Research on Captive Broodstock Programs for Pacific Salmon

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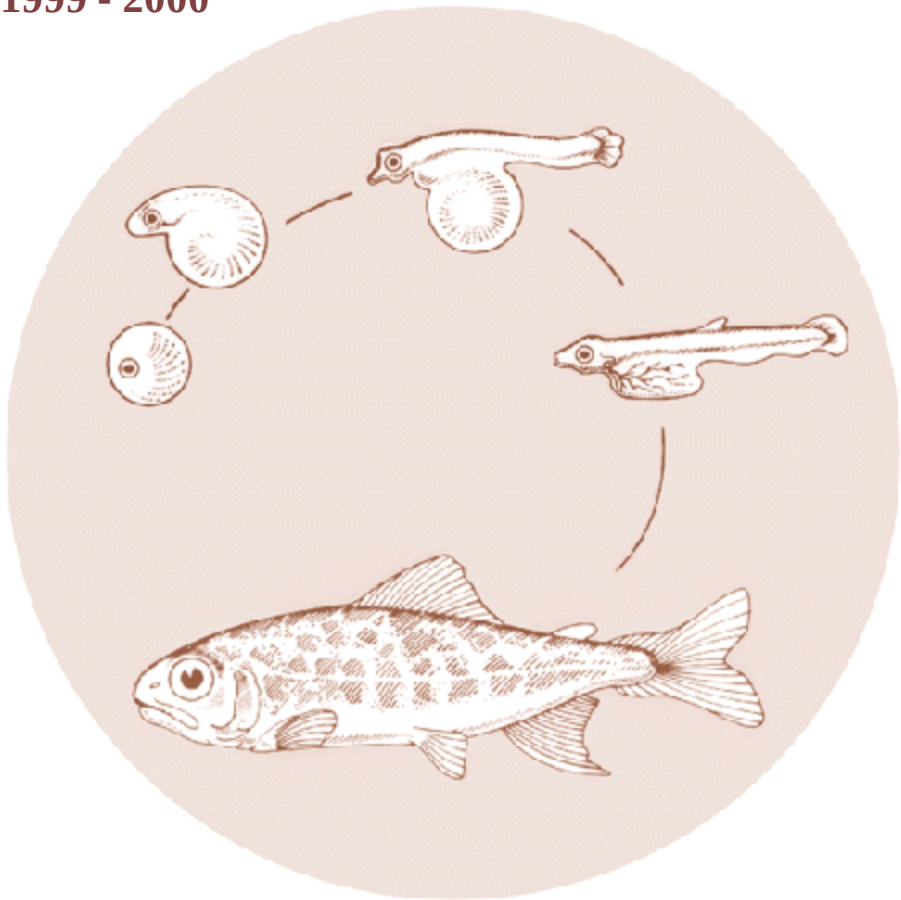

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RESEARCH ON CAPTIVE BROODSTOCK PROGRAMS FOR PACIFIC SALMON

ANNUAL REPORT
(PERFORMANCE PERIOD: 1 JUNE 1999 THROUGH 31 MAY 2000)

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EXECUTIVE SUMMARY

The efficacy of captive broodstock programs depends on high in-culture survival and the fitness of cultured salmon after release, either as adults or juveniles. This report provides information on continuing captive broodstock research designed to improve technology.

Scientific studies are being conducted which cover all major life history stages of Pacific Salmon. Accomplishments of the project during the past year are as follows.

- Research on the effects of diet composition and growth regimes on age and timing of maturity indicate that the incidence of maturation in male spring chinook salmon is increased by growth.
- Body fat levels had no effect on maturation rates in captively reared fish (fish in the study were much larger than wild fish).
- Endocrine changes associated with spermatogenesis suggests that the period when maturation is initiated in males is broader than previously thought, and may extend over a period of 4 months from November through February of the subsequent year during which the fish will spawn.
- Research on reproductive characteristics of captively reared chinook salmon demonstrated that captively reared chinook salmon differed from ocean-reared chinook salmon (released as smolts) in their body composition and morphology.
- Increased current velocities (exercise) in rearing vessels of captively reared chinook decreased maturation rates, but did not result in other detectable changes in reproductive characteristics.
- Research on bacterial kidney disease (BKD) has identified azithromycin as a promising candidate for use as a therapeutic and prophylactic agent for BKD in captive broodstock salmon. Experiments are continuing to determine the efficacy of azithromycin in treating BKD and evaluating potential toxic effects on juvenile and adult development.
- Research on the effects of inbreeding in captive broodstocks is being conducted to determine genetic risks associated with captive broodstocks initiated from small populations.

Peer-reviewed Publications of the Project

Alcorn, S.W., and R.J. Pascho. 2000. Single-dilution enzyme-linked immunosorbent assay for quantification of antigen-specific salmonid antibody. J. Vet. Diagn. Invest. 12:245-252.

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Berejikian, B.A., E.P. Tezak, S.L. Schroder, T.A. Flagg, and C.M. Knudsen. 1999. Competitive differences between newly emerged offspring of captively reared and wild coho salmon (*Oncorhynchus kisutch*). Trans. Am. Fish. Soc. 128: 832-839.

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Shearer, K.D. and P. Swanson. In press. The effect of whole body lipid on early sexual maturation of 1+ age male chinook salmon (*Oncorhynchus tshawytscha*). Aquaculture.

REPORT STRUCTURE

This report is organized by Task number as outlined in the FY 1999 Work Statement. For each Task there is included either a Final Report or a Progress Report .

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

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

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TASK 2[1]. DETERMINE THE EFFECTS OF CONSTANT AND VARIABLE DIETARY PROTEIN AND ENERGY INTAKES ON POST-RELEASE FITNESS OF JUVENILE CHINOOK SALMON

(PROGRESS REPORT: 1 JUNE 1999 THROUGH 31 MAY 2000)

by

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Introduction

It is not yet possible to define a feeding regimen for captively reared stocks similar to their natural regimen which will enhance the post-release fitness of juveniles and improve the reproductive performance of adults. In the natural environment, seasonal differences in food quality and quantity have profound effects on growth and 'wild' attributes, such as external coloration and fin quality. Formulating the right feeds for conservation fish held for long periods in captivity before release is more complicated than formulating diets for farm fish. Recent research in salmonid nutrition shows it is necessary to consider daily dietary protein intake and protein intake relative to total dietary energy level, rather than simply the levels of total dietary lipid.

The proposed approach is to formulate diets and develop a feeding regimen for captively reared chinook salmon, from juveniles to mature adults, which mimic seasonal differences in food availability in the environment. In the second year the work includes enhancing juvenile quality for post-release fitness, and carrying experimental fish into the post-juvenile period.

Different diets and regimens will be evaluated by their effects on fitness for post-release survival, including growth, body conformation, and color. Specific tasks will investigate the effects of varying daily protein intake (g digestible protein/kg/d) and daily energy intake (kcals/kg/d) on protein and energy accretion in growing juveniles before release. Continuing work will investigate the effects of selected dietary treatments on age-at-maturity, and on adult breeding success in years 3 and 4.

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[1] Results of work conducted under Task 1 as described in the FY99 Statement of Work are presented under Task 7 of this report

The natural diets of juvenile salmonids in freshwater consist mainly of aquatic and terrestrial insects. Many published studies, recently reviewed in depth by Higgs et al. (1995), have documented the natural diet of juveniles, both in terms of prey and nutrient intake. The studies show conclusively that the proximate composition of natural diets is approximately 45% protein and 15-17% lipid. Although the natural diet of juvenile chinook salmon in freshwater consists of insects, with lipid levels ranging from 2-39% on a dry weight basis, the four main groups of insects consumed by juvenile chinook average 53% protein and 16.5% lipid. This is similar to the composition of freshwater copepods and small fishes. Wild juvenile chinook are distinctly different from hatchery-reared chinook in body fat deposits, particularly in the amount of fat in visceral stores, and in external coloration and fin quality. These differences are greater among stream-type juvenile chinook than among ocean-type.

The body conformation of chinook salmon reared in captive broodstock programs differs from that of wild fish, mainly in length to girth ratio. Captively reared fish have a lower length to girth ratio, meaning that they tend to be shorter and fatter. This difference is presumably related to dietary energy intake and level of activity. Dietary energy intake is similar between wild and captively reared fish when expressed in terms of proximate composition, or percentage of the diet as protein and lipid. However, dietary energy intake is a function of the proximate composition of the diet as well as feed intake. Wild fish obviously consume less food during certain periods of the year. Recent studies with juvenile chinook salmon (funded by BPA) have identified annual variations in levels of the metabolic hormone IGF-1 and growth hormone (W.W. Dickhoff, NMFS, personal communication). This provides a metabolic rationale for numerous observations made over 40 years concerning the higher level of whole body lipid in hatchery-reared fish compared with that in wild salmon. During periods of declining day length circulating levels of these hormones are low and protein synthesis rates in the body are reduced. Fish convert both dietary protein and lipid into stored body fat during these periods. In contrast, when day lengths increase, body metabolism patterns change, resulting in increases in protein synthesis rates and lypolysis, and thus lower percentage whole body fat levels. When feeding levels are adjusted to correspond with this pattern of anabolism and catabolism, survival to hatchery return increases.

Dietary requirements also change as fish grow and develop. It is well known that the scope for growth of Pacific salmon decreases with fish size (Brett 1979). Thus, the protein needs of salmon, expressed as a percentage of dietary metabolic energy, decrease with fish size. McCallum (1985) reported that juvenile chinook salmon required 1.3-1.6 g protein/kg/d for maintenance, or no growth. The amount of protein intake to support weight gain varies with fish size and dietary energy intake, but growth increases linearly in juvenile salmonids consuming between 6-18 g protein/kg/d (Fairgrieve 1992).

The rationale for enhancing the external coloration of juvenile chinook is based on the hypothesis that appropriate external coloration reduces the chances of fish being seen by predators.

Work Completed

In January 1999, chinook salmon (Clearwater River Strain, 1998 brood year) were obtained as first-feeding fry from the IDFG Clearwater Hatchery and transferred to the fish culture laboratory of the Aquaculture Research Institute, University of Idaho. The fish were fed a commercial salmon starter diet (BioDiet) until they reached an average weight of 13 g on 3 December. The fish were then graded and counted into groups of 25. Each group was bulk-weighed and placed in one of 12 experimental rearing tanks (400 L), using a round-robin procedure, until each tank contained 150 fish. Dietary treatments were assigned randomly, with replicate groups receiving each dietary treatment. The tanks were supplied with temperature-controlled, recycled freshwater (9-11 ^{o}C), and the lighting was maintained under the photoperiod regime of the area.

The fish were fed the first group of experimental diets, which have been assayed by batch for proximate composition. All feeds contained the appropriate levels of protein and lipid. Feeding frequency was four times per day to begin, but decreased to two feedings per day by the end of the reporting period. Feeding was by hand 5 d/wk.

Three dietary treatments were used in this trial: 1) Constant diet, to provide 9 g protein/kg/d; 2) Constant diet with variable feeding level, to provide 6-12 g protein/kg/d with proportional dietary energy intake; and 3) Variable diet adjusted to provide 6-12 g protein/kg/d at a constant dietary energy intake of 75 kcal/kg fish/d. Each diet was fed to three replicate tanks of fish. The feed formulation for the three diets is shown in Table 1. The feed was produced at the Hagerman Fish Culture Experiment Station by compression-pelleting. The variable dietary treatments (#2 and #3) provided the fish with the lowest protein intake during late fall and early winter months, and the highest protein intake during spring and early summer months. This was accomplished by producing different feeds for each seasonal period, and by adjusting feeding level to supply the desired protein and energy intake per fish per day. The constant dietary treatment (#1) was fed at 2% of the biomass in each tank per day during the first period, but this level of feeding was slightly more than the fish would consume, so all feeding levels were reduced to 1.5%. Feed amount was adjusted weekly for estimated growth and adjusted after the fish were weighed and counted based upon actual growth. Feed consumption was recorded daily to monitor feed intake and to permit feed efficiency ratios to be calculated for each growth period.

For the first 10 wk the fish in each tank were bulk-weighed and counted every 2 wk after a 2-d period of fasting (weekend). The average length was determined by measuring the fork-length of a sample of 50 fish from each tank. The average weight and length of fish in each replicate tank were then calculated, as was the variation in length among fish within tanks. The fish have been weighed and counted monthly since the tenth week of the feeding trial to reduce handling stress.

Twelve fish from each dietary treatment group (three per replicate tank) were removed and sacrificed for proximate analysis at selected periods. Proximate analyses will be carried out at Hagerman using standard procedures (AOAC 1990). Muscle tissue

samples will be used to measure the concentration of mRNA associated with actin/myosin. This will determine any response to seasonal changes in photoperiod associated with protein growth and dormancy, thereby providing a direct measurement of true growth (protein accretion). The proximate composition data will be used to calculate protein and energy retention at various stages of growth in the fish associated with dietary treatment and season. Data will be transformed if necessary and statistically analyzed using a computer program (GraphPad Prism, version 2.01, GraphPad Software, Inc., San Diego, CA). Treatment effects will be considered significant at $P<0.05$.

Work to be Completed

Chinook from the smolt feeding study will continue to be reared on the variable dietary protein and energy intake treatments until late June 2000. Juvenile fitness for the post-release environment will then be assessed by measuring:

1) Energy reserves: Total body lipid, expressed on a percentage and on an absolute amount (g/fish) will be measured using AOAC methods, as will the other relevant proximate components, e.g., moisture, crude protein, and ash (AOAC 1990). Individual fish will be analyzed, but data from individual fish will be pooled per replicate tank, making 12 observations (3 per dietary treatment group). Data will be analyzed by ANOVA after appropriate transformations using GraphPad Prism, version 2.0 (GraphPad Software, Inc., San Diego, CA). Treatment effects will be considered significant at $P<0.05$. If warranted, post-hoc tests to determine differences among treatment groups will be used.

2) Fin quality and body conformation: The height of the dorsal fin (Dorsal Fin Index, DFI) will be measured using standard morphometric truss measurement methodology, as described by Winans (1990). Fin-to-body ratios will be calculated using the methods of Kindschi (1987). Individual fish will be measured, but data from individual fish will be pooled per replicate tank, making twelve observations (three per dietary treatment group). Data will be analyzed by ANOVA using GraphPad Prism, version 2.0 (GraphPad Software, Inc., San Diego, CA). Treatment effects will be considered significant at $P<0.05$. If warranted, post-hoc tests to determine differences among treatment groups will be used.

3) External coloration: External coloration is an important aspect of the ability of smolts to avoid predators. External coloration of sockeye in lakes is expected to be similar to other lake-dwelling salmonids, e.g. light on the bottom, darker on the top, and silvery on the sides. In streams, however, a different color pattern is likely to be more evident. Fish will be examined before and during outmigration to determine if external coloration patterns of fins and skin change during this critical period, and to establish the extent and degree of external coloration common in wild fish. External coloration patterns will be captured by digital imaging and evaluated by scoring and by computer comparisons. Data collected on juveniles will be tabulated and statistically analyzed after appropriate transformations. A final report on this portion of the study will be prepared.

TASK 3: ASSESSMENT OF QUALITY OF FISH RELEASED FROM ONGOING CAPTIVE BROODSTOCKS (PROGRESS REPORT: 1 JUNE 1999 THROUGH 31 MAY 2000)

by

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Introduction

Captive broodstocks are presently being reared for ESA-listed chinook salmon in Oregon and Idaho, and sockeye salmon in Idaho. A key to the success of these programs will be consistent production of juveniles with the fitness required for increased post-release survival and adult return. However, so far the only program to release juveniles to the habitat (i.e., the Redfish Lake sockeye program) has met with somewhat variable success. A major strategy for this program is release of presmolts into Stanley Basin Lakes in early fall, with the fish overwintering and outmigrating as smolts the following spring. Over the last few years (1995-1998), over-winter survival of sockeye salmon presmolts released into Stanley Basin Lakes has ranged from only a few percent to about 40% and averaged about 20% (P. Kline, IDFG, personal communication, March 1999).

The dynamics driving over-winter survival in Stanley Basin Lakes are poorly understood. Over-winter survivals of fish released into top salmon producing systems in Canada and Alaska often averages above 60%. However, Stanley Basin Lakes are highly oligotrophic and are located at the highest elevation (6500 ft) and farthest distance (950 miles) from the sea of any sockeye-producing lake in North America. These factors may limit carrying capacity and absolute production thresholds. These factors may also influence the quality of fish rearing in the habitats.

Previous studies on Stanley Basin kokanee suggest that they have greatly depleted springtime lipid levels and may lack energetic reserves (Steinhart and Wurtsbaugh 1995). The exact relationships between how dietary factors that affect kokanee may affect hatchery released sockeye are unknown. Energetic reserves for fish released from the captive broodstocks will depend on diet-related components acquired during culture and the fish's feeding success after release. However, drastically low energy reserves may exacerbate in-lake mortality. Low springtime lipid levels may also leave the fish lacking the energetic reserves necessary to complete migration (Steinhart and Wurtsbaugh 1995).

The consequences of inappropriate energetic reserves can be profound. There is a critical need to determine the quality of fish used for ESA-related recovery programs, both at time of release into the rearing habitat and at outmigration. It is anticipated that these measurements will provide guidance for developing rearing and release strategies to enhance survival both in the habitat and during downstream migration.

In cooperation with the Stanley Basin Sockeye Technical Oversight Committee and the Chinook Salmon Captive Propagation Technical Oversight Committee, fish were sampled in 1999 both during hatchery rearing, and at times of release into and outmigration from rearing habitats. These evaluations concentrated on sockeye salmon, but will expand to include chinook salmon in 2000 and 2001 (first sampling for 2000 occurred on 25 and 26 May 2000). Additional fish were collected in 1999 in cooperation with IDFG from various streams and these fish are being assessed for whole body energy reserves (lipid stores), fin quality, and morphometrics. Samples will be taken again in 2000 and 2001 by IDFG, and subjected to the same analysis.

Assessing relevant parameters will establish a data base by which to compare rearing habitat, downstream, and adult return survival. The goal will be to determine optimum physical and biochemical benchmarks for hatchery-raised fish to increase fish survival. These measurements will aid in development of rearing strategies to enhance survival. This data will also be used to prioritize research projects in feeds and nutrition, and other culture-related areas.

Work Completed

Samples of sockeye salmon from Idaho were collected in 1999 and 2000 (25 and 26 May). Samples were (and will be) stored at –80 ^{o}C at the UI Hagerman Fish Culture Experiment Station prior to analysis. Fish quality analysis by the UI (proximate analysis, fin quality, morphometrics) will follow prescribed methods and will begin in June 2000. In addition, analysis of these fish has been expanded to include trace mineral analysis (conducted by the Holm Center, UI) and whole body fatty acid composition (conducted at the UI Hagerman Fish Culture Experiment Station).

Work to be Completed

Additional samples of salmon from Idaho will be collected in June/July 2000 and 2001. Fish quality analysis by the UI (proximate analysis, fin quality, morphometrics) will follow prescribed methods and will begin in June 2000. In addition, analysis of these fish has been expanded to include trace mineral analysis (conducted by the Holm Center, UI) and whole body fatty acid composition (conducted at the UI Hagerman Fish Culture Experiment Station). This analysis on samples collected to date will begin in Summer 2000, and be completed in Summer 2001 on samples collected in 2001. Data will be compiled, statistically analyzed, and a final report will be written.

Table 1. Composition of experimental diets for chinook salmon captive broodstock studies[1].

INGREDIENT	DIETS 1 & 2 (%)[1]	DIET 3 (%)[1]
Anchovy meal	54.00	54.00
Blood meal	4.00	4.00
Wheat gluten	4.00	4.00
Wheat mids	16.00	12.50 to 19.50
Fish oil	13.00	6.00 to 13.00
Vitamin C	0.30	0.30
Choline	0.50	0.50
TM salt[2]	0.10	0.10
Vitamin premix[3]	3.00	3.00
Krill hydrolysate	5.00	5.00
Astaxanthin[4]	0.10	0.10

Footnotes:
1. Calculated Proximate Composition of Diets 1 & 2 = 48% crude protein (45% digestible protein) and 17.5% crude lipid, expressed on an "as-is" basis (7% moisture). Diet 3 varies to keep energy intake constant while varying daily protein intake (g protein/kg fish/d).
2. Supplies the following per kg dry diet: Zn as ZnSO4.7 H2O, 75 mg; Mn as MnSO4.H2O, 20 mg; Cu as CuSO4.5 H2O, 1.54 mg; I as KIO.C2H8N2 .2HI, 10 mg.
3. Supplies the following per kg dry diet: thiamin mononitrate, 62 mg; riboflavin, 71 mg; niacin, 294 mg; calcium pantothenate, 153 mg; pyridoxine hydrochloride, 50 mg; folic acid, 22 mg; vitamin B12, 0.08 mg; d-biotin, 0.8 mg; myoinositol, 176 mg; retinol acetate, 8818 IU; vitamin D3, 588 mg; (-tocopherol acetate, 670 mg; menadione sodium bisulfite complex, 37 mg.
4. Carophyll Pink, Hoffman La-Roche, Basle, Switzerland.

TASK 4. EVALUATE THE EFFECTS OF EXERCISE-TRAINING ON REPRODUCTIVE PERFORMANCE, BODY COMPOSITION, AND MORPHOLOGY
(FINAL REPORT: 1 JUNE 1999 THROUGH 31 MAY 2000)

by

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Introduction

The natural reproductive performance of captively reared salmonid broodstocks is currently poor, and is frequently associated with incomplete development of secondary sex characteristics, fin erosion, and poor swimming performance. Improving natural reproductive performance is critical for the success of captive broodstock programs in which adult release is a primary reintroduction strategy for maintaining ESA-listed populations.

Exercise is a natural parameter of anadromous salmonid migration, but in this respect, current husbandry practices do not simulate environmental conditions encountered by wild fish. Wild fish are better adapted to exhaustive exercise, have greater red and white muscle mass, and exhibit reduced post-swimming stress acidosis than hatchery-reared non-exercised fish. Tufts et al. (1991) showed that wild Atlantic salmon collected during their spawning migration exhibited extreme, persistent lacto-acidosis, but no mortality after exhaustive exercise. Barrett and McKeown (1988) demonstrated that exercise increased red and white muscle masses, and facilitated mobilization of stored reserves in juvenile steelhead. Young and Cech (1994a and 1994b) showed that exercise conditioning of striped bass increased growth, red and white muscle mass, and improved swimming performance; and Joergensen and Jobling (1993) showed that growth efficiency and healing of wounded fins in juvenile Atlantic salmon was improved by exercise conditioning.

Exercise conditioning, by simulating environmental conditions encountered by wild fish at sea and during their spawning migrations, may have a beneficial effect on

reproductive performance and its associated characteristics. In particular, exercise conditioning of captively reared salmon through maturation may (i) improve body composition, i.e., decrease fat and increase muscle mass, (ii) promote greater development of secondary sex characteristics, and ultimately (iii) improve reproductive performance. Improved female condition should increase their ability to dig nests, and deposit and cover eggs in the gravel. Improved development of male secondary sexual characteristics should increase female willingness (mate selection) to spawn with them (Berejikian et al. 1997).

The objective of this Task was to expose captively reared salmon to exercise-training programs before release, and to measure improvements (or not) in the development of secondary sex characteristics, body composition, and breeding success. The performance of exercised adults was compared with non-exercised adults and anadromous adults from the same stock previously released as smolts, i.e., ocean-reared fish.

Methods and Materials

Study populations and rearing environments

Captively reared chinook salmon -- On 27 January 1999, 122 fall chinook salmon were obtained from a stock (Minter Creek Hatchery population, Pierce County, WA) of fish maintained at the NMFS Manchester Research Station (MRS) for use in broodstock experiments. The fish were divided randomly into two treatment groups: 1) captively reared, exercised (CRE); and 2) captively reared, non-exercised (CRN), with two tanks devoted to each treatment.

The rearing system used in this study was comprised of four circular fiberglass tanks (3.7-m d and 1-m) with center drains, each continuously supplied with seawater treated by filtration and exposure to ultraviolet light, at a rate of ca. 105 $L \cdot min^{-1}$. Minimal linear water velocity necessary for automatic solids removal was maintained in all tanks by introducing the low-pressure influent water through a pipe placed parallel to the surface. In the two exercise tanks, linear velocity was periodically increased by directing high-pressure recirculated water through a submerged nozzle located at the perimeter of the vessel. Frequency and duration of the exercise periods were timer-controlled. Water pressure and velocity were regulated by centrifugal pumps equipped with variable frequency drives. Water velocity was periodically measured with a velocity meter (Swoffer Instruments, Inc., Seattle, WA) at points 0.3 m, 1.2 m, and 1.8 m from the tank center, along lines dividing them into quadrants. Average linear velocity at each tank location for both baseline and periods of forced exercise are presented in Figure 1.

Fish in each tank were weighed, measured, and counted at the start in January (Julian day 27), June (day 112), and September (day 260), for growth rate calculation and adjustment of feeding levels. Water velocity during periods of forced exercise was increased periodically as the fish grew (Table 1). Average baseline swimming speed among the fish in all tanks ranged from 0.23-0.46 body lengths (bl)$\cdot s^{-1}$. During periods of forced exercise, swimming speed increased to 0.85-1.45 bl s^{-1} (Table 1). The fish were

fed to apparent satiation on a daily basis with a high energy (ca. 25% crude lipid) commercial diet until late August 1999, when feeding was terminated.

Ocean-reared chinook salmon -- Adult chinook salmon were collected from a holding facility at the Minter Creek Hatchery on 27 September 1999. These fish will hereafter be referred to as 'ocean-reared' (OR) to distinguish them from the CRE and CRN treatments. Each fish used in the study was anesthetized, weighed to the nearest 1.0 g, and post-orbital-hypural length (POH) was measured to the nearest 1.0 mm. Individually numbered, 2.5-cm Peterson Disk tags (Floy Tag Co., Seattle, WA) were attached to each fish slightly posterior and ventral to the dorsal fin. Fish were loaded into oxygenated transport tanks and trucked for approximately 45 min to the MRS on 29 September 1998. Males and females were held separately in circular tanks (6-m d).

Body composition

On 17 September 1999, the captively reared (CR) fish were individually weighed (g), measured (mm), and examined for signs of maturity. Mature fish were immediately transferred to freshwater for final maturation and spawning. Non-maturing fish were sacrificed on 24 September 1999 by a lethal overdose of MS-222, weighed, body length measured, and gender determined by visual examination of the gonads. The whole carcasses were placed into individual plastic bags and frozen (-20°C) for later proximate analysis. Mature CR and OR fish which were allowed to spawn before sampling were processed in a similar manner, except that any eggs remaining in the body cavity were discarded before freezing. Testes were not removed.

Each fish was prepared for analysis by repeatedly grinding the whole, partially thawed carcasses in a silent cutter (Hobart Corp.) until a smooth puree was obtained. Proximate analysis of fish samples was conducted using standard (AOAC 1990) methods as follows: moisture (sec. 930.15) by oven drying to constant weight (16 h) at 105°C, protein by nitrogen determination using a LECO FP 428 nitrogen analyzer, and crude lipid (sec. 920.39C) by Soxhlet extraction with dichloromethane, and total ash (sec. 923.03).

Whole carcass proximate composition data were tabulated by stage of reproductive development (mature or immature) and subjected to ANOVA for completely randomized experimental designs, followed by Fisher's PLSD test when significant ($\alpha = 0.05$) differences were detected (Zar 1984). Statistical analysis was performed using StatView Version 4.51 (Abacus Concepts, Inc., Berkeley, CA).

Morphology

Captively reared salmonids experience different environmental conditions during adult growth and maturation than wild or conventional hatchery fish, and this may result in differences in parameters that affect reproductive success, including body morphology. Morphological variation has been observed among wild stocks of adult salmonids (Taylor and McPhail 1985; Kinnison et al. 1997) and between wild and hatchery-reared fish (Fleming et al. 1994), and these differences appear to be related to environmental conditions (Swain et al. 1991). Captively reared adult coho salmon have also been

demonstrated to be morphologically different from wild coho salmon (Hard et al. 1999). Although changes in body morphology therefore appear to be a consequence of captive (or hatchery) rearing, it is unclear how these changes might affect reproductive success.

