et al. 1999, King and Pankhurst 1999, Davies and Bromage 2002). High water temperatures have been shown to inhibit the later stages of maturation in salmonids (for rainbow trout see Billard and Breton 1977, Breton et al. 1983, Pankhurst et al. 1996, Pankhurst and Thomas 1998, Chmilevsky 1999, Davies and Bromage 2002: for pink salmon see Beacham and Murray 1988: for Atlantic salmon see Johnstone et al. 1992, Taranger and Hansen 1993, Taranger et al. 1999, King and Pankhurst 1999, King et al. 2003: for Artic charr see Gillet 1991, Jobling et al. 1995) and can cause egg over-ripening before or after ovulation with corresponding effects on embryogenesis and fry survival.

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

Two key differences in three captive broodstock rearing facilities for Snake River spring Chinook salmon (IDFG Eagle Fish Hatchery, Bonneville Hatchery, Oregon, and NMFS Manchester Marine Laboratory) are the type of water (fresh versus saltwater) and rearing water temperature. Ambient water temperature at Eagle Hatchery in Idaho is generally constant at 14°C, unless water is chilled. At the Bonneville Hatchery where Oregon stocks of Snake River spring Chinook salmon are reared, water temperatures are relatively constant at 9°C. At the NMFS Manchester Marine Field Station, seawater temperatures range from 9^{o}C in January to 13.5^{o}C in August. Given the limitations of broodstock facilities with regard to temperature control of fresh water and the expense of

chilling water, it is critical to determine if water temperature is one of the underlying causes of delayed maturation and poor egg quality. A preliminary test with Lemhi River spring Chinook salmon broodstock, has suggested that rearing environment during the freshwater rearing period prior to spawning had only a slight effect on spawn timing. However, comparison between fish reared in the three locations described above suggested that the rearing environment prior to the transfer to fresh water affected subsequent spawning times (Swanson et al. 2003). Therefore, the present study was designed to determine if reducing temperature during the seawater rearing stage can alter spawning time and fertility of eggs in Carson spring Chinook salmon. In addition, several physiological hormones and their gene expression central to the reproductive axis were measured in order to determine the effect of temperature on the timing and physiology of maturation in spring Chinook salmon.

Materials, Methods, and Description of Study Area

Experimental Design

Three-year old Carson spring Chinook salmon were divided into two temperature treatment groups (2 tanks per treatment) during their last 7 months of rearing in saltwater (October 2002-April 2003). The two temperature treatments were ambient (low of 10^{o}C in January, high of 13^{o}C in August) and chilled seawater (low of 4 ^{o}C in January, high of 9^{o}C in August). Temperature profiles for the two treatments are shown in Figure 1. The fish were reared in 20 ft diameter circular fiberglass tanks supplied with filtered and UV-treated saltwater at the NMFS Manchester Marine Field Station, and fed Moore Clark Pedigree Salmon Brood Diet on a standard ration adjusted for rearing temperature according to the manufacturers feed table. Fish were tagged with PIT tags during July 2002 and sexed with a genetic sex marker (Du et al. 1993). During May 2003 fish were transferred to a common freshwater environment maintained at a constant 10-11^{o}C until spawning in August-September 2003. Fish were not fed during this period. To monitor growth history of all individuals during the experimental period, body mass and length were measured during July and September 2002, and February, May, August, and September 2003. Terminal samples were collected from the following fish: three females per tank on September 2002, February and May 2003; all nonmaturing fish in August 2003; and all females at spawning in September 2003. During August 2003 approximately 30 females and males were moved to an artificial stream for behavior studies (see Task 1a). The remaining females were retained in the freshwater tanks and manually spawned. For all terminal samples pituitaries were collected for analysis of gonadotropin gene transcript levels, and plasma was collected for analysis of sex steroids and gonadotropins. Ovarian or skein tissue was fixed in Bouin's solution for subsequent histological analyses. Carcasses, viscera and ovaries or skeins were frozen for analysis of fat content.

Mortality was relatively high during this experiment, with approximately 62% of the females dying during seawater growth from July 2002 to August 2003. This was due to an outbreak of BKD in all the tanks and fish were treated with erythromycin during fall

2002, winter 2003, and spring 2003. The combination of these mortalities and the low numbers of males precluded our planned crosses for determining the interaction of rearing temperature and sex on gamete quality. However, the effect of rearing temperature on egg quality was assessed.

Gonad morphology and histology

For terminally sampled fish, all gonads were removed, weighed, and photographed with a digital camera. Gonadosomatic index (GSI) was calculated as (gonad weight/somatic weight) x100. Pieces of ovarian tissue were preserved in Karnovsky's or Bouin's fixative for 48 h at 4 ^{o}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 hematoxylin and eosin. In females that exhibited varying degrees of ovarian atresia, histology of only the healthy oocytes are included in this report since the objective was to compare reproductive stage and not document in detail any pathological processes. Ovaries were staged according to Naghama et al. (1983). Samples were in various stages of primary and secondary oocyte growth. Oocytes were classified into the following stages: cortical alveoli, lipid droplet, primary yolk globule (yolk first appear, most the cytoplasm contains cortical alveoli and lipid droplets), and early and late secondary yolk globule stage (for details, see Swanson et al. 2003).

Plasma collection and hormone analyses

Fish were anesthetized in buffered tricaine methanesulfonate (MS-222, 0.05%) and blood was collected into 5 cc heparinzed syringes from the caudal vein using 21 gauge needles. Blood was centrifugation at 800 x g for 15 min and plasma was stored at –70 ^{o}C. Plasma was analyzed for follicle-stimulating hormone (FSH) and luteinizing hormone (LH) content by radioimmunoassays (RIAs, Swanson et al. 1989). Plasma levels of estradiol-17β (E2) were determined by a commercially available RIA kit (ICN Chemicals), that has been validated for use on Chinook salmon plasma. For this RIA, E2 is measured directly in plasma without extraction.

Pituitary transcript analyses

Fish were decapitated, pituitary glands were removed and frozen immediately in liquid nitrogen prior to storing at –70 ^{o}C. Levels of gonadotropin subunit transcripts (alpha-1, alpha-2, LH beta and FSH beta) were measured in pituitaries by quantitative real time PCR assays. Primers and probes were designed and created using Applied Biosystem Incorp, (Foster City, CA) "Primer Express" software package. PCR amplification and fluorescence detection was performed using an ABI Prism 7700 Sequence Detector. Acidic Ribosomal Protein-PO was used for normalization of RNA used in reverse transcription reaction and subsequent real-time RT-PCR assays.

Body composition

Carcasses, gonads and viscera were collected separately and stored at –20 ^{o}C until analyzed for fat 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. A subsample of ground tissue was taken for fat analyses (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. Proximate composition values are expressed on a wet weight basis.

Spawning

At spawning, data on body weight, body length, total ovary mass (total number of eggs stripped minus ovarian fluid), fecundity, average egg weight, skein weight and spawning date were recorded. Two females were only partially ovulated; therefore, data for fecundity and skein weight from these individuals were not included in subsequent statistical comparisons. In order to assess egg quality the stripped eggs from each female were treated as follows. Three subsamples of 50 eggs were taken and weighed. These subsamples were then used to determine the average number and size of eggs produced by each female (egg mass = subsample weight/50, egg number = ovary mass/egg mass). A further 3 subsamples of 50 eggs were taken. These 6 subsamples were then each fertilized with 500 microliters of sperm suspension (milt). Because many males matured as age-3 fish, few males were available for spawning with age-4 females. Only one mature male was left for manual stripping. Thus, milt from a single male was used to fertilize all egg lots. Batches of fertilized eggs were incubated in partitioned Heath trays. After 24 h, three of these subsamples were fixed in Stockard's clearing solution and the number of eggs containing dividing and non-dividing blastodiscs were counted. Once the remaining embryos (three batches/female) had reached the eyed-stage of development they were fixed in Stockard's clearing solution and the number of eggs with or without embryos counted. Percent of fertilized eggs and eyed embryos for each sample were determined. To prevent fungal and bacterial growth during embryo development, the eggs were treated with 1 ml formaldehyde for 600 ml water for 15 min each day.

Statistical analyses

Variation between replicate tanks within treatments was analyzed by nested ANOVA (tanks nested within treatments). For spawning parameters, the effect of treatment was then tested by a T-test using the means of each tank. Thus each treatment had an N = 2. For parameters measured over time, the effect of treatment and time was tested by a two-way ANOVA with date and temperature as the two factors. When a factor was significant multiple comparisons were performed using the Tukey HSD test. Comparisons between parameters in September 2002, February 2003 and immature females in August 2003 were performed by one-way ANOVA on pooled data followed by the Tukey HSD test. Proportional data was normalized by arcsin transformation. Analyses were performed using JMP (SAS) software and $p<0.05$ was considered significant.

Results and Discussion

Spawning time

The average number of days to spawning was not significantly different between the two rearing temperatures (Fig. 2). Interestingly, females that were moved to the artificial stream environment appeared to start ovulating earlier than those remaining in the tanks; however, there were insufficient numbers of fish to demonstrate this with any statistical power.

Spawning parameters

Body size at spawning and total ovary mass (stripped eggs plus skein weight) were similar between temperature treatments (Fig. 3a). The percentage of fertilized eggs was significantly higher for females exposed to the ambient temperature compared to those exposed to the chilled temperature. However, the percentage of embryos surviving to the eye-stage did not differ between treatments (Fig. 4). Mean egg mass was similar between the temperature treatments. Although total fecundity tended to be slightly higher in the ambient group, there was no statistical difference in this parameter between temperature treatments (chilled, 1507±296; ambient, 1920±66 eggs, $P=0.194$).

Fish growth history and oocyte stages

There was no significant difference between treatments in growth of fish from July 2002 through to spawning in September 2003 (Fig. 5a,b). Comparison of the growth history between females that either matured or remained immature at age-4 (September 2003) showed that the immature females were significantly smaller from July 2002 onwards (Fig. 6). By August 2003 the females that did not spawn at age-4 had only reached the lipid droplet stage of oocyte development, which was comparable to the oocyte stage recorded for the large females sampled a year earlier (see Fig. 7).

By September 2002 (1 year prior to spawning) females that were large in body mass had ovaries that contained oocytes at the early to late lipid droplet stage (Fig. 7). The small females sampled (i.e. those that had a similar growth history to females that did not mature as 4 years of age) had ovaries containing oocytes at the late cortical alveoli stage. In other words, in September 2002 (one year prior to spawning of age-4 fish) ovaries of the small fish were less developed than those of large females. By February 2003, the large females sampled had commenced vitellogenesis (yolk incorporation) and their ovaries contained oocytes at the primary yolk globule stage (Fig. 8). At this time, the small females sampled (those that had a similar growth history to females that did not mature as 4 years of age) had less developed ovaries with oocytes only at the early lipid droplet stage. Several of the females that were samples had ovaries that had commenced vitellogenesis despite a decline in body growth from September 2002 to February 2003. By May 2003, the large females had ovaries that contained oocytes at the secondary yolk globule stage (Fig. 9). At that time, the majority of the small females sampled (those that had a similar growth history to females that did not mature as 4 years of age) had ovaries that contained oocytes only at the early lipid droplet stage. Only one of the small

females had ovaries with vitellogenic oocytes. Thus, the degree of advancement of egg growth was related to body growth history in most of the females, but there were exceptions to this pattern.

The skeins from ovulated females were devoid of early stage oocytes except for one that contained a small number of very early cortical alveoli stage oocytes among the degenerating post-ovulatory follicles (Fig. 6). There was no evidence that the skeins contained early stage oocytes capable of forming another cohort of oocytes akin to those seen in the interoparous trout. Skein weight was similar between temperature treatments (chilled, 152±88 g; ambient 140±20 g, P =0.85). A high proportion of the stripped females had a relatively large number of 'overripe' eggs retained in the anterior portion of the ovary. This was reflected in a higher skein weight to somatic weight ratio when compared to post-spawning spring Chinook salmon returning to the Rapid River hatchery (Carson, 10±2%; Rapid River, 1±0.1 %). Data from Rapid River spring Chinook salmon were taken from Swanson et al. 2003. High egg retention was observed in females from both temperature treatments.

Body and ovary growth, and hormone levels in terminally sampled females

Mean GSI increased gradually from September 2002 to May 2003 (Fig. 10a, Table 1). By spawning in September 2003, ovary mass had increased considerably and GSI had increased to over 30% for both temperature treatment groups. No treatment effects were found for either GSI or body mass (Fig. 10a,b). The immature females in August 2003 had similar GSI to females sampled 5 months earlier in February 2003.

Plasma FSH increased gradually for both temperature treatment groups from September 2002 to May 2003 (Fig. 10c, Table 1). Females from the chilled water treatment had a significant increase in plasma FSH at spawning which was not seen in the females from the ambient temperature group. Plasma LH was only detectable in spawning individuals and females from the chilled water treatment had significantly higher levels compared to those from the ambient temperature treatment (Fig. 10c).

Plasma E2 increased from September 2002 to February 2003 (Fig. 10d, Table 1). There was a large increase in the variation of plasma E2 for the females sampled in May suggesting plasma E2 levels had increased in some individuals. The immature females in August 2003 had similar plasma E2 levels to those sampled in September 2002 and February 2003.

Levels of pituitary gonadotropin subunit transcripts (Alpha 1, alpha 2 and LH beta) exhibited a similar change with time, increasing from September 2002 to February 2003, and then remaining constant until a large significant increase at spawning (Fig. 11a-c, Table 1). LH beta increased 1000-fold. There were no significant treatment effects on alpha 1, alpha 2 and LH beta transcript levels. The immature females in August 2003 had similar gonadotropin gene transcript levels to females sampled almost one year earlier, in September 2002. FSH beta transcript levels increased from September 2002 to February

2003 (Fig. 11d, Table 1). In May 2003 females from the chilled water treatment group had significantly higher FSH beta transcript levels compared to those from the ambient temperature group. As with the other gonadotropin subunits, FSH beta transcript levels in immature females sampled in August 2003 were similar to those of females in September 2002.

Carcass, visceral, and gonad fat content exhibited a similar change with time, all decreasing significantly at spawning (Fig. 12abc, Table 1). There was a general tendency for females from the chilled water treatment to have higher levels of fat compared to those from the ambient temperature treatment; however, this difference was not statistically significant.

Gastric distention

Severe chronic gastric distention was observed in 50-70% of the females sampled during seawater rearing. This syndrome resulted from a large distention of the stomach causing compression and atrophy of the left ovary (Fig. 13 - 14). This resulted in a significant reduction in the weight of the left relative to the right ovary; a situation not normally seen in wild returning spring Chinook salmon (Fig. 15ab). This syndrome occurred equally in both temperature treatment groups.

Discussion

This study provides evidence to indicate that a reduction in water temperature during the 7 months of saltwater rearing prior to spawning does not significantly alter the timing of spawning in captively-reared spring Chinook salmon. In addition, fish produced by the two rearing temperature regimes did not differ in body size at spawning, ovary mass, egg size or fecundity. The chilled temperature treatment appeared to cause a reduction in the rate of egg fertilization, but this effect was not reflected in a reduction in the proportion embryos surviving to the eyed-stage of development. The females in the two temperature treatments also did not differ in the occurrence of the retention of over-ripe eggs in ovulated females. Of the physiological parameters measured, only the gonadotropins responded to the temperature treatment; females from the chilled temperature treatment had higher plasma FSH, as well as LH and FSH transcript levels compared to those from the ambient temperature treatment. Also, temperature treatment did not appear to affect the occurrence of the gastric distention syndrome. Thus, the results of this study indicate that reducing water temperature for the last 7 months of the seawater rearing-phase had little effect on reproductive physiology or the quality of gametes or embryos.

These findings are contrary to recent studies in Atlantic salmon and rainbow trout that have shown that relatively small elevations in rearing water temperature can delay spawning, reduce egg quality and cause egg retention (Taranger and Hansen 1993, Pankhurst et al. 1996, Davies and Bromage 2002). The absence in the present study of any positive response to chilled water temperature treatment in spring Chinook salmon suggests that the reduction in reproductive performance observed in the captive

broodstock is not related to rearing temperature. However, it should be noted that the chilling of rearing water is logistically difficult and there are limits to the number and type of temperature treatments available for study. It is possible that a change of greater than 2-3 °C below ambient water temperatures would be required to produce a detectable improvement or that the timing of this reduction is important, or that a longer duration of reduced water temperature is needed. It is also possible that the numbers of fish surviving to spawning were too small to obtain statistically valid estimates of spawning time in the two treatments.

One of the most striking result is that by rearing Carson spring Chinook salmon (normally a hatchery returning stock) in a captive environment from smolting onwards we were able to mimic many of the reductions in reproductive performance described in the captive broodstock programs; delayed spawning, reduced egg quality, gastric distention syndrome and high egg retention. Captively-reared spring Chinook salmon are typically much smaller than their wild counterparts at maturation and spawning, and wild returning spring Chinook salmon do not have ovary damage due to gastric distention (Swanson et al. 2003). Poor growth, ovary damage and disease outbreaks affect how the reproductive system develops and it is highly likely that these factors contribute significantly to the reduction in reproductive success seen in these fish. Damage to the ovary caused by gastric distention during the seawater growth phase potentially reduces the number of developing eggs. Damage to tissues that provide a stimulatory environment for oocyte development (e.g. steroid production) in fish with this syndrome may also have a significant effect on oocyte maturation and may result in substantially reduced fecundities compared to wild fish.

In the interoparous rainbow trout, the removal of one ovary stimulates the other ovary to compensate by developing twice as many eggs (Tyler et al. 1994). Thus, a loss of tissue in one ovary in this species may induce changes in the other ovary to compensate for the loss of eggs. However, it is unlikely that semelparous Chinook salmon is able to compensate for the partial loss of an ovary since in Chinook salmon the maturing ovary does not contain a cohort of smaller oocytes destined to form the eggs for the following spawning season. Results from the present study indicate that, unlike the rainbow trout, the skeins of spawned Chinook salmon contain no oocytes in early stages of development. The absence of these early stage oocytes in the skeins at spawning suggest that semelparous fish do not have the appropriate mechanism to recover from the ovarian damaged caused by gastric distention. The cause of gastric distention syndrome has not been determined; however, at least one report in the literature suggested that a component of the diet that affects digestibility or gut motility causes this problem (Lumsden et al. 2002, see Swanson et al. 2003 for more information).

It is interesting that the chilled temperature treatment appeared to produce an elevation in gonadotropins relative to females from the ambient temperature treatment. Several studies have suggested temperature can affect changes in the LH production and secretion (Breton and Billard 1977, Davies et al.1995, Pankhurst and Thomas 1998). For example in males, elevated temperatures reduce LH and delay spermiation (Breton and Billiard 1977). In the present study, fish from the reduced water temperature group had higher

gonadotropin levels than fish from the ambient group, but this difference in gonadotropin levels did not appear to have a significant effect on embryo quality or numbers of ovulated eggs. In mammals, there is evidence that elevated FSH levels prior to ovulation have been associated with poor development of the resulting embryos (e.g. Van Blerkom and Davis 2001). However, the results of the present study in Chinook salmon suggest if anything, there was a positive effect on embryonic development. Survival of embryos to the eyed-stage was similar in the ambient and chilled treatments even though fertilization rates were lower in the chilled groups. Thus, mortality from fertilization to eyed-stage must have been higher in the ambient group for the survival to eyed-stage to be the same between the two groups.

An important factor to keep in mind is that the quality of eggs from artificially spawned fish is highly dependent upon the amount of time between ovulation, stripping and fertilization of the eggs (Springate et al. 1984). Therefore, to confirm relatively subtle effects of the treatments, a large number of females should be tested. In the present study the number of spawning females was severely restricted by mortality during rearing.

The general pattern of oocyte growth and accompanying physiological parameters were similar to those previously reported for spring Chinook salmon (Swanson et al. 2003) and other salmonids (e.g. Slater et al. 1994, Gomez et al. 1999). Small but significant increases in plasma gonadotropin, E2 and pituitary gonadotropin transcript levels were detectable from the fall of 2002 to the spring of 2003. The change in patterns in hormone production was accompanied by a significant increase in GSI and the development of oocytes from the lipid droplet to the primary yolk globule stage. We have previously reported similar increases in these endocrine parameters associated with the lipid droplet stage of oocyte development in the coho salmon (Campbell et al. 2003). Increases in plasma E2 and FSH pituitary transcript levels correlated well with the progress of vitellogenesis. Large increases in plasma LH, alpha 1, alpha 2 and LH beta transcript levels correlated with final maturation and ovulation. This is the first time maturational changes in pituitary gene expression have been reported for the spring Chinook salmon.

The analysis of growth history revealed that poor growth during the seawater rearing period resulted in a failure to mature at four years of age. Oocytes in the females that did not mature at age-4 had developed only as far as the lipid droplet stage. By August 2003, the levels of the reproductive parameters in these immature females were similar to those seen in females during the previous September. Presumably, if these females had continued to grow they would have matured and spawned in September 2004 at 5 years of age. This is consistent with studies in other salmonids that have suggested that the decision to mature at a given age depends on the previous growth history of the individual and that females that fail to make a threshold size/energy store necessary for maturation will arrest oocyte development at the lipid droplet stage (Thorpe et al., 1990, Duston and Saunders, 1999, Taranger et al., 1999).

In general body and organ fat content changes during maturation were similar to those reported for other spring Chinook salmon (Swanson et al. 2003); a general decrease from May 2003 to spawning during the period of greatest ovary growth and removal of feed.

Prior to May 2003 it appeared that whole body and organ fat content remained relatively constant; however, the small numbers of females sampled and high variation in values for these early sample dates reduced the power to find any significant differences. During May, the captive Carson spring Chinook salmon had a relatively high level of visceral fat content (15-20%) compared to returning Rapid River Hatchery adult spring Chinook Salmon (5%; Swanson et al. 2003). Other captive Snake River spring Chinook salmon also exhibit high levels of body fat compared to migrating hatchery adults (Swanson et al. 2003). The subsequent mobilization of fat stores for body maintenance and gonadal growth was similar to that seen for the Rapid River Hatchery spring Chinook salmon and the temperature treatments did not affect this mobilization. From these data it appears that captive broodstock do not lack the necessary fat stores required for body maintenance and ovarian development. However, it is clear that feeding with commercial broodstock diets and rearing in captivity alters fat deposition. It remains to be determined if these large fat deposits contain accumulated contaminants (e.g. Hites et al. 2004) which could affect egg and embryo development.

Summary and Conclusions

In Carson spring Chinook salmon, reducing water temperature 2-3 ^{0}C below ambient during 7 months of saltwater rearing prior to spawning did not substantially affect any of the reproductive parameters studied. However, the low numbers of fish surviving to spawning in this study may have precluded detection of small differences in spawn time or gamete quality. The most significant factor affecting age of maturity, irrespective of rearing temperature, was body growth. Females that were below approximately 800 g body mass at 3 years of age did not mature the following year at 4 years of age. The degree of ovarian development was also related to growth history, irrespective of temperature treatment. In this study, the captive Carson spring Chinook salmon had generally poor survival, growth, ovary growth, and egg quality; all problems reported for the endangered captive Chinook broodstock. This indicates that the problems experienced by these recovery programs are not specific to the stocks under recovery, but is a basic problem with moving spring Chinook salmon into an artificial rearing environment. It may be possible to improve spawning time and ovulation by the use of GnRH implants and photoperiod manipulation. However, if some of the reproductive problems result from the relatively poor growth of females and corresponding poor development of the reproductive axis, the above treatments are only alleviating rather than preventing the problems.

Future studies will be designed to test a longer period of reduced water temperature in the seawater rearing phase and extending the chilled water treatment through the last 5 months of rearing prior to spawning. In addition, photoperiod should be explored as a method to advance spawning time in spring Chinook salmon.

Data Management Activities

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

Table 1. Comparison of parameter levels between the initial September 2002 sample, pooled February samples and immature females sampled in August 2003. Data are mean±S.E. (n).

