Research on Captive Broodstock Technology for Pacific Salmon

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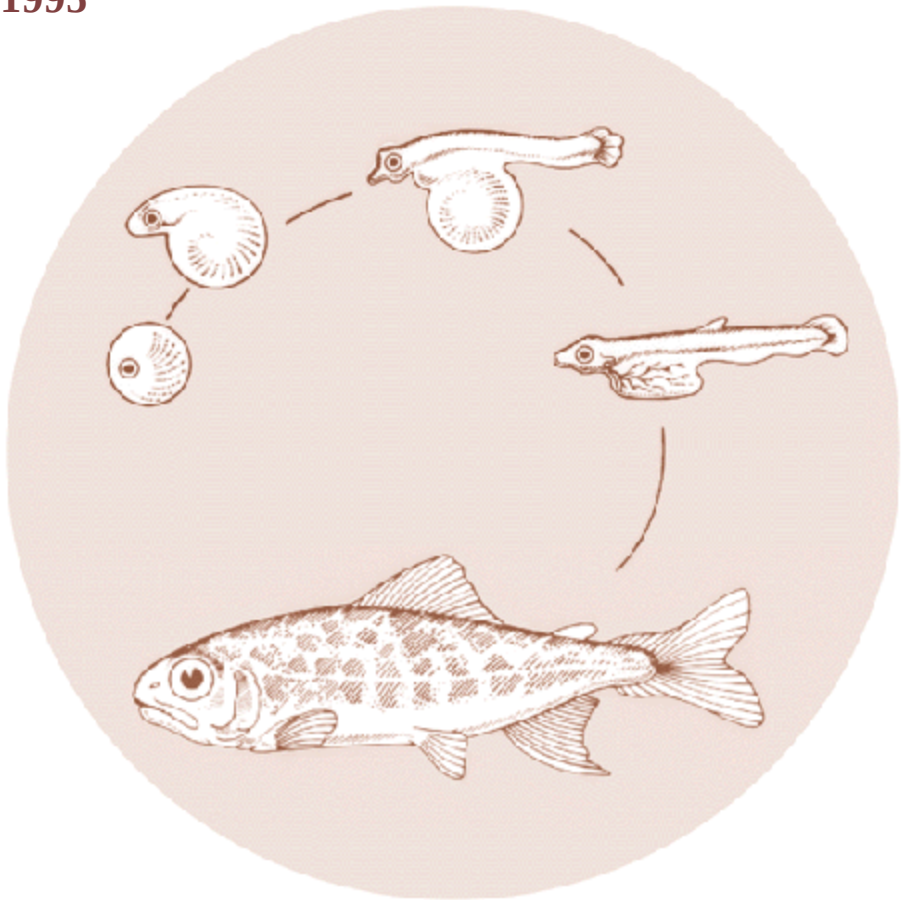

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RESEARCH ON CAPTIVE BROODSTOCK TECHNOLOGY FOR PACIFIC SALMON

ANNUAL REPORT 1995

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EXECUTIVE SUMMARY

This report summarizes research on captive broodstock technologies conducted during 1995 under Bonneville Power Administration Project 93-56. Investigations were conducted by the National Marine Fisheries Service (NMFS) in cooperation with the U.S. Fish and Wildlife Service, University of Washington, and Northwest Biological Science Center (U.S. Geological Survey). Studies encompassed several categories of research, including fish husbandry, reproductive physiology, immunology, pathology, nutrition, and genetics.

Captive broodstock programs are being developed and implemented to aid recovery of endangered Pacific salmon stocks. Like salmon hatchery programs, however, captive broodstock programs are not without problems and risks to natural salmon populations. Captive broodstock programs can sustain high mortality, which may increase a population's risk of extinction if the captive component is a substantial fraction of this population. Rearing systems must be designed and operated to minimize the risk of loss due to disease, poor reproductive performance of adult fish, and poor offspring viability. Additional risks include genetic change imposed on a population by a captive broodstock program, genetic interaction between captive and natural fish in the wild, and ecological impacts of releases of captive fish on natural populations.

The research projects described in this report were developed in part based on our literature review, *Assessment of the Status of Captive Broodstock Technology for Pacific Salmon.* This assessment elucidated the need for standard, efficient hatchery rearing practices to yield maximal numbers of high-quality offspring (i.e., those that are as similar to the founder stock as possible), as well as the need to determine the genetic consequences of captive broodstock programs for natural salmon populations.

The species for which captive broodstock programs are most critically needed are spring chinook salmon *(Oncorhynchus tshawytscha)* and sockeye salmon (*O. nerka):* these species were therefore the focus of this research. However, some of the results will be applicable to other Pacific salmon species, and we have noted instances where this is not the case.