In this study geometric morphometrics were used to determine the effects of exercise on body morphology of CR adult chinook salmon, and to evaluate the morphological differences between CR chinook salmon and OR chinook salmon returning to Minter Creek hatchery.

Geometric morphometrics -- The morphometric analyses presented here are based on landmark data collected from digital images of CR and OR adult chinook salmon. All fish from the four tanks at MRS and a sub-sample of adult OR fish were anesthetized, weighed, measured, and digitally-photographed for morphometric analysis. Thirteen landmarks were digitized from each image by the same person (AL) using the program TPSDIG. All landmarks used were easily identifiable and were the same used those used by Hard et al. (2000). Several specimens were removed from the analysis because they exhibited signs of excess water retention (i.e., bloating).

Unlike traditional morphometric methods, geometric morphometrics records landmark positions in terms of a Cartesian coordinate system. This method is more powerful than traditional methods, and allows the shape of each specimen to be reconstructed and compared with a 'consensus configuration' which represents the mean shape of all specimens (Yaroch 1996). Coordinates of the specimens in the analyses were rescaled by the least-square Procrustes method using the software TPSRELW. This technique overlays coordinates from a group of specimens onto the consensus configuration, which allows differences in shape to be expressed as residuals from the mean shape.

The total morphological variation in a sample can be partitioned into uniform (affine) and non-uniform (non-affine) components. The uniform component of morphological variation describes how shape changes by stretching or shearing. Non-uniform variation among specimens can be further partitioned into local shape deformations at different scales, which are termed partial warps (Bookstein 1991). Partial warp scores make up the relative weight matrix. Non-uniform morphological variation was analyzed using relative warp analysis, which is equivalent to a principal components analysis of the relative weight matrix.

Supplementary morphological characters -- Several morphological characters were measured which were not associated with the landmarks used in the geometric analysis (Table 2); several of these are characteristics associated with sexual maturation and reproductive fitness. These characters were measured from the digital images of specimens using digitizing software (Image Pro Plus v4.0, Media Cybernetics). Measurements were adjusted for body size by using the residual of the character-body size (represented by post-orbital-hypural length) relationship as the adjusted measurement (Reist 1985).

Breeding behavior and success

The spawning experiment was conducted in an outdoor stream channel (45-m long x 6-m wide; 3.0% gradient). Well water was supplied at 80 $L \cdot min^{-1}$, and recirculated to create a flow of approximately 5100 $L \cdot min^{-1}$. A 5-horsepower pump continuously delivered 350 $L \cdot min^{-1}$ of water from the stream through a chiller to maintain temperature between 10.0 and 11.7 °C over the course of the study. A longitudinal wooden divider divided the stream along its entire length into two side-by-side channels. Each channel was further divided cross-sectionally by setting seven metal barriers on top of weirs situated across the channel, perpendicular to the flow. This configuration created 16 replicate sections (5.0-m long by 3.0-m wide). The substrate was composed of gravel (3-10 cm d and 30-50 cm deep), graded to create very similar depth and velocity profiles in each of the 16 single sections. Water depth in each section was approximately 16 cm across the upstream end, 25 cm across the middle of the section, and gradually sloped to 13 cm across the downstream end of the section.

To begin each trial, a single female was placed into an isolated stream channel section of the channel. Each female was then monitored to determine the onset of nest construction. At approximately 0900 h of the morning following a female's initiation of digging a single male was introduced.

The frequencies of male and female spawning behaviours were recorded from the stream channel banks during three 10-min sampling periods (hereafter referred to as 'scans') each day, during the sexually active period of each female. Females were considered sexually active during the period between spawning their first and last nest. Definitions of recorded behaviors closely followed those described in Tautz and Groot (1975). Briefly, male 'crossovers' were performed by the male swimming over the back or caudal peduncle of a female. Quivers were head-to-tail, high frequency vibrations performed by a male next to the female. Females performed 'nest digs' by rolling onto their sides, arching their backs and rapidly executing a series of multiple up-and-down digging movements with their caudal fins. Once a nest depression was established, females performed 'probes' by extending their anal fin and lowering itself into the center of the developing nest, apparently to test its depth and water flow characteristics (Sandercock 1991). Covering digs occurred immediately after spawning and functioned to cover eggs in the nest pocket.

The frequencies of two classes of aggressive behaviour (attacks and lateral displays) were recorded for males and females. Lateral displays occurred when the male or female stiffened its body, extended its pelvic and anal fins and raised its dorsal fin. Attacks were open-mouth pursuits that ended in contact, but included instances where fish charged their mates with their snout, and sometimes included chasing.

Results

Growth and maturation rates of captively reared salmon

Nearly eight months of exercise training were completed before signs of maturity were observed. During this period, fish in both treatments nearly doubled their initial weight (Table 1). Fish grown in the exercise tanks matured at a higher rate (29.2%) than those in the non-exercise tanks (12.7%; $X^2 = 3.86$, 1 df, $P = 0.049$). Inadequate numbers of fish from each treatment matured to determine statistically whether the maturation rate was gender biased (Zar 1984), however 36.8% of the mature exercised fish were males, compared with 42.8% of the mature non-exercised fish. Thus, there did not appear to be a gender bias in the maturation rate associated with the two treatments.

Body composition

Analysis of body composition data from captively reared fish did not support the hypothesis that exercise training increases lean (muscle) mass and reduces lipid accumulation. Among immature fish, exercise had no significant effect on the percentage of body protein. However, whole body lipid stores differed between immature male and fish, and the effects were modified by exercise treatment (Table 3). Exercise increased the percentage of body lipid in females, and reduced the level in males.

Both exercised and non-exercised mature, captively reared chinook salmon were not significantly different from ocean-reared fish with respect to body moisture, protein, and ash content (Table 4). The percentage of whole body lipid was significantly lower in the ocean-reared fish than in either group of captively reared salmon. Among captively reared groups, whole body lipid was not significantly affected by exercise training. Among all groups, mature females were significantly lower in whole body lipid and protein, and higher in moisture than mature males. Body lipid and protein reserves transferred to developing gametes by maturing females and not accounted for by this analysis probably account for the observed differences.

Morphology

For CR fish, sample sizes (Table 5) for all but immature female chinook were too small to apply morphometric analyses. These small sample sizes allow the analysis of morphometric differences between exercised and non-exercised immature female chinook and between CR (male, female, mature, immature, exercised, and non-exercised combined) and OR chinook.

Geometric morphometrics -- No significant effect of exercise on body morphology of immature female chinook was found by MANOVA of the weight matrix (Wilk's 7 = .712, $P = 0.62$). Univariate F-tests also indicated that none of the individual partial warps were significantly different between exercised and non-exercised immature

female chinook (P > 0.19 in all cases). Relative warp analysis revealed that 58.1% of shape variation was accounted for by the first two relative warps. Projection of the immature female chinook specimens on these relative warps revealed no evident separation of groups (Figure 2).

Significant differences in body morphology between CR and OR chinook were evident (MANOVA, Wilk's 7 = .180, P < 0.0001). Relative warp analysis indicated that 60.0% of the total shape variation was accounted for by the first two relative warps, and projection of the specimens onto these warps (Figure 3) revealed strong separation of groups, primarily along the first relative warp.

Shape deformations associated with the first relative warp occur at a variety of scales and reflect morphological variation across the entire form. Figure 4 illustrates the deformations from the consensus configuration that were associated with different points on the two dimensional space of Figure 3. These shape deformations are complex and involve shifts in virtually all of the landmarks, but the areas where deformations are the largest are the snout and lower jaw, the posterior insertion of the dorsal fin, the anterior insertions of the pelvic and anal fins, and the caudal peduncle region.

Supplementary morphological characters -- The analysis of the supplemental morphological characters corroborated the geometric morphometric analysis. Few morphological differences were found between exercised and non-exercised immature females, while CR and hatchery fish did exhibit significant differences in morphology. No significant effect of exercise on the supplemental morphological characters of immature female chinook was found (MANOVA, Wilk's 7 = .925, P = 0.53). Univariate F-tests also indicated that none of the individual partial warps were significantly different between exercised and non-exercised immature female chinook (P >0.22 in all cases), with the exception of snout length, which was marginally significantly different (P = 0.054) between the two groups . Principal components analysis revealed that 61.1% of shape variation was accounted for by the first two principal components. Projection of the immature female chinook specimens on these principal components revealed no evident separation of groups (Figure 5).

Significant differences in supplemental morphological characters between CR and OR chinook were evident (MANOVA, Wilk's 7 = .864, P = 0.0005). Univariate F-tests indicated that four of the variables (snout length, lower jaw length, anterior dorsal fin length, and pelvic fin length) were significantly greater (P <0.005 in all cases) in OR than CR chinook, while two were not significantly different (posterior dorsal fin length [P = 0.26]; pectoral fin length [P = 0.59]) between the two groups. Principal components analysis indicated that 66.5% of the total shape variation was accounted for by the first two principal components. Projection of the specimens onto these principal components (Figure 6) revealed some separation of groups, but the separation was not as clear as in the geometric analysis.

Breeding behavior and success

Soon after the OR adults were transferred to the MRS, they began to deteriorate rapidly and many died before they could be introduced into the channel for observations.

A similar rate of deterioration was observed in fish remaining at the Minter Creek Hatchery. This problem, combined with the low number of maturing CR fish (particularly males), precluded implementation of the experimental design described in the statement of work. Thus, attempts were made to quantify the breeding behavior of as many male-female pairs as possible, and to represent as many pairing combinations as possible.

As a result, a total of 18 pairs were introduced into the spawning channel representing at least one male-female combination of each of the three groups (Table 6). Too few replicate pairs were introduced into the channel to conduct a valid statistical comparison of the breeding behavior and success of each pair-type. The preliminary data suggest that for CR fish exercise may have improved the ability of females to construct nests and deposit eggs (Table 3). However, these parameters must be measured in future studies with adequate replication to determine whether or not females benefit from increased velocities in their rearing vessel.

Discussion

This study demonstrated that CR chinook salmon differed from OR chinook salmon in their body composition and morphology. Increased current velocities in vessels of CR chinook did not significantly affect changes in either body composition or morphology. The low number of maturing CR fish and the rapid physical deterioration of OR fish precluded a valid statistical analysis of the effects of rearing environment on spawning behavior and breeding success.

During the final months of ocean rearing, anadromous salmonids swim for prolonged periods at sub-maximal speeds, and increase food consumption and growth in preparation for upstream migration and spawning (Brett 1995). Sustained aerobic exercise has been reported to increase fish growth and alter body composition and energy content (Christiansen et al. 1989; Houlihan and Laurent 1987; Jorgensen and Jobling 1993). In seawater-reared chinook salmon, sustained exercise increased whole body protein levels and decreased lipid levels as swimming speed increased from 0.5 to 1.5 bl·sec^{-1} (Kiessling et al. 1994). In the present study, however, sustained, low velocity swimming activity (0.2-0.4 bl·sec^{-1}) combined with periodic forced exercise (0.8-1.4 bl·sec^{-1}) had no effect on the whole body proximate composition of either immature or mature male and female chinook salmon, compared with a sustained, low velocity swimming regimen alone. Feed intake in salmon may increase to offset elevated energy demand in exercised fish (Jorgensen and Jobling 1993). The ingestion of more food (protein and lipid) by fish subjected to periodic exercise and fed to apparent satiation may explain the lack of differences in body composition between the two treatments.

A primary objective of the study is to obtain mature adults similar in whole body composition to their ocean-reared cohorts. Statistically significant differences in whole body lipid, but not protein or ash, were observed between captive and ocean-reared salmon. Exercised fish were slightly, but not significantly, lower in whole body lipid than the non-exercised controls (2.9% vs. 3.5%), and both had significantly higher lipid

levels than the ocean-reared group (1.5%). Assuming similar conversion of somatic energy to gonads during maturation among the various treatment groups, differences in somatic lipid content between the captive and ocean-reared groups may be explained by the length of time without food, rather than differences in activity level. Maturing fish cease feeding prior to or upon freshwater entry, relying on body energy stores for activity metabolism and as a source of nutrients for gamete maturation. The difference in body lipid levels between maturing and non-maturing CR chinook indicates that even CR fish undergo reduction in whole body lipid during maturation, although not to the extent of that experienced by OR fish. Any potential benefits of exercise in CR fish may require an earlier cessation of feeding, which is more similar to wild or OR fish. This would allow a longer time for energy stores to be depleted, which must take place before complete development of secondary sexual characteristics (Hendry 1997).

Significant differences in body morphology were noted between captively reared fish and hatchery fish that went to sea. Geometric morphometric analysis revealed complex shape variation between the two groups that involved differences in snout and lower jaw, posterior insertion of the dorsal fin, anterior insertions of the pelvic and anal fins, and caudal peduncle region. Analysis of supplementary morphometric characters suggested that hatchery fish had longer snouts and lower jaws and larger dorsal and pelvic fins than captively reared fish. Previous studies showed that juvenile and adult salmonid body form changes when fish are cultured (Taylor 1986; Fleming and Gross 1989; Swain et al. 1991; Fleming et al. 1994) and that much phenotypic divergence was probably related to environmental conditions (Swain et al. 1991).

Exercise had no effect on the body morphology of immature female chinook, but small sample sizes prevented closer examination of morphological variation among captively reared fish of different sex, maturity status, and exercise status. It is possible that the results from immature females are not representative of the entire captively reared population, but sample sizes did not allow this to be tested.

Morphometric variation can contribute to differences in individual breeding salmon. In a previous study of captively reared chinook salmon, kype length independent of body size explained a significant portion of the variability in courting frequency, suggesting that this character may be important in male breeding success. In other Pacific salmon, certain morphological characters, independent of body size, have been demonstrated to influence male breeding success in; for example, Quinn and Foote (1994) found that hump height was correlated with male sockeye salmon breeding success. Coho salmon males with longer snouts attained greater access to spawning opportunities and greater estimated fertilization success than those with shorter snouts, and caudal peduncle depth was correlated with female breeding success (Fleming and Gross 1994; Hard et al. 1999).

In the breeding behavior portion of this study, the rapid deterioration of the hatchery fish probably resulted from an earlier-than-anticipated run-timing, meaning that fish were sampled during the latter part of the run. Fish that return later have lower energy reserves and a shorter reproductive lifespan than earlier returning fish (Hendry et al. 1998). In future studies involving ocean-reared fish, attempts should be made to

collect returning adults earlier in the run. Studies in subsequent years will circumvent the sample size problems encountered in the current study by increasing the numbers of experimental captively reared populations. The infrastructure improvements (see Task 8) will help to accommodate increased rearing demands.

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Table 1. Fish size and estimated swimming speeds (bl*s^{-1}) for Minter Creek Fall Chinook salmon. Data are means ±SE of two tanks per treatment.

	Day 27		Day 112		Day 260	
	CRE[1]	CRN[1]	CRE	CRN	CRE	CRN
Fish size						
Length (mm)	384.1±0.2	381.0±5.3	407.4±2.5	408.9±0.7	479.0±6.2	472.1±4.7
Weight (g)	839.6±6.7	805.6±25.5	871.7±34.7	916.8±5.6	1593.1±70.3	1517.5±62.8
Swimming speed (bl s^{-1})						
Baseline						
High[2]	0.39±0.01	0.46±0	0.37±0.01	0.43±0.01	0.31±0.01	0.37±0
Low[2]	0.29±0	0.31±0.02	0.27±0	0.29±0.02	0.23±0	0.25±0.02
During exercise periods						
High	1.42±0.02	- -	1.34±0.03	- -	1.45±0	- -
Low	1.04±0.01	- -	0.98±0	- -	0.85±0.08	- -

Notes: [1]Exercised captively reared (CRE); non-exercised captively reared (CRN). [2]High and low indicate ranges of swimming speed based on usual location of fish in tank (between 1.83 and 1.22 m from center of tank).

Table 2. Supplementary morphological characters used in the morphometric analysis.

Character	**ABBREVIATION**	Description
Snout length	SL	Tip of snout to center of eye
Lower jaw length	LJL	Tip of lower jaw to end of maxilla
Anterior dorsal fin length	AFL	Length of anterior edge of dorsal fin
Posterior dorsal fin length	DFL	Length of posterior edge of dorsal fin
Pelvic fin length	PLFL	Length of pelvic fin
Pectoral fin length	PCFL	Length of pectoral fin

Table 3. Proximate composition (% wet wt) of immature Minter Creek Fall Chinook salmon. Data shown are means ± standard errors.

		Moisture	Protein	Lipid	Ash
Treatment	CRE[1]	69.07±0.75	17.37±0.11	12.57±0.86	2.04±0.07
	CRN	70.69±0.72	17.25±0.16	10.80±0.81	2.02±0.04
	p	0.6898	0.5259	0.7083	0.1677
Gender	Female	69.56±0.69	17.20±0.11	12.09±0.79	1.99±0.03
	Male	70.59±0.80	17.54±0.17	10.79±0.83	2.13±0.10
	p	0.2737	0.0925	0.2324	0.0257
Interaction	*p*	0.0143	0.4918	0.0182	0.0358

[c1]Exercised, captively reared (CRE); non-exercised, captively reared (CRN).

Table 4. Proximate composition (% wet wt) of Minter Creek Fall Chinook salmon after spawning. Data shown are means ± standard errors.

		Moisture	Protein	Lipid	Ash
Treatment	CRE[1]	79.38±0.53	15.87±0.37	2.89±0.58[a]	2.06±0.20
	OR	79.94±0.29	16.53±0.24	1.50±0.14[b]	2.29±0.04
	CRN	78.65±0.49	16.32±0.33	3.53±0.36[a]	2.31±0.07
	p	0.0715	0.1972	< 0.0001	0.1575
Gender	Female	79.94±0.31	15.77±0.17	2.18±0.27	2.29±0.06
	Male	78.31±0.28	17.34±0.26	2.73±0.31	2.22±0.06
	p	0.0029	0.0003	0.0344	0.3439
Interaction	*p*	0.8209	0.3112	0.6453	0.7577

[1]Exercised captively reared (CRE); non-exercised captively reared (CRN); ocean-reared adults returning to Minter Creek Hatchery (OR).

Table 5. Sample sizes for groups of adult chinook salmon for morphometric analysis.

Origin	Sex	Maturity	Exercise	Number
Captive	Female	Immature	Yes	39
Captive	Female	Immature	No	32
Captive	Female	Mature	Yes	4
Captive	Female	Mature	No	12
Captive	Male	Immature	Yes	5
Captive	Male	Immature	No	9
Captive	Male	Mature	Yes	2
Captive	Male	Mature	No	6
Hatchery	Female	Mature	N/A	28
Hatchery	Male	Mature	N/A	33

Table 6. Mean (± S.E.) reproductive characteristics of different combinations of ocean-ranched (OR), captively reared exercised (CRE), and captively reared non-exercised (CRN) male and female chinook salmon chinook salmon introduced into the spawning channel. Behavioral frequencies (digs, probes, quivers, crossovers, attacks and lateral displays)were calculated per minute of observation. Duration refers to the time (days) in which females were sexually active, defined as the time between completing her first and last nest.

		Hatchery Female			Exercised female			Non-exercised Female		
	Male type	HAT n=4	CRE n=1	CRN n=3	HAT n=1	CRE n=1	CRN n=1	HAT n=2	CRE n=1	CRN n=4
Female Character										
	Weight (g)	4,463 (624)	3,997	4,493 (965)	3,309	2,900	3,033	2,363 (13)	2,435	1,838 (251)
	Eggs retained	1,948 (896)	2,491	2,436 (785)	11	2,817	299	1,958 (1,057)	2,705	1,812 (164)
	No.spawnings	2.3 (1.3)	3	1.3 (1.5)	5	1	4	0.5 (0.7)	0	0.8 (0.8)
	Digs	0.02 (0.03)	0.02	0.03 (0.02)	0.07	0.00	0.10	0.01 (0.02)	0.00	0.06 (0.04)
	Probes	0.14 (0.16)	0.13	0.03 (0.02)	0.35	0.01	0.39	0.12 (0.17)	0.07	0.60 (0.71)
	Attacks	0.02 (0.03)	0.01	0.05 (0.07)	0.00	0.00	0.01	0.01 (0.01)	0.00	0.02 (0.03)
	Lat Display	0.00 (0.00)	0.00	0.01 (0.01)	0.00	0.00	0.00	0.00 (0.00)	0.00	0.00 (0.00)
	Duration	0.80 (0.3)	--	0.10 (0.1)	2.9	--	3.1	0.52 (0.7)	--	0.10 (0.1)
Male Character										
	Weight	1,996 (1,293)	1,033	1,789 (960)	4,250	1,033	2,727	3,047 (1,720)	2,697	1,332 (322)
	Quiver	0.06 (0.06)	0.08	0.03 (0.01)	0.08	0.02	0.30	0.09 (0.12)	0.03	0.22 (0.21)
	Crossover	0.03 (0.04)	0.03	0.01 (0.02)	0.01	0.00	0.27	0.04 (0.05)	0.03	0.58 (0.92)
	Attacks	0.00 (0.00)	0.00	0.05 (0.06)	0.00	0.00	0.01	0.03 (0.00)	0.01	0.00 (0.00)
	Lat. Display	0.00 (0.00)	0.00	0.03 (0.02)	0.00	0.00	0.01	0.00 (0.00)	0.00	0.00 (0.00)

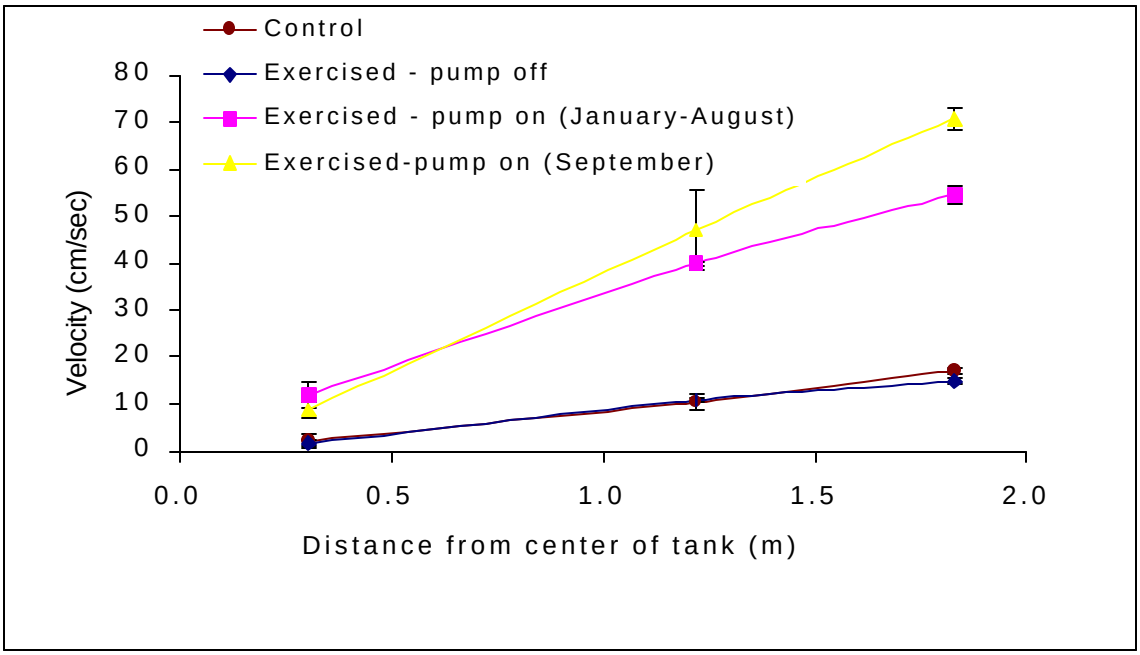

Figure 1. Velocity profiles in exercise and control tanks. Measurements were taken at three standardized distances from the standpipe, at each of four 90 degree intervals around each tank.

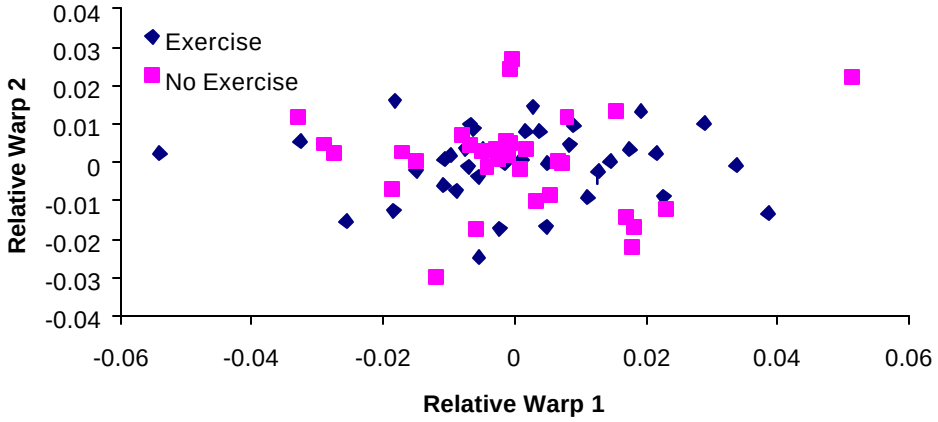

Figure 2. Scatterplot illustrating the relative warp analysis of 71 exercised and non-exercised immature female captively reared chinook salmon.

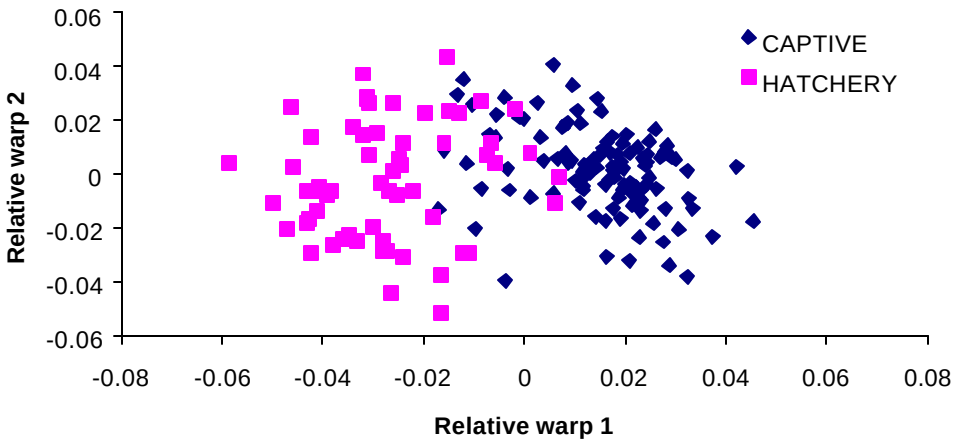

Figure 3. Scatterplot illustrating the relative warp analysis of 170 CR and OR (Hatchery) chinook salmon.

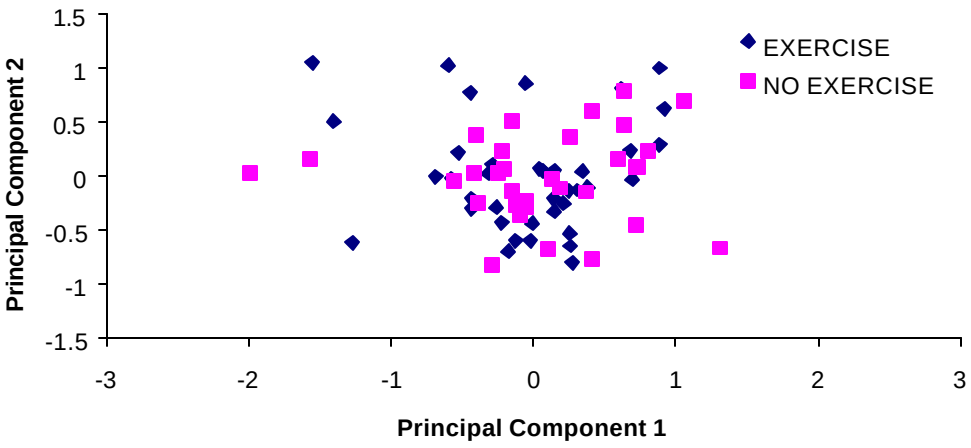

Figure 4. Scatterplot illustrating the principal components analysis of supplementary morphological characters of 71 exercised and non-exercised immature female CR and OR chinook salmon.