Parameter	September 2002	February 2003	Immature
GSI	0.39±.02 (5) [b]	0.87±0.07(12) [a]	0.84±0.1(5) [a]
Plasma E2	1.30±0.59(4) [b]	5.42±0.83(11) [a]	2.52±0.29(3) [ab]
Alpha 1	1.56±0.14(4) [b]	2.20±0.13(12) [a]	1.43±0.13(5) [b]
Alpha 2	1.17±0.06(4) [b]	1.57±0.08(12) [a]	1.13±0.04(5) [b]
FSH beta	8.33±1.13(4) [b]	37.91±5.17(12) [a]	7.24±1.28(5) [b]
LH beta	5.35±2.69(4) [b]	46.09±9.06(12) [a]	7.20±2.08(5) [b]
Plasma FSH	0.24±0.01(5) [b]	0.62±0.07(12) [a]	0.20(1)
Visceral Fat	22.61±2.78(4) [a]	11.82±2.34(12) [a]	10.52±3.07(5) [a]
Gonad Fat	5.78±1.62(4) [a]	9.16±0.85(12) [a]	6.53±1.37(5) [a]
Carcass Fat	18.02±0.89(4) [a]	16.62±0.95(12) [a]	9.90±2.09(5) [b]

Within each parameter a similar letter indicates no significant difference ($P<0.05$)

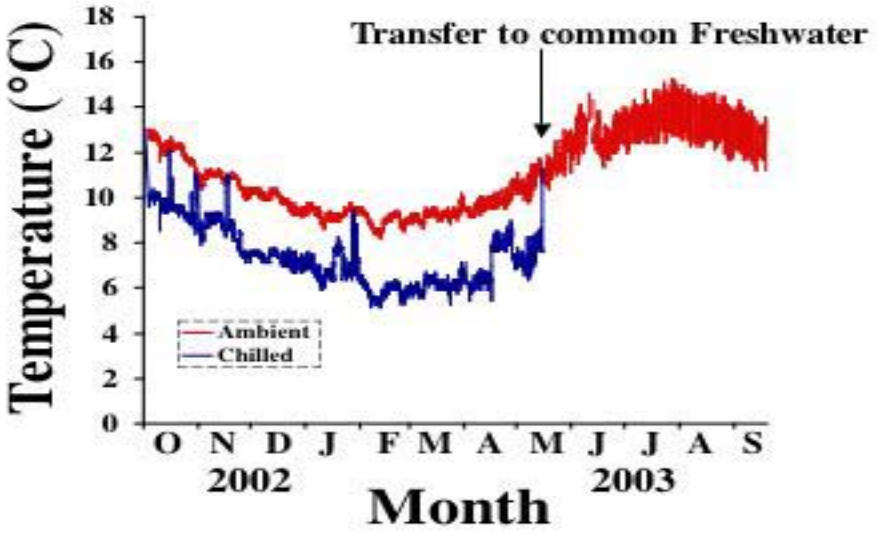

Figure 1. Temperature profile of the ambient and chilled temperature treatment groups during seawater rearing.

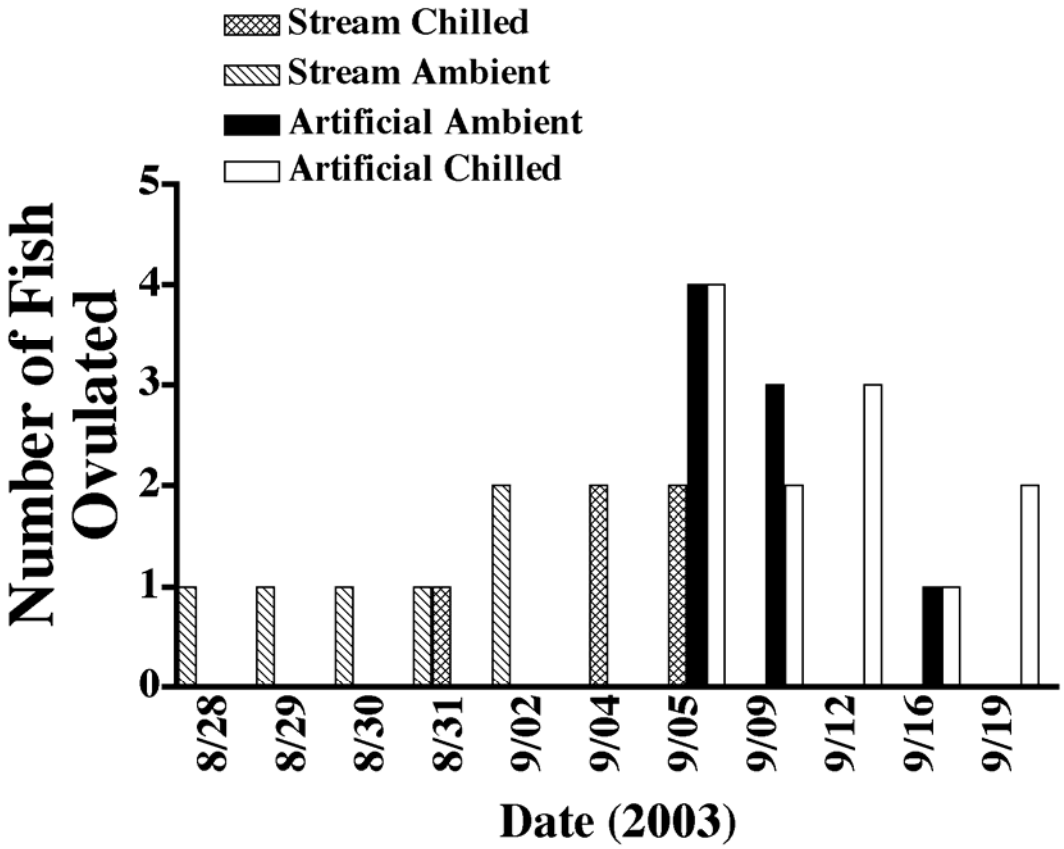

Figure 2. Ovulation dates for females from the chilled and ambient temperature treatments. The ovulation dates for females moved to the stream correspond to the day mortalities were detected, dissected and found to contain ovulated eggs.

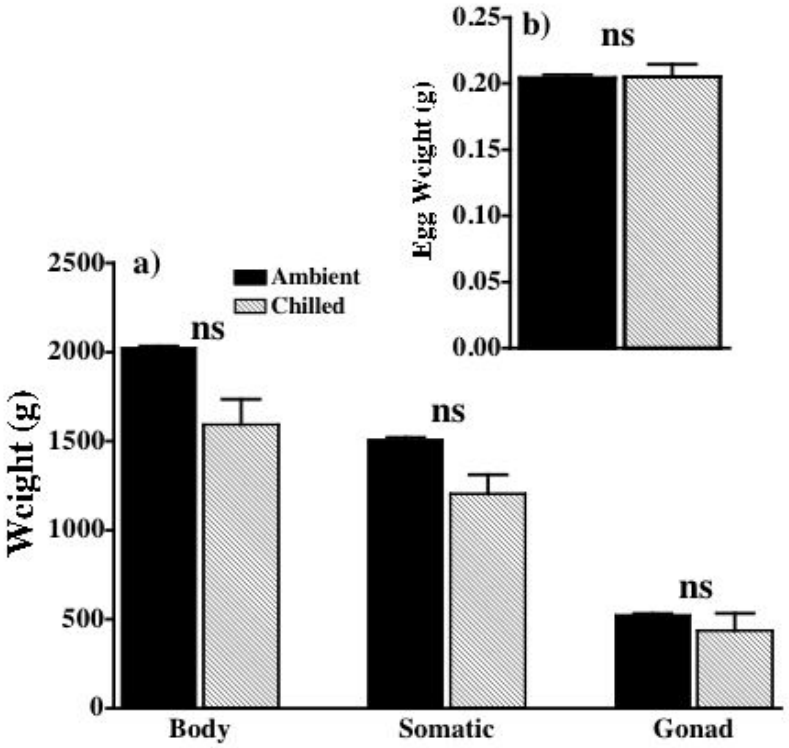

Figure 3. Average body weight, somatic body weight, gonad weight (a) and egg weight (b) for females from the ambient and chilled temperature treatment groups spawned in September 2003. Bars represent mean of replicate tanks±S.E, n=2. There were no significant differences between treatments (ns, P >0.05).

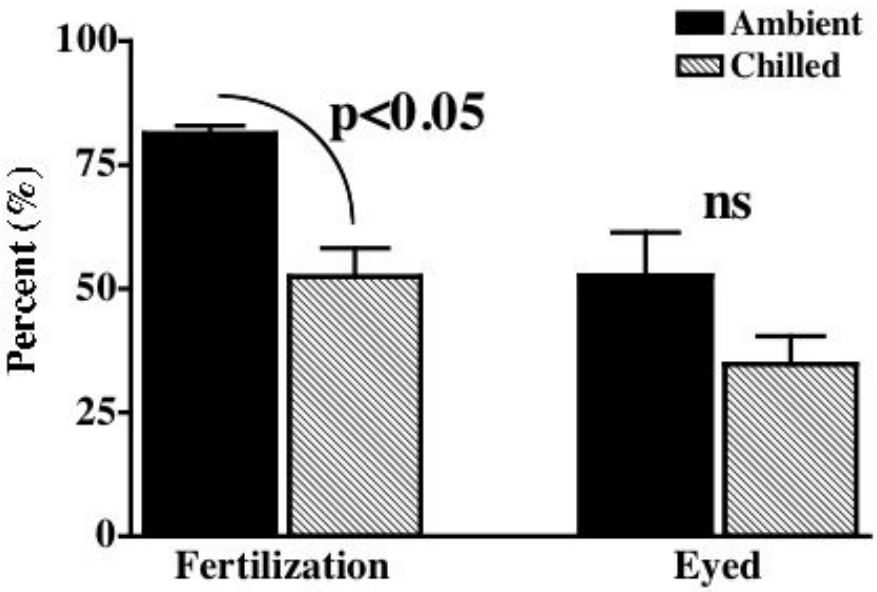

Figure 4. Average percent fertilization and eyed eggs for females from the ambient and chilled temperature treatment groups spawned in September 2003. Egg lots were fertilized with a common male. Bars represent mean of replicate tanks ± S.E, n=2.

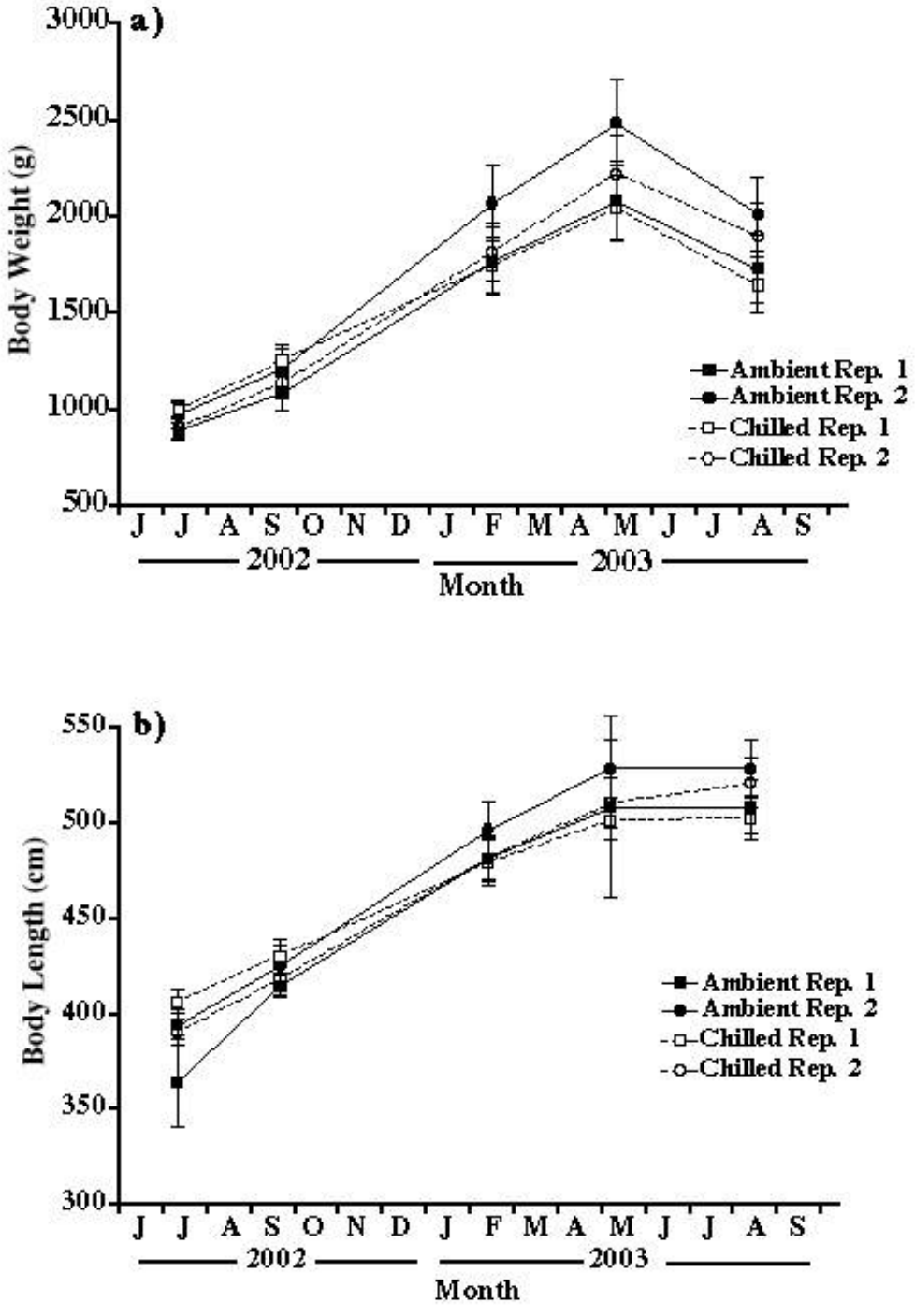

Figure 5. Change in body weight (a) and body length (b) of the females surviving to spawning. Data represent the mean±S.E. of each replicate tank for each temperature treatment. There were no significant differences between treatments (ns, $P > 0.05$).

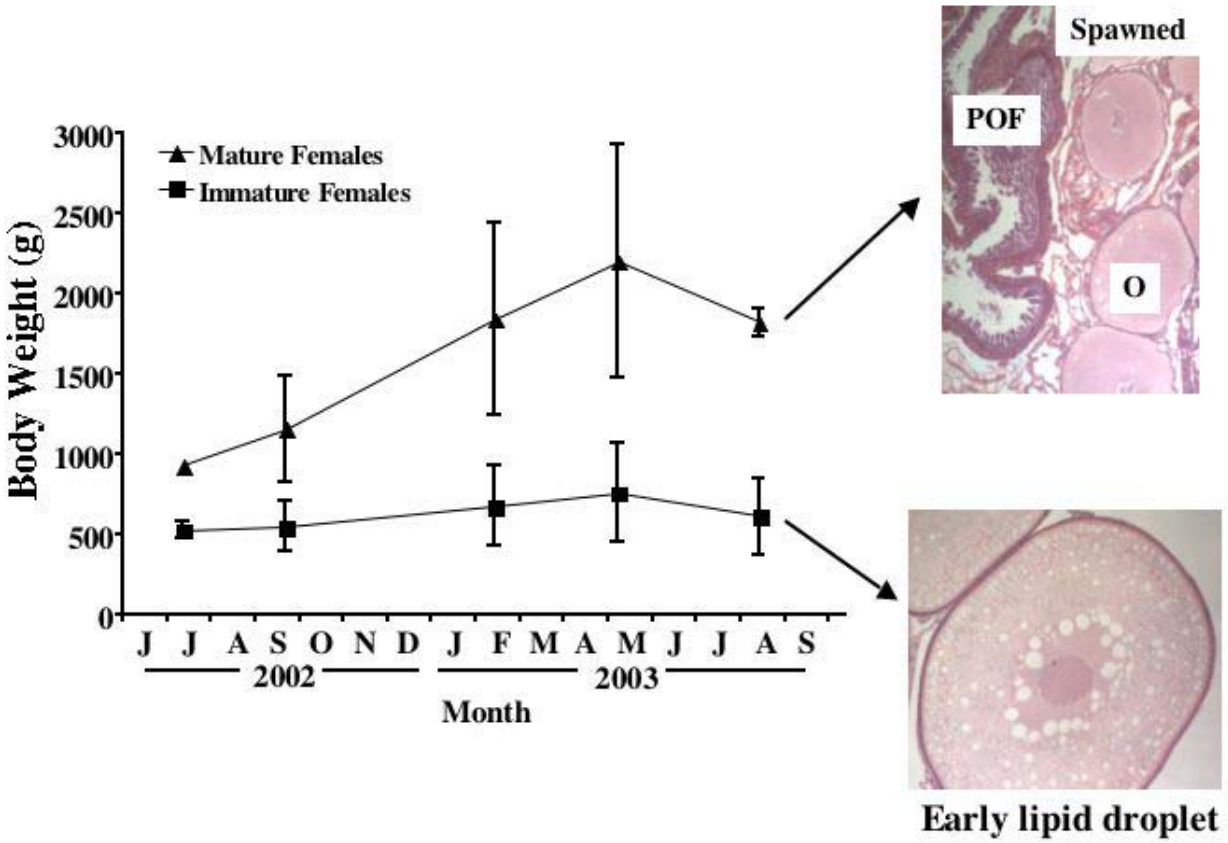

Figure 6. Change in body weight during the rearing period for females that were mature (n=20) or immature (n=5) in August 2003. Data represent the mean±S.E. of each category. A representative picture of the oocyte development stages present in the spawned skeins and immature ovaries for each category are shown. POF-post ovulatory follicle, O-oocyte.

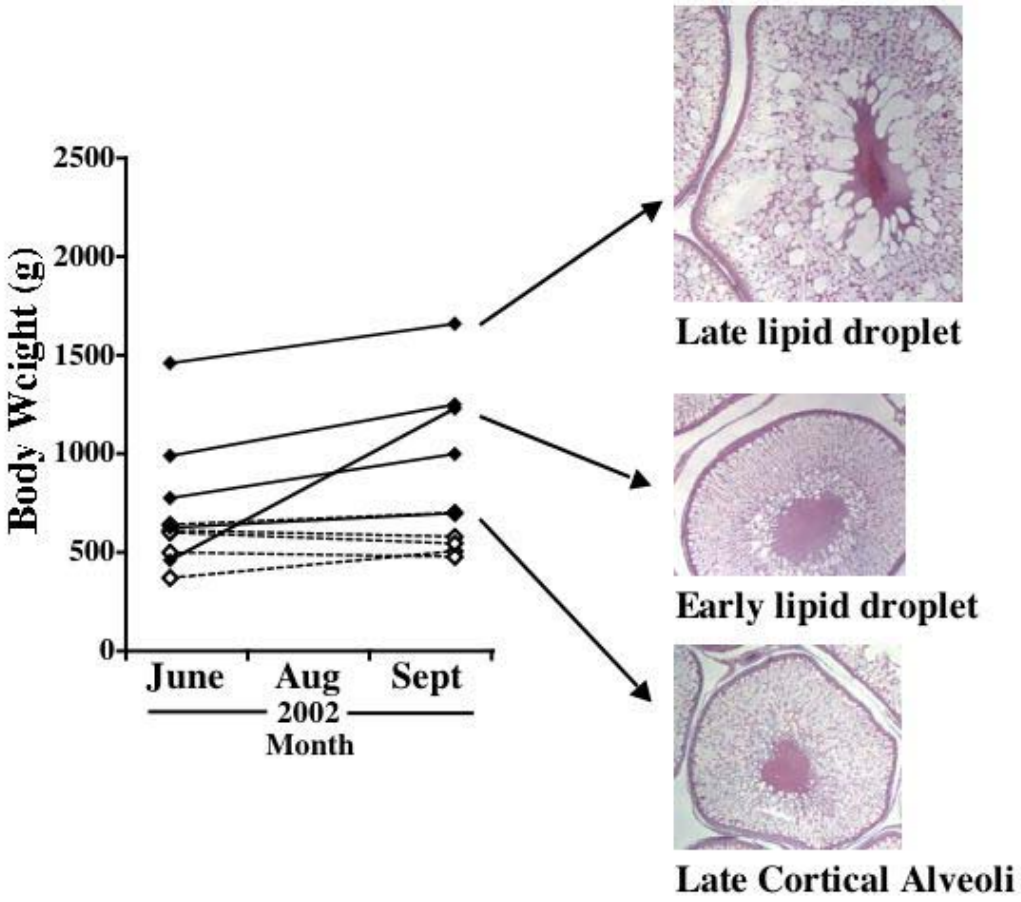

Figure 7. Change in body weight for individual females sampled in September 2002 and females remaining immature in August 2003. Pictures of the oocyte stages present in the ovaries (individuals indicated by the arrows) are shown on the right.

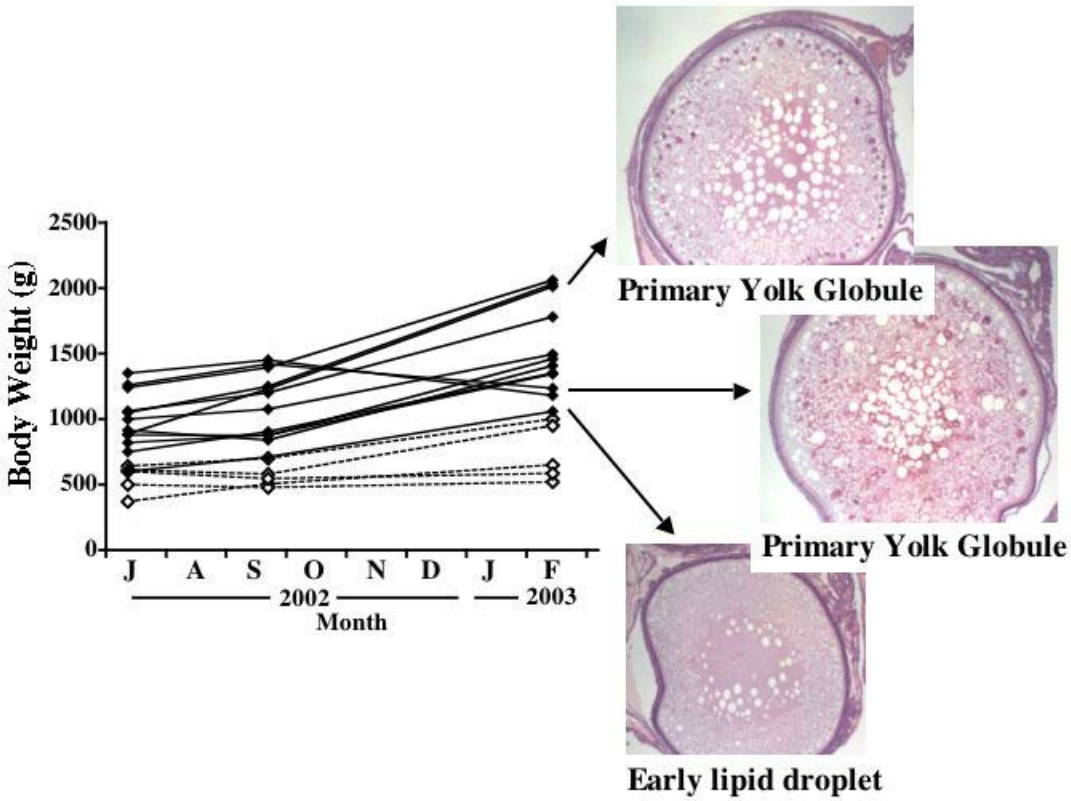

Figure 8. Change in body weight for individual females sampled in February 2003 and females remaining immature in August 2003. Pictures of the oocyte stages present in the ovaries (individuals indicated by the arrows) are shown on the right.

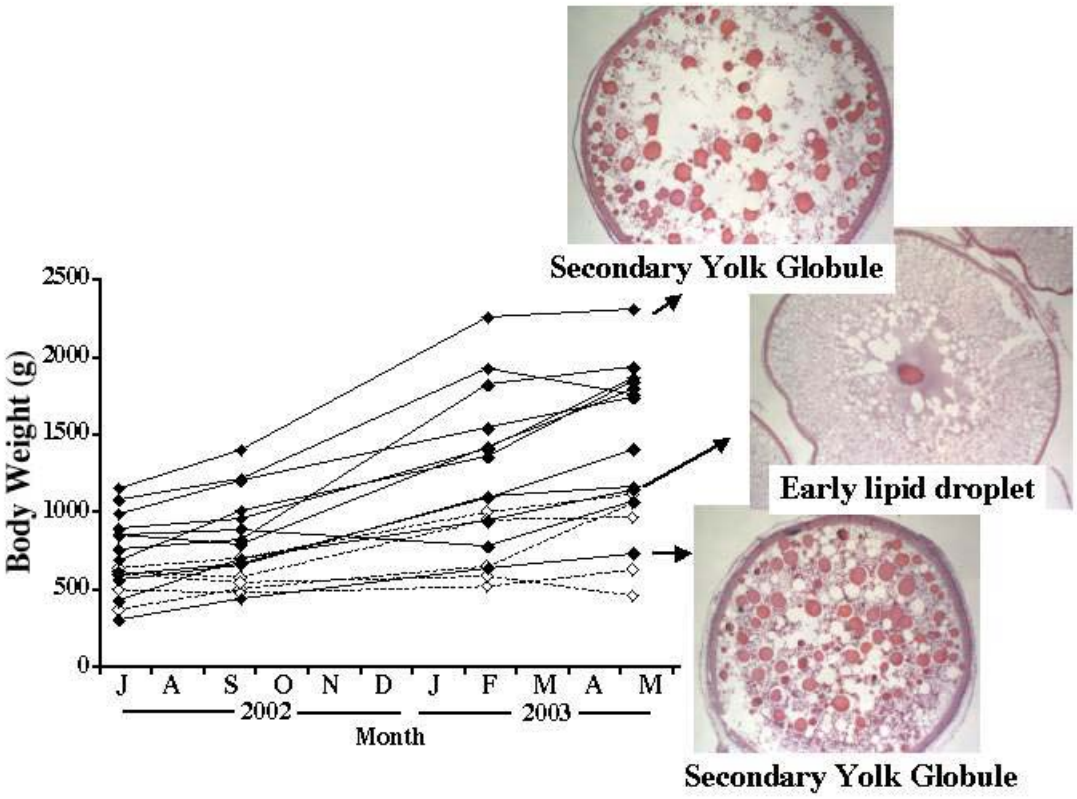

Figure 9. Change in body weight for individual females sampled in May 2003 and females remaining immature in August 2003. Pictures of the oocyte stages present in the ovaries (individuals indicated by the arrows) are shown on the right.