The work was divided into three major research areas: 1) research on sockeye salmon; 2) research on spring chinook salmon; and 3) research on quantitative genetic problems associated with captive broodstock programs. Investigations of nutrition, reproductive physiology, fish husbandry, and fish health were integrated into the research on sockeye and spring chinook salmon. A description of each investigation and its major findings and conclusions is presented below. For ongoing research projects, we report the results through September 1995.

Part I: Fish Husbandry: Effects of Rearing Environment on Survival and Reproduction of Sockeye Salmon

An experiment was performed to compare growth, survival, and reproduction of sockeye salmon broodstock reared in three different environments: 1) tanks containing freshwater, 2) tanks containing filtered and UV-treated seawater, and 3) seawater net pens. For this experiment, 1990-brood Lake Wenatchee sockeye salmon were reared in either freshwater or seawater until 1 month prior to spawning, when fish reared in seawater were transferred to freshwater. During 28 months of rearing, survival averaged about 32% for replicates held in freshwater tanks, 35% for replicates in tanks supplied with pumped, filtered, and sterilized seawater; and 26% for

replicates held in conventional seawater net-pens. Survival of fish in net-pens was significantly lower than that of fish reared in tanks.

In fall 1993, about 15% of the fish reared in fresh water matured at age-3, but no fish from either the seawater tank or seawater net-pen treatments matured. In fall 1994, 79% of fish in the freshwater treatment and 70% of those reared in the seawater tank treatment matured as 4-year-old fish. In contrast, only 8% of 1990-brood fish reared in the net-pen treatment spawned at this time.

Growth differences between treatments were also noted, with average prespawning sizes of 2.26 kg in replicates from freshwater tanks, 1.57 kg in those from seawater tanks, and 1.49 kg in replicates from the seawater net-pen. There were significant differences between average weights of fish in the three treatments: fish reared in freshwater tanks were about 44% larger than fish reared in seawater tanks and 52% larger than those reared in seawater net-pens.

Average fecundity of female 1990-brood Lake Wenatchee sockeye salmon in the three rearing treatments varied as follows: 2,477 eggs/female with 49.6% eyed-egg viability for fish reared to maturity in the freshwater tanks; 1,899 eggs/female with 42.4% viability for fish from the seawater tank treatment; and 1,783 eggs/female with 45.8% viability for fish from the seawater net-pen treatment. There were significance differences in fecundities of female fish in this experiment, and as expected, fecundity was positively correlated with body size.

Current data from our captive rearing experiments suggests the following ranking priority among methods for rearing salmonid captive broodstocks to maturity: 1) circular tanks supplied with pathogen-free fresh water; 2) circular tanks supplied with pumped, filtered, and UV-sterilized seawater; and 3) seawater net-pens. Pathogen-free water (e.g., well or treated water) is limited at most fish culture facilities. However, our results were also encouraging regarding culture to maturity in processed seawater, and it appears reasonable to consider this strategy for additional rearing of some captive broodstocks.

Part II: Endocrine Changes During Maturation of Lake Wenatchee Sockeye Salmon (Brood Year 1990) Reared in Either Fresh Water or Seawater

Very little work has been done to directly compare the effects of seawater versus freshwater rearing environments on spawning success of adult Pacific salmon broodstock. We initiated this study to determine whether gametogenesis in sockeye salmon proceeds similarly in fish reared for a period in seawater compared to those reared continuously in a freshwater environment. Because both oogenesis and spermatogenesis are regulated via the reproductive endocrine system, we monitored circulating levels of pituitary gonadotropins and sex steroids as indices of the maturation process. This study was conducted on fish reared as part of the study described in Part I.

Three-year-old Lake Wenatchee sockeye salmon were reared in either of two environments: 1) 4.1 -m fiberglass tanks supplied with pathogen-free fresh, or 2) 4.1 -m fiberglass tanks supplied with filtered and UV-sterilized. There were three tanks per treatment, and fish were fed to satiation daily through mid-September 1994. At that time, fish in the filtered seawater group were transferred to tanks containing fresh water. It was not possible to maintain a constant water temperature between the two groups. In the freshwater group, water

temperature remained relatively constant at 8-9°C throughout the year. In the seawater group, water temperature ranged from 8 to 14°C.

To follow changes in gonadal development and reproductive hormones in fish reared under these conditions, we individually tagged subsamples of fish in each group. During 1994, fish were measured (weight and length) and blood was collected periodically through spawning in late September or early October. Additional samples of blood and gonads were taken for histology.

There were three major findings from this study. First, endocrine changes associated with sexual maturation in sockeye salmon were similar to what has been reported for other salmonids, and did not differ between fish reared in seawater and fresh water. Second, spermatogenesis and secondary oocyte growth were initiated prior to May in fish that matured during 1994. Third, rearing environment did not affect gametes as assessed by the survival of offspring to hatch; however, eggs from seawater-reared females were smaller.