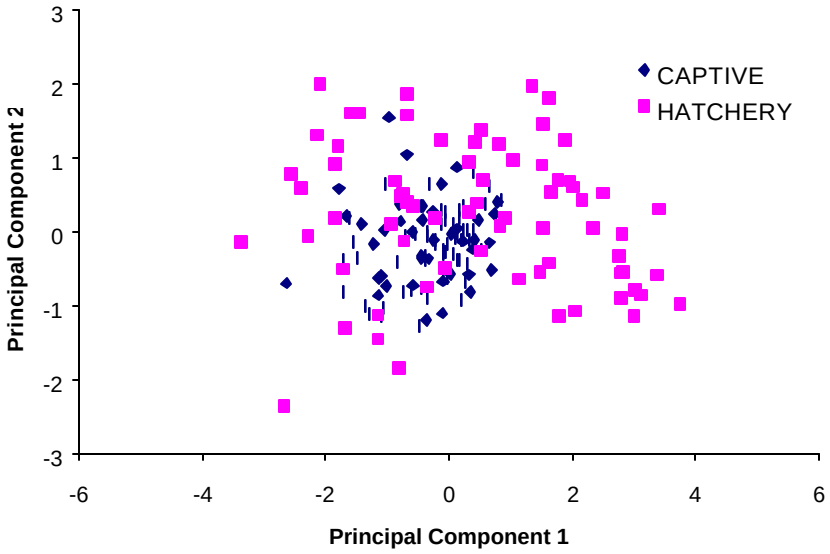

Figure 5. Scatterplot illustrating the principal components analysis of supplemental morphological characters of 170 CR and OR (Hatchery) chinook salmon.

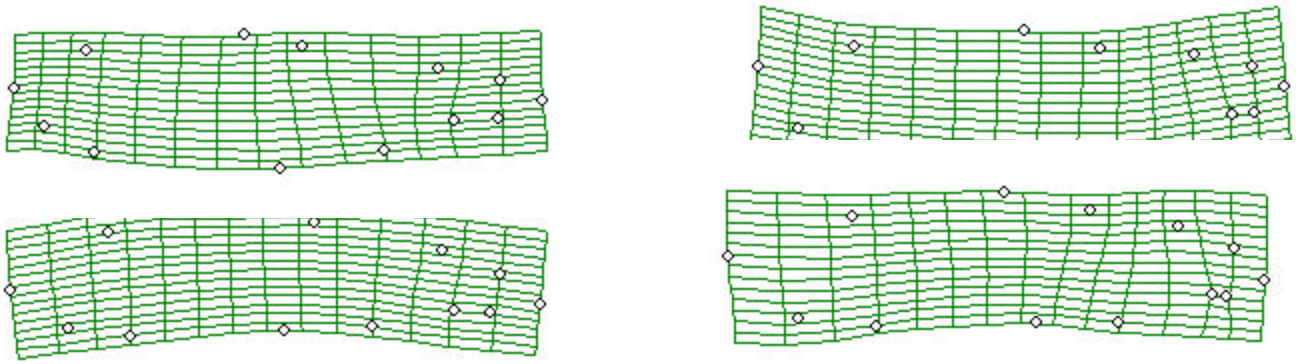

Figure 6. Thin-plate spline representations of shape deformations from the consensus configuration (see text) associated with relative warps (RW) 1 and 2. These images show how the consensus configuration for the entire chinook sample was deformed at the extremes of relative warps 1 and 2. The images are placed to represent the four corners of Figure 3, i.e. the top left figure represents the deformation associated with the top left of Figure 3, which is the lowest value of RW 1 and the highest of RW2. The anterior end of the fish is at the left in each diagram.

TASK 5. EFFECTS OF CAROTENOID DIETS ON ADULT BREEDING SUCCESS. (FINAL REPORT: 1 JUNE 1999 THROUGH 31 MAY 2000)

by

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Introduction

Sockeye salmon reared and spawned indoors under incandescent lights do not develop the bright red coloration and morphology typical of wild salmon. Sockeye reared under the same conditions except exposed to natural sunlight for the last months prior to spawning had better-developed coloration than fish kept inside (unpublished data). Egg quality appeared unaffected. Color and morphology differences between natural and captivity-reared adults reintroduced into spawning areas may lead to poor spawning success for the captively reared fish due to mate-choice selection behavior.

A pilot study was conducted in 1997 to determine if 1) dietary pigment source and/or, 2) the use of near-UV light (lacking in incandescent lights relative to sunlight) prior to spawning could enhance the development of secondary sex characteristics in spawning adult sockeye salmon. In this trial, the dietary treatments were applied for only 10 wk during the early summer, followed by the light treatments in the late summer until spawning in October (during which time the fish were starved). The trial was timed to correspond with the natural activity of the fish, e.g., feeding in saltwater under low near-UV light deep in the sea prior to entering the river, then starving as they migrate up the river and are exposed to high near-UV light intensities. Dietary treatments compared replicate groups of sockeye fed high levels of natural (various pigments from krill) or artificially produced pigments (astaxanthin only) with a control group fed low levels of pigment for the last 2 months of feeding prior to spawning (river migration and starvation). Results of this pilot study indicated that 1) a longer feeding period (or higher levels of pigment) was necessary to obtain a high enough level of pigment in the fish flesh to be available to transfer to the skin and, 2) light in the blue - black spectrum had a significant effect on final spawning coloration, though the coloration was still different than wild sockeye.

Work Completed

Starting in spring 1999, with spawning expected in fall 2000, sockeye salmon were stocked and fed one of three diets: 1) a natural carotenoid-enriched diet containing products derived from Pacific krill, 2) a carotenoid rich diet containing synthetic astaxanthin and, 3) a carotenoid deficient diet. The hypothesis being tested was that levels of pigment in the tissue of conventionally fed sockeye are too low for good skin pigmentation during spawning, and that light in the UV or near-UV range is necessary to induce this color change. In addition, astaxanthin and its esters (produced by chemical synthesis) in some previous diets are in a different form and may not be as effective as natural astaxanthin as a source of pigment for Pacific salmon. Previous work with natural astaxanthin at the University of Washington and current practices in Chile with farmed salmon suggest that fish pigment levels can be effectively increased by feeding certain krill-based products. The diets were to be fed for a full year prior to a 2-month light treatment. Replicate tanks of fish from each of the different dietary treatments were to be exposed to four light treatments: 1) incandescent light only (lacking in wavelengths below 500 nanometers), 2) incandescent plus black light (350-400 nanometers peak), 3) incandescent plus UV b light (300-350 nanometers peak) and, 4) full spectrum light sources (to approximate natural sunlight). These treatments were to be applied during the late summer and early fall until the fish spawn. During this time the fish would not be fed.

The feeding trial was stocked out and begun in winter 1999 as planned. Initially the diets were well accepted by the fish and growth. In early fall 1999 it was necessary to order an additional shipment of the krill hydrolysates used to make the diets. Within 2 wk of providing the diets containing the second shipment of krill hydrolysates, feeding in these groups dropped off. Feeding in the other groups was restricted to maintain equal growth rates among the treatments. During the next 4 months, diets were adjusted first by working with the supplier to get a new batch of the hydrolysates and then by adjusting down the level of krill in the formulations. While partial success was achieved getting fish to feed on lower levels of hydrolysate, optimal growth needed to spawn fish in the second year was not obtained. In addition, during the time that the fish were on reduced rations there was an outbreak of bacterial kidney disease (BKD). In spring 2000, the fish were still too small to spawn by fall so the experiment was ended.

Work to be Completed

At a meeting of captive broodstock researchers held prior to submission of the 1999 proposed work statement, this objective was rated as a low priority. Therefore, there will be no attempt to start the trial over again, or continue this line of research.

Table 1. Composition of experimental diets for pigmentation studies of adult sockeye salmon captive broodstock studies.

INGREDIENT	DIET 1 (%)	DIET 2 (%)	DIET 3 (%)
Anchovy meal	54.00	40.00	54.00
Blood meal	4.00	4.00	4.00
Wheat gluten	6.00	0.00	6.00
Wheat mids	19.00	16.10	19.10
Fish oil	13.00	11.00	13.00
Vitamin C	0.30	0.30	0.30
Choline	0.50	0.50	0.50
TM salt	0.10	0.10	0.10
Vitamin premix	3.00	3.00	3.00
Krill hydrolysate	0.00	5.00	0.00
Krill (frozen)	0.00	20.00	0.00
Astaxanthin	0.10	0.00	0.00

TASK 6. MEASURE THE BREEDING SUCCESS OF CAPTIVELY REARED STOCKS REARED IN DIFFERENT FACILITIES.
(FINAL REPORT: 1 JUNE 1999 THROUGH 31 MAY 2000)

by

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Background

As part of the captive rearing strategy by the Idaho Department of Fish and Game (IDFG) for Lemhi River chinook salmon, captively reared adults are released into streams to spawn naturally. In 1998, the first releases were made of an ESA-listed stock of chinook salmon which had been reared to adults in captivity. Preliminary information from IDFG (P. Hassemer, IDFG, personal communication) indicated that their ability to spawn successfully was less than that of their wild cohorts. As captive rearing technologies improve, it is important to monitor the breeding performance of captively reared chinook salmon to allow for improvements in rearing technology. Information gathered during the monitoring efforts is used to design controlled reproductive success experiments under the Captive Broodstock Research Project (93-056-00).

Work Completed

In 1999, scientists of the National Marine Fisheries Service (NMFS) assisted the IDFG in monitoring the success of captively reared chinook salmon released into Bear Valley Creek, Idaho. Bear Valley Creek is a tributary to Hayden Creek, which flows into the Lemhi River about 28 km north of Leadore, ID. In August 1999, all maturing chinook salmon (brood years 1995-1997) were measured, weighed, and identified by PIT-tag code as part of the ongoing IDFG pre-release sampling program. NMFS scientists emplaced a numbered Petersen disk tag through the dorsal musculature. Thirty-two males and 34 females were released into Bear Valley Creek during the last week in August 1999.

The behavior of fish in the stream was monitored daily by a combination of IDFG and NMFS personnel. Between 30 August and 17 September 2000, NMFS scientists scanned the entire stream section available to the chinook salmon once or twice daily. During each scan, observers walked the stream from the downstream weir to approximately 3.5 km upstream, where high stream gradient inhibited chinook from upstream-migration. The attempt was made to locate as many fish as possible. When a fish was located its "status" was recorded. Each fish observed was assigned a specific status, as follows.

Females:

Test digging **-** digging in a broad area, non-focused, and indicative of exploratory digging for a nest site (see Berejikian et al. in press);

Nest digging **-** focused digging as part of nest construction;

Cover digging **-** placement of gravel on top of a redd in which eggs have been deposited;

Holding away **-** swimming in main current, holding position, not under cover;

Holding near redd (male present) **-** female near a redd or excavated area with a male nearby;

Holding near redd (male absent) **-** female near a redd or excavated area with no male nearby;

Inactive **-** relatively motionless, usually along the stream margin or under cover.

Males:

Courting **-** male attending a female and exhibiting courtship behaviors including crossovers and quivers (Tautz and Groot 1976; Schroder 1981; Berejikian et al. 1997);

Satellite **-** male closely associated with the courting pair but positioned slightly downstream. Satellites had frequent aggressive interactions, but were subordinate to the dominant (courting) male;

Holding near female **-** male positioned close to female but exhibiting no courtship behavior;

Wandering **-** actively swimming or holding position in fast currents, but not competing for access to or courting a sexually active female.

Inactive **-** same as for females.

In addition to the scan sampling, the development of individual redds throughout the stream section was documented from the first observed spawning until no females remained sexually active. Each new redd was numbered, and each day the location and condition (e.g., size and state of development) of each redd was documented. This allowed estimation of possible spawning events over night.

When a female was observed nest digging and was actively courted by a male, an observer monitored the pair of fish until spawning occurred. Where possible spawning event locations, time, and participants in the spawning event were recorded.

To eliminate duplicate reporting of data, IDFG and NMFS collaborators agreed that all data collected were to be summarized for the purposes of producing a final report by IDFG. Therefore, data recorded by NMFS personnel from the above work were provided to IDFG on 12 May 2000 in electronic format (Excel 2000 spreadsheets). NMFS will continue to provide technical support during data analysis and report as requested by IDFG. This task will be continued in future years to determine improvements (or not) in the breeding success of captively reared chinook salmon as changes to culture technologies are made. Information gathered from these studies will continue to be shared between IDFG and NMFS through the Snake River Chinook Technical Oversight Committee process. Results will help to guide future research under this project as it relates to quality of adult salmon.

TASK 7. THE EFFECTS OF GROWTH AND A LOW FAT DIET ON THE INCIDENCE OF EARLY SEXUAL MATURATION IN MALE SPRING CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*) (FINAL REPORT: 1 JUNE 1999 THROUGH 31 MAY 2000)

by

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Summary

The purpose of the experiment was to determine the effects of growth rate, body size and low body fat on the incidence of early sexual maturation in male spring chinook salmon (*Oncorhynchus tshawytscha*). To produce fish of a range of sizes and growth rates, and low body fat, fish (0.5 g) were fed a practical low fat diet (7% fat, 54% protein, 19.7 kJ/g) at six feeding levels (100 = full ration, 88, 76, 64, 52, or 40%) from February 1998 to July 1999. As a control, one group of fish was fed a commercial diet containing less protein and more fat (49.9% protein, 21.8% fat, 22.1 kJ/g) at the 64% level. All diets were fed in duplicate (700 fish/tank). Fish were sampled at approximately monthly intervals to monitor growth, whole body lipid and state of sexual maturity. To monitor smoltification, gill ATPase and plasma insulin-like growth factor I (IGF-I levels) was measured in fish sampled from the 88, 64 and 40% ration groups from March to November 1998. An additional group of 1400 fish that were fed the experimental diet at 100-88% ration was sampled at monthly intervals to determine when maturation was initiated, and characterize the endocrine changes associated with somatic and testicular growth in maturing and non-maturing male fish. Pituitary follicle-stimulating hormone (FSH), plasma 11-ketotestosterone (11-KT) and IGF-I were measured in individual fish in this group from July 1998 to October 1999. The degree of testicular development was determined by histology.

At the end of the experiment in July 1999, mean weights of fish were: 336, 304, 286, 241, 203, 161 and 258 g for the 100-40% ration groups and the commercial feed group, respectively. From spring 1999 to the end of the experiment, maturing males were significantly larger than non-maturing males and females, which were similar within each treatment. Whole body fat levels in fish fed the low fat experimental diet, at all feeding levels, did not differ and remained between 2.5 and 5% from the start of the experiment until September 1998. Subsequently, fat levels increased to between 6 and 8% by July 1999. Fat levels in fish fed the commercial feed increased from first feeding to 8% in September 1998, and continued to increase up to approximately 12% by July 1999. Maturing males in all treatments had significantly higher fat levels than non-maturing males or females from January 1999 until the termination of the experiment in July 1999. The incidence of maturing males ranged from 66 to 93% and was significantly affected by feeding level. The relationship between feeding level and maturation was best described using a quadratic equation, lowest in the fish receiving 40% ration and highest in fish fed at the 76% ration, with maturation rates in fish fed the 100% level slightly lower than that of the 76% level. The incidence of maturation between the fish fed the commercial and experimental feed at the 64% ration levels did not differ, despite large differences in body fat suggesting that body fat levels had no effect on the incidence of maturation in these large fish.

Ration did not appear to alter the process of smoltification. Fish in low (40%), medium (64%), and high (88%) ration groups smolted in the first spring as indicated by increases in gill Na+K+ ATPase levels.

The timing of the onset of maturation was determined by both testis histology and plasma androgen levels. The first histological evidence of the onset of spermatogenesis (presence of type B spermatogonia) was first observed in a small number of males during November 1998 (10 months prior to spermiation in October 1999). However, similar changes were observed in a larger proportion of fish during February 1999, suggesting that the period of puberty (onset of spermatogenesis) could be as great as 4 months. Frequency distribution of plasma 11-KT levels at each monthly sampling indicated that there was a clear bimodality reflecting differences between maturing and non-maturing fish in December 1998. This bimodal distribution of 11-KT levels became more evident in subsequent months. From February 1999 to the end of the experiment, more advanced stages of spermatogenesis were observed. It was not possible to classify fish as maturing or non-maturing based only on gonadosomatic index (GSI) until after May 1999. However, fish with a low GSI showed no testis development (only type A spermatogonia), and had low levels of pituitary FSH and plasma 11-KT. Conversely, fish with a high GSI showed progressive maturation of the testis along with elevated pituitary FSH and plasma 11-KT levels. Plasma IGF-I levels and body weight were not strongly associated with maturation at the early stages of testis growth (December-February); however, as testis development progressed (April-September) higher plasma IGF-I levels and body weight were seen for maturing compared with non-maturing males. Preliminary analysis of data collected during the initial months of the experiment, July-November 1998, indicated a correlation of pituitary FSH levels with fish weight, plasma 11-KT and IGF-I levels. It remains to be determined if these associations represent a direct interaction between the growth and reproductive axis.

The results from this study indicate that the incidence of maturation in male spring chinook salmon is increased by growth. Body fat levels had no effect on maturation rates in fish of the size produced in this study, which were much larger than wild fish. If there is a threshold of size and/or growth rate below which maturation is prevented, it is much smaller than fish produced in this study. Data from previous studies suggest that the threshold of size may be below a weight of 12 g in December. Future studies will address this using commercially available diets. The data on endocrine changes associated with spermatogenesis suggests that the period when maturation is initiated in males is broader than previously thought and may extend over a period of 4 months, from November through February of the previous year during which the fish will spawn.

Introduction

Male chinook salmon (*Oncorhynchus tshawytscha*) have a plastic life history and can sexually mature from 0 to 7 years of age. Early sexual maturation of males (maturation before the youngest females) occurs in wild fish but the incidence is far lower than observed in hatchery-reared fish (Foote et al. 1991, Mullan et al. 1992). Up to 70-96% of male chinook have been reported to mature early when fish are reared in captivity (Hard et al. 1985). Early sexual maturation of male salmon results in reduced effectiveness of enhancement efforts and a financial loss to the salmon farming industry. Age of sexual maturation of male chinook salmon has been shown to be influenced by genetic, biotic, and abiotic factors (Power 1986). The level of energy stores has been shown to influence the incidence of sexual maturation in Atlantic (Rowe and Thorpe 1990b) and Pacific salmon (Silverstein et al. 1997, Shearer and Swanson 2000), but fish size and/or growth rate at specific times of the year appear to be the biotic factor exerting the most influence (Hopkins and Unwin 1997, Silverstein et al. 1997). Previous studies in spring chinook salmon indicate that maturation can be initiated in the late fall approximately 10 months prior to final maturation (Silverstein et al. 1997, Shearer and Swanson 2000). However, the length of this initiation period is not well defined and may extend into late winter. It has been suggested, primarily from studies in Atlantic salmon that the spring preceding autumn spawning is a permissive period. In other words, maturation is then 'permitted' to progress if growth and energy stores are adequate to support maturation. Reducing feeding level or fasting during the spring period has been shown to reduce the incidence, but not prevent early male maturation in Atlantic (Rowe and Thorpe 1990a, Herbinger and Friars 1992, Berglund 1995) and chinook salmon (Hopkins and Unwin 1997). The optimum strategy for preventing early male maturation may be to prevent initiation. Thus, it is necessary to determine whether there is a threshold of growth or body size below which maturation will not be initiated.

The primary objective was to clarify further the relationship between growth and the incidence of maturation by determining if there was a threshold of size or growth rate for maturation, and to determine if rearing fish on a low fat diet could prevent early maturation in relatively fast growing male chinook salmon. Additional objectives were (1) define the period when the maturation process was initiated and to determine the endocrine changes associated with somatic and testicular growth, (2) determine the effects of growth on smoltification, and (3) determine the effect of growth rate and dietary fat level on immunocompetence (see Task 9 of this report, by R. Pasch and S. Alcon).

Materials and Methods

Fish, Rearing Conditions, Diets and Feeding

Chinook salmon eggs were obtained from the Willamette salmon hatchery and incubated at the Northwest Fisheries Science Center, Seattle, WA. After hatching (20 January 1998; see Table 1 for a chronology of the experiment) fish were placed into a single tank (4' d) supplied with water from a recirculation system (8 °C). Fry were fed a commercial salmon starter feed (BioOregon-Starter, 23.1 kJ/g energy, 50% protein, 22% lipid) for one month. Fish (0.5 g) were then randomly assigned (700/tank) into tanks (4' d) and fed the experimental diet at one of six feeding levels: satiation (100% ration) or 88, 76, 64, 52, or 40 % of the satiation level. Each ration was fed to two replicate tanks of fish. An additional treatment consisted of two tanks of fish fed a commercial feed (BioOregon-grower, 22.1 kJ/g energy, 49% protein, 22% lipid) at the 64% level (based on an equivalent dry matter). The experimental diet (Table 2) was formulated to contain 54% protein, 7% fat, and 19.7 kJ/g energy. Fish were fed a single meal, by hand, each morning. The fish were fed 6 d/wk for the first 16 wk and 5 d/wk thereafter. A natural photoperiod was used and temperatures varied seasonally between 8-12.5 °C (Figure 1).

After 18 wk (day 173) the 100 and 88% groups were moved to larger tanks (8' d) and the 76 and 64% groups were moved to larger tanks (6' d). Prior to the move, all fish were given a $KMnO_4$ bath (5 ppm) for 1 h to control *Costia necatrix* infestation. The number of fish in each tank was reduced to 350 on day 207 due to the limitations of the biofilter capacity. Erythromycin (100 mg/kg body wt /d for 2 d) was added to the feeds on two occasions (January-February and April-May, 1999) to control bacterial kidney disease (BKD). Mortality and the amount of feed fed were recorded daily. The main experiment was terminated in July 1999 after 539 d (day 559). At this time eight unsexed fish from each tank were tagged with passive integrative transponder (PIT) tags and one replicate from each treatment was placed into one of two tanks so that the males could be reared to final maturity in October 1999 (day 644).

Sampling and Fat Analysis

Ten fish from each tank were randomly selected for determination of body weight, length and proximate composition at approximately monthly intervals over the first year and approximately every 6 wk thereafter (Table 3). Fish were sexed beginning with the sample collected on day 215. Beginning on day 439, gonads were dissected and weighed to determine gonadosomatic index (GSI). For proximate analysis, fish smaller than 1 g were pooled (10 fish) before analysis. Fish between 1 and 100 g were chopped, dried and ground in a mortar and pestle. Fish larger than 100 g were ground in a food processor and a sub-sample of 100 g of wet material was dried, then reground and sub-samples were taken for analysis. Moisture was determined by drying to constant weight at 105 °C, fat was determined using a Soxhlet device with dichloromethane as the solvent. Moisture and fat analysis were performed on individual fish over 1 g (10 fish /tank). Energy density of feeds was determined using a oxygen combustion bomb calorimeter (model 1108, Parr Instrument Co., Moline, IL.).

Assessment of Smoltification

Beginning on 16 March 1998, samples were collected to monitor smoltification during spring and fall 1998. Samples were taken at approximately 2-wk intervals through 21 June. Sampling was resumed on 6 August and continued through 5 November. Six fish were taken from each of the replicate tanks of the 40, 64, and 88% ration treatments. Fish were killed with a lethal dose of MS222, weighed, and measured. Subsequently the tail was severed and blood was collected in heparinized capillary tubes. Plasma was prepared by centrifuging blood at 3,000 x g for 3 min. Plasma was stored at -80 ^{o}C until analysis for insulin-like growth factor I (IGF-I), according to Shimizu et al. (2000). Gill filaments were clipped and stored in a sucrose, EDTA, imadazole buffer at -80 ^{o}C until Na^{+} K^{+}ATPase activity was assessed using the method of McCormick (1993).

Assessment of Sexual Maturation

To allow for extensive sampling of maturing and non-maturing male fish during the course of the experiment, an additional two tanks of fish fed the highest ration were established. The purpose of this sampling was to monitor key reproductive hormones and testicular development to determine when puberty was initiated in the male fish used in this study. During the initial sorting of fish into experimental groups in February an additional two tanks of fish (0.5 g, 700/tank) were stocked and fed the experimental diet at 100% ration. These fish were reared in parallel to the fish being fed 100% ration in the main experiment. On day 173 these fish were moved to a recirculation system similar to that used by the main experiment and stocked into four tanks (6' d) at 350 fish/tank.

Sampling commenced on July 8 (day 189) and continued at approximately monthly intervals until November 9 (day 313). At this time BKD severely affected these fish and the test was terminated. A new group was created using culled fish from the 80-90% ration groups from the main experiment, and stocked in four tanks (4' d) at 130 fish/tank. Due to the limited number of fish available the sampling period was extended from 4 to 7-wk intervals. These tanks were fed at 88% ration due to loading constraints of the recirculation system. The final major sample was taken on 28 June (day 544) when mature and immature males were clearly distinguishable by GSI and histological appearance of the testis. A small number of fish were maintained until 29 September (day 637) when males were fully mature. At this point the experiment was terminated.

A minimum of 10 males was sampled from each tank on each sampling date. As these groups were of mixed sex, a range of 13-23 fish was taken per tank/sample time over the period of the experiment. After each sampling the number of fish removed was recorded and ration adjusted accordingly. Fish were killed with a lethal dose of MS222, weighed, and measured. Blood was collected via the caudal vein, initially using heparinized capillary tubes after severing the caudal peduncle. When fish were greater than 50 g, blood was collected via heparinized syringes. Pituitaries were frozen in liquid nitrogen and stored at –70 °C. Testes were dissected, weighed, and fixed in Bouin's fixative for 24 h prior to storage in 70% ethanol. Bodies were stored at –20 °C. Blood was centrifuged at 3,000 x g for 3 min and plasma stored at –70 ^{o}C.

Hormone analyses were performed on a portion of the 40 males collected per sample point. Initially GSI was used as a guide to select 16 representative males from the 40 sampled. From January 1999 to the end of the experiment, testis histology was used to distinguish immature and maturing males. A variety of hormone analyses was performed. Plasma 11-ketotestosterone (11-KT) was measured according to Schulz (1984), using a methylene chloride extraction method described in Planas and Swanson (1995). Plasma IGF-I was measured according to Shimizu et al. (2000). Pituitary follicle stimulating hormone (FSH) was measured according to Shearer and Swanson (2000).

Fixed testis were dehydrated through ethanol and imbedded in paraplast, sectioned, and stained with hematoxylin and eosin. Stages of spermatogenesis were determined by light microscopy using Schulz (1984) as a guide. Details of lobule numbering and cell development were as follows: Type A spermatogonia only (–), 1-5 cysts of type B spermatogonia (+), greater than 5 cysts of type B spermatogonia and or spermatocytes (2+), appearance of spermatocytes and spermatids (3+).

Statistics

Tank means were used as the unit of observation unless otherwise stated. Percentage data were arcsine transformed prior to ANOVA, and Fisher's PLSD test was used to conduct multiple mean comparisons. Regression was performed on untransformed data. All analyses were performed using Statview™ (Abacus Concepts, Berkeley, CA).

RESULTS

Growth and Mortality

The feeding regime was designed, assuming equal feed efficiency, to produce fish which differed in weight by 12% between successive feeding levels. It had been planned initially to feed the 100% ration fish to satiation, but this proved impossible due to the limitations of the biofiltration system. Although water quality parameters, such as ammonia, oxygen, hardness, etc., remained within the tolerance range for salmonids over the course of the experiment (Wedemeyer 1996), there were indications that fish were being stressed. When the fish receiving the highest ration (100%) were fed to satiation on Monday (after 2 days of not being fed) from July 1998 onward, all groups failed to consume their allotted ration the next day. Therefore, feeding was adjusted to below satiation by observing feeding behavior on subsequent days. Briefly, the amount fed to the 100% groups was determined by observing the feeding response each day and adjusting the feeding level of the lower ration groups accordingly. On 1 December 1998 mean weights of the fish in each treatment (n=2) were approximately 119, 100, 92, 79, 64 and 51 g for the 100 to 40% ration groups, respectively, and 79 g for the fish fed the commercial feed. Based on the amount of feed, fish fed at the 100 and 40% levels were slightly smaller than expected throughout the experiment (Figure 2, Table 4). From day 439 (March 1999) males were significantly larger than females in all treatments (Figure 3). Maturing males were approximately 50 g larger than non-maturing males or females in all treatments at the end of the

experiment (Figure 4). Differences in condition factor between males and females were observed as early as November (Day 321) (Figure 5). The cumulative total of fish sampled by day 559 (July 1999) was 1,654. Overall mortality was 9.3% and did not differ among treatments. Mortality was attributed to poor water quality (5.9%), BKD (2.2%), and unknown causes (1.2%).