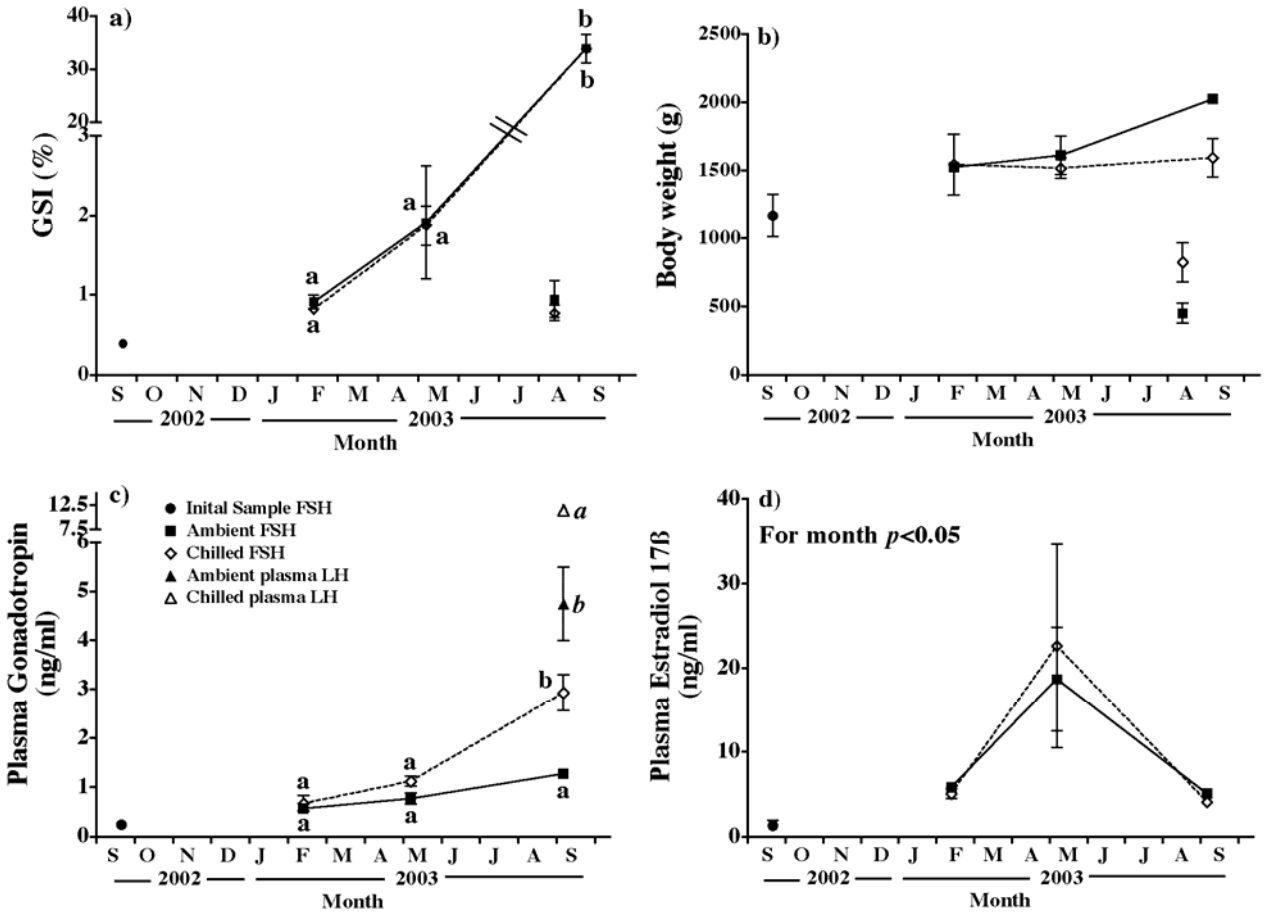

Figure 10. Change in GSI(a), body weight(b), plasma gonadotropins(c), and plasma E2(d) for females sampled from the two temperature treatment groups during the rearing period. For February, May, and September 2003 data represent the mean±S.E. (n=2) of each replicate tank for each temperature treatment. Statistical comparisons were made between data on February, May and September 2003 and points without common superscript letter are significant differences (P<0.05). Comparison between initial samples from a common tank in September 2002, pooled values in February 2003 and immature females in August 2003 are shown in Table 1.

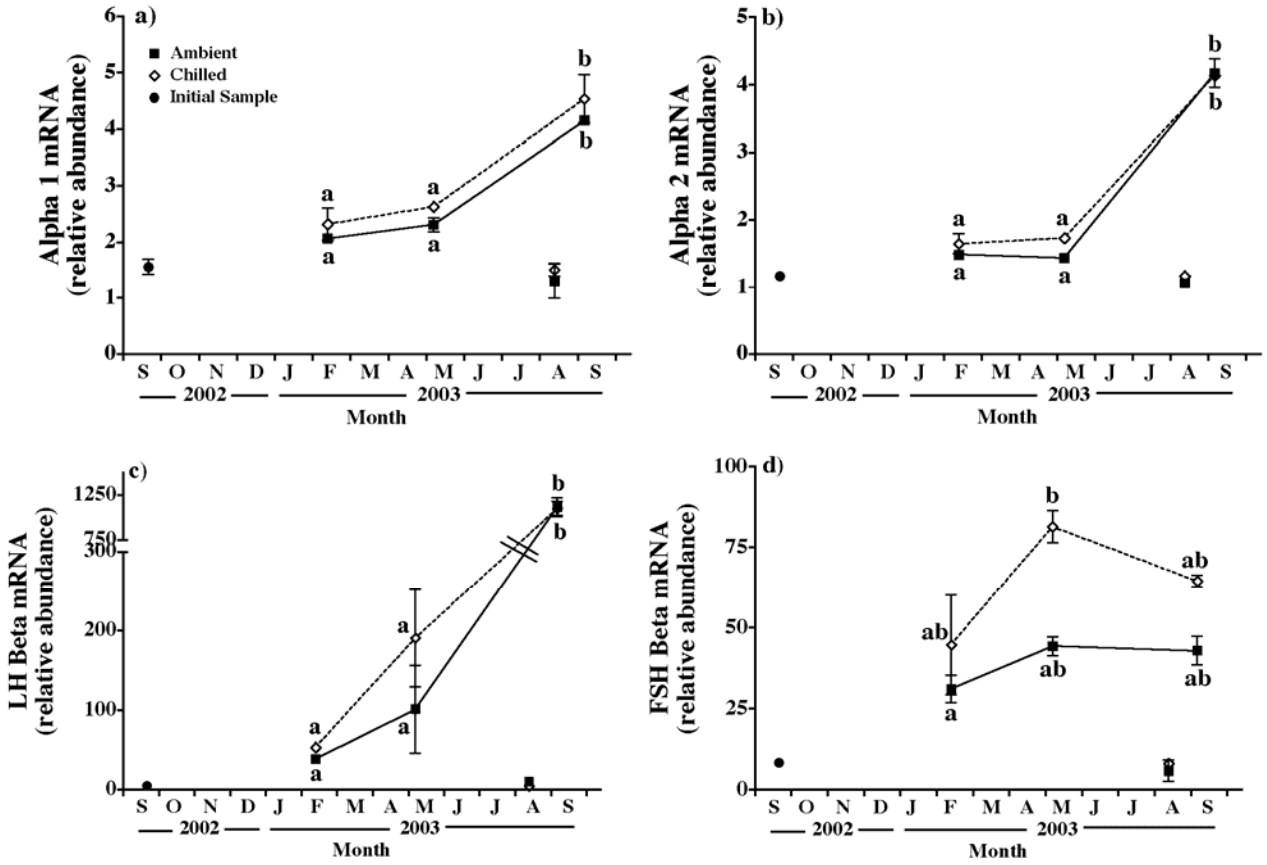

Figure 11. Relative change in pituitary transcript levels of Alpha 1(a), Alpha 2(b), LH beta(c), and FSH beta(d) for females sampled from the two temperature treatment groups during the rearing period. For February, May, and September 2003 data represent the mean±S.E. (n=2) of each replicate tank for each temperature treatment. Statistical comparisons were made between data on February, May and September 2003 and points without common superscript letter are significant differences ($P<0.05$). Comparison between initial samples from a common tank in September 2002, pooled values in February 2003 and immature females in August 2003 are shown in Table 1.

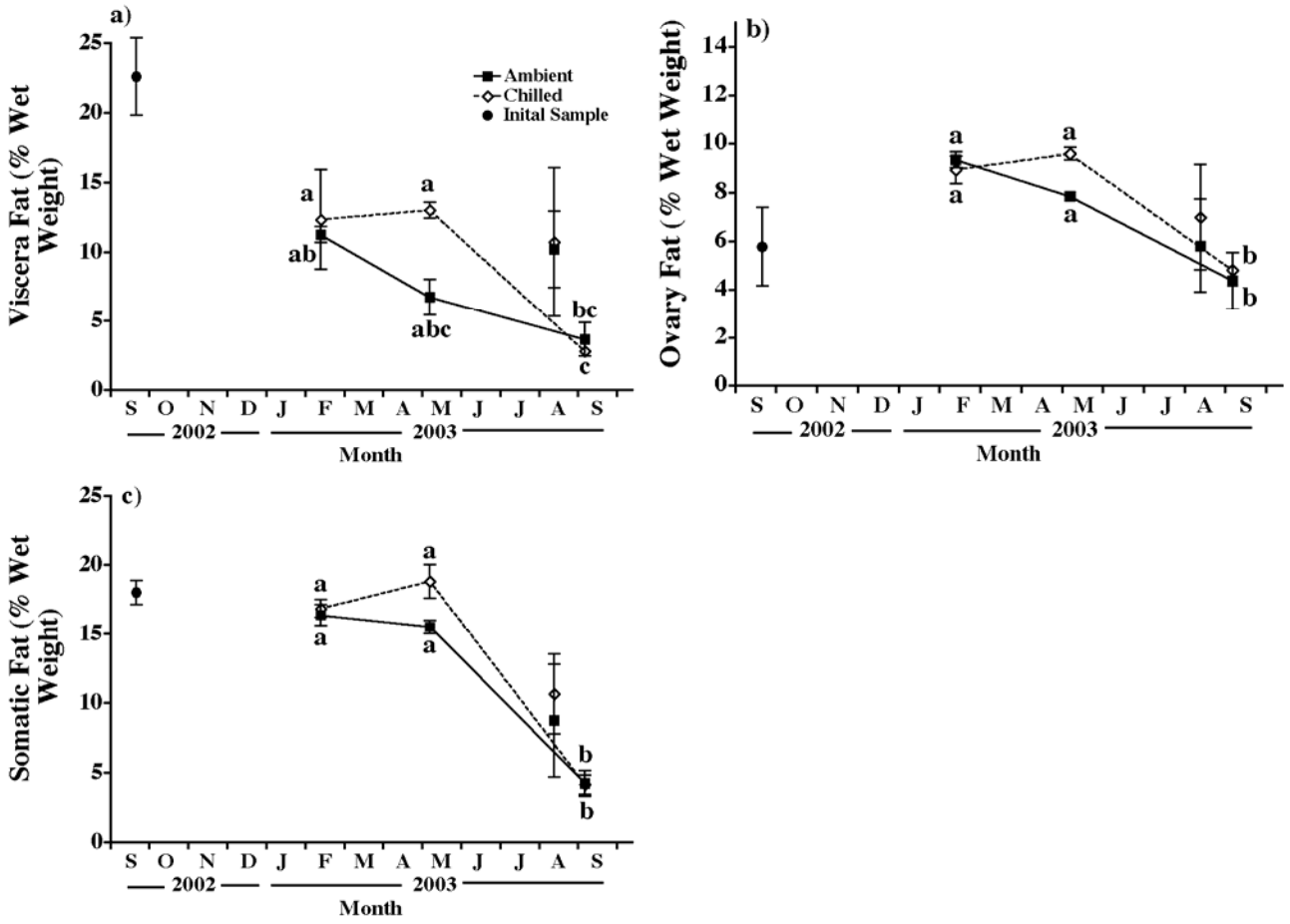

Figure 12. Fat content for viscera(a), gonad(b) and carcass(c) for females sampled from the two temperature treatment groups during the rearing period. For February, May, and September 2003 data represent the mean±S.E. (n=2) of each replicate tank for each temperature treatment. Statistical comparisons were made between data on February, May and September 2003 and points without common superscript letter are significant differences ($P<0.05$). Comparison between initial samples from a common tank in September 2002, pooled values in February 2003 and immature females in August 2003 are shown in Table 1.

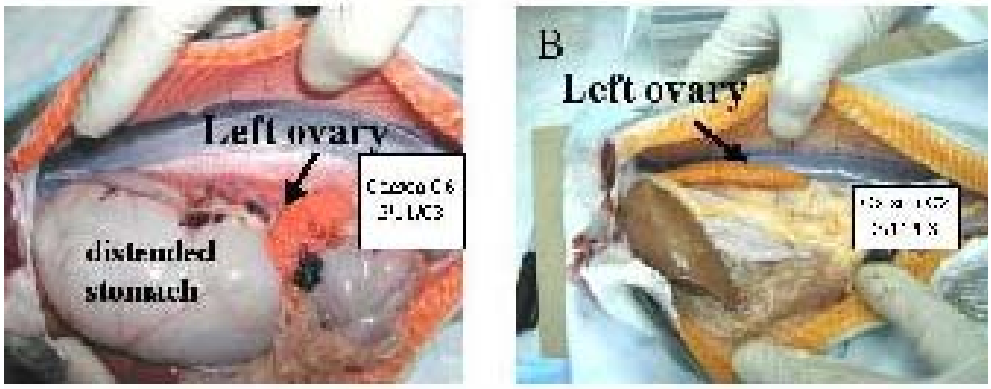

Figure 13. Left ovaries of female spring Chinook salmon sampled on February 11, 2003. a) Fish with gastric distention disorder and necrotic left ovary. Note that the ovary is twisted and contains hemorrhaging and atretic oocytes. The right ovary of this fish was normal. b) Fish with normal left and right ovaries and no gastric distention. Only 2 out of 12 fish sampled had normal left and right ovaries. The remaining fish had varying degrees of gastric distention and necrosis of the left ovary.

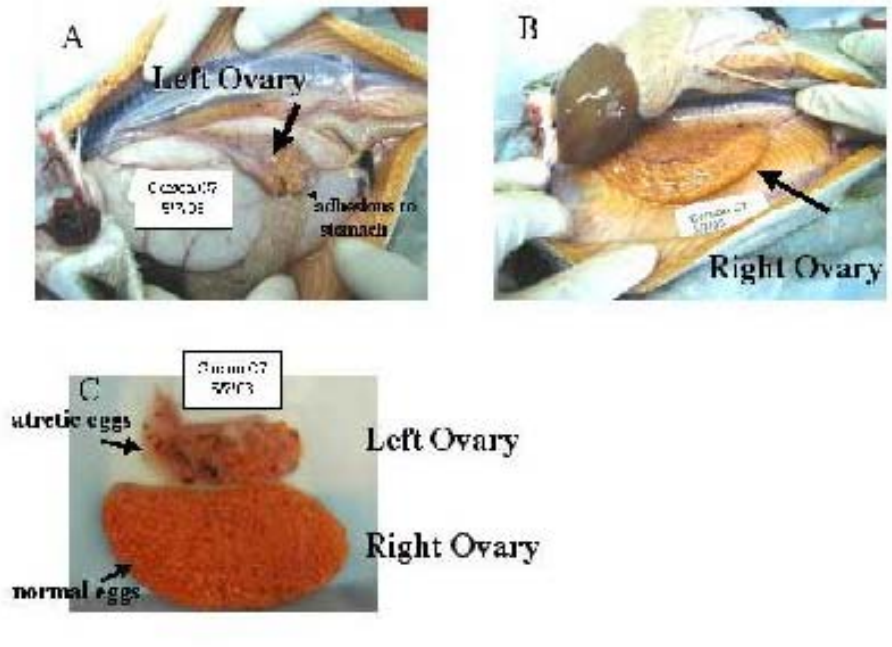

Figure 14. Left and right ovaries of one female (C7) spring Chinook salmon sampled on May 7, 2003. a) Left ovary is almost completely regressed and has many adhesions to the stomach. b) Right ovary is normal. c) Comparison of left and right ovaries.

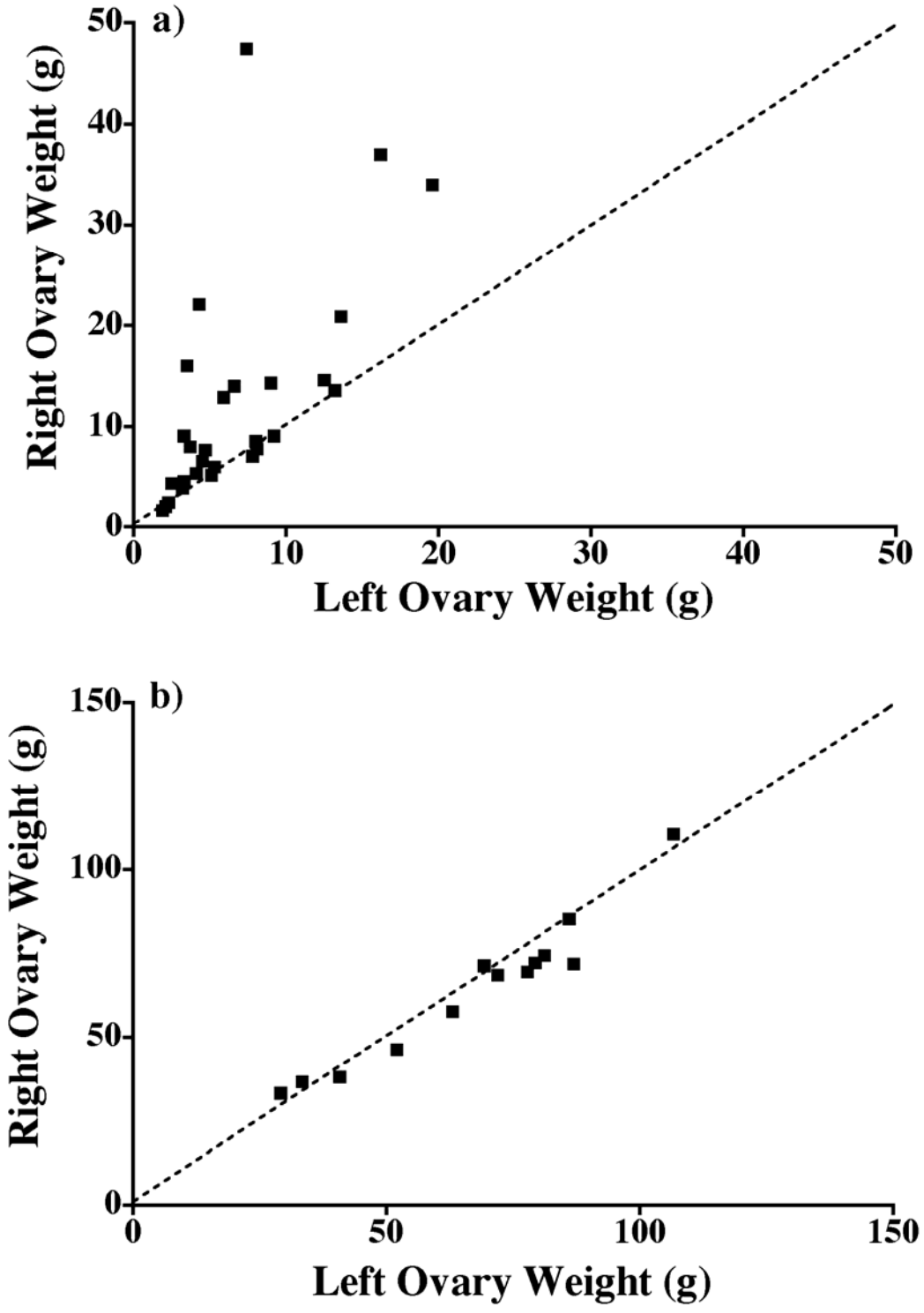

Figure 15. Plot of left ovary weight versus right ovary weight for Carson females sampled over the rearing period(a) and Rapid river females returning to the hatchery sampled in May 2001(b). Data for the latter group was taken from Swanson et al. 2003.

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TASK 3d. MONITORING FERTILITY AND EMBRYONIC DEVELOPMENT IN REDFISH LAKE SOCKEYE SALMON CAPTIVE BROODSTOCK REARED AT THE BURLEY CREEK HATCHERY

By

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Introduction

Production of high quality gametes and offspring is essential for the success of captive broodstock programs for recovery of depleted salmon stocks. Generally, the survival of offspring to the eyed-stage has varied widely in captive broodstock programs, with averages in some programs around 60-70% and others near 40%. The high variance in survival is of great concern because there is more potential for genetic selection. For example, in 1996 the overall survival of Redfish Lake sockeye salmon embryos to the eyed stage was 54.5 +/- 24.6%, with a median of 64% (Table 1). Embryonic survival also appears to vary year to year, and with spawn date within a year. Although females are not always crossed with multiple males to assess male effects on eyed-embryo survival, data from the Redfish Lake sockeye salmon broodstock program suggests that the major source of variation in embryonic survival is due to the female parent (C. McAuley, NOAA Fisheries, personal communication). This is similar to results from single parent crosses in rainbow trout, where the female parent was the major factor affecting the survival of offspring to the eyed stage (Nagler et al. 2000). Unfortunately, in the broodstock programs, poor fertilization rates are often not distinguished from poor rates of survival of embryos as the presence of embryonic cell masses is not always confirmed in the dead eggs. Therefore, it is difficult to determine whether the underlying problem is with the fertility of the egg or with early embryonic development.

Presently, it is not known what factors are responsible for variable survival of offspring and egg fertility in the captive broodstock. In rainbow trout, factors such as nutritional status of the female, egg over-ripening, and chemical composition of the egg have been implicated in affecting the fertility of eggs and survival of embryos (Bromage and Cumaranatunga 1988, Bromage et al. 1992). Practices that ensure appropriate timing of the egg collection appear to have alleviated a great deal of the problem with poor fertility in female rainbow trout (Springate et al. 1984, Bromage et al. 1992). However, problems persist even in the commercial rainbow trout industry where there appears to be a problem with variable egg quality (Nagler et al. 2000). This could be due to age of the females, environmental factors such as temperature, husbandry of captive fish, genetics of the parents, or diet (Brooks et al. 1997). There may be interactive effects of these variables. For example, high water temperature may partially disrupt the process of final oocyte maturation, making it difficult to collect the eggs at the appropriate time.

For a rational development of methods to improve survival of offspring to the eyed stage, it is critical to first determine the underlying basis of the problem in the captive

broodstock using single parent crosses to separate the effects of each parent and a detailed analysis of embryonic development. Therefore, the major aim of studies conducted under Task 3d was to assess fertilization rates and embryonic survival in Redfish Lake sockeye salmon. Females were crossed with multiple males so that the effects of male and female parent on fertility and embryonic survival could be determined. The studies were conducted over two spawning seasons, fall 2002 and 2003.

Materials, Methods, and Description of Study Area

Experimental fish and hypotheses

Fertility and embryonic development (as an index of gamete quality) were monitored during spawning of Redfish Lake sockeye salmon captive broodstock in October 2002 and 2003 using brood year 1999 and 2000 fish, respectively. Fish reared in fresh water at the NMFS Burley Creek Hatchery were used for both studies. Two hypotheses were tested in both studies: 1) The percent of eggs fertilized does not differ from the percent of embryos surviving to the eyed-stage. If this hypothesis was supported, it would suggest that the underlying problem with variable survival of offspring was due to variation in fertility of the egg or sperm, not with subsequent development of embryos. 2) There is no effect of parent (male or female) on fertilization rates or percent of embryos surviving to the eyed stage. If this hypothesis was supported, it would indicate that there is no consistent problem with fertility of males or females, or contribution of one parental sex to the survival of embryos. Additional hypotheses were tested as described below.

The initial study that was conducted in autumn 2002 monitored fertility and embryonic development from gametes collected at two different spawn dates. The initial plan was to compare gamete quality from fish spawned on three dates that were approximately in the early, mid- and late phase of the spawning season. The plan was to test the hypothesis that gamete quality was related to the delay in spawning time. However, two factors caused alteration of this plan. First, approval could not be obtained from the Technical Oversite Committee (TOC) to use the sufficiently large numbers of eggs required to compare three spawn dates. Second, in this particular year, all broodstock were treated with implants containing gonadotropin-releasing hormone analogue (GnRHa), which resulted in a compression of the spawning period. This was done out of concern that this brood year was maturing even later than usual, which would have prevented planting of egg boxes in Redfish Lake prior to the freezing of the lake. Females were implanted on either 16 or 18 October 2002. Males were implanted on one of several dates: 25 or 27 September, or 13 or 16 October 2002. All fish received approximately 75 µg GnRHa per kg body weight. The majority of females were spawned on one of three dates; 25, 30, or 31 October 2002. For this study, adults spawning on 25 and 30 October were used. Since the GnRHa treatment compressed the spawning time, it was not possible to compare spawning time across a wide range of spawn dates as is usual for the untreated

broodstock. The mating design, sampling protocols, and analysis of samples are described below.