We did not find a significant difference in endocrine changes associated with maturation in sockeye salmon reared in either fresh water or seawater. Growth appeared to differ between the two groups, particularly in females, and this resulted in differences in egg size. The results from this study suggest that sockeye salmon broodstock can be reared in either freshwater or saltwater without compromising gametogenesis in maturing fish.

Part III: Effects of Rearing Temperature on Growth, Reproductive Performance, and Immune Function in Captively Reared Sockeye Salmon

The seasonal timing of reproduction in salmonids is regulated primarily by photoperiod and secondarily by temperature. Although there is substantial information on the effects of temperature on embryonic development, the effects of rearing temperature on seasonal timing or age of sexual maturation, gamete quality, and fish health in Pacific salmon have not been rigorously tested through controlled experiments. Response to environmental temperature most likely reflects differences in adaptations to thermal regimes in the native environment, and thus may vary considerably among species and strains. Therefore, published data from rainbow trout (0. *mykiss*) and Atlantic salmon (*Salmo salar)* studies cannot be used to reliably predict the responses of other Pacific salmon to water temperature changes.

Rearing temperatures affect growth, gamete quality and fish health; however, temperatures that support rapid growth often reduce disease resistance, delay maturity and impair gamete quality. Therefore, we are investigating the effects of three rearing temperatures on sockeye salmon growth rate, development, immune function, gamete and embryo viability, and timing and age of sexual maturation.

Sockeye salmon of Lake Wenatchee stock (1994 brood) are being reared in fresh water throughout the life cycle (from fertilized eggs to mature adults) under three water temperature regimes: 1) 12 °C, 2) 8 °C, and 3) 12 °C for 8 months and 8 °C for 4 months. Fry were distributed into six tanks (2 tanks per treatment) and gradually adapted to rearing temperatures. Fish will be reared to maturity in fall 1997 or 1998. To monitor the process of smoltification, samples of blood will be collected for analysis of thyroid hormones, and gills will be collected

for gill ATPase measurements. Ultimately we will assess the effects of rearing temperature on growth, age of maturity, and gamete quality when fish mature during 1997 and 1998.

To measure the effect of rearing temperature on the immune system of sockeye salmon, a number of immunological assays are being developed by the National Biological Service at the Pacific Northwest Natural Science Center in Seattle, Washington. These assays will provide measures of nonspecific and specific immune functions of fish from each of the three temperature groups at various points during rearing.

Aspects of both the humoral and cellular immune functions will be measured in a panel of assays selected to provide a general index of the status of nonspecific defense mechanisms. Progress was made during the current reporting period in modifying and optimizing those assays to meet the requirements of this project to measure immune functions in large numbers of fish.

Unless specified otherwise, broodyear 1992 spring chinook salmon (*Oncorhynchus tshawytscha)* from Carson National Fish Hatchery in Carson, Washington, or broodyear 1993 sockeye salmon (*O. nerka*) from Lake Wenatchee were used for the experiments completed during this reporting period. All fish were raised at the Northwest Biological Science Center in sand-filtered, UV-treated Lake Washington water and fed a pelleted moist diet *ad libitum* 5 days per week.

During this reporting period, a panel of assays to assess nonspecific immune parameters of sockeye salmon was developed. Other investigators have recommended the use of assays for nonspecific host defenses to evaluate fish or the effect of immunostimulants. One of the objectives of this study is to determine if the defense systems of sockeye salmon are affected by immunomodulations related to rearing temperature.

The assays were intended to measure humoral and cellular functions from a single blood sample. However, the small number of phagocytic cells found in the blood of healthy sockeye and chinook salmon made it difficult to efficiently quantify phagocytic functions. Results from subsequent testing have suggested that it may be possible to substitute the anterior kidney as a source of cells for measuring different aspects of phagocytic cell function. The kidney cells would be used in the NBT, phagocytosis, and myeloperoxidase assays.

Numbers of phagocytic cells recovered from the kidneys of individual fish of either species were large enough to supply cells for an accurate analysis in the NBT and phagocytosis assays. A tissue imprint from the anterior kidney is also being evaluated in the myeloperoxidase assay to eliminate variability in the quality of test samples obtained from a blood or kidney smear. We will continue to use blood to measure the percent volumes of erythrocytes (hematocrit level) and leukocytes (leukocrit level).

Serum will also be separated from blood samples for complement fixation and lysozyme assays and for measurement of total or specific immunoglobulin. Previous studies have shown that the level of lysozyme varies among tissue types within a species and among fish species. We will continue to evaluate serum in the lysoplate assay rather than kidney tissue because serum can be stored frozen prior to testing and has less variability in the assay than kidney tissue.