Whole Body Fat

In fish fed the low fat experimental diet, whole body fat levels (Figure 6) remained relatively low during the period from day 50 until approximately day 257 (February to November 1998). In contrast, fish fed the commercial diet, which contained a higher fat level, increased in whole body fat during this period. From day 257 until the end of the experiment (day 559, July 1999) all groups showed increases in body fat. At the last sampling, July 1999, maturing males had significantly higher levels of body fat than non-maturing males and females in all treatments (Figure 7).

Smoltification

At the initial sampling in March plasma IGF-I levels were similar among the treatment groups (Fig. 8). By mid-April through June, plasma IGF-I levels significantly increased to approximately 15 ng/ml in fish fed the 64 and 88% rations. Plasma IGF-I levels in fish fed the 40% ration remained below 10 ng/ml through June. Subsequently, plasma IGF-I levels increased to above 15 ng/ml in all treatments from August through November. Peak values near 30 ng/ml were attained in early September in the fish fed 64 and 88% rations. There was a general decline in IGF-I levels from early September through November. IGF-I levels in the 40% group were generally lower than found in the 64 and 88% groups. There were no consistent differences between the two groups fed at a higher level.

Gill ATPase activities increased in all groups from March through June, with the highest activities found in the fish fed 88% ration in May and June (Figure 9). Both the 40 and 64% ration groups showed a strong peak in activity in the fall, with peak values in the 64% group preceding those found in the 40% group by about a month. There was no fall peak in ATPase activity for the 88% group. Fish in all treatments showed a general decline in ATPase activity by early November.

Initiation and Progression of Sexual Maturation

Due to problems experienced with loading in the water recirculation system and disease outbreaks, the fish used for monitoring endocrine changes during maturation did not grow as large as the fish fed the 100% ration in the main experiment. However, these fish eventually obtained a size equivalent to that of the 64% ration group. From May 1999 (day 509) males where significantly larger than females (Figure 10). Only one maturing female was seen throughout the experiment. In September 1999, 80% of the males were spermiating, which is consistent with the 81% male maturity estimated in June 1999 from GSI and histological appearance of testis samples collected during that period.

Maturing and non-maturing males were distinguished using parameters directly associated with the onset and progression of spermatogenesis, viz, appearance of type B spermatogonia, spermatocytes, and spermatids, and increases in GSI and plasma 11-KT levels.

During November, spermatogonial proliferation was observed in the testes of several of the males sampled (Table 5); however, there was no clear division between non-maturing and maturing in any of the factors associated with testis growth (testis histology, GSI and plasma 11-KT). Thus, for samples taken from July 1998-November 1998 preliminary correlation analysis was performed between factors with hypothesized interactions (Tables 6 and 7). Pituitary FSH and plasma 11-KT levels showed a strong positive correlation during August, September and November. Plasma IGF-I and pituitary FSH levels showed a similar positive association with high correlation during the same months. Plasma IGF-I levels were correlated with body weight during July-October 1998, however this relationship was not significant in November. Pituitary FSH levels were correlated with body weight during July, August and November.

During January and December, there was no distinct bimodality in the GSI data. However, there was a clear bimodal distribution in plasma 11-KT levels, the higher mode coinciding with a few individuals where type B spermatogonia were observed in the testis, and the lower mode generally corresponding to individuals with low GSI values (Figure 12, Table 5). When the data was divided into maturing and non-maturing males based on this bimodal split in plasma 11-KT levels, maturing males had significantly higher GSI and pituitary FSH levels (Figure 12). There was no difference between maturing and non-maturing fish in terms of body weight and plasma IGF-I levels (Figure 12).

In February, maturing males were distinguished from non-maturing males by testis histology (appearance of type B spermatogonia or spermatocytes). The testes of non-maturing males had only type A spermatogonia. Although the GSI data did not show a clear bimodal distribution, individuals that exhibited an advancement in spermatogenesis also had higher GSI (Figure 12b Table 5) than the non-maturing males. Maturing males had significantly higher pituitary FSH and plasma 11-KT levels (Figure 13) than non-maturing fish. However, some of the individuals showing no detectable testis development by histology had relatively high FSH and/or 11-KT levels (see Figures 12b and 12c). There was no significant difference between maturing and non-maturing fish in terms of body weight and plasma IGF-I levels. Some of the individuals showing no detectable testis development did have relatively high FSH and/or 11-KT levels (see Figures 12b and 12c).

Maturing and non-maturing males could clearly be distinguished by April 1999 using testis histology (appearance of type B spermatogonia and spermatocytes) and bimodality of the distribution of GSIs in sampled fish (the lower mode corresponding to non-maturing males, Figures 11 and 12a, and Table 5). From April to September 1999, maturing males were significantly larger and had significantly higher levels of pituitary FSH, plasma 11-KT and IGF-I when compared with non-maturing males (Figure 12).

Effect of Growth and Body Fat on the Incidence of Sexual Maturation

In the main experiment, the mean male GSI increased from 0.16 in March 1999 to 0.29 in May, and 3.49% at the end of the experiment in July 1999 (Table 8). It was not possible to classify males as maturing or not maturing in May as there was no clear bimodality in the distribution of GSI data. In July 1999, males were classified as maturing (GSI>1.0%) or non-maturing (GSI<1.0%) (Figure 14). Of the 744 males sampled in July 1999, 82.3% were considered to be maturing.

The incidence of maturing males in July 1999 differed among treatments (Table 8) and the relationship between feeding rate and percent maturation was best described using a quadratic model (Figure 15). Of the 112 fish that were PIT tagged in July 1999, 4 died of BKD and 9 lost their PIT tags. When fish were examined on October 6 (day 644) there were 53 males and 53 females. Of the males, 45 (85%) were fully mature which was similar to the 82.3% maturation observed in July.

Discussion

Growth and Fatness

The purpose of the experiment was to determine the effects of low body fat stores and growth rate or body size on the incidence of early male sexual maturation in spring chinook salmon. It is generally considered desirable to have rapid growth in fish reared for enhancement or commercial aquaculture. A positive relationship between size at release and adult return rate has repeatedly been reported (Burrows 1969, Ewing et al. 1980). When chinook salmon are farmed, larger juveniles will smolt at 0-age allowing transfer to seawater earlier, thus reducing the length of the production cycle. Large body size is also advantageous in adult fish being released for enhancement purposes. In females, large body size is accompanied by high fecundity, while in males mating success is improved with large body size (Berejikian et al. 2000). Although a high growth rate prior to smoltification in salmon is sometimes advantageous, a number of studies with both Atlantic salmon (Berlund 1995, Friedland and Haas 1996) and Pacific salmon (Clarke and Blackburn 1994, Silverstein et al. 1997) have shown that a high growth rate increases the incidence of early male sexual maturation.

In the present study the rate of maturation in 1+ spring chinook salmon was increased by increasing growth rates or body size, ranging from 66-82%. However, the rate of maturation even in the lowest ration group was much higher than expected for this stock of fish. This was most likely due to the rapid growth and large body size achieved using this experimental diet and rearing regime. In a study of Willamette River spring chinook salmon, Beckman et al. (1998) reported differences in size between wild and hatchery-reared fish. During August, body weights in 0-age wild and hatchery fish were 6 and 12 g, respectively. During November body weights were 10 and 63 g in wild and hatchery fish, respectively. In comparison, 0-age fish fed the 100 and 40% ration levels in the present study were 22 and 12 g in August, and 90 and 40 g in November. Clearly the fish produced in the present study were much larger than wild fish, but

within the range of body size of hatchery fish. From the start of the experiment in January 1998 to November 1999, the specific growth rates of the 100 and 40% ration groups were 1.8 and 1.5%/d, respectively. This is lower than expected for 0-age chinook salmon (2-2.5 %/d) when fed to satiation under hatchery conditions at temperatures similar to those of the present experiment (Brett et al. 1982). The growth rates obtained in the present study were higher than s normally observed in a hatchery, but far less than the maximum that can be achieved by this species. There is little basis for comparison of the growth achieved by the 1^+ fish in the present experiment, since fish of this size would most likely have been released at 0-age. Growth rates in all of the experimental groups were about 0.5%/d between November 1998 and July 1999. This reduction in growth was necessary due to the limitations of the capacity of the biofilter in the water recirculation system. As early as April 1999, maturing males were significantly larger than non-maturing fish of either sex growth. This has been shown previously for both males and females in Atlantic (Simpson 1992, Kadri et al. 1996, Stead et al. 1999) and chinook salmon (Shearer and Swanson 2000).

As rapid juvenile growth is generally achieved by feeding a high ration of an energy-dense diet, high body fat stores are generally associated with a rapid growth rate (Shearer et al. 1997). The low fat diets fed in the present experiment produced fish with low body fat levels during the first year of the experiment (up to January 1999). After this point, endogenous processes resulted in higher body fat levels in these fish regardless of sex or maturation state. This is in contrast with earlier work (Shearer and Swanson 2000) where fish fed low fat diets, and reared under similar conditions, maintained low body fat levels during their second year. One possible explanation is that the fish in the present experiment were considerably larger at a similar age, and there is a general tendency for fish to increase in fat with increased size (Shearer 1994). Another possible reason for the difference in fat between the two experiments is that the fish used in Shearer and Swanson (2000) were the progeny of wild parents (Yakima stock) while hatchery fish from the Willamette stock were used in the present study. There is insufficient published information to resolve this question. An increase in fatness associated with sexual maturation in salmon has been reported previously (Kadri et al. 1996, Stead et al. 1999).

Smoltification

There was a general seasonal change in plasma IGF-I levels, increasing from March through September and then declining through November. This seasonal change was greater than differences found between treatment groups. There were no consistent, significant differences between the feeding groups, though the 40% group generally had the lowest IGF-I levels. Thus, even though there were differences in size between the groups, there were no consistent differences in endocrine growth signal.

All fish displayed development of gill ATPase activities indicative of smolting by mid-May, which is relatively early for spring chinook salmon. Development of smolt characters in this late spring period was probably due to the relatively high growth rates found in these fish compared with wild fish. A second peak in gill ATPase was found in the fall. This is characteristic of fish which are maintained in captivity and has previously been described by Ewing et al. (1979 and 1980) for Rogue River spring chinook salmon. The temporal difference

in peaks of gill ATPase between the 40 and 64% ration groups may be related to growth rates or size, as the 64% group was both larger and had higher plasma IGF-I levels than the 40% group. The relatively low ATPase values found in the 88% group is enigmatic. Further analysis of the data, separating maturing from non-maturing males, may be necessary to determine the cause this difference. In a previous study maturing males exhibit a spring but not an autumn increase in gill ATPase (P. Swanson, NMFS, personal communication).

Initiation and Progression of Maturation

One of the goals of this study was to define more precisely the period when maturation is initiated, and to describe the changes in key reproductive hormones during the onset of spermatogenesis in male spring chinook salmon. Previous studies by Duston and Saunders (1997) and Stead et al. (1999) demonstrated that yearling Atlantic salmon males initiate sexual maturation approximately one year prior to final sexual maturation. This also appears to be the case for male chinook salmon (Silverstein et al. 1998, Shearer and Swanson 2000).

The pituitary-gonadal axis (FSH and 11-KT) -- From the data on reproductive hormones and testis histology maturation had commenced by December 1998, with maturing males having significantly higher pituitary FSH, plasma 11-KT and GSI than non-maturing males. The levels of these both FSH and 11-KT rapidly increased with testis growth (January to September 1999) consistent with their established role in regulating spermatogenesis. FSH from the pituitary stimulates the production of 11-KT by the testis, which in turn stimulates all phases of spermatogenesis (Planas and Swanson 1995; Nagahama et al. 1997).

In the present study, plasma levels of 11-KT were the best indicator of maturation, with a clear bimodal distribution observed as early as December, even when detection of type B spermatogonia was not possible in most fish. At this stage many males destined to mature (final maturation of 80%) did not display any detectable spermatogonial differentiation, yet the hormone known to be involved in this process (11-KT) was clearly elevated in many of these individuals. During November a small number of fish showed some spermatogonial proliferation and differentiation indicating that maturation had commenced in these individuals. However, most individuals showed no evidence of progression of spermatogenesis as the testes contained only type A spermatogonia. In addition, it was not possible at this stage to distinguish maturing and non-maturing groups by bimodal distributions in hormone levels. Correlation analyses during November indicated a positive correlation between pituitary FSH and plasma 11-KT levels, and between plasma 11-KT and GSI. Coupled with the finding of some individuals showing spermatogonial proliferation and differentiation, these correlations support the contention that maturation was underway in November. Furthermore, pituitary FSH and plasma 11-KT were strongly positively correlated several months earlier (during August and September 1998), one year or more prior to spermiation in September 1999. Therefore it is possible that these reproductive hormones initiated maturation as early as August-September. One of the problems with this data is that during, August and November, pituitary FSH and plasma 11-KT levels were also positively correlated with body weight. Thus, the correlation between these two hormones may be a direct result of body weight. The precise relationship among these factors remains to be determined.

Interactions between the growth and reproductive axis -- Previous studies in spring chinook salmon have shown that body adiposity and growth can affect the proportion of males maturing in a population (Silverstein et al. 1998, Shearer and Swanson 2000), and that these factors may influence maturation up to a year prior to spermiation (Silverstein et al. 1998). Recent research has indicated that IGF-I plays an important role in growth and reproduction in fish and may be a critical factor for the interaction between the growth and reproductive axes (see references in Baker et al. 2000). It has been hypothesized that plasma IGF-I may stimulate FSH production by pituitary and/or androgen production by the testis, thus providing a hormonal link between the growth and reproductive axis (Loir and Le Gac 1994, Maestro et al. 1995, Le Gac et al. 1996, Weil et al. 1999, Baker et al. 2000). In the present study, from April 1999 through to September 1999 (spermiation) maturing males had significantly higher plasma IGF-I levels compared with non-maturing males; however, it is not clear whether this is an effect of size or maturity. At this time, maturing males were significantly larger than non-maturing males or females, as has been observed in other studies (c.f., Shearer and Swanson 2000). Plasma IGF-I levels are correlated with body size and growth (Beckman et al. 1998), thus it is likely that the high IGF-I levels in maturing males is due to high growth in these individuals.

From December 1998 through February 1999 in the present study, plasma IGF-I was not significantly different between maturing and non-maturing fish. This suggests that peripheral levels of IGF-I do not have a strong influence on reproduction at this stage. In addition, during this period no significant difference in body weight was detectable between maturing and non-maturing fish, supporting the contention that maturation is initiated prior to the increase in growth typically seen for maturing male chinook (Foote et al. 1991). This does not preclude any effect for growth and/or IGF-I on puberty as the activation of the reproductive axis may have occurred prior to this stage. During the months of August and September, a positive correlation was detected between pituitary FSH and plasma 11-KT levels, and between plasma IGF-I and pituitary FSH levels (Table 6). Plasma IGF-I levels were also significantly correlated with body weight during July-October 1998, but this relationship was not significant in November. Pituitary FSH levels were correlated with body weight during July, August and November 1998. Such relationships may be a direct result of fish size rather than interactions between hormones involved in initiating maturation. Further analyses with larger data sets will clarify these relationships. One other complicating factor is the process of smoltification. During the period of August/September, these fish were also undergoing a fall smolting, which is associated with increases in plasma IGF-I levels and changes in body conformation (Beckman et al. 1998; see smoltification section of this report). It may be difficult to determine the relative roles of IGF-I in smoltification and puberty at this time.

Effect of Growth and Body Fat on the Incidence of Sexual Maturation

Earlier work has demonstrated that both body size and fatness affect early sexual maturation in male chinook salmon (Clarke and Blackburn 1994; Silverstein et al. 1998; Shearer and Swanson 2000), but the precise role of these two factors and their interaction is not clear. In the present study body fatness had no effect on the incidence of maturation. Earlier work (Silverstein et al. 1998) indicated that fatness appears to be more important in small fish. Therefore, in fish which are more comparable in size to wild fish, a threshold of both fatness and size may exist for maturation to proceed. As size or growth rate is emerging as the predominant

correlate with early male sexual maturation, it is intended to continue examination of the relationship between size during the period when maturation is initiated and the incidence of maturation. By combining the results of the present study with data from Silverstein et al. (1998) and Shearer and Swanson (2000), where juvenile chinook were also fed low fat diets, it appears that the threshold size for initiation, in December, the year prior to maturation, may be as low as 10 g (Fig. 16).

Conclusions

- The results of the current study and previous work (Silverstein et al. 1998, Shearer and Swanson 2000) indicate that, although energy storage affects the incidence of early sexual maturity of male chinook salmon, fish size or possibly growth rate is the predominant factor.

- The current study suggests that if there is a threshold size below which maturation will not be initiated it is quite small, possibly less than 10 g in December in 0^+-age fish.

- In 0^+-age spring chinook salmon, initiation of maturation had occurred as early as November.

- Advancement of spermatogenesis was observed by histology in November, but similar stages were observed in a larger proportion of fish and by February 1999. This suggests that the window in which maturation could be initiated may be as wide as 4 months. Maturing males could be consistently identified by histology from February onwards.

- The data on plasma 11-KT levels showed a clear bimodal distribution as early as December and proved to be a useful tool to determine the onset of maturation.

- The advancement of spermatogenesis was accompanied by elevated levels of pituitary FSH and plasma 11-KT, which is consistent with the primary action of these two hormones in regulating reproduction in male chinook salmon.

- During December-February, body weight and plasma IGF-I levels were not significantly different between maturing and non-maturing males. This casts doubt on their proposed involvement in testis development at this stage of maturation.

- Major increases in testis growth were accompanied by increases in fish weight and plasma IGF-I levels, however, the interactions between these factors and testis growth remains to be determined.

- Reproductive and growth hormones were correlated as early as August-September 1998, however, it is not clear whether these represent true interactions or were driven entirely by body size or growth rate alone. It is interesting to note that this may be the period when the interaction between these two endocrine systems is most critical, as it precedes a transition indicative of onset of spermatogenesis (differentiation of type A to type B spermatogonia).

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Table 1. Chronology of the experiment (significant events).

Month		Event
February	1998	start experiment
April		smoltification assessment
March		smoltification assessment
June		immunocompetence assessment
June		transfer fish to larger tanks
July		water quality problems/reduce ration
July		reduce number of fish to 350/tank
August		smoltification assessment
September		smoltification assessment
November		smoltification assessment
November		immunocompetence assessment
January	1999	medicated feed (BKD)
March		immunocompetence assessment
April		medicated feed (BKD)
July		main sampling
October		final sample- terminate experiment

Table 2. Formulation of the experimental diet.

Ingredient	(%)
Anchovy meal	55
Condensed hydrolyzed fish[1]	20
Wheat middlings	14
Whey	2
Krill meal	3
Choline chloride	1
Vitamin mix[2]	2
Mineral mix[3]	1
Carboxymethylcellulose	0.5
Guar gum	0.5

[1] Processing waste and bycatch, inclusion based on dry weight.
[2] Supplied the following per kg premix (vitamin E, 15200 IU; biotin, 158 mg; vitamin B12, 4 mg; folic acid, 2200 mg; inositol, 52800 mg; menadione, 1220 mg; niacin, 29500 mg; d-pantothenic acid, 14100 mg; pyridoxine, 4100 mg; riboflavin, 7040 mg; thiamine, 5720 mg), ADM, Des Moines, IA.
[3] Supplied the following as mg/kg premix (I, 1000; Mn, 10500; Zn, 7450; Cu, 1550; Se, 160), ADM, Des Moines, IA.

Table 3. Date of sampling for weight and body composition.

Date	Day of the year
19 February 1998	50
16 March	75
30 March	89
27 April	117
26 May	146
22 June	173
3 August	215
14 September	257
17 November	321
19 January 1999	366
15 March	439
17 May	502
13 July	559
6 October	644

Table 4. Final July and October fish weights and July whole body fat levels.

Ration	Weight in July (g)			Weight October (g)			Fat July (%)		
	Maturing males	Immature males	Females	Mature males	Immature males	Females	Maturing males	Immature males	Females
100	367±9	293±21	318±7	440±34	377±	491±61	10.2±0.5	8.2±0.6	8.3±0.4
88	332±8	234±27	290±5	420±42		435±26	9.3±0.1	8.3±0.5	9.0±0.3
76	319±7	240±29	259±5	313±14		345±25	9.3±0.4	8.3±0.4	8.4±0.1
64	272±6	171±17	223±5	289±18		293±25	9.3±0.1	6.7±0.6	6.9±0.1
52	249±8	174±14	174±6	290±29	379±5	279±28	9.7±0.1	7.4±0.3	7.3±0.1
40	198±7	137±10	149±4	259±30	249±27	266±11	8.4±0.1	6.9±0.3	6.9±0.3
Control (64)	314±10	220±19	219±6	319±38	246±26	319±28	11.5±0.1	11.7±1.2	11.3±0.5

Table 5. Stages of spermatogenesis observed during experimental period[1].

Date		Immature	Maturing
8 July	1998	-	-
12 August		-	-
8 September		-	-
12 October		-	-
9 November		-	+
16 November		-	+
14 December		-	+
11 January	1999	-	1+, 2+
22 February		-	+, 1+, 2+, 3+
5 April		-	1+, 2+, 3+
24 May		-	2+, 3+
June 28		-	2+, 3+
29 September		-	Spermiating

[1] Criteria for stage of spermatogenesis according to Schulz (1984). Criteria for stage and lobule numbering. Only presence of type A spermatogonia (-), 1-5 cysts of type B spermatogonia (+), greater than 5 cysts of type B spermatogonia and or spermatocytes (2+), appearance of spermatocytes and spermatids (3+).

Table 6. Correlation analysis between pituitary FSH and Plasma 11-KT and between plasma IGF-I and pituitary FSH.

	Pituitary FSH vs Plasma 11-KT			Plasma IGF-I vs Pituitary FSH		
Date	n	Correlation Coefficient	*P* value	n	Correlation Coefficient	*P* value
7/8/98	11	0.606	0.047	11	0.023	0.9479
8/12/98	14	0.788	0.0004	13	0.673	0.0098
9/8/98	13	0.66	0.0122	12	0.754	0.0032
10/12/98	13	0.394	0.1882	12	0.142	0.6687
11/9/98	9	0.702	0.0328	10	0.377	0.2948
11/16/98	14	0.755	0.0011	14	0.627	0.0146

Table 7. Correlation analysis between pituitary FSH and body weight and between plasma IGF-I and body weight.

	Pituitary FSH versus Weight			Plasma IGF-I versus Weight		
Date	n	Correlation Coefficient	*P* value	n	Correlation Coefficient	*P* value
7/8/98	15	0.656	0.0065	11	0.607	0.0465
8/12/98	16	0.779	0.0002	13	0.691	0.0072
9/8/98	14	0.486	0.0782	12	0.708	0.008
10/12/98	13	0.412	0.1657	12	0.702	0.009
11/9/98	11	0.463	0.1569	10	0.612	0.0597
11/16/98	16	0.791	0.0001	14	0.445	0.1126

Table 8. Maturation rates and gonadosomatic index (GSI) (mean ±SEM, n=2) of 1 + age male chinook salmon fed graded levels of a low fat (7%) diet or a commercial control diet (22%).

Feeding rate (%)[1]	Male maturation July (%)	GSI July (%)	GSI October %
100	81.9±2.8	6.2±0.2 c	6.8±0.6
88	87.5±3.0	6.6±0.2 bc	6.1±0.4
76	92.8±1.0	6.6±0.2 bc	6.3±0.8
64	88.0±6.8	6.7±0.3 bc	5.3±0.4
52	75.8±6.0	6.8±0.3 bc	6.4±0.6
40	66.2±8.2	7.2±0.4 b	4.8±0.5
Control (64%)	78.9±0.8	8.2±0.3 a	6.4±0.5
P	0.08	<0.001	0.27

[1] Relative to the 100% rate.

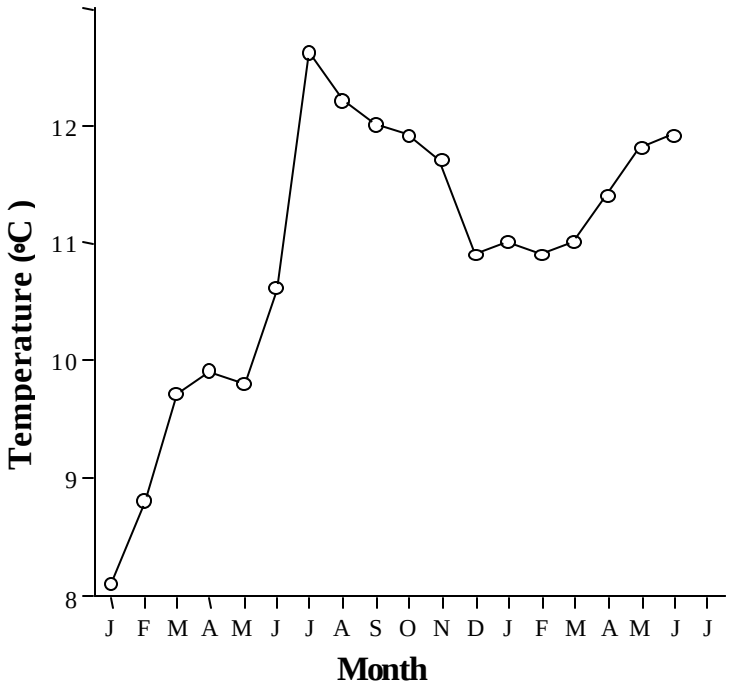

Figure 1. Mean monthly temperature over the course of the experiment.

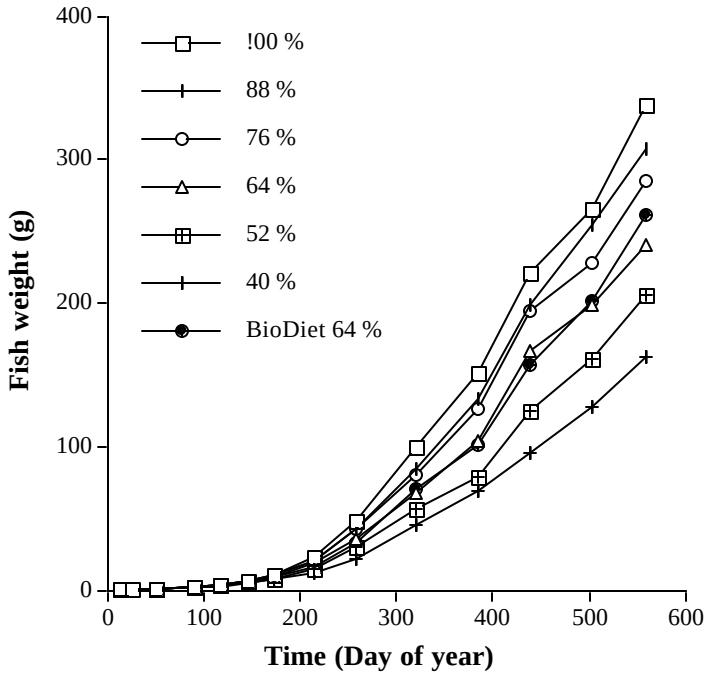

Figure 2. Growth of juvenile chinook salmon fed a low fat diet (7%) at six ration levels or BioDiet (22% fat) at the 64 % ration level. Each data point is the mean of two replicate tanks. Day 0 = 1 January 1998.