In the second study, Redfish Lake sockeye salmon from brood year 2000 spawned in October 2003 were used. The plan was to repeat the study we conducted in fall 2002 where all fish had been treated with GnRHa to induce spawning; however, there was a decision in September 2003 on the part of hatchery personnel not to treat brood year 2000 females with GnRHa. The broodyear 2000 females had been allocated to an experiment to test the effects of exercise on spawning time and the staff wanted the fish to mature without exogenous hormones. This precluded replication of our study design from fall 2002. We did not have the option to use only control fish for our experiment. Thus, we decided to collect gametes from control and exercised adults. This allowed us to evaluate effects of exercise was on fertility and embryonic development. Fish from one spawn date were used. The hypothesis tested was the following: Exercise of adults for a year prior to spawning has no impact on fertility of gametes and/or development of resulting embryos. The details of the exercise regime are described in the Redfish Lake Sockeye Salmon Captive Broodstock Rearing FY2003 annual report (Maynard et al., in preparation). The mating design, sampling protocols and analysis of samples are described below.

Mating design, egg collection, incubation of embryos and sample analyses

Brood year 1999 Redfish Lake sockeye salmon spawned in October 2002 --Males and females for this study were selected randomly from fish spawned on two different spawn dates (October 25 and 30). The only characteristic used to exclude males from the study was if the sperm from that individual was not motile. Milt was collected from males into small plastic bags and atmosphere in the bag was saturated with 95% oxygen. Bags of milt were stored in coolers until used for fertilization of eggs. Eggs from 12 females at each of 2 spawn dates were collected and divided into 3 batches of 100 eggs per female, and dispensed into one-quart ziplock plastic bags. A total of 24 females (300 eggs per female) and 24 males were used in the study. Each batch of 100 eggs was fertilized with 0.1 ml milt from one male. Each female was crossed with three different males. Immediately after adding milt, eggs were gently mixed, a small volume of fresh water was added, and eggs were incubated for 5 min in a cooler on top of a bed of ice with insulation between the bags of eggs and ice. After 5 min, water was decanted and approximately 200 ml of diluted iodophore was added and incubated for 20 min prior to dispensing fertilized eggs into incubation containers.

Fertilized eggs were incubated according to normal hatchery protocols in isolated containers (50 eggs per 10.2 cm diameter circular containers with nylon mesh bottoms) within Heath trays maintained in fresh water at 9-10^{o}C. Each batch of 100 eggs per cross was split into two different cups (A or B; 50 eggs per cup). Cups A and B were incubated in a separate stack of Heath Trays so that when samples were collected from the A cups, the embryos in the B cups were not disturbed. Fertilized eggs and embryos

(25 per time) were randomly sampled at the following times: 12-18 h (first or second cleavage), 12 d (epiboly), and 30 d post-fertilization (eyed-stage) and at hatch. The 12-hour and 12-day samples were collected from cup A, and remaining samples were collected from cup B. The order of collection was intended to avoid disturbing embryos that needed to develop to the eyed stage at sensitive periods of development. Dead (opaque) eggs/embryos were counted. All sampled eggs/embryos were fixed in 35 ml Stockard's to look for the presence of embryonic tissue and to determine the stage of development. Samples were examined under a dissecting microscope. Fertilization rates were calculated from the number of embryos in first or second cleavage after 12 h of incubation.

Eggs from the first spawn date (25 October) were initially sampled at 7 h post fertilization when completion of first cleavage was expected based on published information for that incubation temperature. However, after examination of the samples, no cell division was evident; therefore, it was not possible to collect information on fertilization rates for this spawning date. For the subsequent spawn date, the timing of the first sample collection was altered to 12 h when embryos completed the first or second cleavage. Although the plan included collection of samples at hatch, a massive fungal outbreak precluded collection of samples from both spawn dates.

Brood year 2000 Redfish Lake sockeye salmon spawned in October 2003--During the spawning period in autumn 2003, fertilization rates and development were monitored in eggs/embryos from 12 females on only one spawn date, 22 October 2003. As mentioned above, there was an opportunity to compare fertility and embryo survival from adults that had been part of a study to examine effects of exercise on spawn timing. Therefore, the analyses of fertility and embryonic development were confined to fish from a single spawn date. Six females and males were randomly selected from each of the two treatments (exercise or non-exercise). At the time of spawning, body weights and lengths, total egg number, and weight of 50 eggs were recorded. Mean egg size was determined by calculating individual egg weight from the weight of 50 eggs.

Eggs from 12 females were collected and divided into 9 batches of 50 eggs per female and dispensed into one-quart ziplock plastic bags. A total of 12 females (450 eggs per female) and 12 males were used in the study. Each batch of 50 eggs was fertilized with 0.1 ml milt from one male and incubated in individual cups in Heath Trays. Each female was crossed with three different males in triplicate, with a total of nine incubation cups per female. The mating and incubation design are shown in Figures 1 and 2.

Immediately after adding milt, eggs were gently mixed, a small volume of fresh water was added and eggs were incubated for 5 min in a cooler on top of a bed of ice with insulation between the bags of eggs and the ice. After 5 min, water was decanted and approximately 200 ml of diluted iodophore was added and incubated for 20 min prior to dispensing fertilized eggs into incubation containers. Eggs/embryos were incubated according to normal hatchery protocols in isolated containers (10.2 cm diameter circular containers with nylon mesh bottoms) within Heath trays maintained in fresh water at 9-10^{o}C. Since the previous study showed no significant mortality from fertilization to the

12 d post-fertilization, sample collection at 12 d was not done. This allowed for increasing replication of the crosses without utilizing more eggs for the experiment. Fertilized eggs and embryos (25 per time) were randomly sampled at 18hr (second cleavage) and 30 d post-fertilization (eyed-stage). At each sampling point, obviously dead eggs/embryos were counted. All sampled eggs were fixed in 35 ml Stockard's solution to look for the presence of embryonic tissue and to determine the stage of development. Fertilized eggs were considered to be only those with the presence of the 1st or 2nd cleavage of embryonic tissue at the 18-hour sample collection. Each sampling was scored for survival to 18 h or 30 d post fertilization.

Statistical analyses

Treatment or parent effects on egg fertility or embryonic survival were analyzed by ANOVA. Proportional data (percent survival) was normalized by arcsine transformation, followed by ANOVA. Multiple comparisons were made using Fishers PLSD test. Regression analyses and ANOVA were performed using Statview 512+ software and $p<0.05$ was considered significant.

Results and Discussion

Brood year 1999 Redfish Lake sockeye salmon spawned in October 2002

***Spawning*--** On 25 and 30 October 2002, a total of 16 and 25 females, respectively, were spawned. The usual characteristics to determine whether a given female is ovulated and ready to be manually stripped are the softness of the abdomen and the ability to express eggs with gentle abdominal pressure. Although many females were judged to be soft and ready to spawn, in most cases eggs could not be expressed with abdominal pressure. Several females were killed with a blow to the skull and the abdomen was opened to reveal that most eggs had ovulated. A small cluster of over-ripe unovulated eggs appeared to be blocking the flow of ovulated eggs through the vent (Fig. 3). Therefore, all females that had evidence of ovulation by the softness of the abdomen were spawned. The incidence of the "egg plug" was not recorded; however, it appeared to be evident in a large number of females. The cause of this condition is unknown. Females spawned on these two dates were completely ovulated and no females showed overt signs of having over-ripe eggs.

***Fertilization and embryonic survival*--**The appearance of unfertilized eggs and fertilized eggs 12 h post fertilization (time of 1st cleavage) is shown in Figure 4. The unfertilized eggs have a diffuse blastodisc; whereas, the fertilized eggs are easily identified by the defined boundary of the blastodisc and crevice formed by the 1st embryonic cell division. The percent of eggs fertilized and embryos surviving to 12 or 30 d post-fertilization did not differ between the two spawn dates (Fig. 5). For the 25 October spawning, no significant effects of male or female parent were found on fertilization rates, or embryonic survival (Fig. 6-7). The final percent of eggs surviving to the eyed-stage did not significantly differ from rates of fertilization. This suggests that eyed-egg survival

for this spawn date was largely dependent on initial rates of fertilization. For the 30 October spawning no significant effects of female parent were found for rates of fertilization; however, significant effects on embryonic survival to 12 d or to the eyed stage were found (Fig. 8). Examining hatchery records revealed that the females spawned on October 30th for this experiment came from one of three families, E, F, or I. There were three to six females from each family represented in this study. Analysis of survival data by female family revealed that fertility rates were similar among females from the three families, but females from family F had significantly lower embryonic survival than those from the other two families (data not shown). No effects of male parent on fertilization rates or embryonic survival to the eyed stage were found (Fig. 9). Trends in the data suggest that mortality of embryos largely occurs within the first 12 d of incubation since there was no significant difference in survival from 12 to 30 d post fertilization.

Overall, fertilization rates and percent of embryos surviving to the eyed stage for this study were 85% or greater. This was consistent with the general population of captive broodstock spawned at the Burley Creek Hatchery in 2002 (personal communication, Debbie Frost, NMFS) and higher than observed for previous years. The high rates of embryonic survival cannot be attributed to any single variable. However, it is possible that the synchronization of spawning time with GnRHa treatment and spawning fish within two weeks of the treatment aided in proper timing of egg maturation and collection. The data collected on fertility and embryonic development suggests that eyed-egg survival in this brood year was largely dependent on initial rates of fertilization. However, there was some effect of female parent on embryonic development suggesting that the maternal environment (chemical or genetic make-up of the egg) can affect early embryonic development.

Brood year 2000 Redfish Lake sockeye salmon spawned in October 2003

***Spawning*--** During autumn 2003, Redfish Lake sockeye salmon were spawned from 6 October to 11 November 2003. The peak in spawning occurred on 22 and 23 October 2003. Mean eyed egg survival for the general population of adults spawned on those two dates was 78.2 and 65.6%, respectively. Eyed-egg survival ranged from 63.7 to 79.5% for fish spawned between 15 October and 3 November. Lower rates (34.9-53.4%) of eyed-egg survival were found for fish spawned early in the season (6-10 October) that for those spawned at later dates (personal communication, Debbie Frost, NMFS). For this study, females and males spawned on 22 October were used.

***Effects of exercise treatment on female growth*--** There was no significant difference between exercised and non-exercised females in terms of body weight and length (Fig. 10), mean egg size (Fig. 11), total egg number or relative fecundity (Fig. 12). This suggests that the exercise treatment did not significantly alter body growth of females or allocation of energy to egg production.

***Fertilization and embryonic survival*--** No significant effects of the exercise treatment on fertility of males or females were found (Fig. 13). The exercise treatment also had no

apparent effects on survival of embryos to the eyed-stage (Fig. 14-15). Significant effects of male (Fig. 16) and female (Fig. 17) parent were found on both fertility and embryonic survival, but these could not be attributed to the treatment. Parents with low fertility tended to have embryos with low rates of survival to the eyed-stage. When examined either by parent or treatment, no differences in percent of eggs fertilized and percent of embryos surviving to the eyed stage were found. Again this indicates that eyed-egg survival rates are largely determined by rates of fertilization. Exceptions to this rule are found in individual cases such as one female (F9, Figure 17).
Regression analysis among various parameters only revealed one significant relationship. The percent of eggs fertilized or embryos surviving to the eyed-stage were positively related to total egg number ($R^2 = 0.62$, P=0.0023; Fig. 18 -19), but not relative fecundity (data not shown). No significant regression relationships were found between body length or egg size, and either fertility or embryonic survival. Body weight (unstripped females) was weakly related to embryo survival and fertility (R2 = 0.32, P=0.049; data not shown). This suggests that some factor affecting number of ovulated eggs in addition to body size affects egg fertility. It is possible that the low egg numbers came from fish that were either incompletely ovulated, had low recruitment of eggs into vitellogenesis or had higher levels of atresia than expected for fish of that size. Since individuals with partially ovulated ovaries were not tracked carefully and the degree of egg retention was not quantified, it was impossible to determine which of these variables may explain the observed relationship between egg number and fertility. However, the simplest and most likely explanation is incomplete ovulation of eggs.

It is well established in other salmonids that the timing egg collection relative to ovulation is the major factor affecting egg quality (Bromage et al.1992). The completion of final oocyte maturation and ovulation must occur at the appropriate time to ensure high fertility. If eggs were collected too early or the ovulation and maturation process was incomplete, one would expect reduced egg fertility.

Summary and Conclusions

Several overall conclusions can be made from these studies of gamete quality in Redfish Lake sockeye salmon captive broodstock. First, the number of embryos surviving to the eyed-stage is largely determined by fertilization rates. Few instances of problems with embryonic development were observed in this study. Thus, problems with highly variable or low eyed-embryo survival are most likely due to problems with fertilization. Second, the study on brood year 2000 fish showed that there is variation in fertility in both males and females. The focus of efforts to improve embryo survival should be on controlling factors that affect fertility in both sexes. Third, there was no evidence that exercise altered fertility or embryo survival. Thus, no benefit of this treatment was found. Fourth, the highest fertility and embryo survival were found for brood year 1999 adults that were spawned in 2002. This group also showed the highest degree of synchrony of spawning, most likely due to treatment with GnRHa. Fish in this brood year spawned within 5 d of each other, while fish in brood year 2000 spawned over one month. Taken together, one could speculate that synchronizing spawning improves

gamete fertility, perhaps by making oocyte maturation and ovulation more readily detectable and synchronous within the individual. Finally, in both years, rates of eyed-embryo survival were higher than historically observed for this stock of fish reared in captivity. This was attributed in part to improvements in milt production using GnRHa treatment of males, but could also be due to changes in protocols for collection and fertilization of eggs. Ovarian fluid was not strained from the ovulated eggs and incubation eggs with milt prior to water-hardening was extended. Since this study showed eyed-embryo survival is determined largely by fertilization rates, it is not surprising that eyed-embryo survival was improved by increases in milt volume and changes in fertilization protocols.

Data Management Activities

Data are collected manually by NOAA, and UW researchers onto preformatted data sheets or directly into a Power MacIntosh G4s. Data are entered and summarized on personal computers operated by researchers using Microsoft Excel, Statview 512+, Cricket Graph 1.3.2, and Prism. Data on histology of tissues is recorded using a digital camera and stored as either TIFF or JPG files on a Power MacIntosh G4. Backup copies of data are saved on an external Lacie hard-drive and on CD-ROMs. All data are checked for quality and accuracy before analysis. Analytical processes are described in the text of the annual report.

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Table 1. Survival of embryos to the eyed-stage relative to spawning date in BY1993 Redfish Lake Sockeye Salmon spawned in 1996 (from C. McAuley, NOAA Fisheries).

Week of spawning	Spawning date	Number of females	% Survival to eyed-stage
1	17 October	42	81.0
2	21-23 October	138	65.1
3	28-30 October	18	76.5
4	4-6 November	62	71.4
5	12-13 November	23	45.7
6	21 November	14	31.1
7	25-26 November	90	10.6

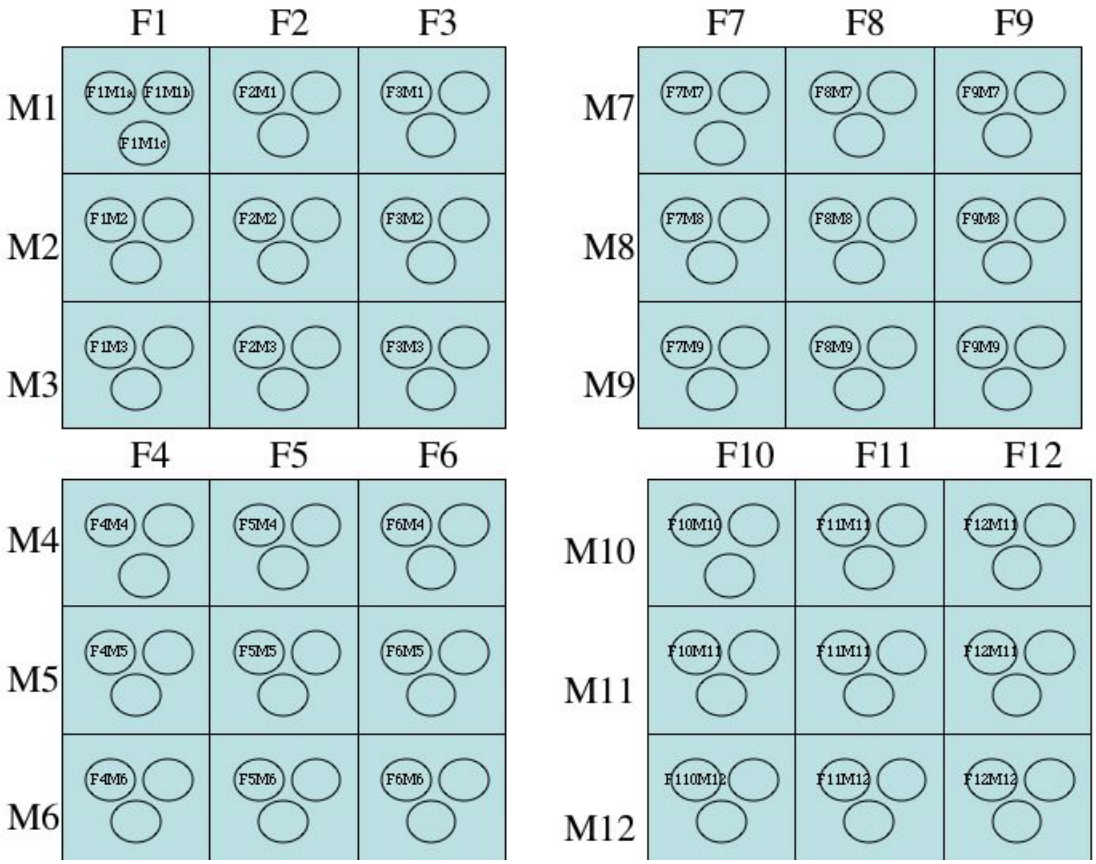

Figure 1. Diagram representing design for crosses of males and females for brood year 2000 with triplicate cups containing 50 eggs.

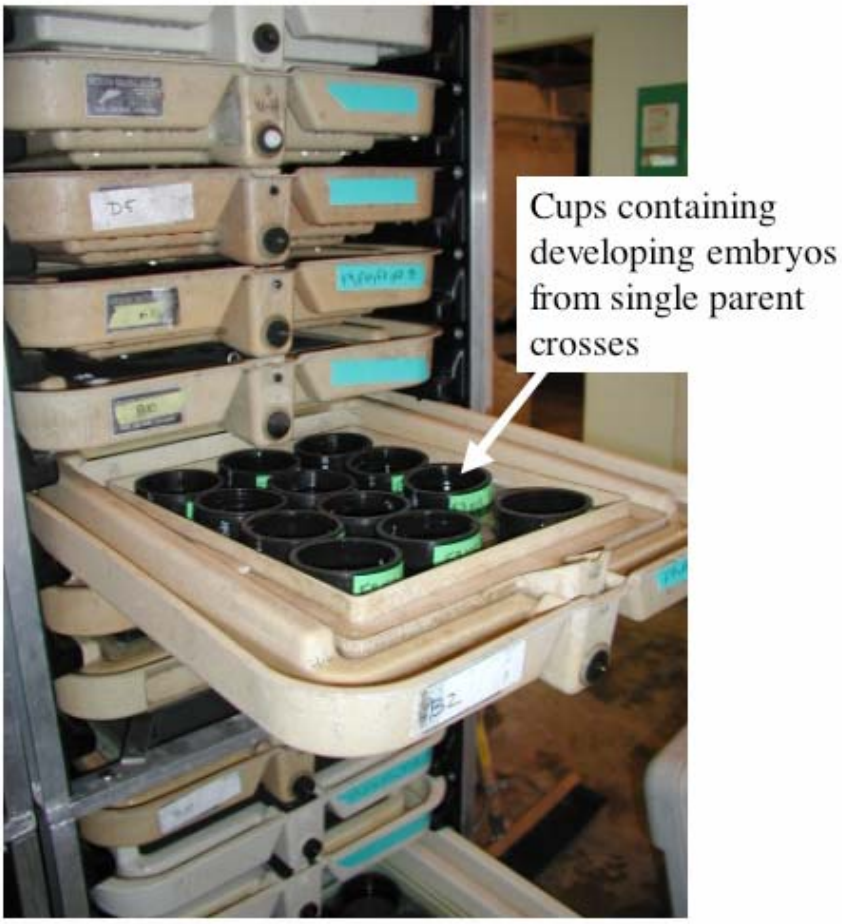

Figure 2. Heath trays containing cups used for incubation of embryos.

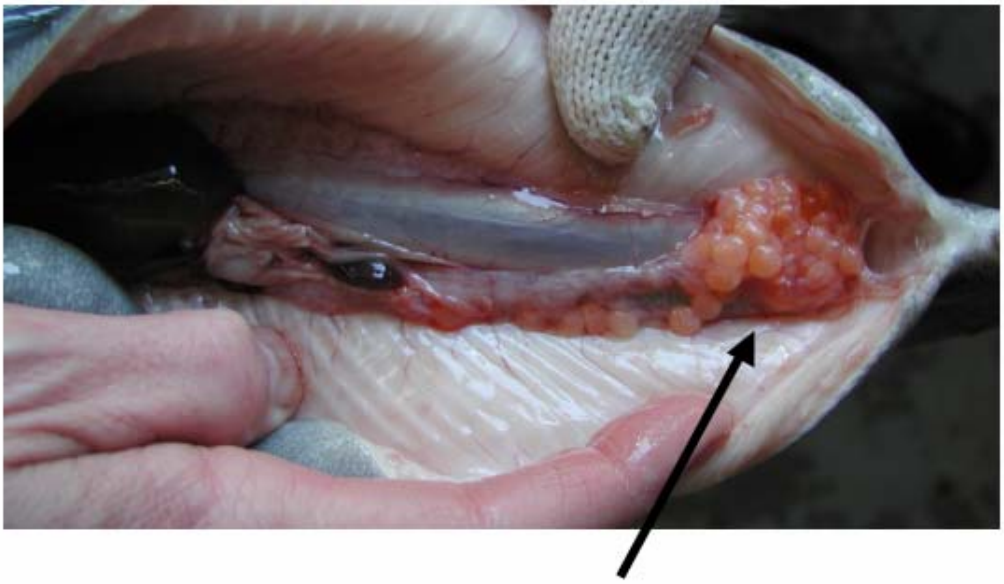

Figure 3. Photograph of post-spawning of brood year 1999 Redfish Lake sockeye salmon female spawned during October 2003. Eggs could not be freely expressed with abdominal pressure. When abdomen was opened most eggs were ovulated, but a cluster of unovulated over-ripe eggs appeared to be blocking the vent.

Figure 4. Comparison of unfertilized eggs (top row) with fertilized eggs (bottow row). Fertilized eggs have embryonic tissue at two-cell stage 12 h post-fertilization. Blastodisc in unfertilized eggs is diffuse. Eggs and embryos were cleared in Stockard's solution prior to examination.

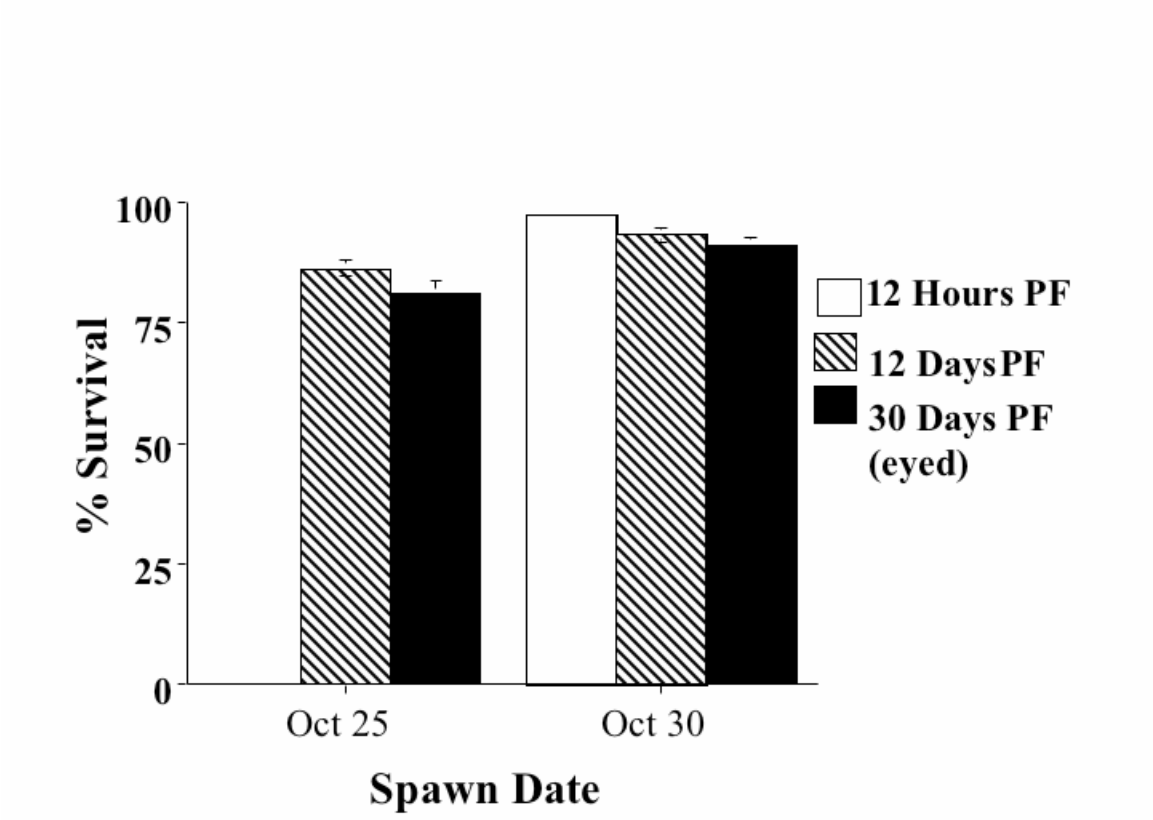

Figure 5. Mean rates of embryo survival 12 h, 12 d and 30 d post-fertilization (PF). Data are mean +/- SEM of crosses of 12 females and 12 males per spawn date for brood year 1999 Redfish Lake sockeye salmon spawned during October 2002. Three batches eggs from each female were fertilized with milt from three different males, and vice versa.