Serum will also be used in Fish-Ig ELISAs to measure total immunoglobulin levels and changes in the levels of immunoglobulin specific to the p57 protein antigen of *R. salmoninarum.* An important consideration in developing the Fish-Ig ELISAs is the choice of microtiter plate. Variation in the adsorption of proteins to the wells of microtiter plates from different manufacturers has been reported. Our results agreed with reports that the use of the Costar 3590 provided the greatest sensitivity among seven varieties of microtiter plates in an ELISA for a soluble antigen fraction of *R. salmoninarum.*

Other researchers coated the wells with rabbit immunoglobulin, which may have different binding characteristics than the BSA-DNP conjugate we used: binding to the wells by the BSA-DNP conjugate was primarily facilitated by adsorption of the BSA moiety. Comparisons have been made of the binding of BSA and human immunoglobulin to microtiter plates from several manufacturers, and the Costar 3590 microtiter plate adsorbed both BSA and human immunoglobulin well in these comparisons. Overall, the data suggest that the protein adsorption characteristics of the Costar 3590 microtiter plate will be acceptable for coating wells with either mouse immunoglobulin or the p57 antigen.

Experiments to examine the effects of rearing temperature on immune function are scheduled to begin during 1996. During the spring and late summer of 1996 through 1998, the immunological competence of fish in each temperature group will be assessed by measuring selected factors related to specific and nonspecific host defense mechanisms. Results from these measurements will provide relative estimates of the general immune status of fish from each temperature group, as well as their ability to resist infection by *R. salmoninarum.* Testing will be done annually during these two periods to separate the effects of season from those of developmental stage.

Testing of the susceptibility of fish to infectious'disease is scheduled to begin during 1996. During the spring and late summer of 1996 through 1998, subgroups of fish from each temperature group will be tested for their susceptibility to infection by either of these two fish pathogenic microorganisms: 1) *R. salmoninarum*, the causative agent of bacterial kidney disease; or 2) infectious hematopoietic necrosis virus (IHNV), which causes infectious hematopoietic necrosis (IHN). The principal challenge method for the *R. salmoninarum* and IHNV challenges will be a waterborne exposure.

Part IV: Progress in Comparing the Efficacy of Azithromycin and Erythromycin as Therapeutic Feed Additives to Control Bacterial Kidney Disease in Chinook Salmon

One of the major causes of mortality of captive broodstock has been bacterial kidney disease (BKD). Erythromycin has been the primary antibiotic used by fish culturists in attempts to prevent and control BKD in salmonids; however, this drug has not been an effective chemotherapeutant against this disease. Azithromycin is a new macrolide antibiotic that concentrates in polymorphonuclear leukocytes, macrophages, and fibrocytes. These cellular elements have been reported to sequester and protect *Renibacterium salmoninarum (Rs),* the causative bacterium of BKD. Because of the need for alternative therapeutants for treatment of BKD in endangered or threatened salmonid fish, we initiated studies to test the effectiveness of azithromycin for reducing mortality due to BKD in either chinook or sockeye salmon.

A study was conducted to compare the effectiveness of azithromycin to erythromycin in reducing mortality due to BKD in chinook salmon. Approximately 2,000 1994-brood fall

chinook salmon were placed in rectangular holding tanks and maintained on a semi-moist salmon grower feed for 17 days. We prepared test feeds by milling two nonmedicated control feeds, comprising treatments A and B, and three medicated feeds, comprising treatments C, D, and E. We added powdered azithromycin dihydrate to treatments C and D and erythromycin phosphate to treatment E. Target concentrations in fish were based on mean weight and density of fish per tank

On 1 June 1995, fish from the 20 tanks assigned to treatments B, C, D, and E were challenged by immersion for 24 hours in tanks containing 40 liters of static water supplied with oxygen. Stock *Rs* was collected, cultured, and quantified, and the isolate, ATCC-33209 (fourth passage), was cultured in duplicate flasks prior to the challenge. Duplicate samples of the log-phase cultures were preliminarily quantified by fluorescent antibody technique (FAT) at serial dilutions (v:v) of $1\ 0^{-5}$, $1\ 0^{-6}$, and $1\ 0^{-7}$ in phosphate-buffered-saline containing peptone saline. Simultaneously, quadruplicate aliquots of stock *Rs* were inoculated onto KDM 2 plates to determine final bacteria concentrations at a later date.

Based on the FAT counts, we projected final tank concentrations to be $3.0 \times 1\ 0^{6}$ *Rs* cells/ml. Upon quantifying the KDM 2 plates after a 6-week incubation period, we determined that the stock *Rs* concentration was 3.87×10^{9} *Rs* cells/ml, resulting in final tank challenge concentrations of 2.90×10^{6} *Rs* cells/ml. We added 30 mL of stock *Rs* to 300 mL of PBS-peptone in flasks to each of the challenge tanks. The five control flasks, destined for the non-challenged control group replicates (treatment A), received 300 mL of PBS-peptone only.