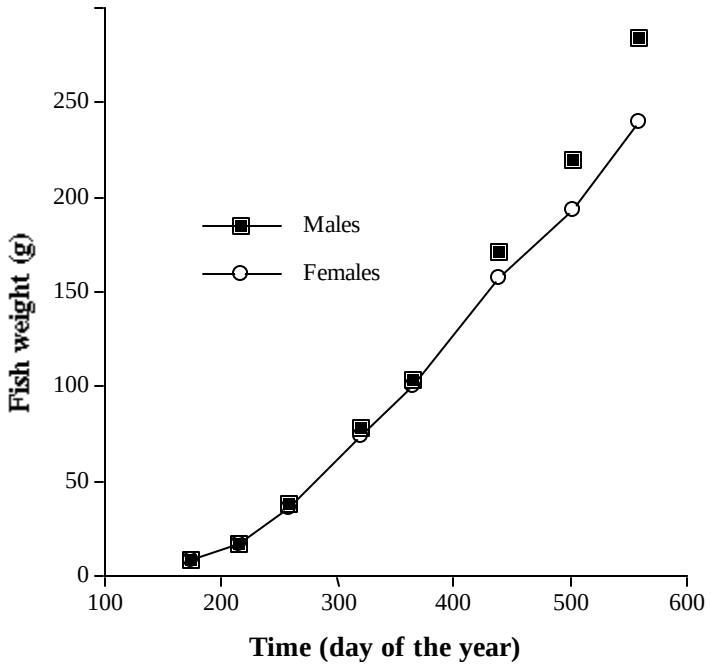

Figure 3. Mean weights of males and females (all treatments combined).

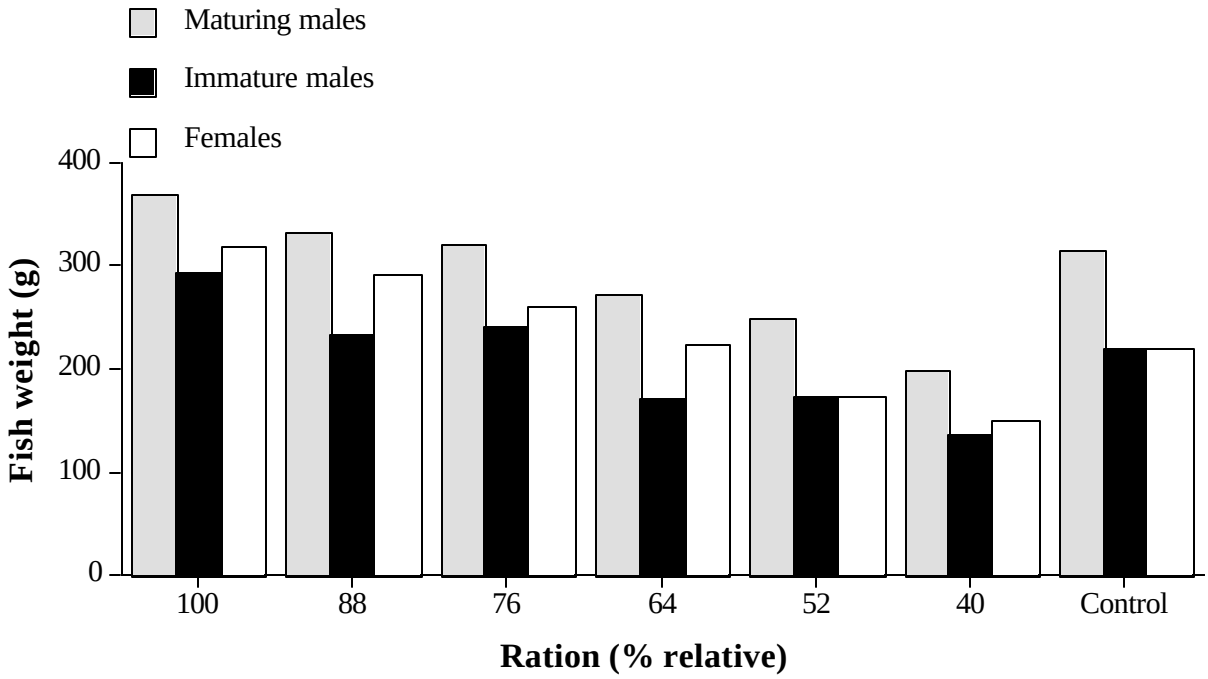

Figure 4. Weights of 1^+ chinook by treatment, sex and state of maturation in July 1999. BioDiet was fed at the 64% level.

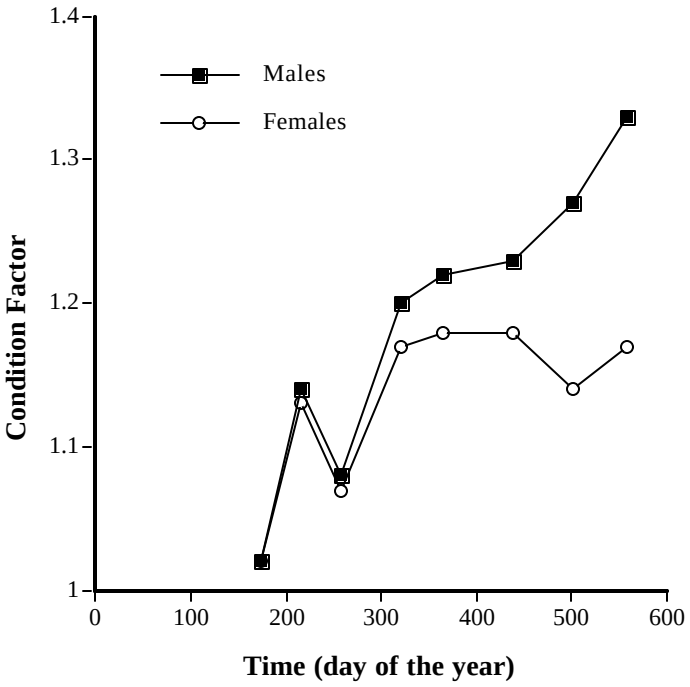

Figure 5. Condition factors of juvenile male and female chinook salmon during the course of the experiment.

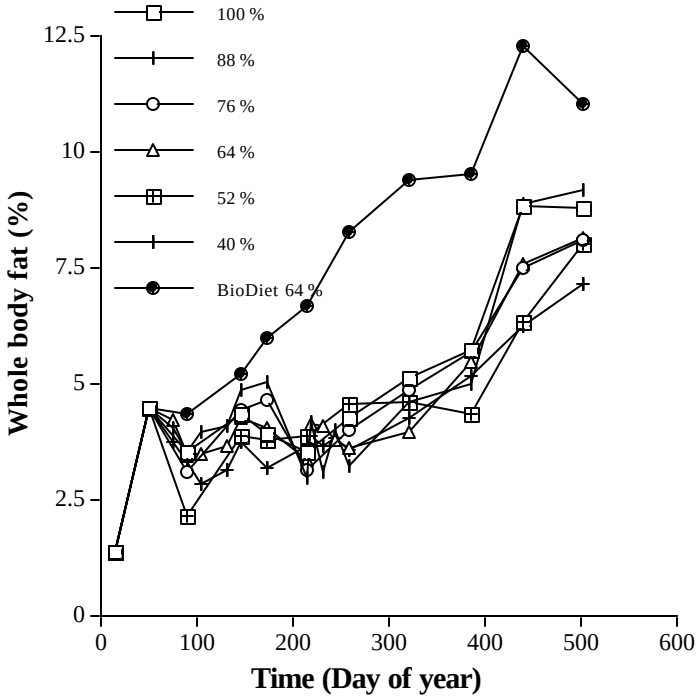

Figure 6. Whole-body fat of juvenile chinook salmon fed the experimental low fat (7%) at various ration levels (100-40%) or BioDiet (22%) diet at the 64% level. Each data point is the mean of 2 replicate tanks (n=10 fish/tank). Each tank mean consisted of 10 fish.

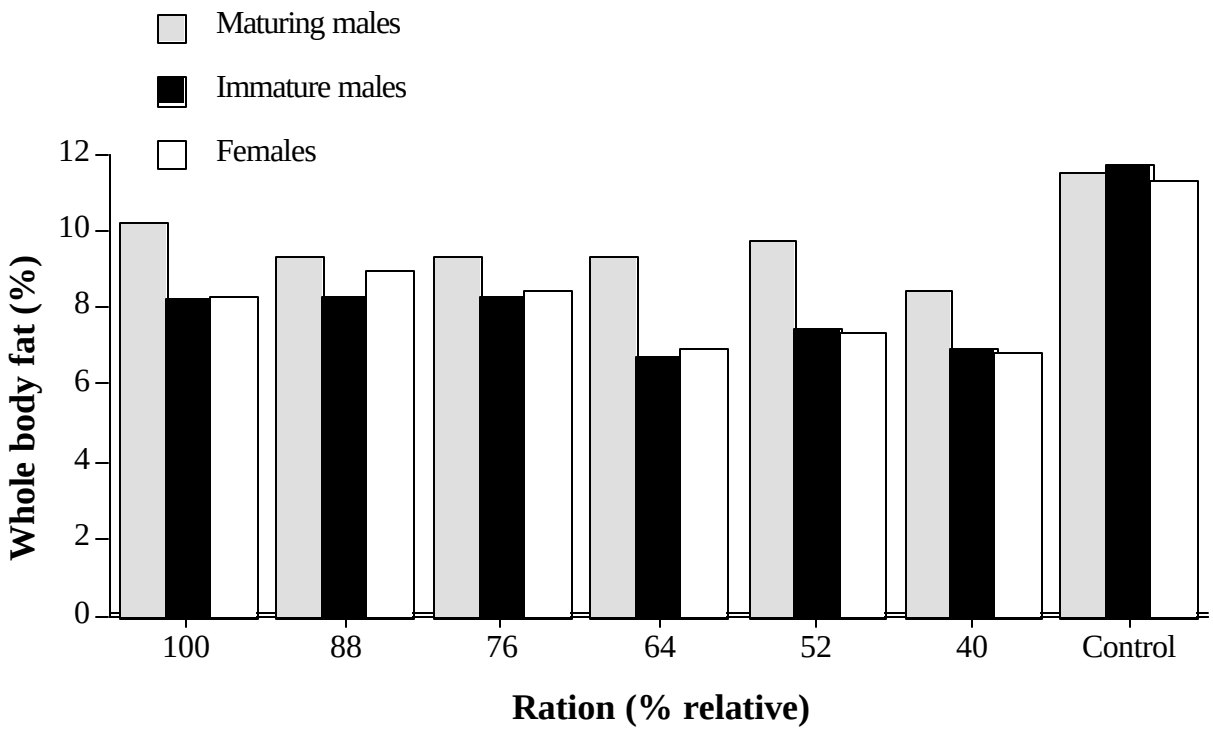

Figure 7. Whole-body fat levels by treatment, sex and state of maturation in July 1999. BioDiet was fed at the 64% level. Data are mean of 2 replicate tanks.

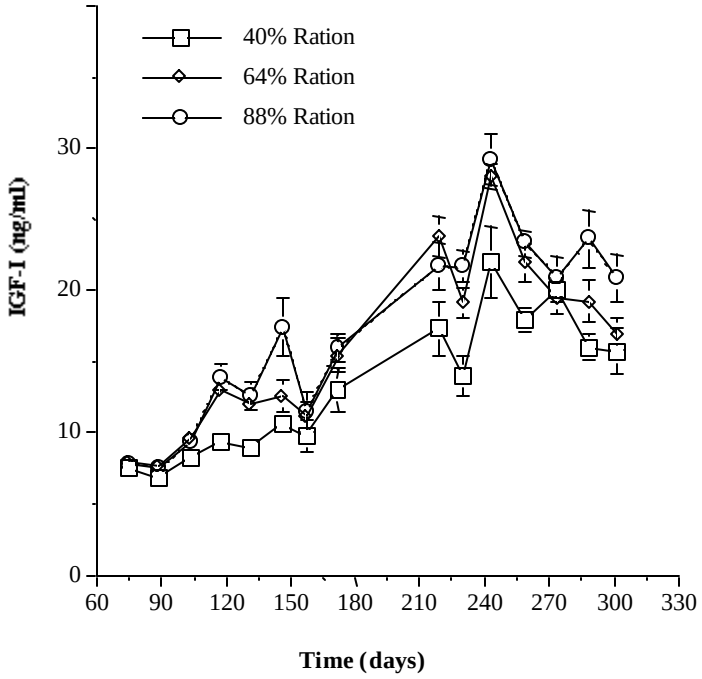

Figure 8. Plasma IGF-1 concentrations in 0-age chinook salmon fed the experimental low fat (7%) diet at 88, 64 or 40% ration. Data are mean ± SEM, n =12.

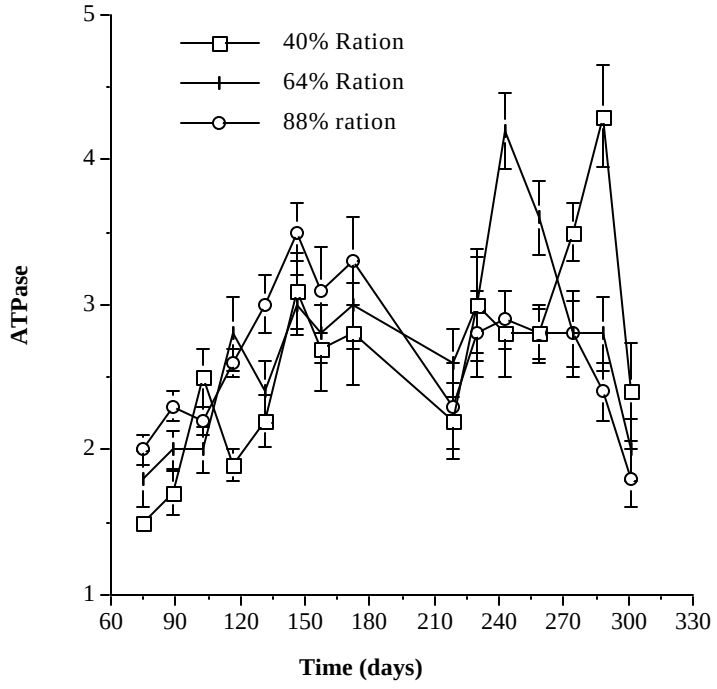

Figure 9. Gill ATPase concentrations in 0-age chinook salmon fed fed the experimental low fat (7%) diet at 88, 64 or 40% ration. Data are mean ± SEM, n =12.

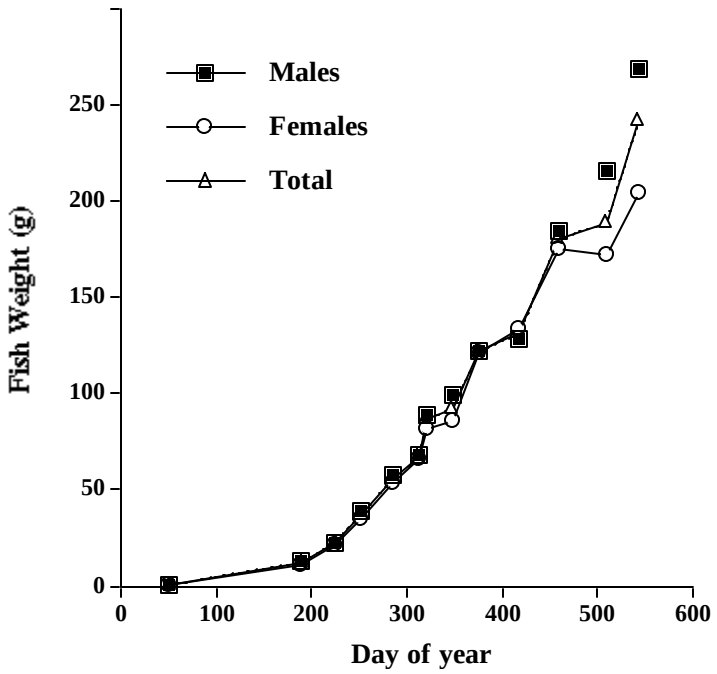

Figure 10. Growth of juvenile chinook salmon fed the experimental low fat diet (7%) at 100-88% ration level that were used for maturation sampling. Data points are the mean of two or four replicate tanks.

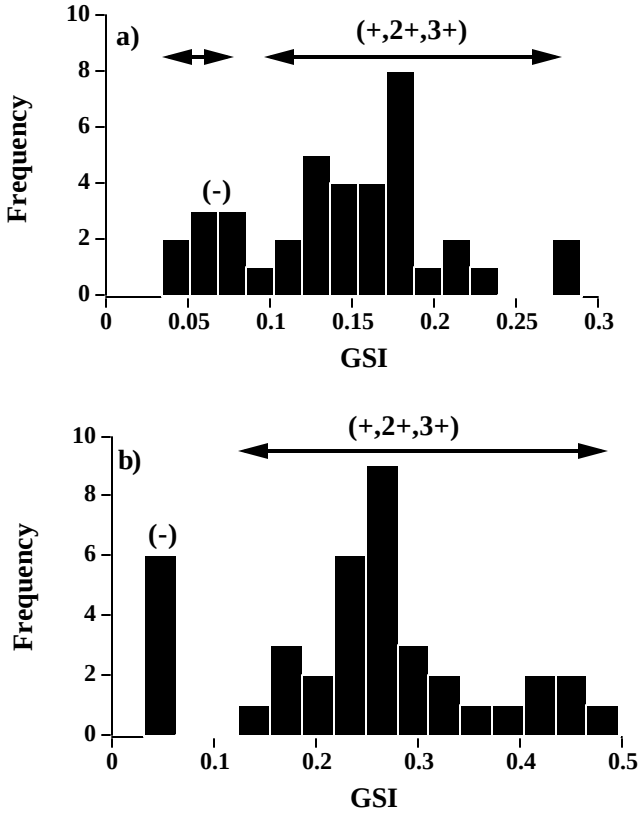

Figure 11. Frequency distribution of male gonadosomatic index (GSI) in, a) February 1999 and, b) April 1999. Arrows and figures in brackets indicated the status of testis development found in a representative sample from these groups (see Table 1 for criteria for testis development).

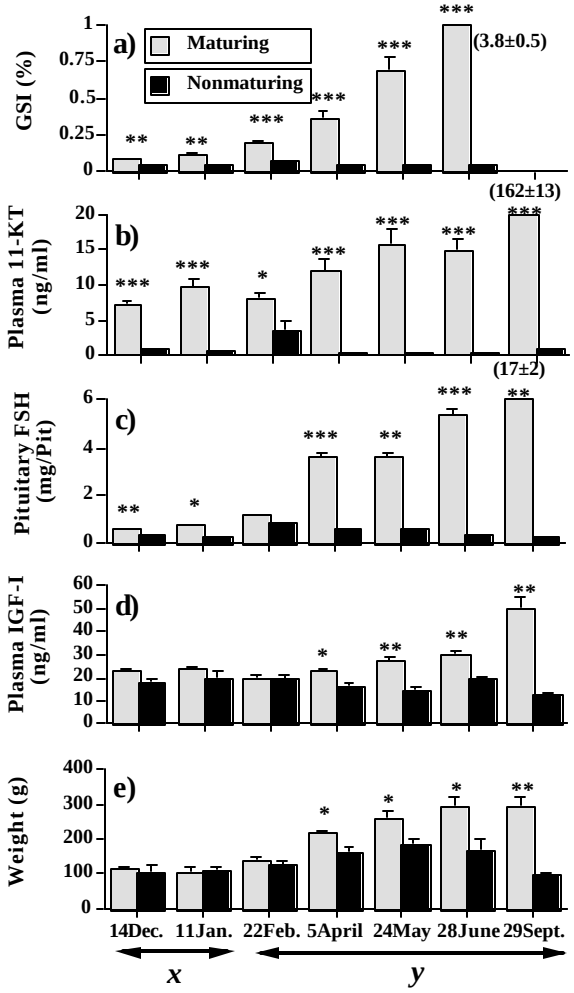

Figure 12. Levels of various parameters measured in maturing and non-maturing chinook salmon males; a) GSI, b) plasma 11-KT, c) pituitary FSH, d) plasma IGF-I, and e) body weight. * denotes significant difference (*=P<0.05, **=P<0.01, ***=P<0.001) between maturing and non-maturing males at the same time point. x and y shows time points where 11-KT levels (x) or testis histology and GSI (y) were used to determine maturing and non-maturing males. Numbers in brackets indicate the off-scale value±SEM of the maturing group at the time point indicated.

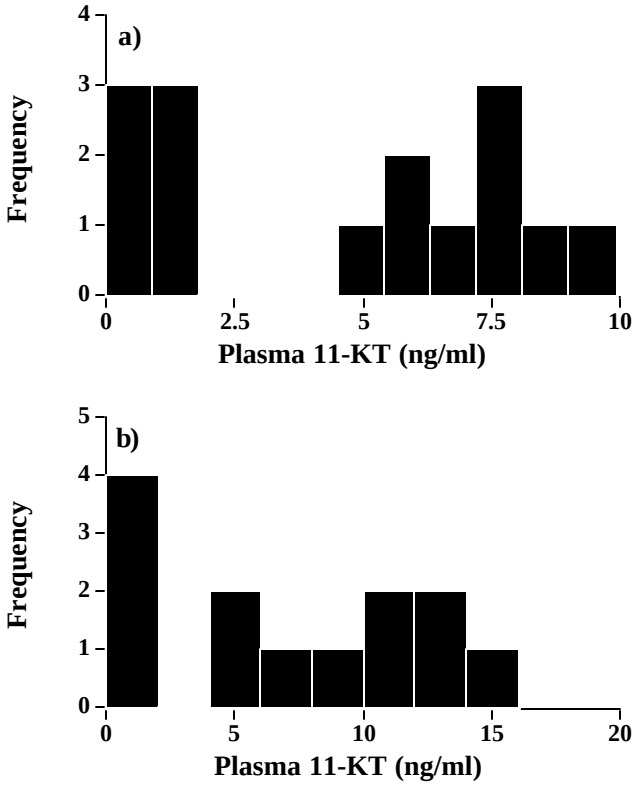

Figure13. Frequency histogram of plasma 11-KT levels in male chinook during, a) December 1998 and b) January 1999.

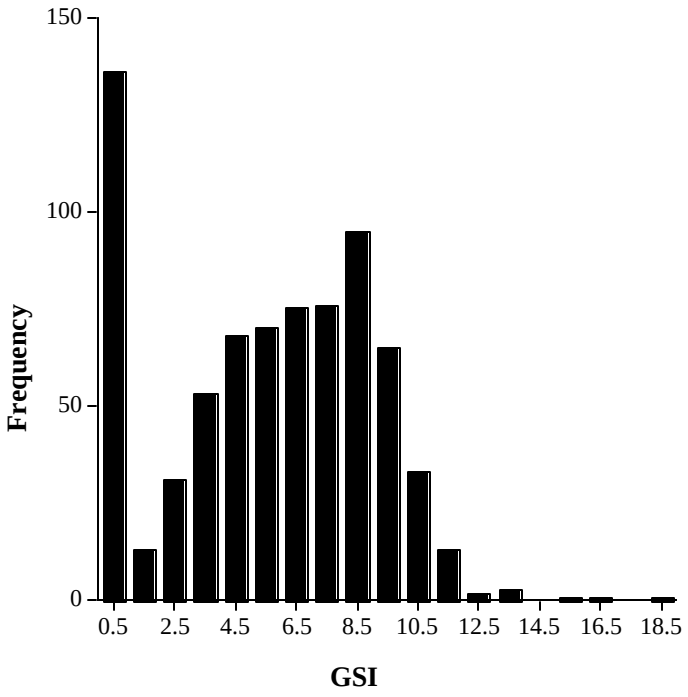

Figure 14. Frequency distribution of male gonadosomatic indexes (GSI) in July 1999. Fish with a GSI over 1.0 were considered to be maturing. Data is from all treatments. Of 744 males, 82.3% were maturing.

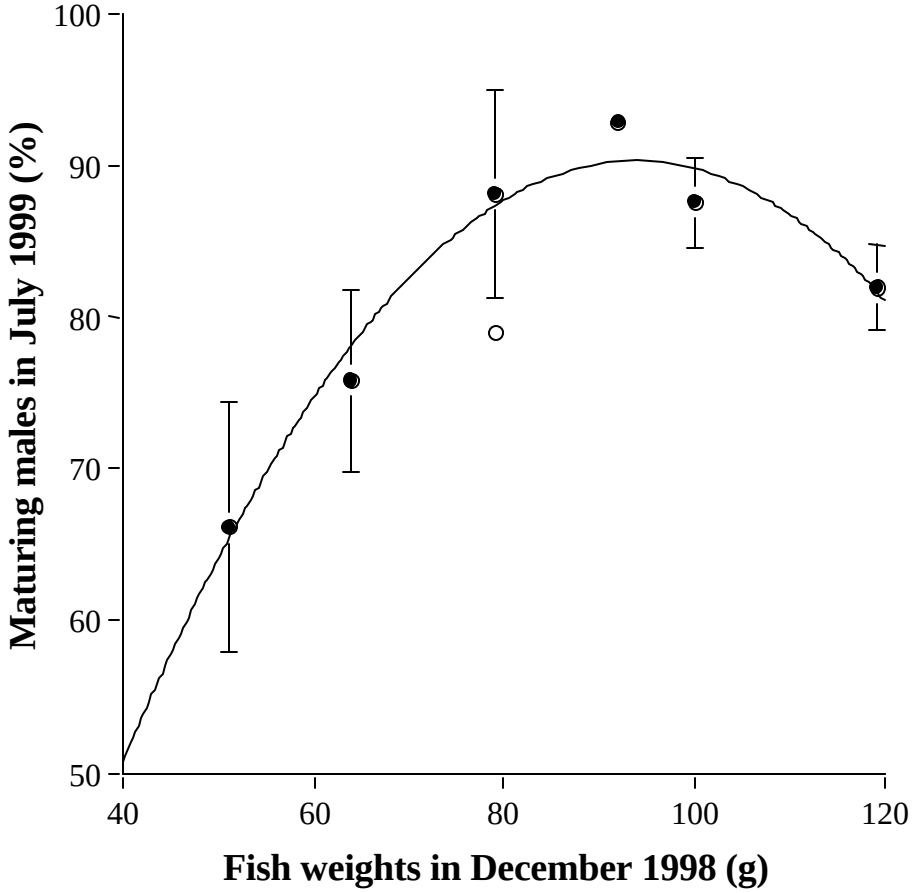

Figure 15. The relationship between fish weight in December 1998 and the incidence of 1+ age male sexual maturation (mean ±SEM) for spring chinook salmon fed the experimental low fat (7%) diet (filled circles) or BioDiet (22% fat, open circle).

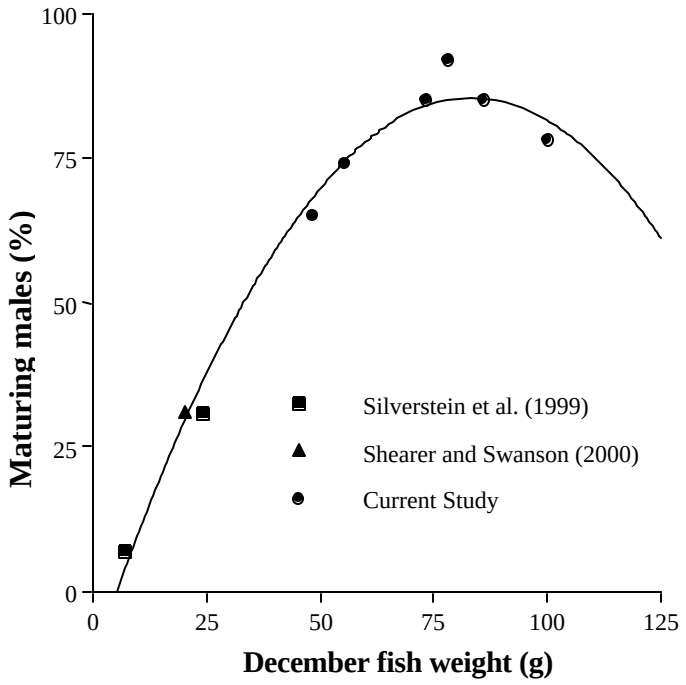

Figure 16. The relationship between the weight of male spring chinook salmon in December and the incidence of sexual maturation. Shearer and Swanson (2000), Yakima stock. Silverstein et al. (1998) and current study, Willamette stock. All fish were fed experimental low fat diets (<7%) and reared under similar laboratory conditions.