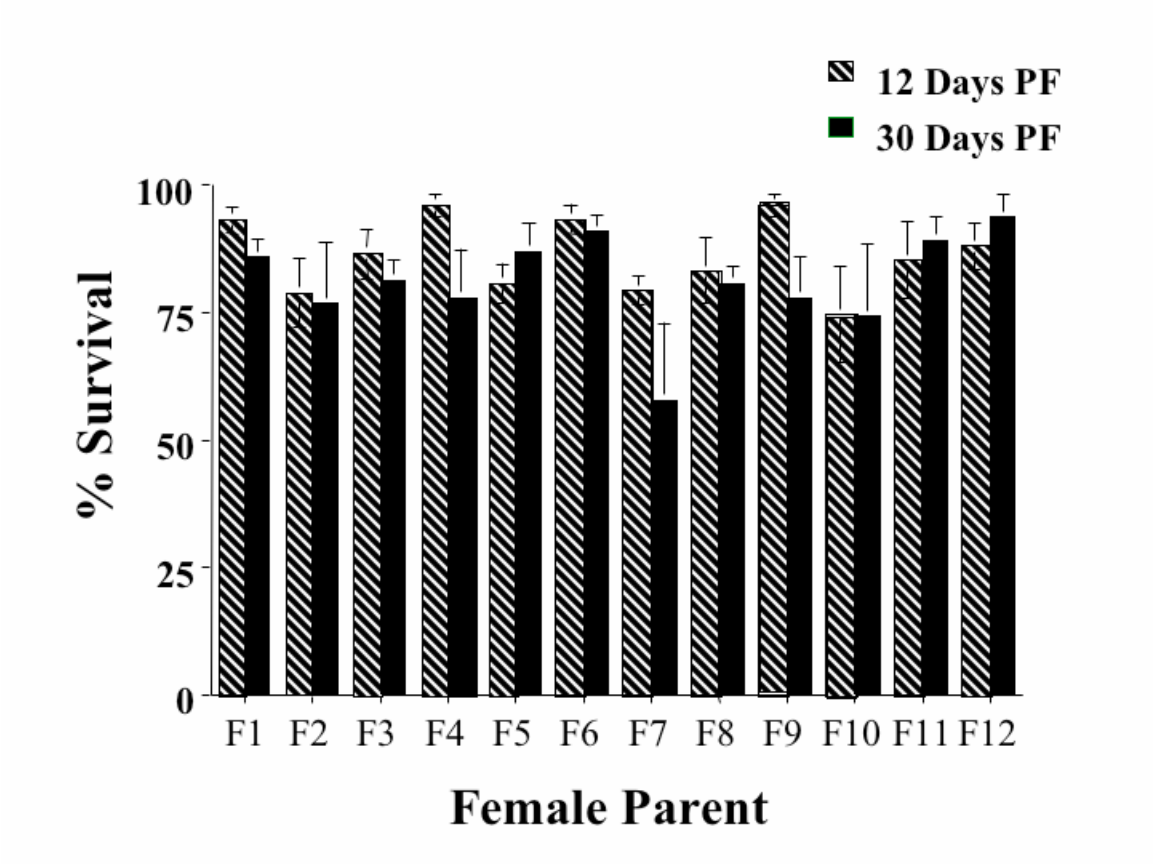

Figure 6. Embryo survival 12 d and 30 d post-fertilization expressed by female parent. Data are mean +/- SEM of crosses with 3 different males per female for brood year 1999 Redfish Lake sockeye salmon spawned on 25 October 2002. No significant effects of female parent on embryo survival were found.

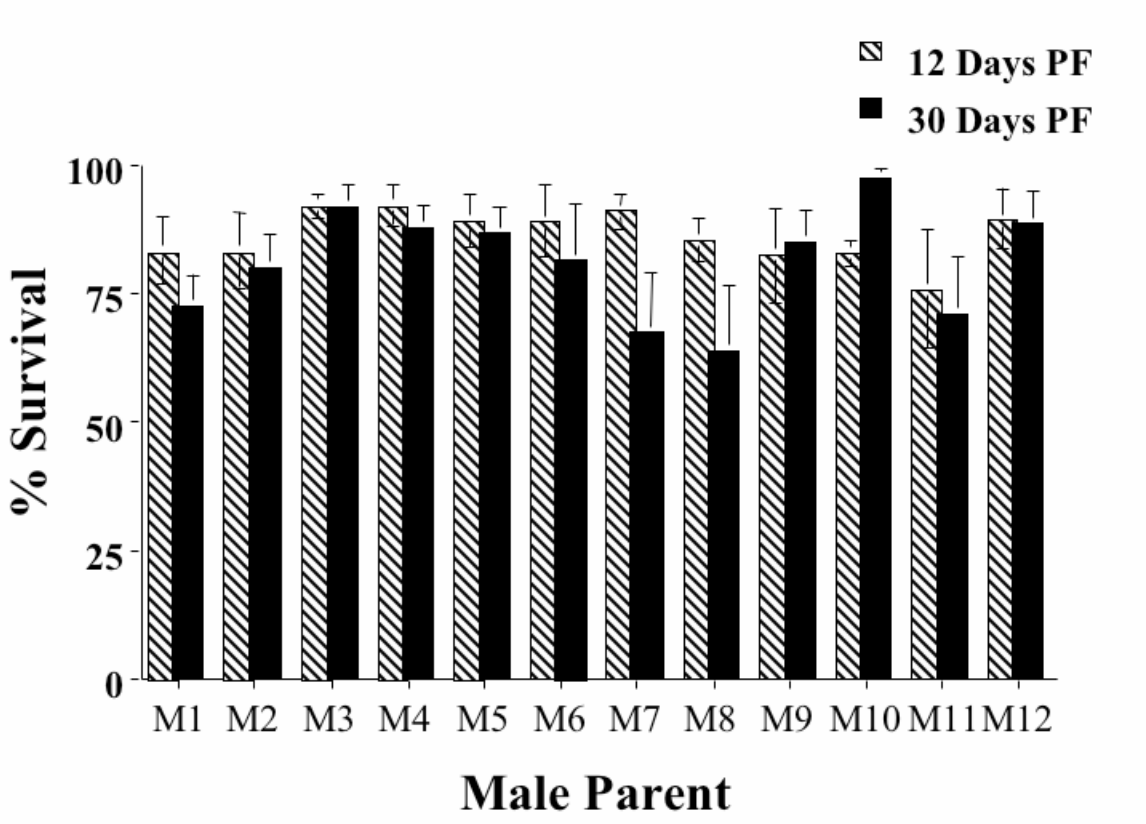

Figure 7. Embryo survival 12 d and 30 d post-fertilization expressed by male parent. Data are mean +/- SEM of crosses with 3 different females per male for brood year 1999 Redfish Lake sockeye salmon spawned on 25 October 2002. No significant effects of male parent on embryo survival were found.

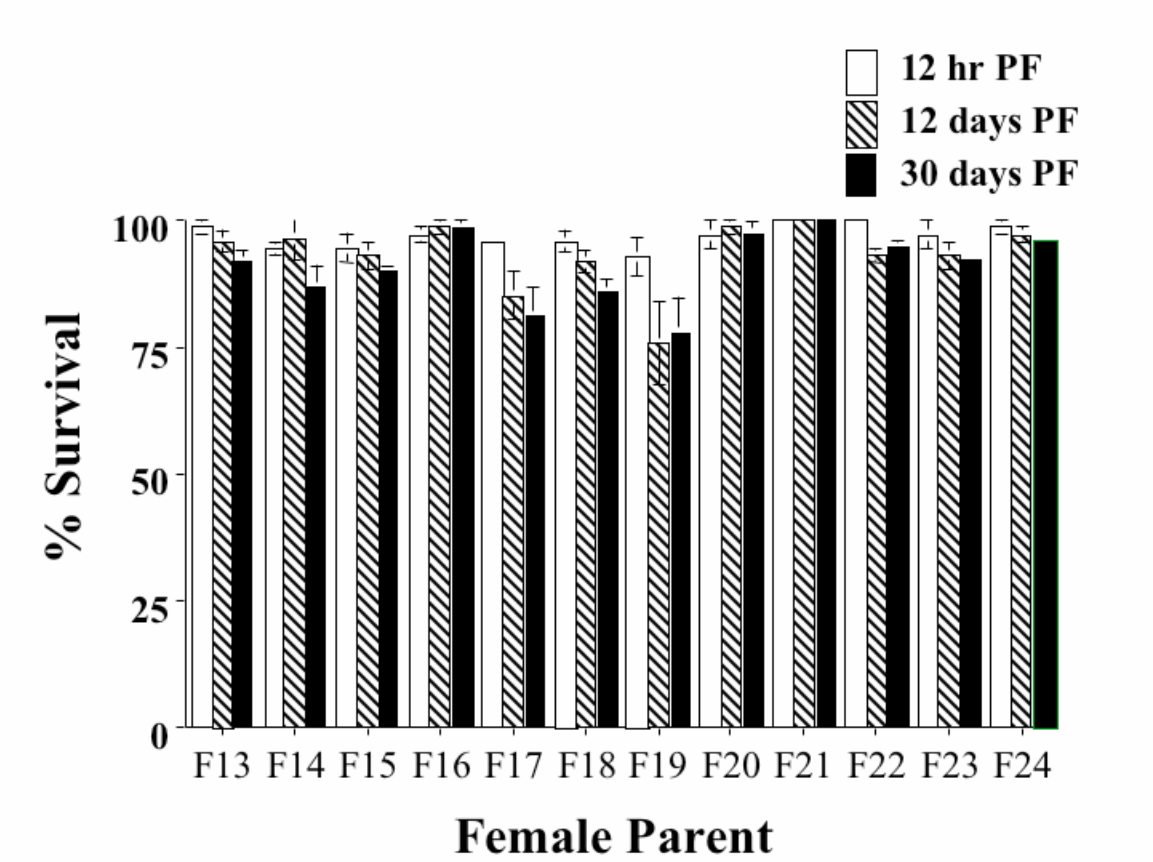

Figure 8. Embryo survival 12 h, 12 d and 30 d post-fertilization (PF) expressed by female parent. Data are mean +/- SEM of crosses with 3 different males per female for brood year 1999 Redfish Lake sockeye salmon spawned on 30 October 2002. No significant effects of female parent on fertilization rates (12 h PF) were found. There were significant effects of female parent on survival to 12 d (P=0.003) and 30 d PF (P=0.0006), but no effect on fertilization rates.

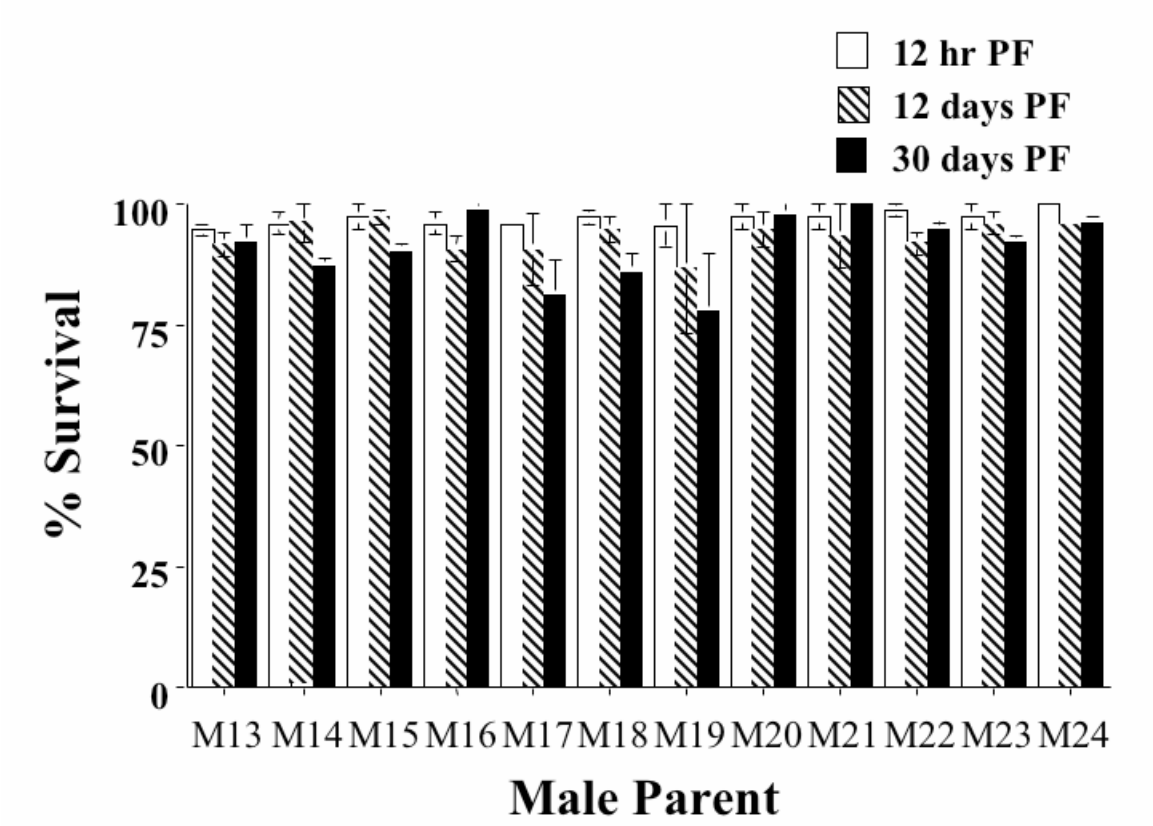

Figure 9. Embryo survival 12 h, 12 d and 30 d post-fertilization expressed by male parent. Data are mean +/- SEM of crosses with 3 different females per male for brood year 1999 Redfish Lake Sockeye salmon spawned on 30 October 2002. No significant effects of male parent on survival at any stage were found.

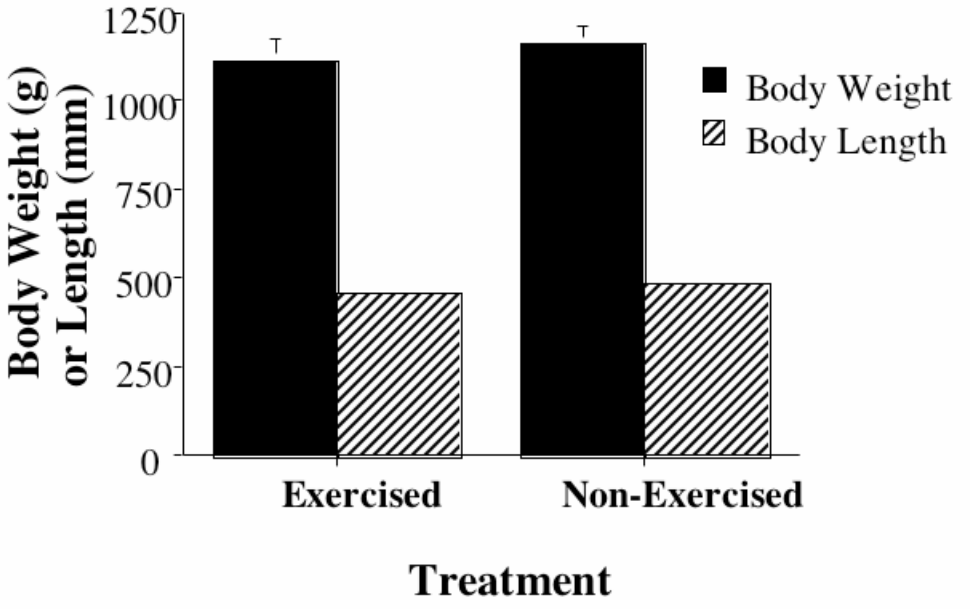

Figure 10. Body weight and length for brood year 1999 Redfish Lake sockeye salmon females spawned on 21 October 2003. Data are mean +/- SEM of six females per treatment. No significant effects of treatments were found on body size.

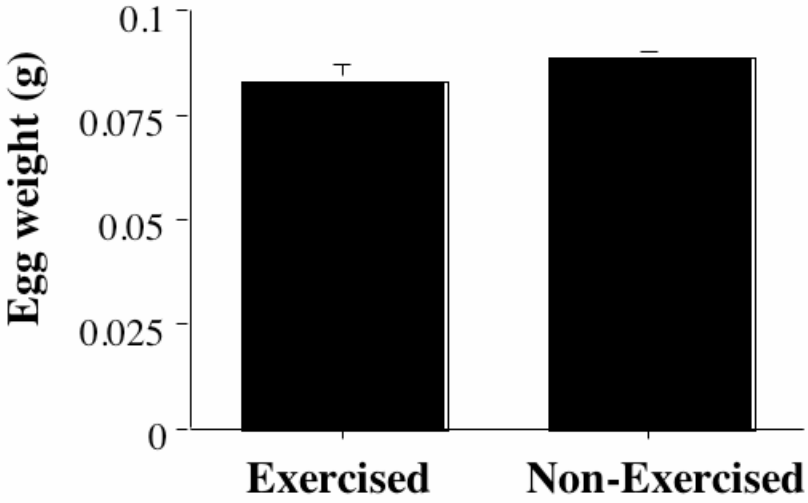

Figure 11. Egg size for brood year 2000 Redfish Lake sockeye salmon females spawned on 21 October 2003. Data are mean +/- SEM of six females per treatment. No significant effects of treatments were found on egg size.

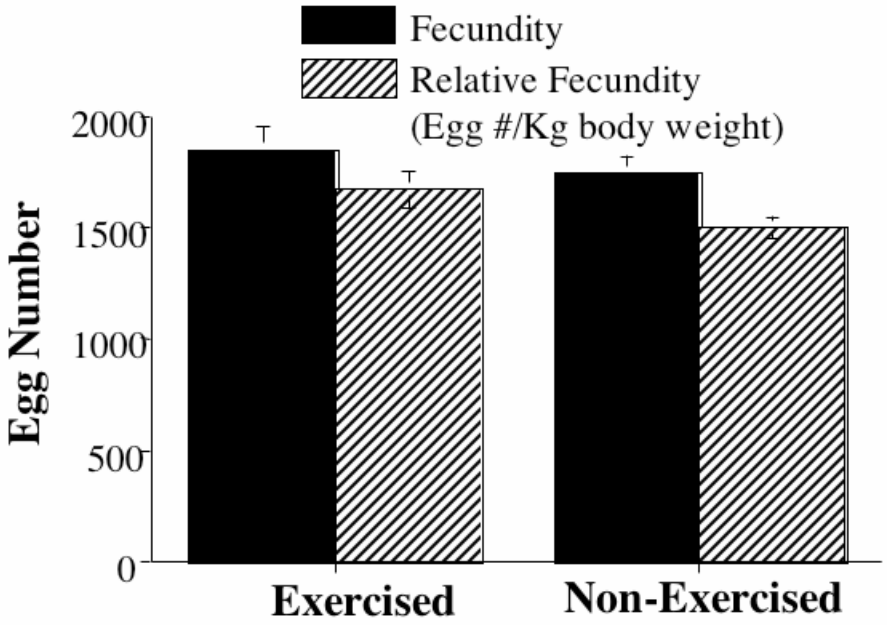

Figure 12. Total egg number and relative fecundity on brood year 2000 Redfish Lake sockeye salmon females spawned on 21 October 2003. Data are mean +/- SEM of six females per treatment. No significant treatment effects on egg number or relative fecundity were found.

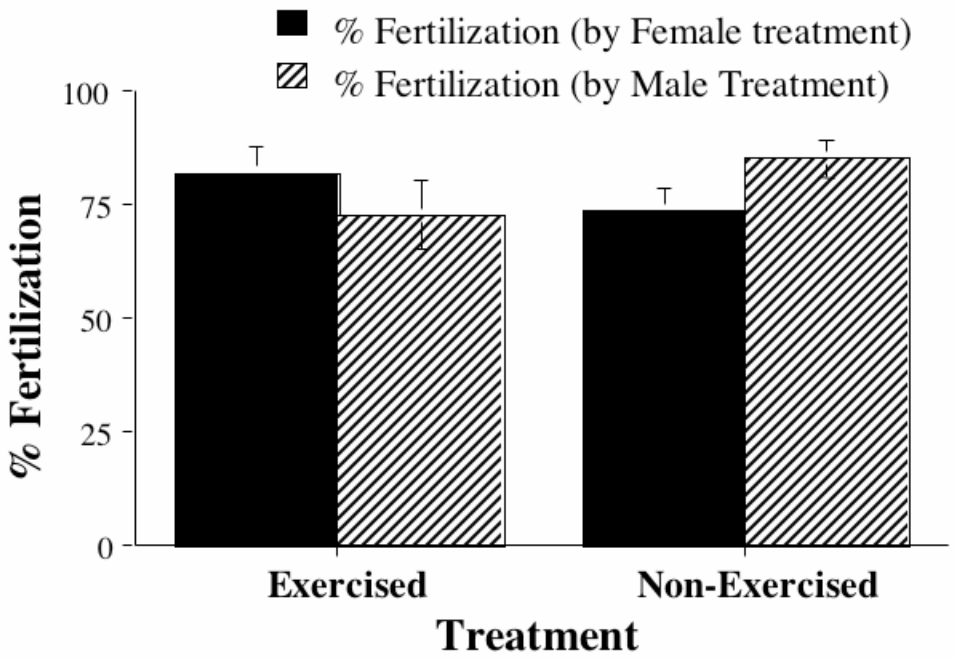

Figure 13. Percent of eggs fertilized by male or female treatment for brood year 2000 Redfish Lake sockeye salmon spawned on 21 October 2003. Data are mean +/- SEM for six males or females per treatment. Three batches of eggs from each female was fertilized with milt from three different males and each of these crosses were done in triplicate. Milt from each male was used to fertilize three different females, and again these crosses were done in triplicate. Thus, this was a 3x3 factorial design with triplication. There was no significant effect of treatment of fertility of males or females.

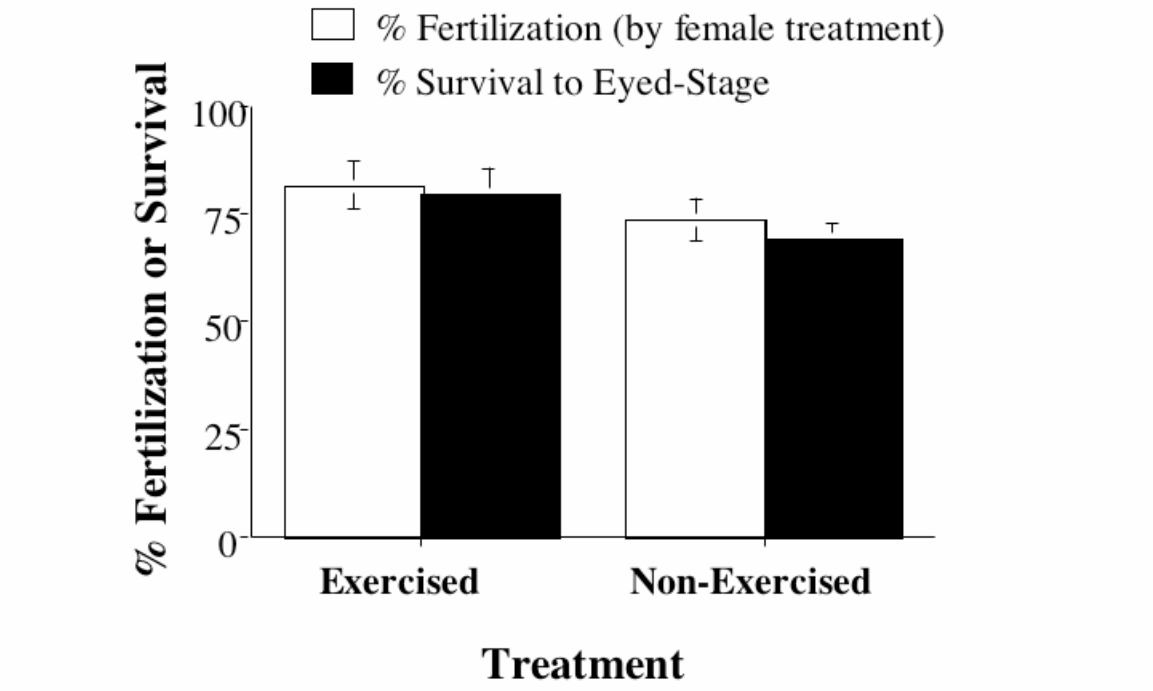

Figure 14. Percent of eggs fertilized and surviving to the eyed stage by female treatment for brood year 2000 Redfish Lake sockeye salmon spawned on 21 October 2003. Data are mean +/- SEM for six females per treatment. Three batches of eggs from each female were fertilized with milt from three different males and each of these crosses were done in triplicate. Milt from each male was used to fertilize three different females, and again these crosses were done in triplicate. Thus, this was a 3x3 factorial design with triplication. There was no significant effect of treatment on fertilization rates or survival to the eyed stage.