The PBS-only control flasks were poured into the five control tanks before adding bacteria to challenge tanks. Control tanks were covered with plastic during the challenge, and fish were carefully observed, with oxygen provided continuously during the 24-hour, static challenge. No mortalities occurred during this time.

On 9 June 1995, fresh water was turned off and raw, unfiltered seawater was introduced to the tanks. Mortalities caused by osmoregulatory stress began occurring in all tanks 4 days later, and eventually ranged from 15.1 to 20.0% among treatments. However, these differences were not statistically significant. No disease organisms or gross pathological signs were observed in saltwater transition mortalities (BKD analyses pending).

Before this study began, we discussed the possibility of vaccinating against *Vibrio anguillarum* or treating with antibiotics in the event that a vibriosis epizootic was to occur during the study. We elected to not vaccinate or treat fish with antibiotics to prevent confounding of the independent variable being studied. After a period of 7 days with no mortalities, we detected *V. anguillarum* in wet mounts and in TSA agar cultures from mortalities examined on 29 June 1995. The diagnosis was confirmed by bacterial growth inhibition around 6.4-mm concentration disks, which were permeated with a vibrio-specific phosphate.

Unfortunately, vibriosis mortality has continued since the original episode, with cumulative mortality in all treatments currently converging at approximately 85%. Kidney tissue samples from all vibriosis mortalities not severely decayed are being prepared for BKD analyses.

This was the first investigation of azithromycin antibacterial activity in fish to our knowledge, and the duration of antibacterial protection observed from its use for a single feeding period was considerably longer than that described in any published or unpublished literature that we are aware of. The comparatively high intracellular activity of azithromycin to other antibacterial drugs, combined with our observations on the prolonged duration of antibacterial activity, are two necessary prerequisites for effective chemotherapy to control BKD.

Although mortality due to vibriosis has hindered our ability to discern efficacy of azithromycin against BKD, the prolonged (nearly 3.5 months post-medication) period of anti-vibrio activity in fish fed 50 mg/kg for 2 weeks was particularly encouraging, and strongly suggests that further investigation of this drug is warranted. Subsequent studies have been initiated in sockeye salmon. We plan to rear fish that survive the BKD exposure to maturity to determine whether the azithromycin treatment has any negative effect on gamete quality. Fish from these studies will mature during fall 1997.

Part V: Nutrition Research

Effects of Feeding a Nutritionally Enhanced Diet During the year before Spawning on Survival and Reproductive Performance of Sockeye Salmon

In our literature review, we found evidence suggesting that the natural diet of sockeye salmon differs substantially from that of coho or chinook salmon during the adult, marine phase of the life cycle of the fish. Presumably, this diet results in different intake of a number of essential nutrients, including carotenoid pigments. This evidence is supported by anecdotal reports of beneficial results with Pacific salmon when krill or squid is included in the diet fed to adults.

Although the causative dietary components have not been identified, we believe that the first phase in developing a sockeye salmon broodstock feed is to compare reproductive performance between fish fed conventional and enhanced diets. The enhanced diet will be fortified with all of the nutrients and ingredients suspected of being beneficial to the fish. If reproductive performance of the fish is increased, a second phase of work can begin to identify specific components of the enhanced diet responsible for increased reproductive performance.

In this experiment, we formulated and are testing an enhanced diet containing elevated levels of vitamins, a dietary protein and energy ratio that mimics the natural diet of the fish, and a significant proportion of unprocessed crustacea, mainly frozen krill.

Sockeye salmon of the Lake Wenatchee stock (1991 brood year) were reared in fresh water from March 1995 to the end of the experiment. All fish were implanted with PIT tags and allowed to recover from this procedure for several weeks. Fish were then transferred to the Seward Park Hatchery and placed in circular concrete ponds.

Each pond was divided into four pie-shaped sections, with water delivered to each section through a manifold system along the outside wall of each pond. Water flow was directed from the outside wall toward a center standpipe drain to prevent feed from drifting between sections.

The fish were individually weighed upon transfer, and triplicate rearing groups were assigned to the two experimental diets. Because fewer fish were available for the study than originally planned, monthly sampling was dropped from the experimental design. Thus, only initial and final sampling will be done. A disease outbreak occurred in late May, causing some mortality. The bacterial pathogen responsible for the mortality was identified as *Aeromonas hydrophilus*, and after the fish were promptly treated with Medadyne (oxytetracycline hydrochloride) by intraperitoneal injection, mortality stopped.