TASK 8. EXPANSION OF THE INFRASTRUCTURE AT THE MANCHESTER MARINE RESEARCH STATION FOR FUTURE RESEARCH ON ADULT QUALITY
(PROGRESS REPORT: 1 JUNE 1999 THROUGH 31 MAY 2000)

by

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Introduction

A major focus of the captive broodstock research group is to improve the quality of adult salmon grown in captive broodstocks through manipulation of environmental parameters (e.g., diet, feeding regimens, and exercise). This research requires a substantial culture facility, which contains adequate numbers of tanks for valid experimental replication, and the tanks and water supply must be large enough to support culture to maturity. Such a facility must also provide for the maximum of experimental control of potentially confounding factors. Currently, no such facility is available for such research.

The Manchester Research Station (MRS) currently supplies filtered, sterilized sea-water to populations of ESA-listed chinook and sockeye salmon. Effluent from these tanks is available for use in culturing salmon captive broodstocks for research on adult quality. The fish culture system at the MRS will be expanded to accommodate 12 large, identical rearing vessels dedicated to research. This approach takes advantage of the existing infrastructure and realizes cost savings over development of a completely new facility.

A major goal of the Captive Broodstock Technologies Project (current and future) is to determine the effects of experimental rearing strategies (e.g., dietary manipulations, growth regimens, exercise, therapeutic disease treatments) on survival and the quality of

adults for release and artificial spawning. This general area of research has been determined to be of very high priority by the various technical committees overseeing current captive broodstock programs for ESA-listed salmon. This complex and integrated research requires populations of experimental fish, together with the substantial investment in infrastructure to carry out valid comparative long-term experiments. The MRS has the environmental authorization to take and return large volumes of seawater to Puget Sound. This is because of the installation of a unique depuration system which operates full-time.

A further advantage of expanding the research resources at MRS is that it will be integrated with one of the major captive broodstock rearing facilities for ESA-listed stocks. This will increase the capacity for information exchange between ongoing research and practical stock restoration.

Work Completed

During the FY99 Performance Period, the following items were completed.

1. Facility plan and drawings;
2. Excavation of a tank pad area;
3. Excavation for the pump vault;
4. Installation of a seawater delivery to the pump vault;
5. Installation of a concrete pump vault; and
6. Installation of an electrical panel and control center.

Work to be Completed

The basic design of the proposed facility is as follows. Effluent seawater (approximately 4000 L/min) from the ESA-listed captive broodstocks at the MRS will be collected in a concrete vault. A maximum of 2000 L/min of the water will be pumped from the vault to an array of 12 large rearing tanks situated on a concrete pad. The water will exit the tanks, enter open drains in the concrete pad, and enter the existing Ozone Depuration System, before being discharged into Puget Sound.

During the next performance period, the following items will be completed:

1. Installation of electrical wiring necessary to power pumps and controllers;
2. Installation of one active and one back-up pump, with controllers;
3. Preparation and construction of a concrete pad with open drains;
4. Installation of sand filters and UV sterilization units; and
5. Installation of rearing tanks (3.70-m d x 1.52-m deep) with covers.

TASK 9[1]. EFFECTS OF RATION LEVEL ON IMMUNE FUNCTION IN CHINOOK SALMON (FINAL REPORT 1 JUNE 1998 THROUGH 31 MAY 2000)

by

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Introduction

Losses from infectious diseases can have a dramatic impact on the success of salmonid aquaculture programs. Fish health management for salmonids often takes a preventative approach by minimizing the introduction of pathogenic microorganisms through careful selection of healthy broodstock, improvements to fish husbandry practices, and rigorous disease surveillance (Ahne et al. 1989; Rohovec 1980). When avoidance of a pathogen is not possible, vaccination or prophylactic and therapeutic use of antimicrobial compounds are used to control some diseases (Press and Lillehaug 1995; Schnick et al. 1997).

Many fish health management practices also reduce the adverse effects that certain environmental factors may have on a fish's ability to resist disease. Salmonids possess many of the immune functions used by higher vertebrates to resist infection by pathogenic microorganisms (Ellis 1977; Ingram 1980). The physiological processes of disease resistance interact with the endocrine system, and thus can be impaired by inattention to aquaculture practices that stress the fish, including poor water quality, improper rearing temperatures, crowding, and nutritional problems (Pickering 1993). Stress is known to have a deleterious effect on survival of salmonids because of the maladaptive effects of cortio-steroid production on their immunocompetence (Barton and Iwama 1991; Weyts et al. 1999).

Recent advances in the characterization of the immune functions of fish, and the development of new methods for their study, will encourage further examination of different aquaculture practices relative to fish health and disease resistance. For example,

[1] Work on Task 9 as outlined in the FY99 Statement of Work has been initiated, and will be reported in 2001.

in a previous report we described a number of assays developed by the U.S. Geological Service at the Western Fisheries Research Center (WFRC) in Seattle, Washington to measure nonspecific and specific immune functions of salmonids. They have been combined into a panel of hematological, immunological, and serological assays that can be used to provide a general index of the immune status of fish reared under different conditions.

There is strong evidence that growth rate may have a significant role in the regulation of smoltification by the salmonid endocrine system (Dickoff et al. 1997). In addition, successful smoltification and greater smolt-to-adult survival are associated with larger fish at the time of release (Mahnken et al. 1982). Because of the relationship of the endocrine and immune systems changes in growth rate might also effect disease resistance. Little is known, however, whether the rate of growth can affect the ability of salmonids to produce an immune response. Consequently, this study used the WFRC panel of assays to examine the general immune status of chinook salmon (*Oncorhynchus tshawytscha*) from diet groups fed different levels of a standardized ration. Results suggest that certain functions of the macrophages are affected when the amount of ration (and growth) is increased.

Methods

Fish

The effect of diet level on the immune system was investigated as part of a larger project to determine the effects growth rate and a low fat diet on early male maturation in chinook salmon (described in Task 7 of this report). Brood-year 1997 chinook salmon of the Willamette River (OR) stock were reared from the eyed egg stage at the Northwest Fisheries Science Center (NWFSC) in Seattle, WA. After hatching the fish were placed in a single tank (4' d). Throughout the experiment the fish were reared on a natural photoperiod in tanks supplied with water from a recirculated system between 9 °C and 12.5 °C. One month after hatching, the 0.5-g fish were randomly distributed among 14 tanks (4' d), 700 fish per tank. The fish were assigned to an experimental diet at one of 6 feeding levels, see below, or to a commercial diet (2 tanks per feed level group). After 18 wk (day 173), the fish in the 100% and 88% feed level groups were transferred to larger tanks (8' d) and the fish in the 76% and 64% feed level groups moved to larger tanks (6' d). Prior to the move all fish were given a 5 ppm MgO bath for 1 h to control *Costia necatrix* infestation. On day 207 the number of fish in each tank was reduced to 300 due to limitations of the biofilter. To control outbreaks of bacterial kidney disease, erythromycin (100 mg/kg/d) was added to the feed for 28 d beginning 2 January 1999.

Diet formulation and feeding

The fry were fed a commercial salmon starter feed (BioOregon-Starter) for 1 month. The experimental diet was a semi-moist pellet that contained 54% (w/w) protein and 7.4% (w/w) lipid (Table 1). The fish were fed the experimental diet to satiation (100% ration) or 88, 76, 64, 52 and 40% of the satiation amount. The seventh treatment group was fed a commercial feed (BioOregon-Grower) at the 64% ration level, based on an equivalent dry matter content. The fish were fed once a day by hand for 6 d/wk for the first 16 wk of the study and 5 d/wk thereafter.

Table 1. Formulation of the experimental diet.

Ingredient	(%)
Anchovy meal	55
Condensed hydrolized fish[1]	20
Wheat middlings	14
Whey	2
Krill meal	3
Choline chloride	1
Vitamin mix[2]	2
Mineral mix[3]	1
Carboxymethylcellulose	0.5
Guar gum	0.5

[1] Processing waste and bycatch.
[2] Supplied the following per kg premix (vitamin E, 15200 IU; biotin, 158 mg; vitamin B12, 4 mg; folic acid, 2200 mg; inositol, 52800 mg; menadione, 1220 mg; niacin, 29500 mg; d-pantothenic acid, 14100 mg; pyridoxine, 4100 mg; riboflavin, 7040 mg; thiamine, 5720 mg), ADM, Des Moines, IA.
[3] Supplied the following as mg/kg premix (I, 1000; Mn, 10500; Zn, 7450; Cu, 1550; Se, 160), ADM, Des Moines, IA.

Bacteria

Aeromonas salmonicida (ATCC 33658) was used as a metabolic stimulant in the nitroblue tetrazolium (NBT) assay. A lyophilized preparation was resuspended in 5 mL of Trypticase soy broth (TSB) and incubated at 28°C for 16 h. An aliquot of the bacterial suspension was streaked for colony isolation on trypticase soy agar (TSA) and incubated at 28 °C for 24 h. Two flasks containing 700 mL of TSB were inoculated with an isolated colony and placed on a shaking incubator at 28 °C for 16 h. The bacterial suspension was centrifuged at 1000 × g for 20 min at 25 °C and the supernatant discarded. The bacteria were resuspended in Dulbecco's phosphate-buffered saline (PBS), pH 7.4 and centrifuged at 1000 × g for 15 min at 25 °C. The concentration of

viable *A. salmonicida* in the suspension before centrifugation was determined by the culture of serial 10-fold dilutions on TSA. The plates were incubated for approximately 24 h at 28 °C and individual colonies were enumerated to determine the concentration of cells in the stock solution. The pellet was resuspended in PBS + 3% (v/v) formaldehyde and incubated for 16 h at 25 °C. The bacteria was washed in PBS, pH 7.4 by centrifugation at 10 000 × g for 15 min at 25 °C and resuspended in PBS, pH 7.4 + 0.3% (v/v) formaldehyde and stored at 4 °C. The day before use, an aliquot of the killed bacteria was washed three times in PBS, pH 7.4 by centrifugation at 2000 × g for 40 min at 4 °C. The washed cells were resuspended to a concentration of 2×10^9 bacteria/mL in Macrophage-SFM Medium (Life Technologies) without the phenol red pH indicator supplemented with 0.2% (w/v) NBT, and 100 IU/mL penicillin, and 100 µg/mL streptomycin (MØ-PRF medium).

Staphylococcus aureus (ATCC 12600) cells were grown for the phagocytosis assay in TSB at 37 °C for 24 h with agitation. The bacterial suspension was centrifuged at 2500 × g for 20 min, then the pellet was resuspended in PBS, pH 7.4. The concentration of viable *S. aureus* in the suspension was determined by the culture of serial 10-fold dilutions on TSA. The plates were incubated 20 h at 37 °C and individual colonies were enumerated to determine the concentration of cells in the stock solution. The bacterial cells were combined with an equal volume of PBS, pH 7.4 containing 6% (v/v) formaldehyde and stored at 4 °C. The formalin-killed *S. aureus* cells were labeled with fluorescein isothiocyanate (FITC) as described by Cantinieaux et al. (1989).

Blood and Tissue Samples

During September 1998, December 1998, and March 1999, fish were sampled from the 100%, 64%, and 40% ration-level groups to measure selected parameters of their basal immune functions. Fish from the group receiving the commercial diet were sampled during December 1998 and March 1999. During each sample period; 5 fish were removed from each tank by repeated dip-netting every other day for 5 d (3 sample dates) and were immediately anesthetized with tricaine methanesulfonate (MS-222). The weight and fork length was determined to calculate the condition factor. The condition factor was calculated according to the method described by Piper (1982).

After severing the caudal peduncle, a blood sample was taken from the caudal vein for the hematocrit, leucocrit, and plasma protein determinations. Duplicate smears of whole blood were made for each fish on glass microscope slides which had been previously washed with 70% (v/v) ethanol containing 10% (v/v) hydrochloric acid. The remainder of each blood sample was allowed to clot overnight at 4 °C and centrifuged at 5000 × g for 20 min at 4 °C. The serum was stored at -80 °C.

On each sample day a five-fish pool of anterior kidney tissue was prepared for each ration level subgroup. Kidney samples and reagents were maintained on ice, or at 4 °C, unless otherwise noted. The anterior kidney was aseptically removed and a small portion from the center of the sample was used to make several kidney imprints on each of two glass microscope slides. The tissue imprints were air-dried, then fixed in absolute

methanol for 5 min. One of the slides was stained using the Diff-Quik stain (Baxter Healthcare Corp) according to the manufacturer's instructions, and the other slide was stored at 4°C for the myeloperoxidase assay. The remaining tissue was placed in a siliconized glass petri dish containing 15 mL leucocyte isolation media (LIM); Leibovitz's L-15 medium (Life Technologies) supplemented with 10% (v/v) fetal calf serum (FCS), 1.65 g/L glucose, 5 IU/mL ammonium heparin, 100 IU/mL penicillin, and 100 µg/mL streptomycin. Kidney tissues from five fish were pooled in one petri dish. The tissue pool was processed by first pressing it through a stainless steel screen to disrupt cell clumps. A single cell suspension was created by repeatedly drawing in and expelling the cell suspension from a 10 mL syringe. The cell suspension was passed through a glass wool mat to remove any remaining large aggregates. A single cell suspension of leucocytes was prepared by passing the processed tissue pool through a 34%-51% Percoll (Sigma) discontinuous gradient as described by Braun-Nesje et al. (1981). The leucocytes were washed once in Leibovitz's L-15 medium containing 20 IU/mL heparin (L-15/heparin) by centrifugation at 1000 × g for 10 min. The cell pellet was resuspended in a small volume of L-15/heparin and an aliquot was mixed with trypan blue exclusion dye for cell enumeration using a hemocytometer. The cells were observed microscopically at 200 × magnification to enumerate the viable leucocytes.

During the September and December 1998 sampling periods the fish from each subgroup were numbered and placed in a sealed bag on ice and transported to the WFRC prior to organ removal. During the March 1999 sampling period the anterior kidney imprints and the single cell suspension of anterior kidney pools were prepared immediately after bleeding at the NWFSC.

Hematocrit and leucocrit -- Hematocrit values for individual fish were determined according to the method of Wedemeyer and Nelson (1975). Leucocrit values for individual fish were determined according to the method of Wedemeyer et al. (1983).

Total plasma protein -- The protein concentration of each plasma sample was determined by its refractive index using a clinical refractometer (Spartan) according to the manufacturer's instructions. The instrument was calibrated each day before use with a 6 g/dL protein standard (Sigma).

Complement fixation -- The reagents used in the complement assay were maintained on ice or at 4 °C unless otherwise noted. Rabbit red blood cells (RaRBC) were obtained from defibrinated rabbit blood (Remel) by repeated washing in veronal-buffered saline supplemented with 0.1% (w/v) gelatin, 0.1 M EGTA, and 0.1 M $MgCl_2$, (GVB-EGTA-Mg, Sigma) by centrifugation at 1000 × g for 15 min until the supernatant was colorless. The concentration of RaRBCs was adjusted to exactly 1% (v/v) in GVB-EGTA-Mg (1% RaRBC). The exact cell concentration is determined by spectrophotometric analysis; 1.5 mL of distilled water is added to 0.5 mL of an approximately 1% (v/v) suspension of RaRBCs and the cells were allowed to lyse at 4 °C. Three milliliters of GVB-EGTA-Mg were added to the lysed red blood cell solution and the absorbance at 540 nm was determined. A 1% (v/v) RaRBC solution yields an OD_{540} of 0.385 (data not shown). The stock suspension was then adjusted to

exactly 1% (v/v) RaRBC using GVB-EGTA-Mg and the concentration was rechecked to ensure accuracy.

To determine the concentration of complement in the test plasma, a 1:25 (v/v) dilution of each chinook plasma sample was prepared in GVB-EGTA-Mg. Doubling dilutions from 1:25 to 1:200 (v/v) of each test plasma in GVB-EGTA-Mg were prepared in duplicate in 2 columns of a 96-well v-bottom plate. The final volume of each well was 125 µL. To one of the two columns for each test plasma was added 125 µL/well of GVB-EGTA-Mg (serum background) and 125 µL/well of 1% (v/v) RaRBC was added to the other column (serum test). On each plate there were 8 control wells, 4 wells of 125 µL/well 1% RaRBC added to 125 µL of 0.1% (w/v) saponin (positive control) and 4 wells of 125 µL/well 1% RaRBC mixed with 125 µL GVB-EGTA-Mg (negative control). Also included on each plate were 4 wells of the positive background control of 125 µL 0.1% (w/v) saponin mixed with 125 µL GVB-EGTA-Mg and 4 wells of 250 µL GVB-EGTA-Mg for the negative background control. Each plate was incubated for 60 min at 15 °C in a humid chamber. The plates were then centrifuged at 1000 × g for 5 min at 4 °C and 200 µL supernatant from each well was transferred to the corresponding well of a 96-well flat-bottom plate. The absorbance of each well was measured at 540 nm.

Lysozyme assay -- Lysozyme activity was measured in serum samples from individual fish using a lysoplate assay based on the method described by Lie et al. (1986) and modified by Ellis (1990), except that an 0.2 mm agarose gel support medium (FMC BioProducts) was substituted for the glass plates. Nine microliters of each chinook plasma sample were placed in separate wells (3 mm d) in a 1% (w/v) agarose gel of 0.067 M PBS, pH 6.5 containing 0.5 mg dry weight /mL *Micrococcus lysodeikticus* (Sigma). Control wells containing either 1, 10, 40, or 80 µg/mL chicken egg white lysozyme (HEWL, Sigma) were included on each assay. Chinook plasma samples were tested in duplicate and four replicates of each HEWL concentration were used. The gels were incubated for 16 h at 25 °C then illuminated using a light box. The diameter of the lysis zones was determined visually. In cases where there were multiple clearance zones the diameter of the largest zone was measured.

Differential cell count -- The blood smears from each fish were fixed in absolute methanol for 5 min. The smears were stained using the Diff-Quik stain according to the manufacturer's instructions. The blood smears were examined by light microscopy at 1260× magnification. Three hundred leucocytes were categorized as either lymphocytes, polymorphonuclear leucocytes, monocytes/macrophages, or unknown based on their morphology (Rowley 1990). The number of thrombocytes per 300 leucocytes was also tabulated.

Nitroblue Tetrazolium (NBT) Assay

Reagents -- Metabolic modulators were prepared in MØ-PRF medium containing 0.2% (w/v) NBT (Sigma), 100 IU/mL penicillin, and 100 µg/mL streptomycin (NBTC). The modulators were the metabolic stimulants 0.5 µg/mL phorbol myristate acetate (PMA, Sigma) or *A. salmonicida* (As, ATCC 33658), or the metabolic inhibitor 10 mM

iodoacetamide (IA, Sigma). Undiluted dimethyl sulfoxide (DMSO) and 2 M potassium hydroxide (KOH) were used to solubilize the reduced NBT (formazan).

NBT reduction assay -- The gradient-purified leucocytes from each tissue pool were adjusted to a concentration of 5×10^6 cells/mL in L-15/heparin. All incubations were in a humid chamber at 15 °C. For each tissue preparation, 200 µL aliquots of the gradient-purified leucocytes were placed in each of 12 wells of a 96-well microplate (Nunc) and incubated for 2 h. The non-adherent cells were removed by gentle immersion of the entire plate three times in PBS, pH 7.4. Three control wells of each tissue preparation received 200 µL of NBT solution only (NBTC). The 6 metabolic stimulant wells received 200 µL of NBT solution supplemented with either 4×10^8 bacteria/well *A. salmonicida*, or 0.5 µg/mL PMA (As and PMA, respectively). The 3 metabolic inhibitor wells received 200 µL of NBT solution supplemented with 10 mM iodoacetamide (IA). The microplate was incubated for 30 min, then washed three times as described above. The cells were fixed by immersing the plate three times in absolute methanol. The plate was air-dried, then 120 µL of 2 M potassium hydroxide (KOH) was added to each well followed by 140 µL/well of dimethyl sulfoxide (DMSO). The contents of the each well were triturated vigorously to solubilize the reduced formazan. The absorbance of each well was determined at 630 nm.

Phagocytic activity

After enumeration, the gradient-purified leucocytes were suspended at 1×10^6 leucocytes/mL in Leibovitz's L-15 medium mixed with an equal proportion of Macrophage Serum-Free Medium (Life Technologies, L-15/MØ medium) and 0.1% (v/v) fetal calf serum (FCS). Each well of a chambered microscope slide (Nunc) received 400 µL of a cell suspension. Each cell suspension was loaded in triplicate. Unless otherwise stated, all incubations were done at 15 °C in a humid chamber. Following a 2-h incubation, the non-adherent cells were removed by washing each chamber 2 times with 500 µL L-15/MØ medium pre-warmed to 15 °C. Each chamber received 200 µL L-15/MØ medium containing 1% (v/v) FCS and 5×10^8 bacteria/mL of FITC-labeled *S. aureus*. The slides were incubated for 1 h. The non-phagocytized bacteria were removed by washing each chamber 4 times with 500 µL L-15/MØ medium prewarmed to 15 °C. The cells were fixed by the addition of 200 µL/chamber PBS, pH 7.4 containing 1% (w/v) paraformaldehyde. The slides were incubated for 30 min before the fixative was poured out. The plastic chambers and silicon gasket were then removed from the slide. A small drop of Fluoromount G (Southern Biotechnology Assoc., Inc.) mounting media containing 1 µg/mL of the DNA fluorochrome 4'-6'diamidino-2-phenylindole (DAPI; Sigma) was placed in each chamber area and a glass coverslip placed on the slide. The slides were stored for 16 h at 4 °C in a light-proof box. The cells were observed by fluorescence microscopy with a 420 nm long wavelength band filter and concomitant low-light-level phase contrast microscopy using the Zeiss Axiophot photo microscope at 800× magnification. To determine the proportion of phagocytic cells, 100 macrophage (MØ) cells (glass-adherent leucocytes with U-shaped nuclei) from each chamber area were categorized as phagocytic or non-phagocytic. Phagocytic MØs were those which

had 2 or more FITC-labeled *S. aureus* within the periphery of the cell. Data was presented as the mean percent of MØs which were considered phagocytic among the replicate chambers.

Myeloperoxidase activity

Assay reagents -- Commercially prepared Tris-maleate buffer, pH 6.3, phenylenediamine hydrochloride-catachol indicator reagent, and 3% (v/v) hydrogen peroxide were used according to manufacturer's instructions (Sigma).

Myeloperoxidase assay -- To stain the imprints for myeloperoxidase activity, 50 mL of Tris-maleate buffer placed in each of six Coplin staining jars and warmed to 37 °C. Hydrogen peroxide and phenylenediamine hydrochloride-catachol solutions were added to the buffer and mixed thoroughly. Immediately after mixing, the anterior kidney imprint slides were randomly distributed among the jars and incubated for 30 min in the dark. The slides were washed for 15-30 sec in gently running tap water and allowed to air dry at room temperature. Slides were examined by light microscopy at 1260× magnification to determine the degree of myeloperoxidase staining.

Antigen Specific Plasma Immunoglobulin Response

An ELISA for antigen-specific immunoglobulin (Alcorn and Pascho 2000) was used to quantify the humoral response of individual fish to the hemocyanin protein of keyhole limpets (*Megathura crenulata*, KLH, Sigma).

In June 1998, fish from the replicate tanks of the 100%, 64% and 40% ration level treatment groups were injected with the KLH protein and maintained at the same ration level. To test the effect of ration level during the immune response a second group of fish from each replicate tank of the 100% ration level were reared at the 40% ration level following injection with the KLH. During November 1998 anti-KLH study, fish from the replicate tanks of the 88%, 64% and 40% ration-level-treatment groups were injected with the KLH and maintained at the same ration level.

Injection of test fish with the KLH protein -- Prior to injection, 10 fish from each ration level subgroup were bled for preinjection plasma samples. Fifty fish from each ration level subgroup were injected with 100 µL or 250 µL of 50 µg KLH protein emulsified with Freund's incomplete adjuvant (FIA) in June and November 1998, respectively. Each injection group was maintained in a separate 137-L tank at the appropriate ration level. On each bleeding date ten fish were sacrificed from each tank and bled via the caudal vein. Bleeding dates were 42, 49, 56, 63 and 70 d after immunization. Plasma samples were prepared as described above and stored at -80 °C.

Production of the standard serum -- Hyperimmune serum from 10 rainbow trout (approximately 500 g) obtained from a commercial trout hatchery were vaccinated with the KLH protein to prepare the serum standards for the anti-KLH ELISA. The fish were maintained at ambient temperature in sand-filtered, UV-treated Lake Washington

water at the WFRC and fed to satiation 5 d/wk on a commercial diet (BioOregon). Each fish was injected with a 1 mL emulsion of Freund's complete adjuvant and 100 µg KLH. Each fish was given a booster injection of 1 mL emulsion of FIA and 20 µg KLH at 1.5, 8 and 9.5 months after the primary immunization. All fish were exsanguinated 29 d after the final injection. The serum from the fish with the greatest antibody response to the KLH protein was used as the standard serum for the anti-KLH ELISA.

Anti-KLH ELISA -- Commercially-prepared wash buffer, antigen coating buffer, ABTS peroxidase substrate system, and ABTS stop solution (Kirkegaard and Perry Laboratories, Inc.) were used according to the manufacturer's instructions. All volumes were 200 µL per microplate well unless otherwise stated. Each wash step consisted of rinsing microplate wells 5 times with 200 µL wash buffer. A standard dilution set was included on each microplate and consisted of 1:50, 1:100, 1:200, 1:400 and 1:800 (v/v) dilutions of rainbow trout anti-KLH protein (RBT anti-KLH) in Tris-buffered saline (TBS), pH 8.0, containing 1% (v/v) Tween-20 and 3% (w/v) bovine serum albumin (T-TBS+3% BSA). Each dilution was tested in triplicate. Each ELISA also included internal standards consisting of 1:100 (v/v) dilutions in T-TBS+3% BSA of serum from a rainbow trout immunized with KLH; internal standards were tested in duplicate. Each test fish plasma sample was diluted 1:50 (v/v) in T-TBS+3% BSA and tested in duplicate. Microplate (Costar) well surfaces were coated with 1 µg per mL KLH for 16 h at 4 °C, then unbound protein was removed by the wash procedure. The test plasma, internal standards, and standard dilution set were placed in the appropriate wells on each microplate and incubated for 2 h at 15 °C. The microplates were washed and supernatant of a mouse hybridoma culture producing immunoglobulin to salmonid IgM (Warr 1.14, DeLuca et al. 1983) was diluted 1:1000 (v/v) in T-TBS+3% BSA and added to each well. The plates were incubated for 1 h at 25 °C before the unbound monoclonal antibodies were removed by the wash procedure. A 1:5000 (v/v) dilution of goat anti-mouse IgG (H+L) conjugated to horseradish peroxidase (HRP conjugate, Calbiochem) in T-TBS+3% BSA was applied to each well. The plates were incubated 1 h at 25 °C before the unbound conjugate was removed by the wash procedure. The ABTS substrate was added to each well and incubated for 15 min at 37 °C. Color development was stopped by the addition of 50 µL per well of a 4×-strength stop solution. The absorbance of each well was measured at 405 nm.

Data analysis

General analyses -- Measurements of the morphometric and immunological parameters within sample periods were compared by analysis of variance (ANOVA, $p>0.05$) except for the antigen specific plasma immunoglobulin response. When an environmental or experimental effect had a significant F-ratio in the ANOVA (p 0.05), the Scheffe's procedure for post-hoc paired comparisons was used to determine which pair(s) of means were significantly different. Apart from ration level and diet formulation, it was assumed that all of the tanks were treated similarly. The percent values for the hematocrit, leucocrit, NBT, and phagocytosis assays were normalized using the arc sine transformation prior to statistical analysis.