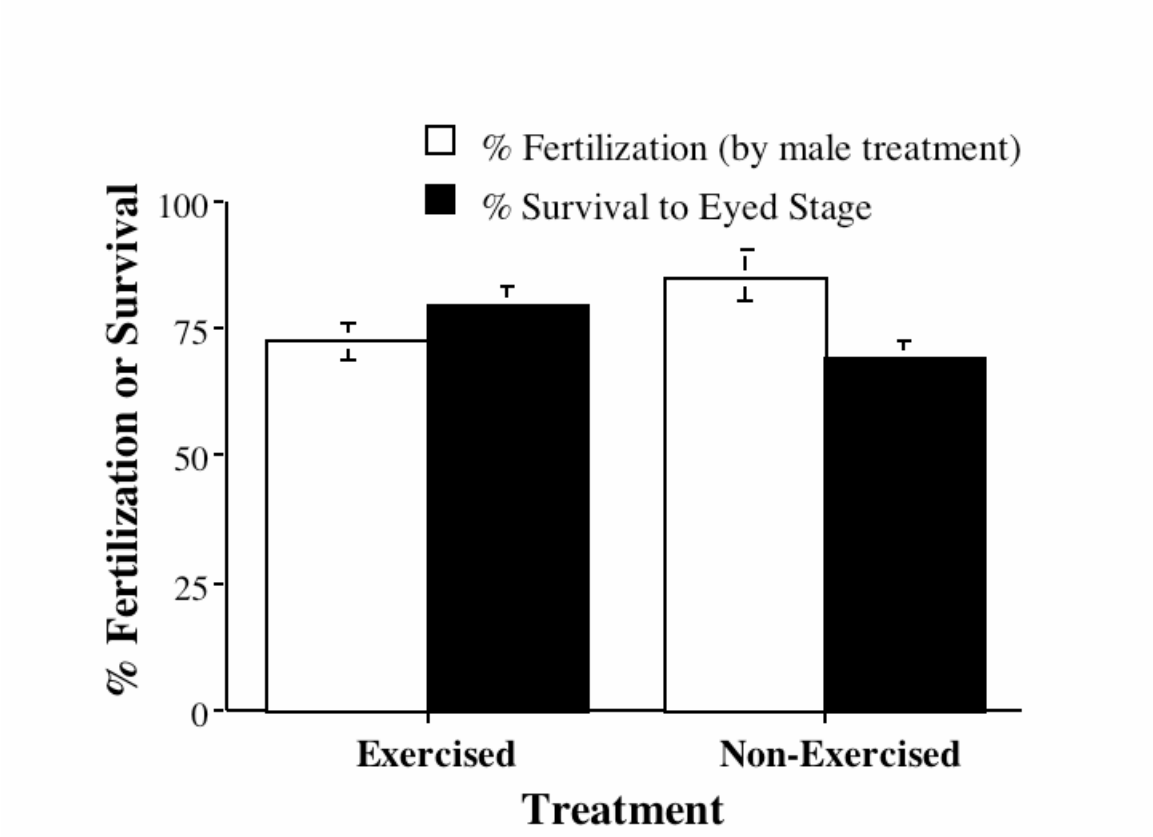

Figure 15. Percent of eggs fertilized and surviving to the eyed stage by male treatment for brood year 2000 Redfish Lake sockeye salmon spawned on 21 October 2003. Data are mean +/- SEM for six females per treatment. Three batches of eggs from each female were fertilized with milt from three different males and each of these crosses was done in triplicate. Milt from each male was used to fertilize three different females, and again these crosses were done in triplicate. Thus, this was a 3x3 factorial design with triplication. There was no significant effect of male treatment on fertilization rates or survival to the eyed stage.

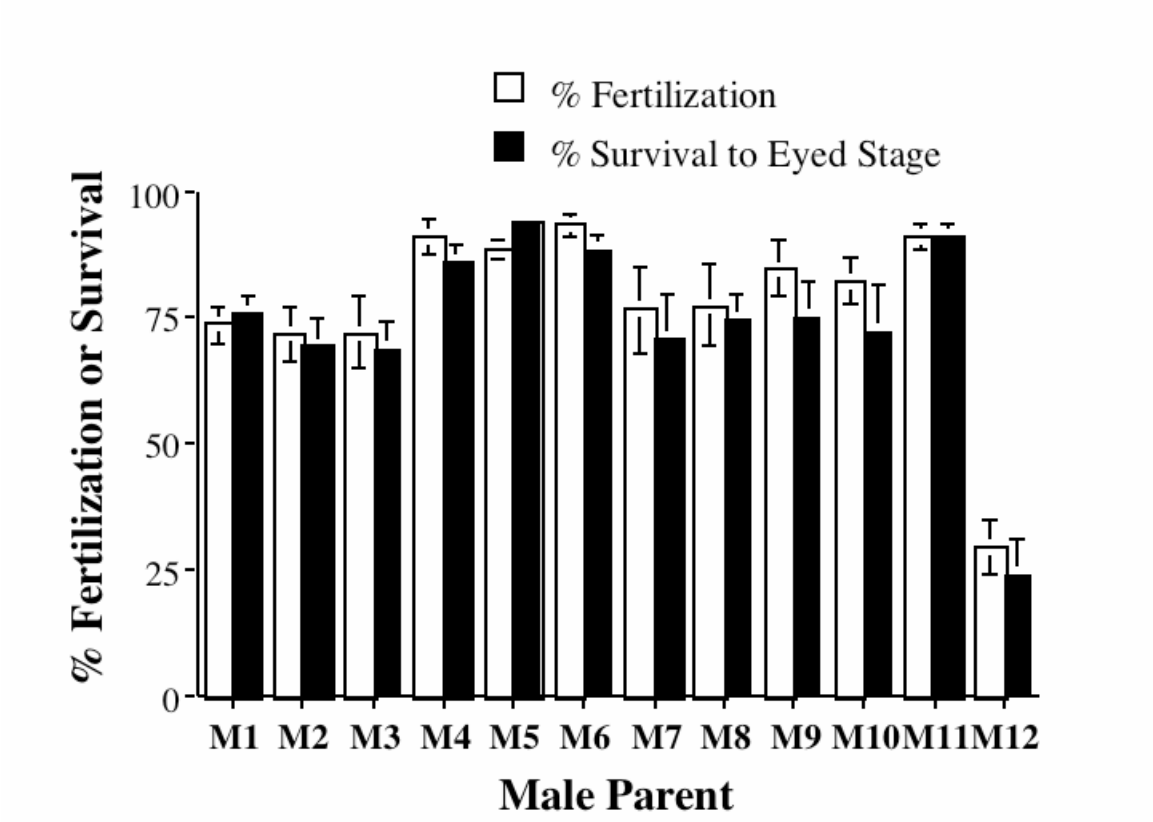

Figure 16. Percent of eggs fertilized and embryos surviving to the eyed stage by male parent for brood year 2000 Redfish Lake sockeye salmon spawned on 21 October 2003. Data are mean +/- SEM for crosses for with three different females. Significant effects of male parent on both fertilization and survival to eyed stage. For clarity of the figure, significant differences are not indicated.

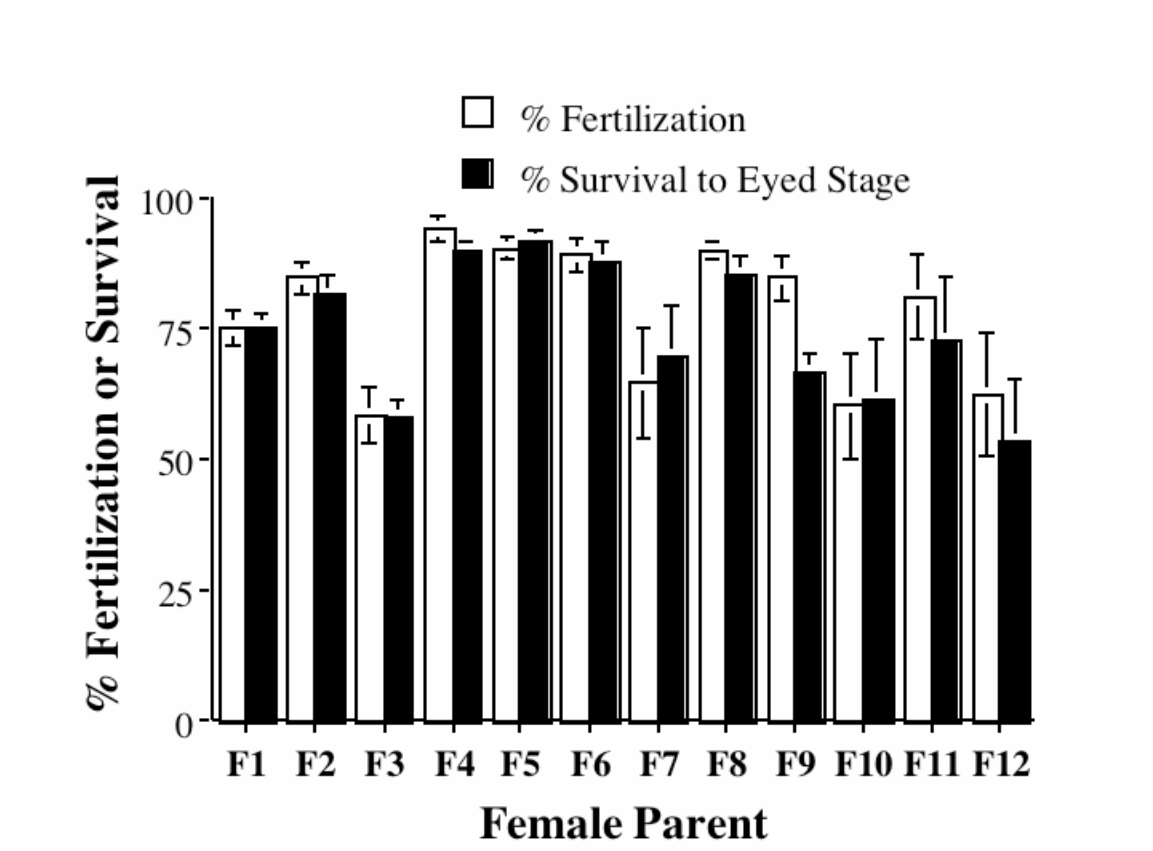

Figure 16. Percent of eggs fertilized and embryos surviving to the eyed stage by female parent for brood year 2000 Redfish Lake sockeye salmon spawned on 21 October 2003. Data are mean +/- SEM for crosses for with three different males. Significant effects of female parent on both fertilization and survival to eyed stage. For clarity of the figure, significant differences are not indicated.

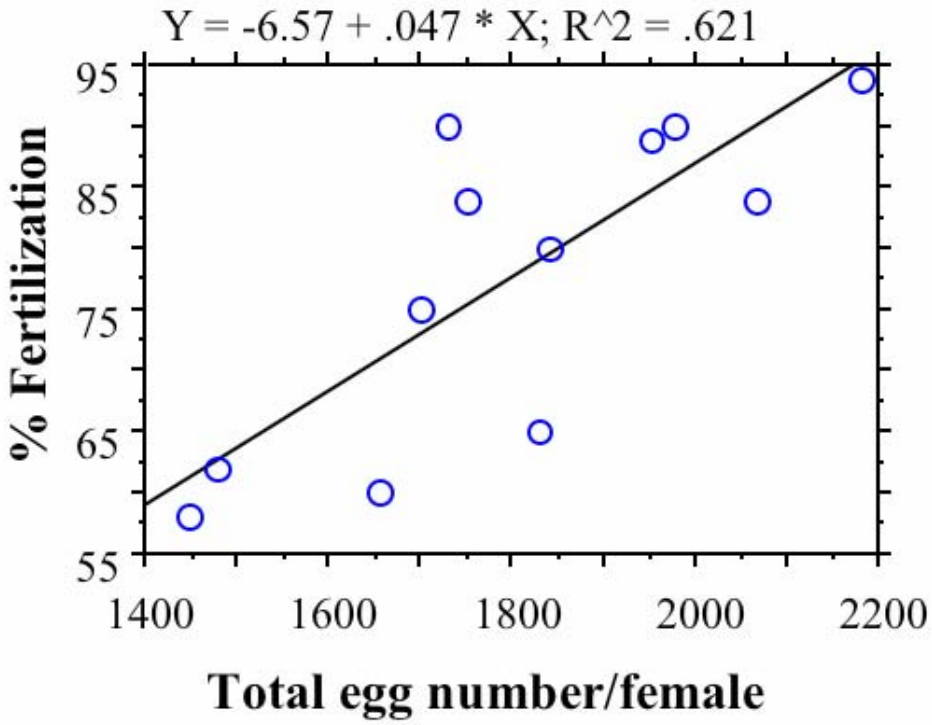

Figure 18. Regression relationship between total egg number per female (N = 12) and percent of eggs fertilized. Each data point represents the mean from crosses of one female with three different males.

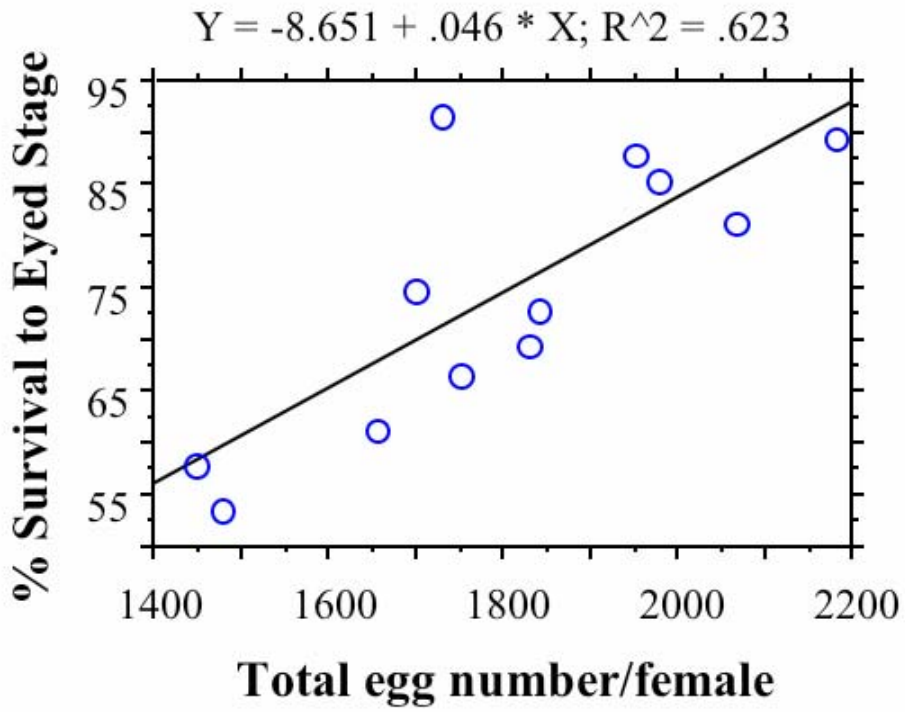

Figure 19. Regression relationship between total egg number per female (N = 12) and percent of embryos surviving to the eyed stage. Each data point represents the mean from crosses of one female with three different males.

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OBJECTIVE 4 – IMPROVING IN-CULTURE SURVIVAL OF JUVENILES

TASK 4. USE OF ANTIBIOTICS AND NEW VACCINES TO REDUCE MORTALITY FROM BACTERIAL KIDNEY DISEASE IN CHINOOK SALMON

By

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

Introduction

Bacterial kidney disease (BKD) is the principal bacterial disease confounding the successful culture of Pacific Northwest salmonids. Epizootics of BKD and chronic BKD infections have hampered and continue to negatively impact captive rearing programs of endangered Chinook and sockeye salmon.

While erythromycin administration has been widely used in captive broodstock rearing to combat BKD, recent experiments have revealed that erythromycin can reduce viability and fecundity (Fairgrieve et al. 2004[a]). An alternative macrolide antibiotic, azithromycin, has improved pharmacokinetics compared to erythomycin, displaying rapid uptake and long-term tissue retention (Fairgrieve et al. 2004[b]), without the palatability problems and toxic effects observed with erythromycin (Moffitt and Kiryu 2001, Schreck and Moffitt 1987). Meanwhile, the utility of azithromycin in preventing or treating BKD has not yet been definitively demonstrated. Studies of vaccines against BKD have identified the utility of a compound whole cell vaccine in treating and preventing BKD (Rhodes et al. 2004, Rhodes et al. 2004). The approach used here will assess the combined prophylactic impact of broodstock treatment with antibiotic (erythromycin or azithromycin) with or without subsequent juvenile fish vaccination with the compound whole cell vaccine.

Azithromycin exhibits characteristics suggesting it may be effective against intracellular bacteria, such as *Renibacterium salmoninarum*, the causative agent of BKD. These characteristics include chemical relatedness to erythromycin, accumulation in cell types susceptible to invasion by *R. salmoninarum* (Bandín et al. 1993, Gutenberger et al. 1997, Peters et al. 1992), slow excretion from salmonid tissues (Fairgrieve et al. 2004[b]), and transfer of antibiotic to progeny *in ovo* (Fairgrieve et al. 2004). However, use of azithromycin alone to control BKD is likely to have limitations. Because azithromycin targets ribosomal subunits, it may not be effective against dormant or quiescent *R. salmoninarum*. Profligate use of azithromycin presents a risk of drug-resistant bacteria, compromising future application of this antibiotic. Vaccination invokes a different strategy against BKD by stimulating a host immune response against *R. salmoninarum*. A compound vaccine consisting of a commercially available product, Renogen (Aqua Health, Canada), and a killed, attenuated isolate of *R. salmoninarum*, MT239 (Bruno 1988, O'Farrell et al. 2000, Senson and Stevenson 1999), has therapeutic value against

BKD (Rhodes et al. 2004, Rhodes et al. 2004). Coordinated employment of both azithromycin and vaccination is a logical progression in our studies on BKD prevention and control.

Materials, Methods, and Description of Study Area

A single cohort of fish were used in all experiments (Fairgrieve et al. 2004[b]). Fall Chinook salmon fry (George Adams stock) were obtained in January 1999, transferred to salt water six months later, and reared to maturity in fall of 2002. In September 2002, female fish received a single intraperitoneal injection of vehicle (deionized water), erythromycin (Gallimycin-100, 40 mg per kg body weight), or azithromycin (Zithromax, 40 mg per kg body weight). Male fish were not treated. In September and October of 2002, gametes were collected from ripe fish, single male-female crosses were made, and fertilized eggs were incubated at 10°C until hatching (December 2002) and swim-up (January 2003).

Experiment 1: Efficacy of broodstock antibiotic treatment on pre-smolt offspring against BKD

Fry of sham-injected or antibiotic-injected broodstock were held in 10-12°C water until April 2003 (mean length = 56.9 mm; mean weight = 2.14 g). Fish were challenged by intraperitoneal injection with a virulent isolate of *R. salmoninarum* at three challenge doses (100 cells per fish, 1,000 cells per fish, or 10,000 cells per fish) or with the peptone-saline vehicle only. Duplicate tanks for each challenge group and broodstock treatment group were used, with 25-29 fish per tank. For the highest challenge dose, progeny from erythromycin-injected broodstock were not used. Fish were checked daily for mortalities up to 104 d post-challenge. Selective KDM plates were inoculated from the kidneys of mortalities to confirm presence of *R. salmoninarum* growth.

Experiment 2: Efficacy of broodstock antibiotic treatment and vaccination against BKD before smoltification in offspring

Fry of injected broodstock were held at 10-12°C water. For each broodstock treatment group, approximately 200 fish were intraperitoneally injected with the phosphate-buffered saline (PBS) vehicle (mock vaccinated) and approximately 200 fish were injected with the compound vaccine (vaccinated) at four months after swim-up (Rhodes et al. 2004). All fish were PIT tagged at five months after swim-up and held in fresh water until June 2003, when they were transferred to salt water. In October 2003, half of the fish in each broodstock and vaccination treatment (~100 fish) each received an intraperitoneal injection of the peptone-saline vehicle (mock challenge) or of a virulent *R. salmoninarum* isolate (challenge). Fish were fed at 1.5% body weight and mortalities were removed daily. Gross pathology observations, gender, length, weight, and kidney weight were recorded at necropsy, and the entire kidney (anterior and posterior kidney) was collected for ELISA.

Experiment 3: Efficacy of broodstock antibiotic treatment and vaccination against BKD after smoltification in offspring

Fry of antibiotic-injected broodstock were held at 10-12°C water and all fish were PIT tagged at five months after swim-up and held in fresh water until June 2003, when they were transferred to salt water. In September 2003, approximately 200 fish of each broodstock treatment were intraperitoneally injected with the phosphate-buffered saline (PBS) vehicle (mock vaccinated) and another ~200 fish were injected with the compound vaccine (vaccinated). In October 2003, half of the fish in each vaccination treatment (~100 fish) each received an intraperitoneal injection of the peptone-saline vehicle (mock challenge) or of a virulent *R. salmoninarum* isolate (challenge). Fish were fed at 1.5% body weight and mortalities were removed daily. Gross pathology observations, gender, length, weight, and kidney weight were recorded at necropsy, and the entire kidney (anterior and posterior kidney) was collected for ELISA.

Results and Discussion

Work completed

Experiment 1: Efficacy of broodstock antibiotic treatment on pre-smolt offspring against BKD

Due to a 24-hour failure in the water chiller at day 66 post-challenge, survival analysis was performed only up to 66 d post-challenge. No difference in survival was observed among the mock-challenged groups or challenged groups except for the highest dose group. Among fish challenged with 10,000 virulent *R. salmoninarum* cells per fish, progeny of broodstock treated with azithromycin exhibited significantly greater survival than progeny of sham-treated broodstock (P=0.0005). The results indicate that broodstock treatment with azithromycin immediately prior to spawning can protect against acute challenge with *R. salmoninarum*.

Experiment 2: Efficacy of broodstock antibiotic treatment and vaccination against BKD before smoltification in offspring

The fish challenge portion of the experiment has been completed. All of the necropsies of the challenged fish have been completed (N = 599), and 273 of the kidney ELISA values have been determined. Raw data analysis (i.e., unadjusted by ELISA values) shows survival is significantly greater among fish vaccinated as pre-smolts ($P < 0.0001$). ELISA determinations for the remaining mortalities will permit a more refined survival analysis.

Experiment 3: Efficacy of broodstock antibiotic treatment and vaccination against BKD after smoltification in offspring

The fish challenge portion of the experiment has been completed. All of the necropsies from the challenged fish have been completed (N = 601), and ELISA determinations for 204 fish have been performed. Due to the two-factor design of this experiment, ELISA determinations for the remaining mortalities are needed for an accurate analysis.

Work to be completed

While experiment 1 has been completed, data analysis of experiments 2 and 3 are still in progress. All fish challenge studies have been completed. All of the kidney ELISA determinations for the challenged fish will be completed. Due to the high survival among the mock challenged fish and the likelihood of consistently low ELISA values, a subsample of 20% of the mock challenged survivors from each treatment group will be necropsied and kidney tissue will be analyzed by ELISA. If there is a lack of homogeneity of ELISA values within a group, additional necropsies and analyses (up to 100% of the group) will be performed. All laboratory work is expected to be completed by July 2004, and data analyses completed by August 2004. Completion will not require additional funds. A manuscript describing these experiments will be prepared by November 2004.

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OBJECTIVE 5 – EVALUATE EFFECTS OF INBREEDING AND INBREEDING DEPRESSION

TASK 5. DETERMINE THE EFFECTS OF CONTROLLED INBREEDING ON SURVIVAL, DEVELOPMENT, AGE STRUCTURE, AND OTHER ASPECTS OF THE LIFE HISTORY OF CHINOOK SALMON

By

Jeffrey J. Hard and Kerry A. Naish

Introduction

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

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

Even if inbreeding depression leads to higher risk of extinction, it is difficult to evaluate this risk relative to other risks, such as catastrophic loss or domestication of animals in captivity, and population fragmentation or local extinction in the wild. This is particularly true in light of recent evidence that inbreeding depression may reduce fitness sharply at intermediate levels of inbreeding (Frankham 1995), and its extent is likely to vary in different environments (Pray 1994, Reed et al. 2002).

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

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

Materials, Methods, and Description of Study Area

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

* 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.

We have reached conclusions for the first two hypotheses, in both cases rejecting the null hypothesis (H_{01} and H_{02}) in favor of the second alternative hypothesis (H_{a12} and H_{a22}). These conclusions are discussed later in this report. The third hypothesis will be addressed conclusively upon maturation of 2002- and 2003-brood University of Washington Hatchery (UWH, Lake Washington, Puget Sound) Chinook salmon. The completed return of first-generation inbred Grovers Creek Hatchery (GCH, Kitsap Peninsula, Puget Sound) stock Chinook salmon in fall 2003 marked the first opportunity to complete the evaluation of inbreeding depression over the life cycle in released fish upon a single generation of inbreeding. We summarized these analyses in this and last year's annual reports.