Frequent handling is thought to be stressful on the fish; therefore, less handling may reduce mortality over the course of the experiment. Because the central goal of this experiment is to examine the effect of diet on sexual maturation and gamete quality, it was necessary to modify the sampling schedule to increase the chance that sufficient numbers of fish would survive to the spawning stage.

At spawning in early October 1995, all fish in the tank will be identified by PIT-tag number and weighed to determine growth and survival to spawning. The first nine mature males and females removed from each dietary treatment group will be used in a spawning matrix. The remaining fish will be sacrificed and spawned. If the results of this experiment suggest a positive effect of diet on survival of adults to spawning, and on quality and hatchability of offspring, a new study will be designed to begin in fall 1995. This new study will involve fortification of the vitamin and mineral supplements used in the previous years' study.

Artemia as Food for First-feeding Sockeye Salmon *(Oncorhynchus nerka)*

The use of live prey organisms, primarily zooplankton, as food for larval fish has generally been limited to fish species which cannot be reared on prepared feeds. Brine shrimp *(Artemia sp.)* and rotifers *(Brachionus plicatilis)* are two commonly cultured prey organisms for feeding fish larvae. These prey organisms are generally fed to the fish larvae until they can be weaned on to less labor-intensive prepared feeds.

Several possible advantages exist in using zooplankton as a food source for salmonids: growth rate may be increased, fish may be better adapted to feed on live food after their release to the wild, and there may be nutritional benefits in terms of carotenoid and amino acid intake. The purpose of this study was to evaluate the use of Artemia as a live food source for sockeye salmon, with weight and length gain, and survival over a period of 8 weeks as the criteria for evaluation.

Approximately 1,000 Lake Wenatchee stock sockeye salmon fry (1994) were obtained. On 21 February 1995, fry reached the first feeding stage and were counted, weighed, and randomly distributed (50 fish/tank) into 12 12-L tanks. Each tank was supplied with 1 .O L/minute of dechlorinated city water that increased in temperature from 8 to 11°C over the experimental period (8 weeks).

A starter diet commonly fed to first-feeding salmon in enhancement hatcheries and was used as the standard in this study. All diets were fed on an equal, dry-weight basis at a feeding rate in excess of what the fish could eat. This feeding rate varied slightly due to Artemia availability (poor hatch rate on some days reduced the amount that could be fed to all tanks).

At the start of the trial and after 8 weeks, 10 fish were removed for determination of whole-body proximate and elemental composition. Feed samples from each treatment were taken weekly for proximate and elemental analysis. For fish and for diet samples, moisture, protein, crude lipid, and ash contents were measured, and mineral composition was determined. Average weight. instantaneous growth rates, and survival were determined weekly for the duration of the feeding trial.

Sockeye salmon fed adult Artemia were significantly larger than fish fed other test diets at the end of 8 weeks. Fish fed Biodiet starter were significantly higher in average weight than fish fed third-instar Artemia nauplii, but not than fish fed first instar Artemia nauplii. While significant differences were found among dietary treatments at the end of 8 weeks, fish grew well on all diets for most of the study

Compared to an earlier study in which coho salmon fry were fed Artemia-based diets, sockeye fry receiving adult Artemia did not show improvement in length and weight after 2 weeks of feeding. It was only after the sockeye, which were approximately one-half the weight of the coho fry at first feeding, reached a weight of 0.35-0.4 g that differences in average weight among dietary treatment groups were apparent. This suggests that adult Artemia, which are 10- to 15-mm in length, may be too large for first-feeding sockeye to efficiently catch and consume.

The proximate composition of fish after 8 weeks of feeding showed trends similar to those seen in the diets, with the exception of moisture. Fish fed the adult Artemia had a higher percent body protein and a lower percent body lipid than fish fed the other dietary treatments.

The results of this study show that sockeye fry thrive when fed Artemia, regardless of the stage of growth of the Artemia. First-feeding fry grew equally well when fed Artemia or Biodiet until they reached a size at which they could effectively prey on adult Artemia, approximately 0.4 g. The results are sufficiently encouraging for further trials to be conducted, and we plan both to refine the Artemia feeding strategies and to use adult Artemia as a carrier for therapeutic compounds used to treat or prevent BKD.

Effect of Dietary Fat Content and Growth Regime on Age of Sexual Maturation of Sockeye Salmon Reared in Captivity

One objective of captive broodstock programs for recovery of depleted salmon stocks is to get a high yield of viable offspring from what is usually a small number of founding stock. A major obstacle to meeting this objective is the inappropriate timing of sexual maturation. Sockeye salmon typically mature at 3, 4, or 5 years of age, but in cultured broodstock it may be possible to influence the age of maturation by modified rearing practices, mainly by alterations in feeding level and/or diet formulation. However, nothing is known about the effects of these alterations on maturation timing in sockeye salmon.