Complement fixation -- For the complement assay the dilution of each test plasma sample that would lyse 50% of the RaRBC (ACH_{50}) was determined based on the method described by Ingram (1990). Briefly, the mean absorbance values for each dilution of the test plasma, and for the positive and negative controls, were corrected by subtraction of the mean absorbance of their respective background wells. The percentage of RaRBCs lysed at each of the test plasma dilutions was calculated by the equation:

$$\% \text{ RaRBC lysis (y)} = \frac{\text{OD of test plasma dilution - OD of negative control}}{\text{OD of positive control}} \times 100$$

A plot was made of the $\log_{10}$ y/(100-y) and the $\log_{10}$ of each test plasma dilution. The inverse-$\log_{10}$ of the y-intercept of the line was equivalent to the ACH_{50} of the test plasma sample. The distribution of ACH_{50} values for each temperature were compared by ANOVA as described above.

Lysozyme activity -- Clearance zone curves derived from the HEWL control wells included in each test were compared to compensate for day-to-day variations in the lysoplate assay. A correction factor was calculated for each test using the formula described by Pascho et al. (1987), except mean and grand mean values were based on clearance zone measurements for the 1, 10, 40, and 80 µg/mL HEWL control wells. When appropriate, each zone diameter for the test samples and the HEWL control samples was multiplied by the correction factor. A correction factor was applied to test samples from three analyses; where appropriate, clearance zone diameters for those test samples were multiplied by either 0.973, 0.987, or 1.043. Lysozyme activity was compared among ration-level groups, and between sample periods, using the ANOVA.

NBT reduction assay -- For the NBT assay, untreated cells (NBTC) were considered to have a basal level of activity. Cells treated with PMA were considered 100% stimulated. Cells treated with IA were considered to be totally inactivated. To correct for differences in the background absorbance between plates, the mean absorbance for the wells treated with IA was subtracted from the mean absorbance for the treatment groups. The percent activation of the basal and naturally stimulated cells in

each tissue preparation was calculated by dividing the mean OD_{630} of the NBTC and As-treated test sample wells, respectively, by the mean OD_{630} of the PMA treated wells. Comparisons of kidney tissue preparations were based on the difference in the percent activation of the NBTC and the As exposed cells (percent change).

Myeloperoxidase assay -- A total of 100 cells were evaluated for degree of myeloperoxidase staining based on the following criteria. Cells exhibiting no staining were assigned a value of "0". Cells with cytoplasm stained up to 1/3 (dark granules) were assigned a value of "1", 1/3 to 2/3 were assigned a value of "2", and 2/3 to all were assigned a value of "3". The average weighted value (AWV) for each slide was figured by multiplying the score value (0, 1, 2, 3) by the number of cells counted in that category, then dividing by the total number of cells counted for that fish. The comparison of groups was based on the AWV by ANOVA.

Anti-KLH ELISA -- For the anti-KLH ELISA, the 1:50, 1:100, 1:200, 1:400 and 1:800 dilutions of the RBT anti-KLH were assigned 16, 8, 4, 2 and 1 antibody activity units/mL to the KLH protein, respectively. A standard curve was developed for each plate by the relationship of the mean absorbance of each dilution of RBT anti-KLH to the assigned antibody activity units/mL. The antibody activity units/mL of the internal standard and each test plasma sample was determined by reference to the standard curve derived from the RBT anti-KLH dilutions on the same microplate, and the dilution factor. The results of the entire plate were disregarded if the correlation coefficient (r^2) of the standard curve is below 0.95, or the calculated antibody activity units/mL of the internal standards was outside a predetermined range.

To measure the antigen specific plasma immunoglobulin response the antibody activity units/mL values were log transformed before a positive-negative threshold was determined for each tank. The positive-negative threshold was defined as the mean antibody activity units/mL of the preinjection plasma samples plus twice the standard deviation of the mean. Post-injection plasma samples with an antibody activity units/mL value greater than the positive-negative threshold were categorized as positive. The relationship of the week after antigen injection and the antibody activity units/mL of the positive plasma was determined by regression analysis. The y-intercept was set at zero as it was assumed that the fish had no prior exposure to the antigen. A Student's *t*-test of the slope values was used to compare the antibody response of the fish reared at the different ration levels for each sampling period. Significance was set at 0.05.

Results

Fish

In July 1998 a change in water quality caused an increase in mortality in the population of fish reared on the BioDiet feed but did not effect the other treatment groups. Contamination of the recirculating water system with *Renibacterium*

salmoninarum caused increased mortalities in all treatment groups with maximum mortalities occuring during January and February 1999. In an effort to control the bacterial kidney disease the fish were treated for 28 d with erythromycin (100 mg/kg/d) beginning on 2 January 1999.

At the time of the first immunological sampling period the fish had been maintained at different ration quantities for 31 wk. At each of the immunological testing periods, the fish fed at the 100% ration level were larger (p=0.0029) and heavier (p=0.0257) than the fish reared at the 64% ration level, except there was no difference in length during the fall sample period (p=0.0630, Table 2). The fish reared at the 64% ration level were consistently larger (p=0.0003) and heavier (p=0.0023) than the fish reared the 40% ration level (Table 2). The fish reared on BioDiet ration were similar in size (p=0.3460) and weight (p 0.8837) to fish reared at the 64% ration level (Table 2). The fish reared on BioDiet ration were larger (p=0.0043) and heavier (p=0.0001) than fish reared at the 40% ration level but shorter (p=0.0001) and weighed less (p=0.0001) than fish reared at the 100% ration level. An effect on the length of the fish by sample date was detected during the winter 1998 (p=0.0016) and spring 1999 (p=0.0004). The measured weight of the fish was effected by sample date during each sample period (p=0.0212) and a within group tank effect was detected during the winter 1998 (p=0.0005). During the spring 1999 sampling period there was no effect of sex on the length (p=0.5087) or weight (p=0.4788) of the fish in any of the 4 feed groups.

Table 2. Morphometric measurements of chinook salmon reared on an experimental diet at 3 ration levels and a commercial diet. Values are mean ± SE for 30 samples.

Sample period	Feed group	Length (mm)	Weight (g)	Condition factor
Fall 1998	100%	170 ± 3.7	62 ± 3.5	1.21 ± 0.013
	64%	159 ± 3.0	49 ± 2.5	1.17 ± 0.010
	40%	136 ± 3.2	29 ± 1.8	1.12 ± 0.010
Winter 1998	100%	208 ± 4.3	113 ± 5.9	1.21 ± 0.019
	64%	186 ± 3.5	78 ± 3.9	1.18 ± 0.012
	40%	161 ± 3.3	49 ± 2.7	1.15 ± 0.011
	BioOregon	181 ± 4.8	76 ± 4.7	1.23 ± 0.018
Spring 1999	100%	262 ± 2.1	214 ± 8.8	1.19 ± 0.040
	64%	240 ± 2.4	166 ± 5.1	1.19 ± 0.013
	40%	198 ± 5.2	95 ± 6.6	1.16 ± 0.026
	BioOregon	230 ± 5.8	158 ± 9.9	1.23 ± 0.016

During the fall 1998 sampling period the fish reared at the 100% ration level had a greater condition factor than the fish reared at the 64% ration level (p=0.0257, Table 2).

The fish reared at the 64% ration level had a greater condition factor than those reared at the 40% ration level (p=0.0023, Table 2). During the winter 1998 and spring 1999 sampling periods there was no difference in the condition factor for the fish reared on the different ration levels except the fish reared at the 100% ration level had a greater condition factor than the fish reared at the 40% ration level during the winter 1998 (p=0.0323). The condition factor of the fish reared on the BioDiet ration was similar to the fish reared on the different ration levels except it was greater than that for the fish reared at the 40% ration level during the winter 1998 sampling period (p=0.0031, Table 2). An effect of sampling date on the condition factor was detected during the fall 1998 (p=0.0001) and spring 1999 (p=0.0048) sampling periods. A tank effect within treatment group on the condition factor was detected in the winter 1998 sample period (p=0.0465).

Blood and plasma samples

Hematocrit -- Fish reared at the 40% ration level had an increased hematocrit compared with the fish reared at the other two ration levels during the spring 1999 (p=0.0496, Table 3). The fish reared at the 100% ration level had a greater hematocrit than the fish reared on the BioDiet ration during the winter 1998 sampling period (p=0.0057). No other differences in the hematocrit of fish reared at the different ration levels or diets were detected. An effect of sample date during the fall 1998 sample date (p=0.0001) and a within group tank effect during the winter 1998 sample period (p=0.0001) on the hematocrit were detected.

Table 3. Immunological parameters of blood plasma taken from chinook salmon reared on an experimental diet at 3 ration levels and a commercial diet. Values are the mean ± SE for 30 samples.

Sample period	Feed group	Sex	Hematocrit	Protein concentration	C' activity (ACH_{50})	Lysozyme (zone size)
Fall 1998	100%	C[1]	47.1 ± 1.10	6.2 ± 0.18	30 ± 1.2	8.4 ± 0.25
	64%	C	46.2 ± 0.72	5.9 ± 0.09	30 ± 1.7	7.9 ± 0.20
	40%	C	46.0 ± 0.95	5.6 ± 0.10	26 ± 2.7	8.6 ± 0.33
Winter 1998	100%	C	50.0 ± 0.59	7.2 ± 0.13	29 ± 0.9	8.6 ± 0.21
	64%	C	47.9 ± 0.95	6.5 ± 0.18	26 ± 1.4	9.0 ± 0.31
	40%	C	47.9 ± 0.74	5.8 ± 0.14	25 ± 1.1	9.2 ± 0.25
	BioOregon	C	46.4 ± 0.81	7.5 ± 0.16	27 ± 1.2	9.8 ± 0.33
Spring 1999	100%	C	46.5 ± 1.01	7.1 ± 0.20	27 ± 1.0	9.0 ± 0.48
		M	46.6 ± 1.56	7.8 ± 0.39	26 ± 1.1	9.2 ± 0.90
		F	45.9 ± 1.30	6.6 ± 0.12	29 ± 1.2	8.9 ± 0.57
	64%	C	46.4 ± 0.97	7.0 ± 0.21	26 ± 1.2	9.6 ± 0.42
		M	47.5 ± 1.14	7.7 ± 1.1	25 ± 1.9	9.6 ± 0.57
		F	45.2 ± 1.59	6.1 ± 0.21	26 ± 1.6	9.6 ± 0.65
	40%	C	50.0 ± 0.88	6.7 ± 0.19	27 ± 0.9	9.3 ± 0.33
		M	50.1 ± 1.20	7.1 ± 0.32	25 ± 1.3	8.7 ± 0.39
		F	49.8 ± 1.30	6.4 ± 0.19	28 ± 1.1	9.7 ± 0.49
	BioOregon	C	48.1 ± 0.85	8.2 ± 0.21	28 ± 1.4	8.9 ± 0.40

M	46.0 ± 1.12	8.8 ± 0.26	27 ± 2.4	9.3 ± 0.83
F	50.9 ± 1.19	7.5 ± 0.20	28 ± 1.7	8.7 ± 0.41

[1]C = combined (both sexes), M = male, F = female.

Leucocrit -- During each sampling period there was a trend toward an increasing leucocrit with increasing feed ration level (Figure 1). The fish reared at the 100% ration level had a greater leucocrit than the fish reared at the 64% ration level (p=0.0163) and at the 40% ration level (p=0.0028) during the fall 1998 sampling period. No difference in the leucocrit was detected between the fish reared on the BioDiet ration and any of the different ration levels at both the winter 1998 and spring 1999 sampling periods. An effect of sample date on the leucocrit was detected in the fall 1998 (p=0.0001) and winter 1998 (p=0.0042) sample periods. There was no gender effect on the leucocrit in the spring 1999 sampling period (p=0.0849).

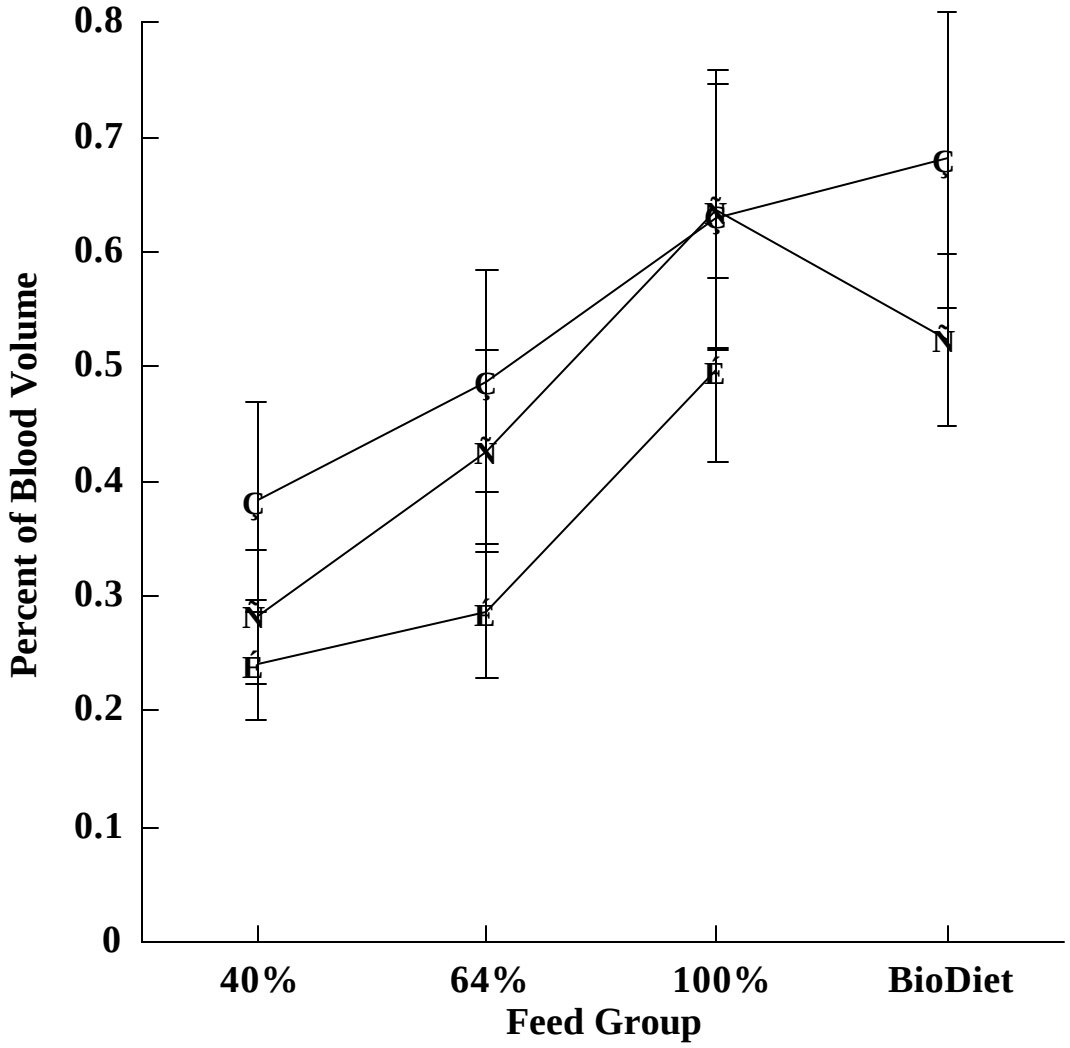

Figure 1. Leucocrit values of of chinook salmon reared on three ration levels of an experimental high protein, low fat diet and a commercial diet during the fall

1998 (E), winter 1998 (G) and spring 1999 (C) sampling periods. Values are mean ± SE for 30 samples.

Total plasma protein concentration -- There was a trend indicating that greater rations of feed increased the concentration of total plasma protein (Table 3). During the fall 1998 sampling period the fish reared at the 100% ration level had a greater plasma protein concentration than the fish reared at the 40% ration level (p=0.0014). During the winter 1998 sampling period the fish reared at the 100% ration level had a greater concentration of plasma protein than the fish reared at the 64% ration level (p=0.0184). The fish reared at the 64% ration level had a greater plasma protein concentration than the fish reared at the 40% ration level (p=0.0059) during this same sampling period. The fish reared on the BioDiet feed had a greater concentration of plasma protein than fish fed any of the different ration levels (p=0.0004) except the fish reared at the 100% ration level during the winter 1998 sampling period (p=0.7058). Regardless of the ration level or feed type, the male chinook salmon had a greater concentration of plasma protein than the female fish (p=0.0001, Figure 2). The plasma protein concentration was affected by the sample date during the fall 1998 (p=0.0001) and winter 1998 (p=0.0283) sampling periods.

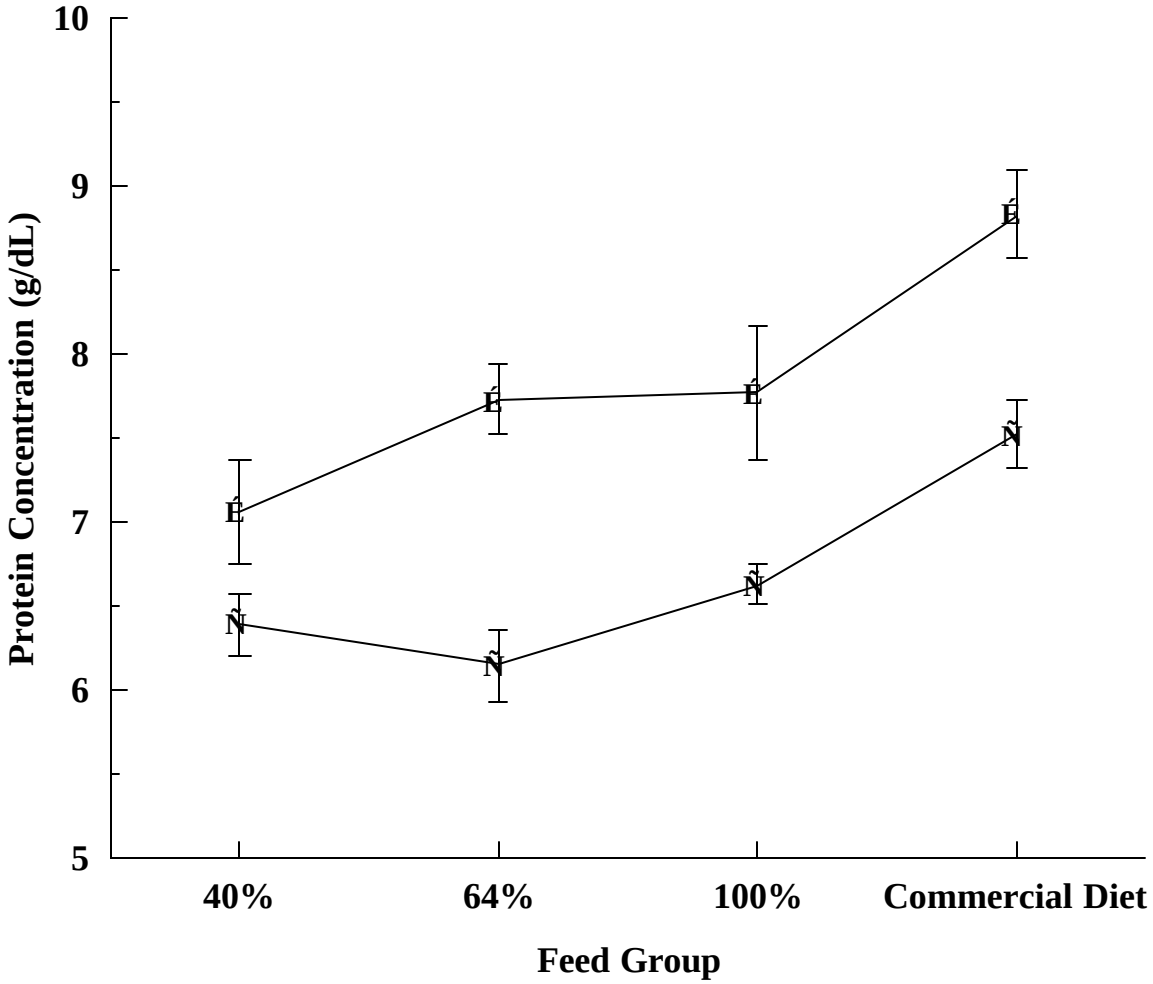

Figure 2. The plasma protein concentration of male (E) and female (G) chinook salmon reared on three ration levels of an experimental high protein, low fat diet and a commercial diet. Values are mean ± SE for fish sampled during the spring 1999.

Complement (ACH_{50}) -- The mean ACH_{50} values for all treatment groups were between 24 and 30 (Table 3). No differences (p=0.05) were detected between any treatment groups at any sampling period.

Lysozyme -- Relative lysozyme concentrations were determined by lysis of *Micrococcus* cells suspended in an agarose matrix. The mean diameter of the zone of clearance for all treatment groups was between 7.9 and 9.8 mm (Table 3). During the winter 1998 sampling period the fish fed at the 100% ration level had lower concentrations of lysozyme than the fish fed the BioDiet formulation (p=0.0148). No other differences (p=0.05) were detected between any treatment groups at any sampling period. During the winter 1998 sampling period a within treatment tank effect was detected (p=0.0007).

Differential leucocyte counts -- Lymphocytes, polymorphonuclear leucocytes (PMN) and monocyte/macrophages constituted a mean of 90.6-96.1, 3.0-7.8 and 0.6-1.4 percent, respectively, of the circulating leucocytes in all treatment groups during all sampling periods. During the winter 1998 sampling period the fish reared at the 40% ration level had a greater percentage of lymphocytes (p=0.0373) and a lower percentage of PMN (p=0.0326) than the fish reared on the BioDiet feed. No other differences in ratios of the circulating leucocyte populations were detected among treatment groups within a sampling period. The number of thrombocytes per 100 circulating leucocytes ranged between 0.7-105.3 with a mean of 27.8-54.0 for each treatment group during each sampling period. No differences in thrombocyte concentrations were detected between treatment groups during any sampling period. An effect of sampling date on the percentage of lymphocytes (p=0.0003) and PMNs (p=0.0001) was detected during the fall 1998 sampling period, and on the percentage of monocyte/macrophages during the spring 1999 sampling period (p=0.0464). The number of thrombocytes per 100 leucocytes was altered by a within treatment group tank effect during the spring 1999 sampling period (p=0.0013).

Nitroblue tetrazoleum reduction

The increase in NBT reduction by adherent anterior kidney leucocytes from the basal level to that induced by the addition of bacteria is the stimulation index. During each sampling period the NBT stimulation index of the treatments were similar. The mean NBT stimulation index for the treatment groups throughout the study was between 15.1 and 18.2. During the fall 1998 and winter 1998 there was an effect of sampling date on the NBT stimulation index (p=0.0014 and p=0.0414, respectively). An effect of tank within treatment group on the NBT stimulation index was detected during the fall 1998 sampling period (p=0.0146).

Phagocytosis

During each of the sampling periods there was a trend toward a decreased percentage of phagocytic anterior kidney macrophages with increased feed availability (Figure 3). A greater percentage of anterior kidney macrophages from the fish reared at the 40% ration level were phagocytic compared with the macrophages from the fish reared at the 100% ration level during the fall 1998 (p=0.0051) and spring 1999 (p=0.0189) sampling periods. During the winter 1998 sampling period the there was a greater percentage of phagocytic macrophages from the fish reared at the 64% ration level than from the fish reared at the 100% ration level (p=0.0159). No difference in the percentage of phagocytic macrophages was detected from the fish reared on the BioDiet feed compared to any of the ration level treatment groups. An effect of sample date on the percentage of phagocytic macrophages was detected during the winter 1998 sampling period (p=0.0001). The percentage of phagocytic macrophages was also affected by a within treatment group tank effect during the winter 1998 (p=0.0001) and spring 1999 (p=0.0010) sampling periods.

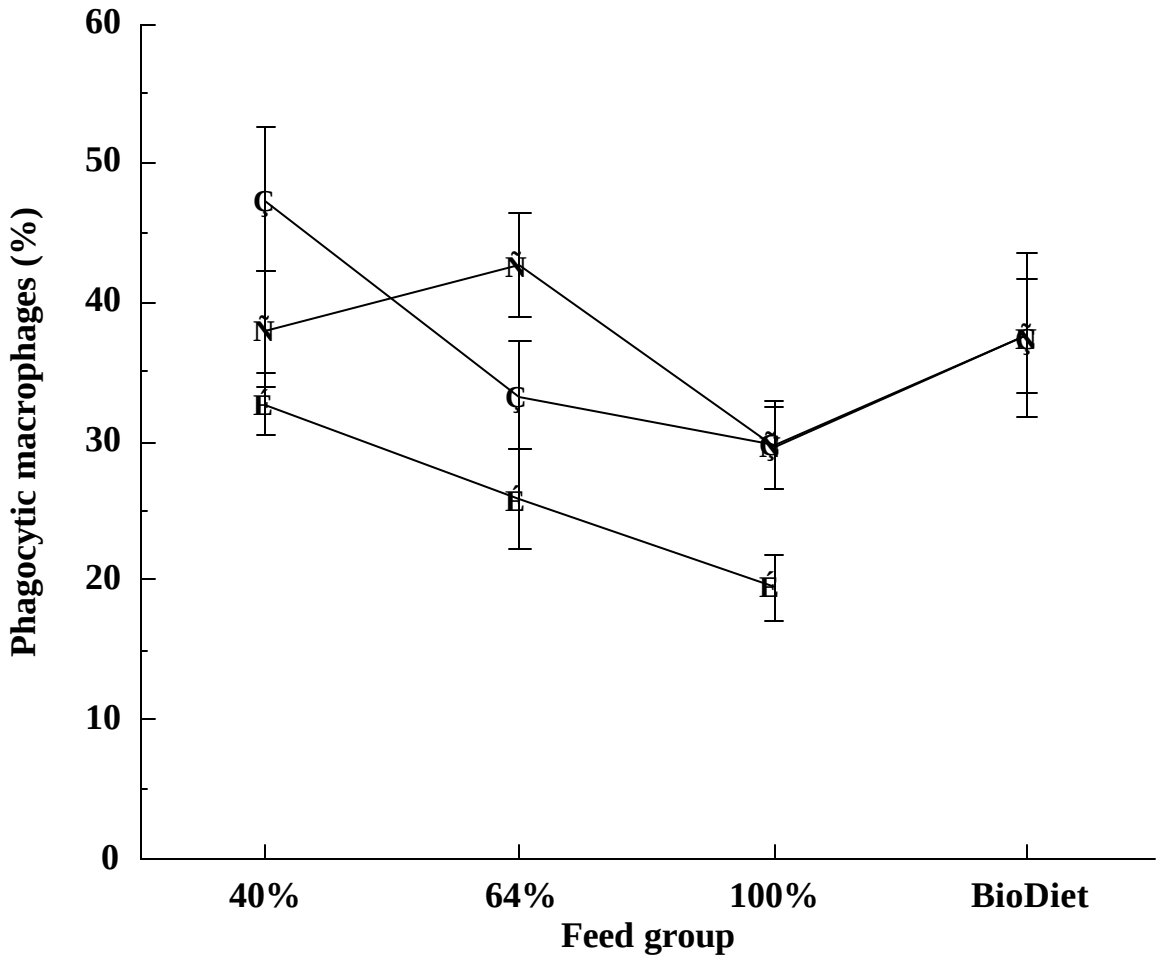

Figure 3. The percentage of glass-adherent anterior-kidney macrophage cells which ingested 2 or more fluorescent-labeled *S. aureus* cells from chinook salmon reared on three ration levels of an experimental high protein, low fat diet and a commercial diet during the fall 1998 (E), winter 1998 (G) and spring 1999 (C) sampling periods. Values are mean ± SE.

Myeloperoxidase

During the spring 1999 sampling period the anterior kidney leucocytes from the fish reared at the 40% ration level had greater myeloperoxidase concentrations than those found in fish reared on the 64% (p=0.0474) and 100% (p=0.0001) rations (Table 4). The anterior kidney imprints from fish reared on the BioDiet feed had greater myeloperoxidase staining than those from fish reared at the 100% ration level during the same sampling period (p=0.0007). During the first 2 sampling periods there was no difference in the myeloperoxidase staining of the anterior kidney imprints from any treatment group. An effect of sampling date on myeloperoxidase staining was detected during the fall 1998 (p=0.0001) and winter 1998 (p=0.0156) sampling periods.

Table 4. Myeloperoxidase staining of anterior kidney blots taken from chinook salmon reared on an experimental diet at 3 ration levels and a commercial diet. Values are mean AWV ± SE for 30 samples.