We established the initial breeding design for this study in 1994 from 150 (30 male, 120 female) adult Chinook salmon as they returned to GCH near Suquamish, Washington. We used a paternal half-sib breeding design (often referred to as North Carolina Design 1, Falconer and Mackay 1996) to establish the initial family structure. We released the 1994-brood progeny of these fish either from GCH or reared them in marine net-pens at Manchester. Captive fish matured between 1995 and 1999 and anadromous releases returned to GCH during the same period. We mated adults in each of these years to create first-generation inbred (F_2 generation of the study) fish to be reared at and released from UWH on Lake Washington. These fish returned to UWH between 1999 and 2003.

In 2003-2004, work continued to address the stated hypotheses through analyses of biological data from fish cultured at the University of Washington's School of Aquatic and Fishery Sciences Hatchery (UWH) or at the Manchester Research Station (MRS). In May 2003, we transferred 3,600 2002-brood Chinook salmon to MRS for marine culture to maturity; in May 2004, we transferred approximately 3,400 2003-brood Chinook salmon to MRS. One complete generation of experimental inbreeding in one population (GCH) was complete with the maturation of adult inbred progeny (through age-5) in November 2003. Current work is focused largely on the UWH population, which previously served as an experimental control for the GCH population. This report summarizes current progress and ongoing analyses.

Results and Discussion

To date, project investigators have:

- Collected fall Chinook salmon broodstock from adults returning to Grovers Creek Hatchery (Puget Sound, Washington) in 1994.
- Established, using a conventional quantitative genetic breeding design (Falconer and MacKay 1996), an experimental population at Grovers Creek Hatchery structured of 96 full-sib families nested within 30 half-sib families.
- In 1995 released to sea 257,093 of these fish from Grovers Creek Hatchery, each identified with full-sib family-specific coded-wire tags.
- Cultured approximately 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 (F_2 generation of the study) from matings of 1994-brood parents at UWH. The experimentally inbred lines correspond to a minimal increment in inbreeding (randomly mated control), a moderate increment in inbreeding (half-sib parents, corresponding to an approximate increase in inbreeding, ΔF, of 12.5%), and a substantial increment in inbreeding (full-sib parents, corresponding to an approximate increase in inbreeding, ΔF, of 25%).
- Released to sea from UWH 10,654 1997-brood CWT smolts, composing a total of 28 families in six experimental groups.

- Established at MRS in marine netpens 3,618 1997-brood PIT-tagged smolts, composing a total of 28 families in six experimental groups.
- Released to sea from UWH 85,111 1998-brood CWT smolts, composing a total of 70 families in four experimental groups.
- Established at MRS in marine netpens 2,088 1998-brood PIT-tagged smolts, composing a total of 70 families in four experimental groups.
- Collected biological and coded-wire tag information from approximately 1,600 adults returning to UWH between 1999 and 2003 (total return exceeding 4,000). These tags have now been decoded. We constructed few matings in these years from these fish because too few experimental females were available for a full mating design. We did, however, create six matings of control and first-generation inbred GCH stock fish at UWH in fall 2002 for an experiment to raise non-inbred and inbred fish to maturity to evaluate reproductive behavior and mating success in these two groups. We also created approximately 105 matings of UWH stock Chinook salmon at UWH in both 2002 and 2003, which provide an opportunity to initiate inbreeding in an independent population as well as a means of estimating the genetic basis of phenotypic variation in life history. Finally, in 2003 we established 8 rearing groups of meiotic gynogens to evaluate inbreeding depression in UWH stock individuals under higher rates of inbreeding than are possible under conventional breeding.

We summarized preliminary data on the effects of one generation of inbreeding to adulthood in GCH individuals up to age-4 at maturity in last year's report. In this report we incorporate GCH returns through age-5, and reevaluate the preliminary conclusions on inbreeding in this stock in light of the additional information.

Inbreeding effects on marine survival

In a previous report, we summarized analyses evaluating inbreeding on survival rate in GCH stock Chinook salmon returning to UWH by 2002 (i.e., through return of age-4 adults). By contrast with analyses of effects of inbreeding expressed during early life history in 1998-brood fish, which failed to reveal significant effects of inbreeding among all groups, analyses through adulthood found appreciably lower survival among progeny of full-sibling parents relative to half-sibling or unrelated parents. We attributed our inability to detect significant effects of inbreeding at the juvenile stage primarily to low power reflecting an insufficient number of families within each group. Retrospective power analyses showed that the greatest power among three separate nested ANOVAs was approximately 10%, indicating that many more families within each group would be required to detect a significant effect of inbreeding, given this pattern of variation. There was also no linear relation evident between the level of inbreeding and inbreeding depression in survival, and no linear relation evident between variance of the survival rate and the level of inbreeding at any developmental stage**.**

However, ultimately inbreeding depression should be evaluated over the entire life cycle. The most appropriate measure of inbreeding depression is a comparison of lifetime reproductive success between related and unrelated individuals. In the absence of this

information, evaluation of marine survival rates does provide a useful indicator of the pattern of fitness differences between free-ranging inbred and non-inbred individuals in the wild environment. Our releases from UWH of 1997- and 1998-brood inbred and control GCH stock Chinook salmon resulted in recoveries of these fish at UWH as adults in 1999-2003. 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 GCH population), and these fish were in poor condition at time of release from UWH due to an *Aeromonas salmonicida* epizootic. No 1997-brood study fish had been recovered at the UWH rack or in fisheries by 2003, according to the PSMFC coded-wire tag database and UWH records.

The 1998-brood release of study fish, which were progeny of 4-year-old females returning to GCH, were composed of more fish in good overall condition at time of release. Most of the fish from this release returned to UWH in 2001 and 2002. Of 1,069 total adults that returned to UWH in 2002, we identified from coded-wire tags 20 adults as GCH stock study fish; an additional 31 were UWH stock controls. In 2003, only 3 age-5 GCH adults returned to UWH, and all of these were progeny of unrelated adults. We completed a preliminary analysis of DNA genotypes of the GCH adults returning to UWH between 2001 and 2003, an important development in our evaluation of the groups' survival rates. This analysis indicates that some fish had originally been misclassified by group based on the coded-wire tag information. The misclassification based on tag data probably reflects the mixing of juvenile fish from different groups during rearing before marking (e.g., through movement between incubation trays, or jumping or accidental transfer between adjacent rearing tanks). A total of 7 fish are known to have been misidentified, and all of them originally thought to be inbred were noninbred controls: 6 fish identified as progeny of full-sib parents and 1 fish identified as progeny of half-sib parents were, based on the corrected data, progeny of unrelated parents.

Based on analysis of these data through 2003 (i.e., through age-5 recoveries, or an essentially complete cohort), the return of adults to the UWH pond from the full-sib group was less than one fifth the return from both the half-sib and unrelated groups, which had similar return rates. The corrections to the data increased the disparity in survival between full-sib inbred and non-inbred control fish—and therefore an increase in the estimate of the degree of inbreeding depression. The return rate for the GCH stock study fish to the UWH pond varied from less than 0.06 % for the full-sib group to 0.35 % for the half-sib group and nearly 0.39 % for the unrelated control group, while the return rate was 0.90 % for UWH stock study control fish (Fig. 1). The differences in return rates of the four groups of fish were highly significant ($G = 282.6$, $df = 3$, $P < 0.001$). As reported last year, much of this significance was due to the higher returns of the endemic UWH stock fish. Excluding that group from analysis, the differences in return rates among the three GCH stock fish were still highly significant ($G = 87.1$, $df = 2$, $P < 0.001$), primarily reflecting the lower returns of the full-sib group relative to the half-sib and unrelated groups. Collectively, these data indicate that free-ranging GCH stock study fish introduced to UWH had lower survival rates to return than did the local UWH stock control fish, but that inbreeding in progeny of full-sib study fish within the GCH stock

further reduced survival substantially. Therefore, the patterns of estimated survival within the GCH stock study fish are consistent with inbreeding depression: progeny of fish that were full siblings (approximate increment in F of 0.25) survived to return at much lower rates than did progeny of fish that were half siblings (F ~ 0.125) or unrelated individuals (F ~ 0). The pattern is far from linear, suggesting a possible threshold effect of inbreeding on survival when F is between 0.125 and 0.25 (Fig. 1).

The corrections applied to the recovery data from the DNA analyses do not materially alter the conclusions we reached for developmental asymmetry or growth and size to adulthood in GCH stock Chinook salmon (data not shown). As summarized in last year's report, the pattern of asymmetry in several paired traits (primarily gill raker and fin ray counts) among the inbred and control groups was an unexpected one. Fish from the control (unrelated) matings had the highest degree of fluctuating asymmetry. The control fish did, however, exhibit lower levels of directional asymmetry. These results are difficult to interpret, but one possibility is that the fluctuating asymmetry indices are biased by the presence of directional asymmetry (Graham et al. 1998), which may account at least in part for these contrasting patterns. Another possibility is that the indices of fluctuating asymmetry are not very sensitive indicators of developmental instability. However, the substantial broad-sense heritabilities estimated for fluctuating asymmetry and directional asymmetry in these paired traits do indicate that genetic and/or common environmental variation among families contribute substantially to expressed variation in asymmetry, a finding that contrasts sharply with those of Bryden and Heath (2000).

The comparison of size of adult male and female fish at age summarized in last year's annual report indicated that GCH stock study fish generally grew at lower rates while at sea than did UWH stock fish. Among the GCH stock inbreeding groups, no clear differences in growth were detectable, and incorporating data from the three five-year-old GCH fish that returned in 2003 from unrelated parents did not change this result. The general trends in growth within the GCH stock study fish were opposite those expected if inbreeding depression reduced growth. It is conceivable that this pattern reflects a situation in which survival and rapid growth to adulthood are strongly linked to inbreeding level (i.e., slower-growing inbred fish survived at lower rates than slower-growing noninbred fish), but other mechanisms are possible because of the limited power of the size analysis.

Composition and matings of UWH parents returning in 2002 and 2003

We mated representative UWH-stock adult Chinook salmon returning to UWH in 2002 and 2003 to create half- and full-sibling families which, upon their maturation, will be used to create inbred and outbred lines. The primary purpose of these UWH matings was to establish an independent population sufficiently large to evaluate inbreeding depression—in effect, replication for the work already underway with the GCH population. Between 16 October and 6 November 2002, we bred 36 adult males to 126 females from the UWH stock to create 126 2002-brood full-sib families in a hierarchical breeding design (mean of 3-4 females per male). We selected these adults at random

from the middle three weeks of the six-week run (mean spawning date, 27 October) (Fig. 2). Mean male size (± SD) was 724.6 ± 117.1 mm and 4.27 ± 2.07 kg; mean female size was 772.8 ± 71.1 mm and 6.15 ± 1.68 kg. The size distributions are shown in Figure 3; mean fecundity was 4936.4 ± 936.5 eggs (distribution shown in Fig. 4).

In October and November 2002 we also had an opportunity to breed six males to six females from the GCH stock to create six additional full-sib families: three noninbred and three first-generation inbred. Insufficient adults were available in 2002 to establish second-generation inbred individuals; the purpose of these crosses is to provide a small group of first-generation inbred and non-inbred adults in which we could eventually evaluate adult reproductive behavior. Specifically, we hope to determine if closely related adults recognize and avoid each other during courting and breeding. We had an opportunity to evaluate this question in 1998; unfortunately, the adults we identified for the behavioral study were lost when a pump failed in a raceway at the Grovers Creek Hatchery. We expect the 2002-brood adults to be available for another attempt at this experiment by autumn 2006.

Between 13 October and 16 November 2003 we mated 37 adult males to 125 females from the UWH stock to create 125 2003-brood full-sib families in a replicate hierarchical breeding design. We selected these adults at random from the middle four weeks of the six-week run (mean spawning date, 24 October) (Fig. 2). Mean male size was 820.2 ± 107.7 mm and 6.02 ± 2.47 kg; mean female size was 789.4 ± 68.0 mm and 6.29 ± 1.75 kg. The distributions are shown in Figure 3; mean fecundity was 4160.7 ± 929.2 eggs (distribution shown in Fig. 4).

To explore the feasibility of determining effects of inbreeding rates higher than those possible from sexual reproduction within a generation, in 2003 we attempted to make presumptive genetic gynogens from 2003-brood UWH fish. Gynogenesis is a form of asexual reproduction (parthenogenesis) in which development in an egg is activated by sperm without fusion of the haploid egg and sperm nuclei to form a diploid zygote. Gynogens can be created by initiating egg development with inactivated sperm (e.g., through UV irradiation) and interrupting cell mitosis or meiosis. The first type of gynogen is mitotic, formed from the recombination of two haploid daughter cells before the first mitotic division. Mitotic gynogens are completely inbred (F = 1.0), but the survival rate of these fish is typically very low. Indeed, we earlier attempted to create several mitotic gynogens from UWH broodstock, but their survival to the eyeing stage was only a fraction of 1 %. The second type of gynogen is meiotic, formed from retention of the second polar body during meiosis. The rate of inbreeding in meiotic gynogens depends directly on the recombination rate (generally it is in the range of 25-75%), and their survival can be considerably higher than that of mitotic gynogens (from generally less than 20% to perhaps as high as 70% of control progeny, in some cases).

After considering the alternatives and the "failure" of the mitotic gynogens, we established 13 meiotic gynogen matings in October and November 2003. The details of the treatments are summarized in Table 1. Egg lots from each female were "fertilized" with UV-irradiated milt from a single male and the resulting embryos heat shocked for 15

min at 27.5 C within 10 min after fertilization. All ultraviolet (UV) treatments of male sperm were 300μW for 180 second, with milt diluted to 1 % by volume. Four families had enough survivors at hatching in December 2003 to continue into culture. We split each of these families into two groups, one which experienced the conventional hatchery rearing regimen and one that was treated after fertilization with methyl-testosterone (MT) and subsequently fed for 100 d with feed treated with MT. The objective of the MT treatment is to masculinize the treated fish to increase the proportion of phenotypic males in the treated group. The survival rates of the gynogen crosses to the eyeing stage are summarized in Table 2. We PIT tagged 100 fish in each of the eight groups in April 2004. If these fish survive to maturity, we will mate them with full-sibling females (sisters) to produce progeny that are more highly inbred (a 50-100 % increase in inbreeding coefficient) than is possible with breeding of simple relatives alone.

Although the fitness of gynogens may be affected by factors independent of inbreeding because of the nature of the genome manipulation and environmental treatments, the performance of these fish should provide an additional point of reference for characterizing inbreeding depression—a glimpse of the magnitude of inbreeding depression at higher inbreeding levels without the additional generations usually required to achieve those levels.

Size and growth of 2002- and 2003-brood progeny

We ponded 2002-brood fry between 6 January and 11 February 2003 in 34 half-sib and 103 full-sib families for the UWH stock and six full-sib families for the Grovers Creek stock. The 34 half-sib and 103 full-sib UWH stock families showed detectable genetic variation in size and growth throughout the sampling period between April 2003 and April 2004 (Fig. 5). After accounting for variation among parents in spawning date, heritabilities and genetic correlations estimated for size exhibited interesting patterns over this period; the heritabilities varied from 0.09 to 0.70 for length and from 0.10 to 0.44 for weight in the different samples, while the corresponding genetic correlation between length and weight varied between 0.92 and 0.99 (Table 3). These estimates indicate variable genetic control of expression of size in different samples, which may reflect not only environmental anddevelopmental variation, but also a common group of genes influencing length and weight.

The heritability estimated for growth rate over this year-long period was 0.54 (Table 3). Interestingly, growth rate was negatively genetically correlated with initial weight (-0.86), suggesting a genetic propensity for larger fry to grow at lower rates during their first year. The six families of 2002-brood GCH stock juvenile Chinook salmon growing (Table 4) in a common environment, seawater raceways, differed significantly in length and weight during each of the six samples taken between PIT tagging in April 2003 and sampling in April 2004 (length: $F = 117.66$, $df = 5$ and 589, $P < 0.001$; weight: $F = 19.05$, $df = 5$ and 589, $P < 0.001$). ANOVAs confirmed evidence of substantial family effects (e.g., at PIT tagging, $F = 15.20$, $df = 10$ and 610, $P < 0.001$; for the last sample, $F = 5.62$, $df = 10$ and 308, $P < 0.001$). The growth trajectory of these fish is depicted in

Figure 5. The growth of the fish in weight also showed substantial family variation over this period ($F = 5.46$, $df = 5$ and 98, $P < 0.001$).

We ponded 2003-brood UWH fry between 5 January and 15 February 2004. At ponding we had sufficient fish for evaluation in 105 full-sib families sired by 30 fathers. Juveniles were sampled from each of the families on three dates between January and April 2004 at approximately six-week intervals (total fish sampled each date = 2,651). The growth trajectories for these fish are depicted in Figure 6. From 12-26 April, we sampled 25 fish from each of the full-sib families for PIT tagging and transfer in May to seawater raceways for grow-out to maturity. The total number of fish measured on each date was approximately 2,600. After accounting for variation among parents in spawning date and for rearing tank effects through ANCOVA, we estimated substantial heritabilities for length and weight, and the genetic correlation between these traits. These genetic parameters were moderate to high over this period; the heritabilities varied from 0.34 to 0.68 for length and from 0.26 to 0.66 for weight in the different samples, while the corresponding genetic correlation between length and weight varied between 0.91 and 0.93 (Table 5). In addition, the heritabilities for exponential growth in weight between the sample periods and the genetic correlations between growth rates in different periods were moderate to high after the initial sample period. The heritabilities for growth rate varied from 0.15 to 0.54; the genetic correlation between different growth rates varied widely, from 0.03 to 0.80 (Table 6). As in the 2002 brood, these results collectively indicate substantial genetic control of juvenile size at this stage involving common genes influencing length and weight. However, these estimates also reflect a large environmental component of variation to size and growth, even in the less variable environment of a conventional hatchery. The genetic parameter estimates for body size obtained here for the UWH stock are similar to those obtained for related traits from the 1994-brood GCH population measured at an equivalent developmental stage (centroid size, a multivariate analog of length: 0.91 ± 0.02, weight: 0.99 ± 0.06, genetic correlation: 0.94 ± 0.03; Hard et al. 1999).

For each of the 8 split gynogen groups, we sampled and PIT tagged 100 juveniles on 16-17 April 2004. Mean lengths and weights (± SD) of the methyl-testosterone treated gynogens at time of sampling are summarized in Table 7 (untreated gynogens did not differ significantly from the other 105 untreated families; data not shown). The treated groups showed differences in length and weight at time of sampling after accounting for variation in spawn date (Hotelling-Lawley Trace = 1388.9, $df = 2$ and 601, $P < 0.001$). Univariate ANOVAs indicated substantial variation in both traits (length: $F = 14.56$, $df = 3$ and 602, $P < 0.001$; weight: $F = 25.00$, $df = 3$ and 602, $P < 0.001$).

On 12 May 2004, we transferred by tank truck to MRS 3,425 2003-brood UWH and GCH stock PIT-tagged Chinook salmon (mean weight, approximately 8 g). On 13 May, we began releasing to Portage Bay 105,000 2003-brood UWH stock coded-wire-tagged fish from UWH in 105 families of 1,000 fish (mean weight has not yet been compiled, but these fish are of similar size to the PIT-tagged groups). Volitional release is continuing, and remaining fish will be forced to leave the UWH pond by 1 June.

Work to be completed in 2004-2005

(i) Continuing culture of 2002- and 2003-brood GCH and UWH stock juvenile Chinook salmon in seawater raceways and netpens at MRS. In the case of 2002-brood GCH stock, these fish represent first-generation inbreds that we intend to culture to maturity for evaluation of reproductive behavior and success in relatives and non relatives. The UWH stock fish represent an attempt to initiate close inbreeding and evaluate inbreeding depression in an independent population that exhibits higher smolt-to-adult survival rates than GCH when released from UWH.

(ii) Collection of one- and two-year old 2003- and 2002-brood males maturing in autumn 2004 and returning to UWH, and estimation of survival and body length and weight data. We will rear and mark these fish at UWH. We intend to cryopreserve representative milt from males returning from the inbreeding matings, for later establishment of inbred UWH lines when adult females return (expected between 2005 and 2008).

For our analyses, we 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 described here and by Lynch (1988), Lynch and Walsh (1998), and Hard and Hershberger (1998). We plan to compare survival and growth among the 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).

Summary and Conclusions

In this study we are evaluating the consequences of inbreeding in two hatchery populations of ocean-type Chinook salmon and evaluating three basic hypotheses:

1. Inbreeding does not reduce fitness of Chinook salmon,
2. Inbreeding has no predictable linear effect on fitness in Chinook salmon, and
3. The effects of inbreeding in Chinook salmon are insensitive to environment.

To our knowledge this study is the first attempt since Ryman (1970) to investigate inbreeding depression in an anadromous salmonid over its entire life cycle. With the complete return of the Grovers Creek Hatchery (GCH) stock to the University of Washington Hatchery in 2003 we have reached conclusions on the first two of these hypotheses for the GCH population. We conclude that the effects of inbreeding expressed during early life history do not reveal significant effects of inbreeding. Survival during embryonic development in our study was highly variable; although we found some trends in survival consistent with inbreeding depression, our study was not powerful enough to

confirm the significance of these trends. (Retrospective power analyses showed that the greatest power among three separate nested ANOVAs was approximately 10%, indicating that many more families within each group would be required to detect a significant effect of inbreeding for juvenile traits, given this pattern of variation in the traits.) Bilateral asymmetry in several paired traits among the inbred and control groups showed an effect of inbreeding on fluctuating asymmetry, but in the direction opposite that expected. Fish from the control (unrelated) group had the highest degree of asymmetry (they did, however, exhibit lower levels of directional asymmetry). These results may reflect the possibility that the fluctuating asymmetry indices are biased by the presence of directional asymmetry; alternatively, they could indicate that the indices of fluctuating asymmetry evaluated here are not very sensitive indicators of developmental instability.

Our analyses of survival in the natural environment from smolt to adult detected lower survival among progeny of full-sibling parents relative to half-sibling or unrelated parents. There was no linear relation evident between the level of inbreeding and inbreeding depression in survival. In fact, the relationship between inbreeding level and marine survival showed evidence of a threshold effect, with survival declining after one generation of inbreeding only when the increment in inbreeding was equivalent to brother-sister mating. Collectively, these data indicate that free-ranging GCH stock study fish introduced to UWH had lower survival rates to return than did the local UWH stock control fish, but that inbreeding in progeny of full-sib study fish within the GCH stock further reduced survival significantly. The lack of consistent evidence that growth in the marine environment was reduced by inbreeding is an unexpected result, but this outcome could indicate a strong positive correlation between growth and survival of inbred fish.

Size and growth of juvenile 2002- and 2003-brood UWH stock fish, which are not yet inbred, appears to be influenced by genetic factors. Marked full- and half-sib family variation in length and weight in comparable samples and in exponential growth in weight between sample periods indicates generally moderate to high heritabilities for these traits and highly positive genetic correlations between length and weight. These results imply that a common set of genes influences juvenile growth in length and weight in both fresh and seawater.

To date, the study results point to the potential for anadromous Chinook salmon to respond rapidly to close inbreeding, with adverse consequences for marine survival and, possibly, growth. These results would support recommendations for initiating artificially propagated populations with sufficient, outbred broodstock and implementing carefully monitored breeding practices to minimize rates of inbreeding during a program's duration. Future results from the study should provide additional insight into how inbreeding depression and domestication affect performance and fitness in the different environments characterized by representative hatchery and captive broodstock programs.

Data Management Activities

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

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Table 1. Summary of treatments of 2003-brood University of Washington Hatchery (UWH) stock Chinook salmon used to create meiotic gynogens. All ultraviolet (UV) treatments of male sperm were 300μW for 180 sec, with milt diluted to 1 % by volume. The embryo treatment to initiate gynogenesis involved heat shock of 27.5 C for 15 min beginning 10 min after fertilization.

Date	Female	DNA No.	Male	DNA No.	Batch	Water temp. (C)
10/31/03	973	1	999/1000	11/12	4	13.3
10/31/03	974	2	999/1000	11/12	2	13.3
10/31/03	975	3	999/1000	11/12	3	13.3
10/31/03	978	4	999/1000	11/12	2	13.3
10/31/03	981	5	999/1000	11/12	5	13.3
10/31/03	983	6	999/1000	11/12	1	13.3
10/31/03	984	7	999/1000	11/12	1	13.3
10/31/03	985	8	999/1000	11/12	4	13.3
10/31/03	986	9	999/1000	11/12	5	13.3
10/31/03	988	10	999/1000	11/12	3	13.3
11/04/03	1174	15	1193/1194	20/19	2a	12.7
11/04/03	1177	14	1193/1194	20/19	1a	12.7
11/04/03	1184	13	1193/1194	20/19	1a	12.7

Table 2. Summary of initial samplings and embryonic survival rates for 2003-brood University of Washington Hatchery (UWH) stock Chinook salmon used to create meiotic gynogens. Survival rates were estimated at the eyeing stage. We treated a subsample of four of the gynogens with methyltestosterone (MT); we carried these four groups and the four untreated gynogen groups into rearing.