The trend in commercial salmon feeds is toward high-fat, extruded feeds, even for fingerlings, and dietary fat levels of some commercial feeds are 22% or greater, compared to 12-15% in commercial feeds a decade ago. As described in our literature review, dietary energy level is known to control body fat reserves, which in turn is one of the factors affecting maturation time. Related problems concerning captive rearing of sockeye salmon are death of fish before spawning, uncertainty about whether fish will mature after 3 or 4 years, and

asynchronous maturation of males and females. The effects of feeding rate and dietary energy levels on these issues in sockeye salmon are unknown.

Fertilized eggs from 1994-brood Lake Wenatchee sockeye salmon were reared to approximately 10 g average weight (summer 1995). In fall 1995, they will be divided into eight dietary treatment groups. Dietary treatments will be composed of two feeding levels: restricted (pair-fed) and apparent satiation, and four diets: three experimental and one commercial The three experimental diets will be formulated to contain similar protein levels but different fat levels: 10, 15, and 20%.

The commercial diet treatments will be included as controls to permit comparison of the results with those previously obtained in connection with efforts to rear Redfish Lake sockeye salmon. These fish are expected to mature and spawn in fall 1998, with signs of precocious maturation expected in summer and fall of 1996.

Effects of Supplementation of Commercial Diets with Natural Carotenoids on Reproductive Performance of Sockeye Salmon in Captive Propagation

Most pelleted rations developed for salmon are produced for carnivorous species (e.g., rainbow trout, coho, chinook, and Atlantic salmon) and may not be adequate for species such as sockeye salmon that feed primarily on carotenoid-rich invertebrates. We could find no published study that demonstrates a significant correlation between egg carotenoid content and survival of juvenile fish. However, there is general consensus among fish farmers and biologists that superior quality and viability are synonymous with high pigmentation in eggs.

One hundred 1991 -brood Lake Wenatchee sockeye salmon adults were individually PIT tagged, measured, weighed, and randomly divided into two groups of 50 fish each. Groups were placed into replicate circular tanks, and treatment groups were fed an open-formula salmon diet modified by replacing 10% of the fish-meal component of the diet with whole frozen krill or by adding synthetic astaxanthin to achieve approximately 50 ppm supplemental carotenoid in the diet. Fish were fed test diets twice per day to apparent satiation starting on 12 April 1995.

Starting in late April, low-level fish mortality was recorded in all tanks. The mortalities were inspected for pathogens, but no single pathogen was identified as the main cause of death. However, many of the mortalities showed signs of bloating, which suggested an osmoregulatory problem. Subsequently, blood samples were taken from live fish that exhibited bloating, and the samples were analyzed for calcium, magnesium, sodium, and phosphorus content. No evidence of excessive serum calcium or sodium, which would have suggested osmoregulatory failure, was found. Chronic mortality persisted throughout the study until the numbers of remaining fish were below the number needed to continue the experiment. We terminated the experiment on 23 June 1995.

Part VI: The Effect of Whole-body Lipid Stores on Early Maturation of Male Spring Chinook Salmon (*Oncorhynchus tshawytscha*)

In our review of literature, we determined that one critical problem for captive rearing of chinook salmon is loss of fish due to early sexual maturation of males. In many salmonid species, males may mature early relative to females, with the incidence varying among species, stocks, and rearing conditions for cultured fish. The chinook salmon has a high degree of plasticity in its life cycle compared to other Pacific salmon species. Early, or precocious, male maturation can occur at several stages of the life cycle, at 1 or 2 years of age. In a captive broodstock program, it is undesirable to produce mature males at a time when females of the same stock are not mature. In addition, selective mortality of precocious males could reduce the effective breeding population size (N_e) of a captive broodstock. Thus, there is a critical need to develop methods to minimize precocious male maturation in captive broodstock programs for endangered fish species.

The time of sexual maturation is controlled by genetic, abiotic (e.g., photoperiod, temperature, salinity) and biotic (e.g., diet, growth rate, energy stores) factors. The relative importance of these factors and how they interact are poorly understood. Because genetic selection should be minimized in a captive broodstock program for depleted stocks, rearing strategies that minimize expression of the trait should be developed. Research to date, primarily from Atlantic salmon, indicates that growth rate, size, and levels of stored energy at specific times of year or critical periods of the life cycle are important factors affecting the incidence of precocious maturation. It may be possible to minimize the incidence of precocious male maturation through alteration in rearing conditions, growth rates, and diet. However, before methods that minimize the rate of precocious male maturation can be developed, research is necessary to determine how stored energy levels (body fat content), growth rates, or rates of energy deposition at critical developmental stages either permit or prevent the onset of maturation in chinook salmon.