Sample period	Feed group	Sex	Myeloperoxidase
Fall 1998	100%	Combined	1.1 ± 0.20
	64%	Combined	1.1 ± 0.20
	40%	Combined	0.9 ± 0.20
Winter 1998	100%	Combined	0.5 ± 0.07
	64%	Combined	0.4 ± 0.06
	40%	Combined	0.4 ± 0.07
	BioOregon	Combined	0.4 ± 0.06
Spring 1999	100%	Combined	0.9 ± 0.10
		Male	0.9 ± 0.15
		Female	0.9 ± 0.14
	64%	Combined	1.4 ± 0.13
		Male	1.4 ± 0.15
		Female	1.3 ± 0.21
	40%	Combined	1.9 ± 0.16
		Male	2.4 ± 0.15
		Female	1.4 ± 0.21
	BioOregon	Combined	1.7 ± 0.14
		Male	1.6 ± 0.20
		Female	1.7 ± 0.22

Antigen specific plasma immunoglobulin response

Of the 400 chinook salmon injected with the KLH protein in June 1998, only 19 fish produced a response greater than the threshold; 5 fish from both the 100% and 64% ration-level groups, 6 fish from the 40% ration-level group, and 3 fish from the group of fish that were switched from 100% to 40% ration level after injection. Only one of the fish, from the 40% ration level, produced a strong response with 1506 units of antibody activity/mL. All of the other fish were weak responders, with less than 50 units of antibody activity/mL. Of the 300 fish injected in November 1998, only 1 fish from each of the 88% and 64% ration-level groups produced a detectable response. The units of

antibody activity/mL were 62 and 58, respectively. Due to the poor antibody response in both anti-KLH studies no statistical analysis was performed on these data sets.

Discussion

The goal of this study was to determine if certain nonspecific immune functions of chinook salmon are affected by different growth rates. It was part of a larger study examining if growth rate also affects the incidence of early maturation in male chinook salmon. Because male maturation is partly controlled by energy stores (Rowe and Thorpe 1990), the test diet was formulated with relatively low levels of fat so as not to promote early maturation.

Dietary lipids are the source of essential fatty acids that contribute to the permeability and other characteristics of cell membranes. Studies with catfish have shown that intracellular bacterial killing by macrophages correlates to dietary levels of n-3 series fatty acids, particularly long-chain, highly unsaturated fatty acids (Sheldon and Blazer 1991). The experimental diet contained 54% protein and 7% fat, while the BioDiet control feed contained about 50% protein and 22% fat. Recommended levels of dietary lipid for salmonids range between 9% and 15%, depending on the age of the fish and the protein level of the diet (35-50%; Piper et al. 1982). Accordingly, the whole body lipid levels in fish from the 64% ration group remained very low, ranging between 3% and 5% during the September and December 1998 sampling periods, and 7.5% during the spring 1999 sampling period. In contrast, whole body lipid levels for the fish fed the BioDiet averaged over 8% during the fall 1998 sample period, about 10% during the winter 1998 sample period, and near 12% during the spring 1999 sample period (K.D Shearer, NMFS, personal communication). Although the fish from the BioDiet control group had the greatest levels of plasma protein and body lipid, they responded in a manner similar to fish fed the experimental diet at an equivalent rate (64% ration) in all of the immunological assays.

Among the nine parameters of the chinook immune system measured, the effect of ration level was restricted to the plasma protein concentrations leucocrit levels, the percent of phagocytic macrophages, and myeloperoxidase activity. Measures of erythrocyte properties, including the hematocrit are often used in the assessment of fish health. The hematocrit values obtained from the fish in this study were all within the normal range for salmonids (Wedemeyer et al. 1990), suggesting that the fish were not overtly stressed during the course of the experiment. However, the use of the hematocrit as a fish health diagnostic tool has been questioned (Houston 1997). Differences in the fishes life stage, ratio of mature versus immature blood erythrocytes, methods of sample gathering, storage, and preparation (centrifugation) can all affect the hematocrit value. As the fish in this study were all of the same life stage and the samples were collected and treated in a similar manner, the reported values probably reflect accurately the fact that none of the ration levels had a measurable effect on the hematocrit.

Fish mortality due to *R. salmoninarum* began after the December 1998 sampling period. During that sampling period no clinical signs typical of BKD were observed in

any of the experimental or control groups. All experimental groups were treated with medicated feed containing 100 mg erythromycin/kg body weight for 28 d, beginning 2 January 1999. The final (March 1999) sampling period was approximately 45 d after the completion of the antibiotic therapy. During sampling it was observed that about 12% of the fish sampled had granulomatous lesions in their kidney characteristic of BKD. The blood and plasma parameters of the fish with kidney lesions were similar to their healthy cohorts except for the lysozyme values. Lysozyme concentrations in fish have been reported to increase after injection of bacterial products (Chen et al. 1996) and in response to bacterial infection (Møyner et al. 1993). Lysozyme clearance zones produced by the plasma from the fish with lesions tended to be larger compared with the values obtained from cohort fish in the same treatment group, except those fish fed the commercial diet (data not shown). The clearance zones of the fish reared on the commercial diet which had lesions were smaller than the mean value for all fish in that treatment group. These results indicate that most of the standard hematological parameters used in this study were not sufficient to detect fish in the early stages of bacterial kidney disease, but lysozyme levels may be a useful indicator of this disease.

Following their formation in the hematopoietic anterior kidney, phagocytic cells of the fish immune system, including macrophages and PMNs, are thought to emigrate throughout the fish. The number of macrophage cells in the peritoneal cavity of rainbow trout have been shown to increase after the injection of protein, incomplete Freund's adjuvant and live or killed bacteria (Afonso et al. 1998). Fish PMNs have been shown to migrate *in vitro* in response to serum factors such as complement (Griffin 1984) and exogenous factors including some bacterial pathogens, LPS, muramyl dipeptide, chitin, β-glucans, and various synthetic molecules (review by Secombes 1994). The decrease in the percent of phagocytic macrophages in fish reared at the greater ration levels may indicate the macrophages were able to emigrate from the hematopoietic anterior kidney earlier in the maturation cycle than the macrophages in fish maintained on the lower ration levels. Under this hypothesis the fish reared on the high ration diet would have a greater percentage of mature phagocytic cells throughout the fishes body. If the phagocytic cells in the high ration group were then better placed for the trapping of invading microbes then their counterparts in low ration level fish, high ration level fish could show an increased ability to resist the onset of disease. Conversely, the decreased percentage of phagocytic macrophages in high ration level fish may indicate suppression of macrophage activity. Under this hypothesis the increased diet may down regulate the production of phagocytic cells, slow the maturation of phagocytic cells until after they have emigrated from the anterior kidney, or inhibit the ability of the mature macrophages to phagocytize the target particles. Fish reared on a high ration diet may show the effects of immunosuppression under this hypothesis. Blazer et al. (1984) reported similar results from study in which they fed groups of rainbow trout either a commercial formulation or a synthetic, laboratory-prepared diet to examine nutritional effects on specific and nonspecific immune functions for 17 wk. The ability of peritoneal macrophages to phagocytize latex beads was used as a measure of nonspecific immunity. The authors found that fish fed the commercial diet had a higher growth rate compared with fish fed the synthetic diet, but that macrophages from the fastest growing fish phagocytized fewer latex beads than macrophages from the fish fed the synthetic diet. The results of the

current study suggest that the dietary effects observed by Blazer et al (1984) may have been partially related to growth rate, but nutritional differences in the two formulations may have also been a factor. The differences measured in the phagocytic index among the ration-level groups in the current study were influenced by growth rate, not nutritional factors, as each group received an identical diet. None the less, both the kidney macrophage activity in the current study and the peritoneal macrophage activity reported by Blazer et al. (1984) were associated with a high growth rate.

Both neutrophils and macrophages were found in the anterior kidney, and both cells types produced superoxide anion, which was measured by the NBT assay. However, as macrophages adhere to surfaces more readily than neutrophils (Ptytycz et al. 1989), it is believed that the NBT assay was principally measuring the superoxide anion production by macrophages. In contrast, the myeloperoxidase assay is a measure of the bactericidal potential of neutrophils since macrophages produce little, or no, myeloperoxidase (Zelikoff and Enane 1991). The production of myeloperoxidase increased inversely to the ration level during the March 1999 sampling period, a time when brood year 1997 spring chinook salmon would normally smolt. The results of the cellular assays indicate that ration level may have an inverse effect on the protective abilities of the primary non-specific leucocytes, the macrophages and neutrophils, during at least some life stages of the fish. Unlike neutrophils, however, the ability of macrophages to destroy internalized bacteria was not effected by ration level. Several steps are involved in intracellular bactericidal activity, including bacterial recognition, binding, internalization, formation of the phagolysosome and intracellular destruction by reactive oxygen species, including superoxide anion. The regulation of these activities is either controlled by separate processes, or they have different sensitivities to diet levels.

The keyhole limpet hemocyanin molecule was chosen as an antigen to measure the antibody response because it is a large molecule and thought to present a high number of antigenic epitopes. The reason for the lack of antibody response to KLH is unknown. There was a concern that the use of Freund's complete adjuvant, which includes immunopotentiating *Mycobacterium* cells, would stimulate the immune response to a degree that slight differences between fish reared at the various ration levels would be lost. Considering the poor response that was obtained using Fruend's incomplete adjuvant (FIA) the complete adjuvant should be used with this antigen in chinook salmon in any future studies. An equal concentration of the 57 kD antigen of *R. salmoninarum* (p57) in FIA produces a strong antibody response in salmonids. The difference in the response to KLH and p57 may be due to the limited repertoire of antibodies available to fish (Pilström and Bengtén 1996). Antigens associated with fish pathogens may illicit a stronger antibody response due to evolutionary selection of genes which produce reactive immunoglobulins and the ability to produce a strong specific immunological response. The few fish that did produce a response to KLH indicate that an antibody response is possible to this molecule in chinook salmon.

The results of this study indicate that ration levels may not have a profound affect on the immune system of pre-smolt spring chinook salmon. The fact that length, weight, and plasma protein concentration did increase in proportion to the ration level indicates

that greater ration levels allow for increased amounts of available metabolic energy. As most of the immunological functions measured did not respond to even extreme differences in ration level, the immune system of fish can be considered to be fairly robust in regards to available metabolic energy. However, fish reared at the lower ration levels had fewer numbers of all types of circulating leucocytes, but the ability of these cells may have been enhanced by the decreased diet level, as indicated by the results of the phagocytosis and myeloperoxidase assays. The ration amount may not be as important as the ration composition on affecting the immune response of fish. A number of factors, including vitamins, minerals, protein, lipid, and carbohydrates, have been shown to affect the immune response of fish (Blazer 1992; Waagbø 1994).

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TASK 10. EFFICACY AND TOXICITY OF TREATMENTS FOR BACTERIAL KIDNEY DISEASE IN CHINOOK SALMON (PROGRESS REPORT: 1 JUNE 1999 THROUGH 31 MAY 2000)

by

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Introduction

Bacterial kidney disease (BKD) is the major infectious disease preventing successful culture of salmonids in the Pacific Northwest. Between 1993-94 this disease was responsible for catastrophic losses of endangered Redfish Lake sockeye salmon held as captive broodstock. Long-term prophylactic administration of erythromycin has become common in recent years to reduce losses due to BKD. Results of preliminary studies with Lake Wenatchee sockeye salmon suggest that erythromycin may have a negative effect on gamete viability. Recent experiences at Manchester Research Station with Catherine Creek, Lostine River, and Lemhi River spring chinook salmon have shown currently mandated treatment regimens may elicit fatal toxicity reactions. Until alternative therapeutics, such as azithromycin, are identified and proven efficacious for treating BKD in captive broodstock, or until an effective vaccine becomes available, use of erythromycin will continue.

First, the efficacy of erythromycin and azithromycin in treating BKD will be investigated in a BKD challenge experiment. Second, in order to design treatment regimens which avoid toxic reactions and reductions in reproductive success attributable to erythromycin, it is first necessary to identify the cause of each toxic reaction and the condition under which it appears. This experiment will be carried out in large-scale rearing studies, together with supporting laboratory analyses.

BKD-caused epizootics continue to impact captive rearing programs of both sockeye and chinook salmon significantly (Schiewe et al. 1997). There is no vaccine available to protect salmon from infections with *Renibacterium salmoninarum*, the causative bacterium of BKD. Erythromycin has been the primary antibiotic used by fish

culturists in an attempt to prevent and control *R. salmoninarum* (Elliott et al. 1989), administered orally through feed or by injection of maturing adults. However, while use of erythromycin usually results in short-term health improvement of infected fish, it fails to eliminate the infection completely, and symptoms of disease often return after treatment ends.

Azithromycin is a promising candidate for use as a therapeutic and prophylactic agent for BKD in captive broodstock salmon. This new macrolide antibiotic concentrates in polymorphonuclear leukocytes, macrophages and fibrocytes (Peters et al. 1992). *R. salmoninarum* has been shown to invade these cell types, which in turn protect the organism from the host humoral immune system (Bandin et al. 1993; Gutenberger et al. 1997). Azithromycin has strong bactericidal activity against *R. salmoninarum in vitro* and has shown efficacy *in vivo* in prior experimental BKD infections of salmonids.

There are two principal experiments in the work plan: A) compare the efficacy of azithromycin and erythromycin in the treatment of acute BKD in fall chinook salmon; and, B) evaluate the long-term prophylactic use of azithromycin and erythromycin in the development of acute toxicity syndrome and effects on gamete development and quality. The progress of each are reported separately below.

Experiment A

The main objective is to determine the relative efficacies of the two macrolide antibiotics, erythromycin and azithromycin, in the treatment of acute BKD, that is, when disease symptoms first appear. To carry this out, test fish were infected by intraperitoneal injection of a virulent strain of *R. salmoninarum.* This progress report includes efforts from the 1998-1999 and 1999-2000 work plan under this subtask.

Experiment B

The three main objectives are as follows:

(i) Determine how captive fall chinook salmon broodstock gonad development, gamete viability, and survival of the progeny though the swim-up stage are affected by long-term prophylactic administration of erythromycin or azithromycin.

(ii) If the experimental fish are found to be naturally infected with *R. salmoninarum***,** the effects of two erythromycin treatment regimens (two or four treatments annually) on BKD incidence will be measured from the first feeding fry through mature adult life history stages.

(iii) Determine whether erythromycin toxicity responses are related to treatment frequency. This aspect of the study will include a measurement of residual tissue concentrations and will document underlying tissue damage associated with the syndrome. While the study focuses on erythromycin as it is the current antibiotic of choice and the one apparently causing the severe toxic side effects, the long-term effects of azithromycin treatment will also be assessed concurrently.

Work completed

Experiment A

In both years, approximately 2,000 Minter Creek fall chinook salmon were selected for the study. The one major difference between 1998 and 1999 studies was that the fish in the latter study were pre-vaccinated against *Vibrio* after smolting (Alpharma Alpha Dip 2100 *Vibiro anguillarum-ordalii* bacterin, delivered by intraperitoneal injection). In both studies, the fish were acclimated in pathogen-free seawater at the Manchester Research Station for 4 wk prior to *R. salmoninarum* challenge. Fish were fed a standard unmedicated diet. During adaptation, randomly selected fish were screened for the presence of BKD by ELISA and FAT of kidney tissues, and by RT-PCR of blood samples (Rhodes et. al. 1998). Prior year pre-screening showed that ~25% of the fish were infected with *R. salmoninarum* in the absence of clinical signs of disease.

In both years challenge studies included two groups of fish. The group designated for 'challenge' were inoculated intraperitoneally (IP) with a dose of *R. salmoninarum* (ATCC strain 33209) designed to produce clinical acute BKD (approximately 1 x 10^6 bacteria/12 g fish). In both years this method successfully induced fulminating BKD in the study chinook salmon with significant mortality starting 3-wk post-injection. The group designated 'unchallenged' were inoculated IP with an equivalent volume of phosphate buffered saline to duplicate the stress of handling and inoculation of the challenged fish. This group also served as a negative control for any deleterious effects of subsequent antibiotic treatment, activation of quiescent pre-existing BKD, or the appearance of other unrelated disease and mortality.

After inoculation the fish were randomized and transferred into tanks (1.6 m d), with each tank containing 75 fish (1998) or 100 fish (1999). Ten days post challenge (a time period when first mortalities from this challenge usually occur), fish were switched to a feed with antimicrobial supplement, as follows:

<u>1998</u>

Duplicate groups of *R. salmoninarum*-challenged and unchallenged fish were fed:

- BioDiet + no medication,
- BioDiet + erythromycin (dosage to equal 100mg/kg/d for 28 d; standard INAD treatment protocol),
- BioDiet + azithromycin (10 mg/kg/d for 14 d).
- BioDiet + azithromycin (30 mg/kg/d for 14 d).

<u>1999</u>

Triplicate groups of *R. salmoninarum*-challenged fish were fed:

- BioDiet + no medication,
- BioDiet + erythromycin (dosage to equal 100mg/kg/d for 28 d; standard INAD treatment protocol),
- BioDiet + azithromycin (30 mg/kg/d for 14 d).

As in the 1998 study, groups of unchallenged and challenged fish were fed identical diets but, due to tank number limitations, unchallenged/unmedicated and unchallenged/erythromycin groups were carried out in duplicate only. The health of the fish were monitored, and all mortalities necropsied to verify death by BKD. Kidney tissues were examined by FAT to determine levels of *R. salmoninarum* (more sensitive ELISA and RT-PCR assays were to be performed on fish with inconclusive FAT). Representative fish from all treatments were routinely sampled and analyzed for the presence of the bacterium to monitor changes in bacterial load after treatment. In the 1999-2000 study, treatments were repeated on surviving fish after 12-16 wk, and the experiment continued 5 wk beyond that point in time.

Experiment B

Phase 1: First feeding to smolt -- During January 1999, approximately 3,150 George Adams fall chinook salmon were transferred to the Big Beef Creek Hatchery. Prior to initiation of exogenous feeding, 400 fish were randomly stocked into each of 14 isolation tanks (two tanks per treatment). Using this experimental design, the following feedings were carried out:

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

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

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

Tank 1: Forty fish from Treatments 1-7, replicate A

Tank 2: Forty fish from Treatments 1-7, replicate A

Tank 3: Forty fish from Treatments 1-7, replicate A

Tank 4: Forty fish from Treatments 1-7, replicate B

Tank 5: Forty fish from Treatments 1-7, replicate B

Tank 6: Forty fish from Treatments 1-7, replicate B

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

- No treatment.
- Erythromycin administered orally at a rate of 100 mg/kg/d for 2 d, twice per year until mature (November-December 2001).
- Erythromycin administered orally at a rate of 100 mg/kg/d for 28 d, four times per year until mature (November-December 2001).

To date, the erythromycin treated groups have received either one (of two), or three (of four) treatments scheduled for the 1999-2000 reporting period.

Work to be Completed

Experiment A

1998-1999 study -- All samples from the study have been analyzed. Stringent statistical analysis on the data still needs to be performed before broader conclusions can be drawn. It is anticipated that all statistical analysis will be completed by 1 October 2000. In addition, *Vibrio* infections in all groups led to *Vibrio* vaccination of all fish in the 1999-2000 study.

1999-2000 -- This experiment was recently completed and laboratory analysis of samples should be finished by 1 August 2000. Data analysis and preparation of an interim report and recommendations will be completed by 31 December 2000. A final report incorporating data from both studies will be prepared during the June 2000-May 2001 reporting period.

Experiment B

Phase 1: First feeding to smolt stage -- All samples collected during this phase are currently being analyzed. All laboratory work will be completed by 1 October 2000. Data analysis and preparation of an interim report and recommendations will be completed by 31 December 2000.

Phase 2: Smolt to maturity -- Samples will be collected in September 2000 and September 2001 to evaluate the effects of the various treatment regimens (i.e., pre- and post-smolt treatments) on BKD prevalence, drug clearance rates, and organ histology. Maturing females will be spawned towards the end of 2001 to evaluate the effects of the various treatment regimens on reproductive success. Laboratory work will be completed by June 2001. The final report will be prepared during the June 2001-May 2002 reporting period.

TASK 11. INBREEDING.
(PROGRESS REPORT: 1 JUNE 1999 THROUGH 31 MAY 2000)

by

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Introduction

A number of wild salmon populations exist in low abundances. It is not yet known to what extent inbreeding has led to, and continues to impede viability in these populations, which aspects of the life cycle are affected most, and whether inbreeding can limit the effectiveness of recovery efforts involving 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 1983), and experimental work has shown a strong link between the degree of inbreeding and fitness loss (Ralls 1988). A recent study (Saccheri 1998) has 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 to non-anadromous fish, especially brook and rainbow trout. Nevertheless, studies on these species have found adverse effects of close inbreeding on survival and growth (Hard and Hershberger 1995).

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

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

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

Approach

This is an ongoing research project, and three basic hypotheses are currently being tested:

- H_{01}: Inbreeding depression does not reduce viability or alter life history characteristics of chinook salmon.

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

  H_{a12}: Inbreeding depression has effects throughout the life cycle.
- H_{02}: The degree of inbreeding has no predictable effect on inbreeding depression in chinook salmon.

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

  H_{a22}: The relationship between inbreeding and inbreeding depression is nonlinear.
- H_{03}: Inbreeding depression in chinook salmon does not vary between captive (i.e., protective culture throughout life cycle) and hatchery (i.e., protective culture from embryo to smolt) environments.

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

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

In 1999-2000, work continues to address these stated hypotheses by, 1) completing collection of biological data from 5-year-old, first-generation adults returning to their site of release, and, 2) evaluating survival and growth in experimentally inbred and control captively reared progeny cultured in marine netpens. Work is ongoing in Puget Sound, Washington at Grovers Creek Hatchery, the site of the initial releases and returns of the 1994 brood fish; the Manchester Research Station (MRS), the site of captive rearing in marine net-pens, and the University of Washington's School of Fisheries Hatchery (UWSFH), the site of releases of inbred progeny. One complete

generation of experimental inbreeding will be accomplished with the maturation of adult inbred progeny in October 2003.

Work Completed

To date, this study has:

- established, using a conventional quantitative genetic breeding design, an experimental hatchery population of 1994-brood Puget Sound fall chinook salmon structured of 96 full-sib families nested within 30 half-sib families;
- determined the genetic and environmental components of variation in body morphometry of these fish at the parr-smolt stage of development (Hard et al. 1999);
- released 257 093 of these fish to sea from Grovers Creek Hatchery. Each fish is identified with full-sib family-specific coded-wire tags;
- cultured several hundred 2, 3, and 4-year-old PIT-tagged fish from the same cohort to maturity in marine net-pens at the MRS;
- established 3618, 1997-brood PIT-tagged smolts in seawater net-pens at the MRS. These fish are from a total of 28 families of 3-year-old adults in 3 experimentally inbred lines in seawater net-pens;
- established 2088, 1998-brood, PIT-tagged smolts in seawater net-pens at the MRS. These fish are from a total of 70 families of 4-year-old adults in 3 experimentally inbred lines;
- released 10 654, 1997-brood and 85 111, 1998-brood coded-wire-tagged smolts from these experimental lines to sea from the UWSFH.

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 of 12.5%), and a substantial increment in inbreeding (full-sib parents, corresponding to an approximate increase in inbreeding of 25%). Preliminary data on the effects of one generation of inbreeding, measured on stage-specific survival and growth during early life history, was summarized in a previous report covering three years of work between 1996 and 1999.

Maturation of 1994-brood parents

The current work augments the information provided by this study in two ways. First, 1999 marked the return and maturation of 5-year-old 1994-brood adults to Grovers Creek Hatchery. These are the last of the first-generation progeny of the broodstock collected in 1994 to establish this study. They represent the completion of a half-sib/full-sib family breeding design instigated with the 1994 return to the hatchery. This breeding design is commonly used in animal and plant breeding to estimate genetic parameters that describe a population's ability to respond to genetic manipulation (Falconer 1989; Lynch and Walsh 1998). The design permits estimation of genetic and environmental components of variance for a variety of phenotypic traits, as well as providing a convenient means of establishing different levels of inbreeding in experimental groups

within the population. This design is described in detail by Hard and Hershberger (1998) and Hard et al. (1999). Hard and Hershberger (1998) also summarized much of the data collected from the source parents.

In September and October 1999, adults were sampled and marked with coded-wire tags (CWTs) and released in 1995 as they returned to Grovers Creek Hatchery. From these returns marked fish were sampled using body size and the absence of an adipose fin to determine which fish were part of the inbreeding experiment. Data collected included age, sex, fork length, and round weight, as well as family-assignment information from their decoded tags. For females, total fecundity was estimated from volumetric measurements of the egg mass and collected individual samples of eggs for egg-size measurements. In addition, tissue samples were collected for later genetic analysis and took three digital photographs of each adult for morphometric measurements. Experimental fish were mated and families established in incubation units at the UWSFH, but the experiments were terminated after hatching because an insufficient number existed for a robust comparison of inbred and outbred fish.

Survival and Growth of Captively Reared Experimental Progeny

Between June 1999 and May 2000, immature chinook salmon were marked with Passive Integrated Transponder (PIT) tags and grown out in marine net-pens at the MRS. They were fed 2-4 times daily at an average feeding rate that varied from 1.5% to 3.0% mean body weight per day. The fish were sampled every 3-4 months during this period to estimate survival, length, and weight. By June 1999, two broods of fish, including one brood (1997-brood progeny of three-year-old 1994-brood parents) of experimentally inbred fish, were under culture at the MRS. Unfortunately, marine mammals (primarily seals and sea lions) had killed the few (<100) 1994-brood fish remaining at Manchester during winter 1998-1999, and river otters eliminated the 1997-brood population in February 2000.

For each PIT-tagged population, survival was calculated directly from the number of live fish at each sampling event, as all fish were counted and measured during each sampling event and sampling was with replacement. Fish length (fork length to the nearest mm) and weight (wet weight to the nearest g) were measured for each fish in the census. For both populations, lengths for all fish in a sample were measured and also weighed, but where necessary some weights were estimated from linear regression of weight on length. All PIT-tagged fish were sized during each census.

Work to be Completed

Work over the next year will focus on two groups of experimental fish:

- collection of biological data (as above, for the 1994-brood adults) from CWT 1997- and 1998-brood inbred progeny maturing in September and October 2000 (either maturing in marine net-pens at the MRS, or returning to the UWSFH; and

- culture and collection of survival and growth data from 1998-brood inbred progeny growing in marine net-pens at the MRS.

Analyses of these data will focus on survival to adulthood and life-history characteristics of fish maturing in the captively reared groups, and in the adults returning in 2000 from the hatchery releases. If sufficient fish are available, data obtained on body morphometry of approximately 20 1998-brood offspring sampled from each full-sib family in each experimental will be collected and analyzed to evaluate first-generation effects of inbreeding, if any, on juvenile body conformation. Collectively, these data will provide a comparison of first-generation inbreeding effects on early life-history traits and, through examination of the control population, an assessment of relative environmental influences in consecutive generations on these traits.

To evaluate the initial consequences of inbreeding in the 1998-brood captively reared fish, survival and growth will be compared among the three experimental groups: progeny of parents mated at random but excluding matings between siblings (control), progeny of full-sibling parents (corresponding to an average increase in the inbreeding coefficient, F, of 0.25 in one generation), and progeny of half-sibling parents (corresponding to an average increase in F of 0.125 in one generation). For each trait, variation within and among these groups will be analyzed with general linear models and analyses of variance. For each trait, the coefficient of inbreeding depression (Lande and Schemske 1985) will be computed to provide direct comparison of inbred and control groups. Where it is possible to do so from the survival data, the number of lethal equivalents per gamete will be estimated together with the cost in survival associated with one generation of full-sib mating using the methods described by Morton et al. (1956); Ralls et al. (1988); and Kalinowski and Hedrick (1998).

A summary of the comprehensive genetic and phenotypic analyses of the biological data collected from the parents (excluding the morphometric data, which will be require considerable effort to digitize and analyze) will be provided in the next report, due in June 2001. A final report on this project, when one full generation of experimental inbreeding is implemented with the maturation of adult inbred progeny, will be submitted in October 2003.

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