Female	Spawn Date	No. dead embryos	No. live embryos	Survival rate (%)
973	10/31/03	2908	32	1.09
974	10/31/03	3470	963	21.72
975	10/31/03	3363	889	20.90
978	10/31/03	2596	904	25.83
981	10/31/03	1312	1178	47.30
983	10/31/03	1465	1018	40.99
984	10/31/03	1202	1204	50.04
985	10/31/03	3665	516	12.34
986	10/31/03	2622	431	14.12
988	10/31/03	2088	575	21.59
1174	11/03/03	2931	387	11.67
1177	11/03/03	2599	1336	33.95
1184	11/03/03	4980	217	4.18
983 MT	10/31/03	44	747	38.71
986 MT	10/31/03	6	405	13.91
988 MT	10/31/03	22	331	22.53
1177 MT	11/03/03	224	552	34.66

Table 3. Summary of genetic and phenotypic parameters for length, weight and growth in 2002-brood University of Washington Hatchery (UWH) stock juvenile Chinook salmon. Samples were taken two weeks after ponding (sample 1), seven weeks after ponding (sample 2), and fourteen weeks after ponding (sample 3). Each estimate is based on a sample of 2,600 fish from 30 half-sib and 105 full-sib families. Genetic parameter estimates were computed with a restricted maximum likelihood (REML) algorithm (Hard et al. 1999). nd, not determined.

Sample dates	Mean lg (mm) ± SD	h^2 (lg) ± SE	Mean wt (g) ± SD			Growth rate ± SD	h^2 (wt) ± SE	r_A ± SE
1/20-3/4/04	76.6 ± 5.5	0.70± 0.07	5.3 ± 1.3	0.44 ± 0.06	0.92 ± 0.02	0.54 ± 0.49	0.08 ± 0.02	-0.86 ±0.17
2/23-4/8/04	100.8 ± 63.1	0.25± 0.04	33.5 ± 8.2	0.26 ± 0.04	0.99 ± 0.01			
4/12-26/04	123.3 ± 80.6	nd	66.4 ± 15.4	nd	nd			
1/20-3/4/04	213.1 ± 119.8	0.09± 0.03	198.6 ± 50.9	0.10 ± 0.02	0.99 ± 0.01			
	Mean wt (g) ± SD							
2/23-4/8/04	5.3 ± 1.3	0.44± 0.06						

Table 4. Summary of samples of mean lengths (mm) and weights (g) for six families of 2002-brood Grovers Creek Hatchery (GCH) stock juvenile Chinook salmon between April 2003 and April 2004.

Trait	April 2003 Mean ± SD (n)	June 2003 Mean ± SD (n)	Sept 2003 Mean ± SD (n)	Dec 2003 Mean ± SD (n)	Feb 2004 Mean ± SD (n)	April 2004 Mean ± SD (n)
Length (mm)	104.6 ± 12.1 (313)	138.4 ± 14.7 (349)	170.9 ± 13.9 (333)	204.5 ± 21.5 (169)	232.3 ± 31.9 (156)	250.5 ± 27.9 (162)
Weight (g)	13.5 ± 5.0 (313)	33.8 ± 14.5 (349)	65.4 ± 16.2 (333)	118.9 ± 30.6 (169)	185.1 ± 53.8 (156)	216.0 ± 63.4 (162)

Table 5. Estimates of genetic and phenotypic parameters for length and weight in 2003-brood University of Washington Hatchery (UWH) stock juvenile Chinook salmon. Samples were taken two weeks after ponding (sample 1), seven weeks after ponding (sample 2), and fourteen weeks after ponding (at PIT tagging, sample 3). Each estimate is based on a sample of 2,600 fish from 30 half-sib and 105 full-sib families. Genetic parameter estimates were computed with a restricted maximum likelihood (REML) algorithm (Hard et al. 1999).

Sample dates	Mean lg (mm) ± SD	h^2 (lg) ± SE	Mean wt (g) ± SD	h^2 (wt) ± SE	r_A ± SE
1/20-3/4/04	40.8 ± 2.2	0.68 ± 0.30	0.6 ± 0.1	0.49 ± 0.29	0.93 ± 0.05
2/23-4/8/04	57.3 ± 4.5	0.34 ± 0.19	2.0 ± 0.5	0.26 ± 0.21	0.91 ± 0.06
4/12-26/04	76.6 ± 5.4	0.62 ± 0.21	5.0 ± 1.2	0.66 ± 0.23	0.92 ± 0.04

Table 6. Estimates of genetic and phenotypic parameters for growth rate of 2003-brood University of Washington Hatchery (UWH) stock juvenile Chinook salmon during three samples taken two weeks after ponding in January-February 2004 (sample 1), seven weeks after ponding (sample 2), and fourteen weeks after ponding in April 2004 (sample 3). Exponential growth in weight (wt) between dates t_2 and t_1 was estimated as $\ln(wt_2/wt_1)/(t_2-t_1)$. Each estimate is based on a sample of 2,600 fish from 30 half-sib and 105 full-sib families. Genetic parameter estimates were computed with a restricted maximum likelihood (REML) algorithm (Hard et al. 1999); unfortunately, approximate SEs could not be estimated.

Sample period	Mean growth ± SD	h^2	Samples compared	r_A
1,2	0.035 ± 0.007	0.15	1,2 & 2,3	0.03
2,3	0.023 ± 0.010	0.30	1,2 & 1,2,3	0.80
1,2,3	0.029 ± 0.004	0.54	2,3 & 1,2,3	0.54

Table 7. Summary of length and weight statistics for 2003-brood UWH Chinook salmon gynogenetic crosses treated with methyltestosterone (MT). Each sample (151 fish from tanks 1-3, 153 from tank 4) was taken from 16-17 April 2004.

Tank (family)	Mean lg (mm) ± SD	Mean wt (g) ± SD
1	90.7 ± 8.5	0.5 ± 0.1
2	86.3 ± 7.0	0.4 ± 0.1
3	87.5 ± 7.6	0.4 ± 0.1
4	88.2 ± 8.8	0.5 ± 0.1

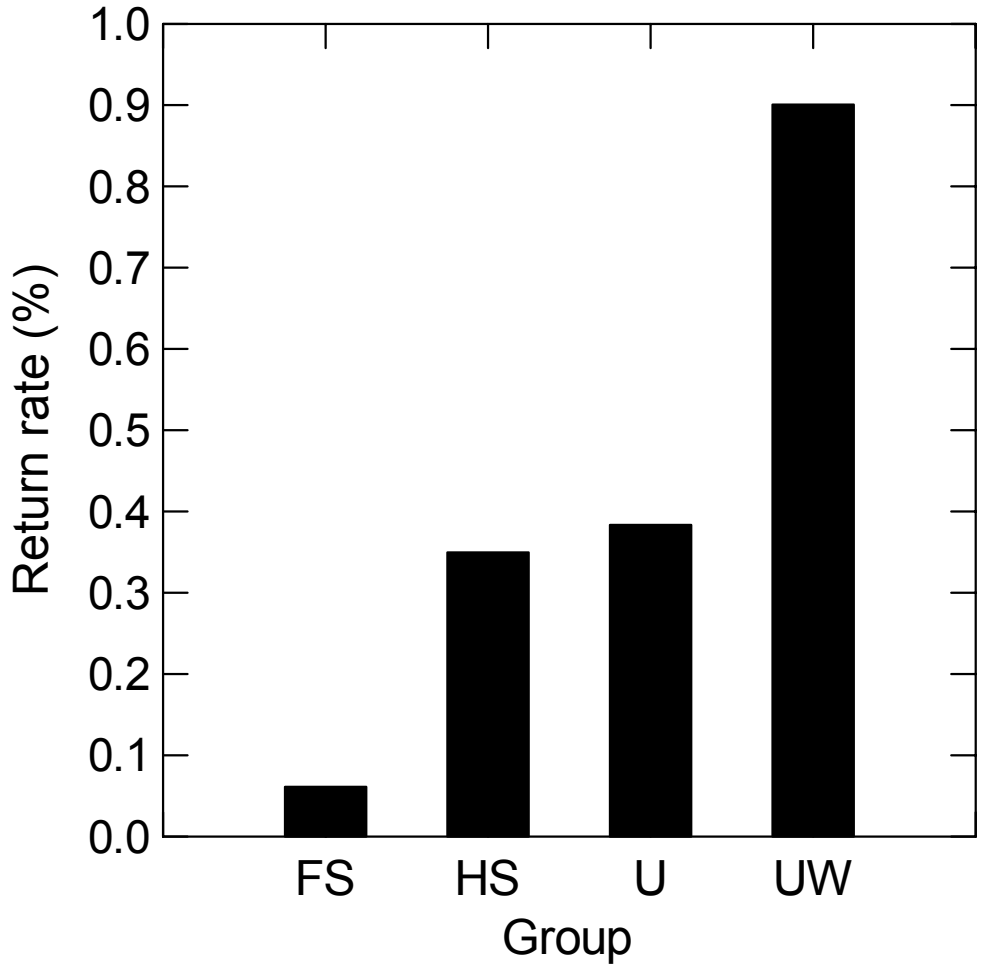

Figure 1. Smolt-to-adult survival rates (%) of 1998-brood Grovers Creek Hatchery (GCH) experimentally inbred stock and University of Washington Hatchery (UWH) control stock Chinook salmon to the UWH rack through age-5 (1998-2003). Data are based on coded-wire tag data and preliminary DNA genotyping analyses. GCH stock groups: FS, progeny of full siblings; HS, progeny of half siblings; U, progeny of unrelated individuals. UW, UWH stock unselected control.

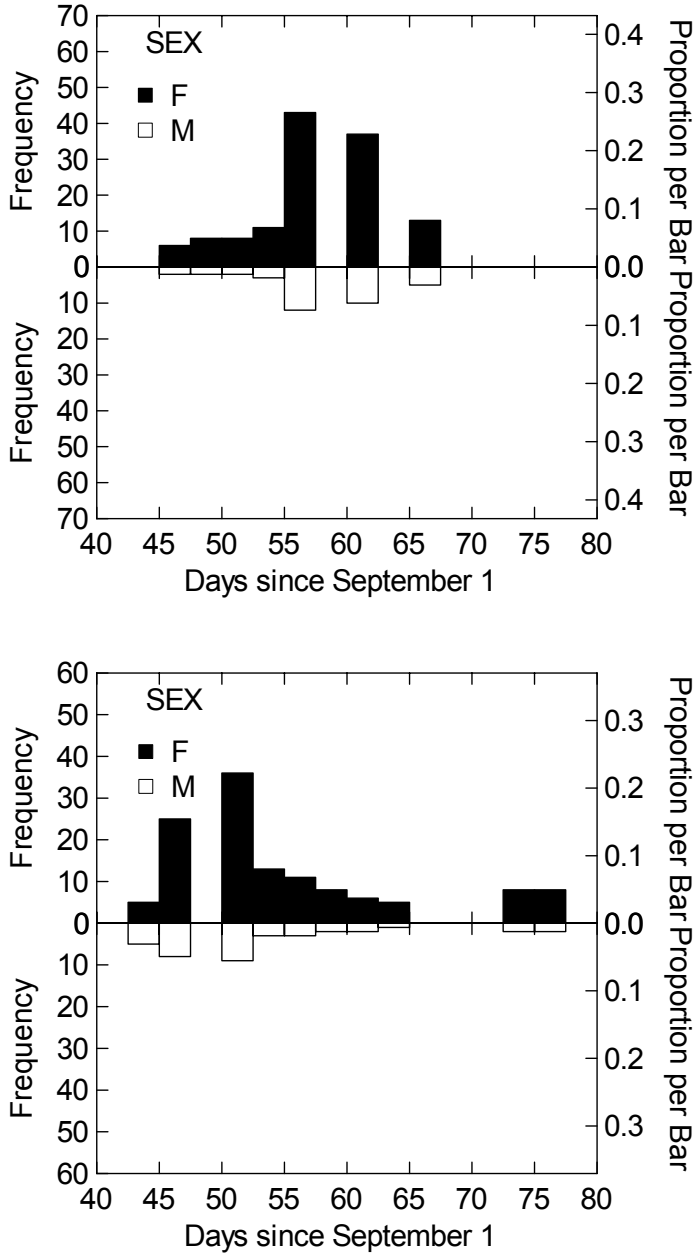

Figure 2. Frequency distribution of spawning dates of adult Chinook salmon at the University of Washington (UWH) used as broodstock for inbreeding in 2002 (left) and 2003 (right). In 2003 broodstock were selected from throughout the distribution (13 October to 16 November) in approximate proportion to their abundance.

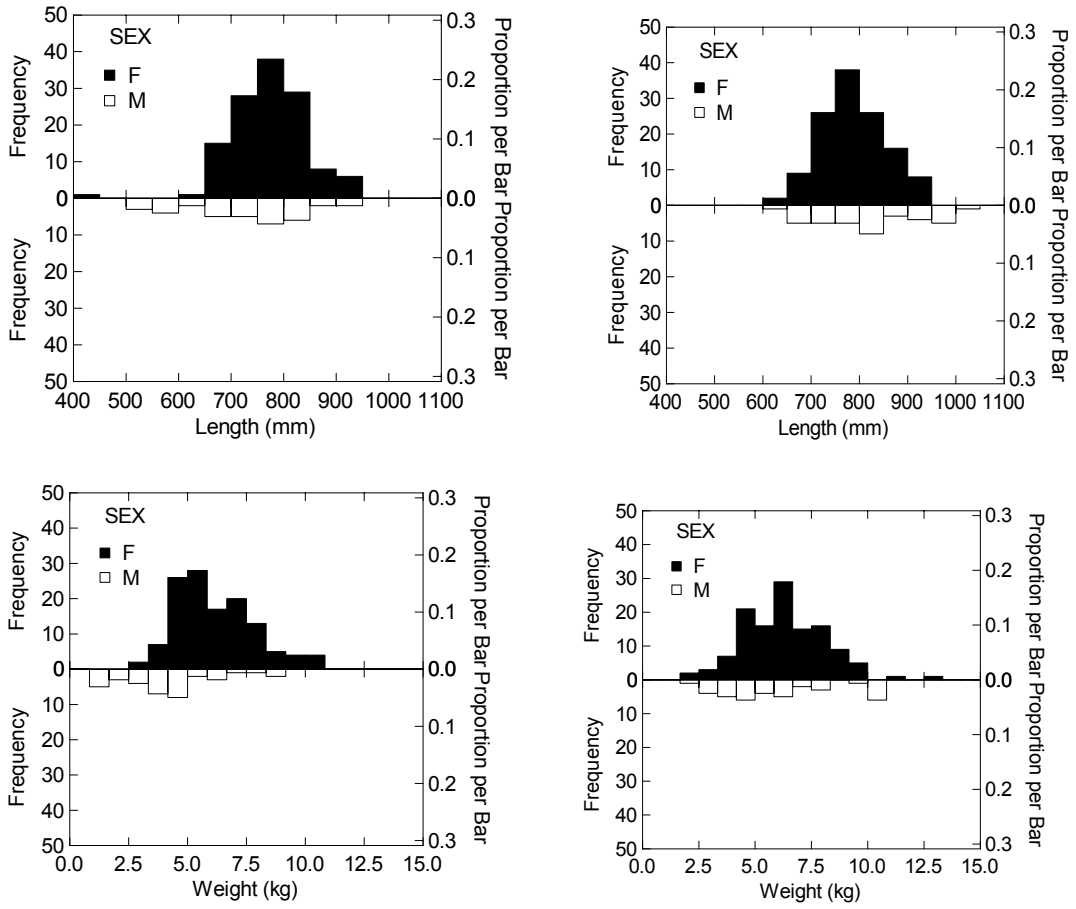

Figure 3. Comparison of fork lengths (top panels) and weights (bottom panels) among 2002-brood (left panels) and 2003-brood (right panels) adult male and female hatchery Chinook salmon used as broodstock for inbreeding at the University of Washington Hatchery (UWH).

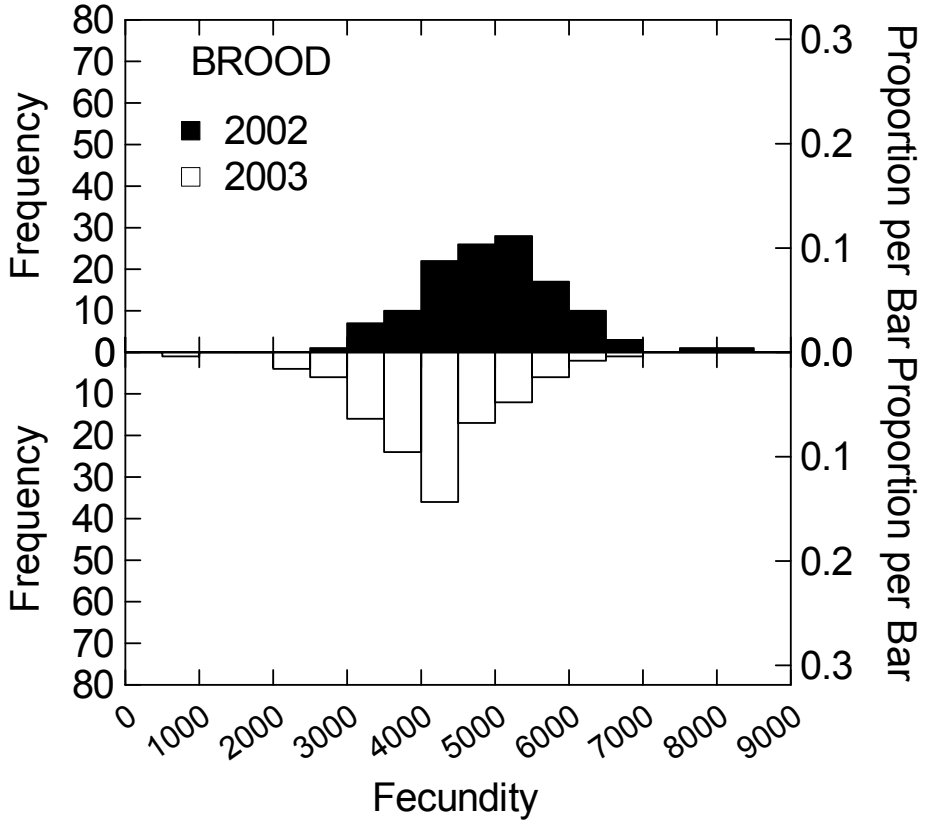

Figure 4. Frequency distributions of fecundities of female Chinook salmon at the University of Washington Hatchery (UWH) used as broodstock for inbreeding in 2002 (top) and 2003 (bottom).

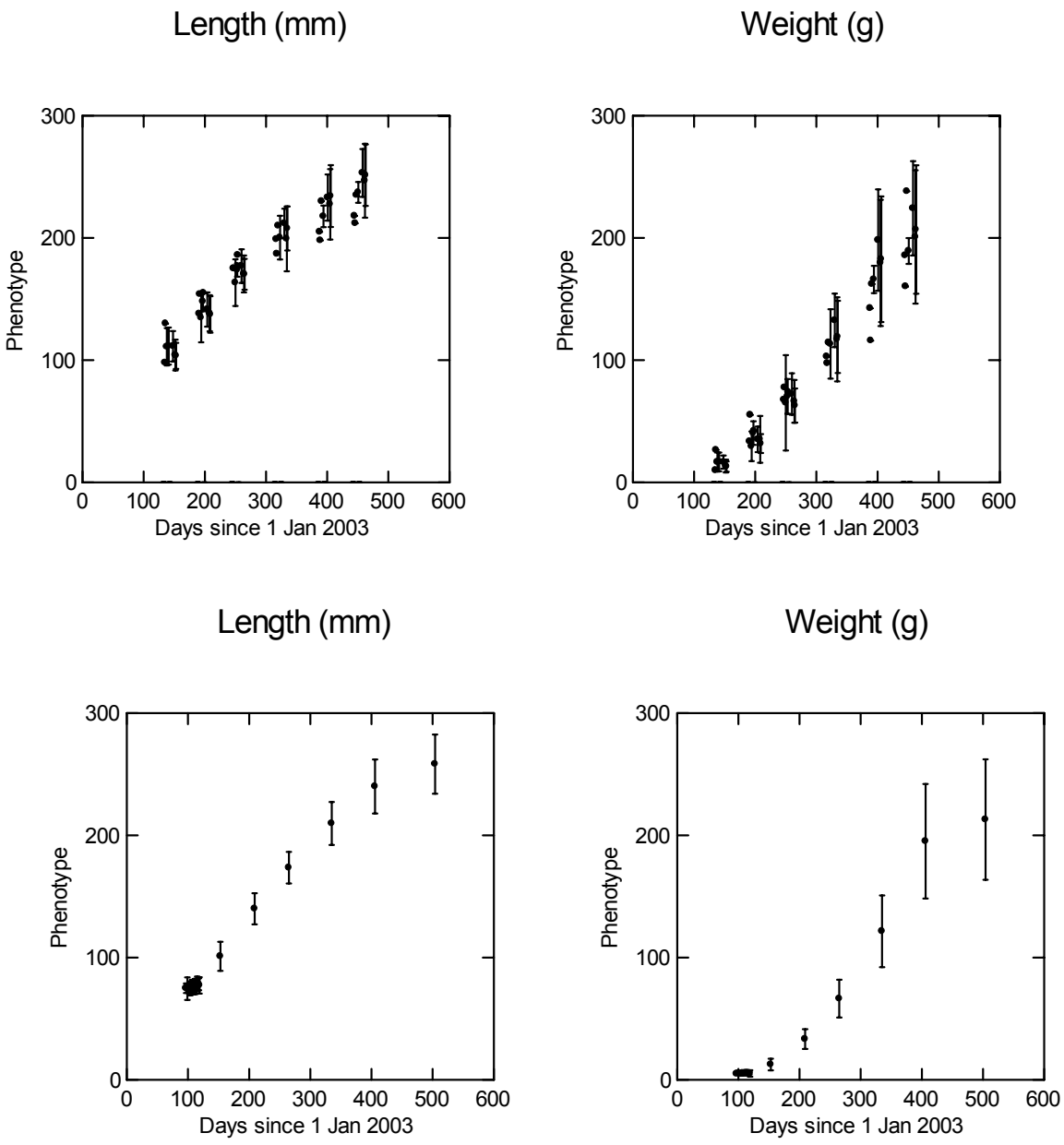

Figure 5. Patterns of growth in length (left) and weight (right) of juvenile 2002-brood Grovers Creek Hatchery (GCH) stock (top panels) and University of Washington Hatchery (UWH) stock (bottom panels) Chinook salmon between samples taken during PIT tagging in April 2003 and samples taken in April 2004. Data are means ± 1 SD over all families.

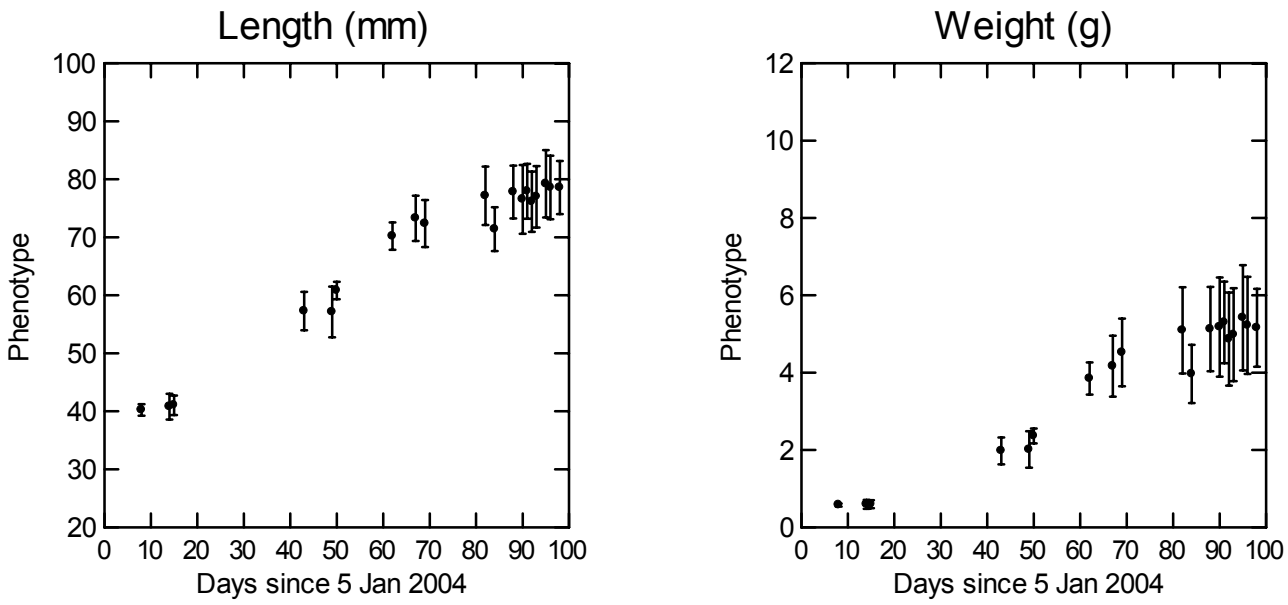

Figure 6. Patterns of growth in length (left) and weight (right) of juvenile 2003-brood University of Washington Hatchery (UWH) stock Chinook salmon between samples taken in January and April 2004. Data are means ± 1 SD over all families.

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