To develop a diet and growth regime that minimizes early maturation of male spring chinook salmon, we proposed two key areas of investigation. First, we proposed to manipulate body-fat levels through diet, and determine if there is a threshold of fat at a particular season or developmental stage that triggers early male maturation of chinook salmon. Second, we propose to determine if there is a window, or sensitive period, during which fish respond to increased growth rates by maturing, or conversely, if there is a period when low growth rates can prevent maturation, as has been suggested for Atlantic salmon. We will attempt to develop a diet and growth regime that sustains somatic growth and provides sufficient stored energy for appropriate life-cycle transitions, but minimizes early male maturity. During this reporting period, we completed a study on the effects of body fat levels on maturation in spring chinook salmon.

We designed an experiment to examine the relationship between the level of whole-body lipid and early maturation in 1+ spring chinook salmon *(Oncorhynchus tshawytscha).* Fertilized eggs were obtained from a wild stock of Yakima River chinook salmon and were hatched in December 1992. Fry were fed a commercial diet from February until August 1993 and then fish were divided into groups of 320 and fed one of five experimental diets containing 4, 9, 14, 18 or 22% lipid and 82, 77, 73, 69, or 65% protein for 13 months.

Fish were reared on natural photoperiod and ambient temperature, and pair-fed to a level based on the tank with the lowest feed consumption. Fish were weighed monthly and subsamples were collected to determine body composition, pituitary follicle-stimulating hormone (FSH) levels, plasma insulin-like growth factor I (IGF-I) levels, and stage of gonadal development.

Throughout the experimental period, the mean fish weight was similar among treatment groups. However, from December 1993 through the end of the experiment (September 1994), maturing males were significantly larger than nonmaturing fish. Initial lipid levels in experimental fish were near 6%, which is similar to levels observed in wild fish of the same stock captured in the Yakima River during August 1993. Fish fed diets containing more than 4% lipid increased in whole-body lipid content during the first 2 months of feeding and maintained relatively constant levels during the course of the experiment. Whole-body lipid levels for the dietary treatment groups averaged 5.6, 7.1, 8.2, 9.4, and 9.6%.

Based on histological examination of the testes of experimental fish, primary spermatocytes were first observed in maturing males during November 1993, indicating that maturation was initiated at this time. In addition, pituitary FSH levels were significantly higher in maturing than nonmaturing males at this time and throughout the study period, and levels increased as spermatogenesis proceeded.

The percentage of maturing males was significantly influenced by whole-body lipid, increasing from 34% in fish fed the 4% lipid diet to 45% in fish fed the 22% lipid diet. These data suggest that whole-body lipid levels influence the rate of maturation of male spring chinook salmon. In addition, both endocrine and histological indicators suggest that maturation was initiated almost a full year prior to the time the fish will spawn.

There were three major findings from this study. First, we were able to successfully manipulate whole-body lipid levels and control size of juvenile spring chinook salmon with a diet and feeding regime. Second, maturation of 2-year-old male chinook salmon was influenced by whole-body lipid levels during autumn, when maturation was initiated. Third, we confirmed that a critical period when maturation is initiated in male spring chinook salmon is a full year prior to spawning. Future studies will examine the interactive and independent effects of growth rate and body fat levels on age of maturation in spring chinook salmon.

VII: Induction of Ovulation and Spermiation in Sockeye Salmon using Gonadotropin-releasing Hormone Analog (GNRH-A) in Controlled-release Devices

During the final stages of sexual maturation, many adult Pacific salmon that are reared in captivity die before they can be spawned. In addition, asynchronous maturation of male and female fish may occur. In a captive broodstock program, it is imperative that methods be developed to prevent loss of gametes at these final stages of rearing. Historically, a variety of hormone treatments such as pituitary extracts, gonadotropin, or gonadotropin-releasing hormone (GnRH) have been utilized to artificially induce ovulation or spermiation, and thus prevent loss due to prespawning mortality. These hormone treatments have also been used to synchronize ovulation or spermiation in different groups of fish. This technique would be advantageous in a captive broodstock program for supplementation for the following reasons: 1) it may be necessary to synchronize spawning of captive adult fish with wild adults returning to spawning grounds to make the appropriate genetic crosses, 2) production of gametes could be maximized since hormonal induction of maturation can prevent large egg-losses because of prespawning mortality or incomplete ovulation, and 3) induction of early ovulation and spermiation may be beneficial in providing offspring with an earlier start in freshwater growth.

Although manipulation of gametogenesis (gonadal growth) has been achieved in some species through chronic treatment of fish with various preparations of gonadotropins and/or steroids, this technology is not presently used on cultured salmonids. However, hormonal

induction of final oocyte maturation, ovulation, and spermiation is widely used on salmon broodstock to prevent losses due to prespawning mortality and to advance or synchronize spawning time.

GnRH or superactive analogues of GnRH (GnRHa) are most commonly used for induction of ovulation and sperm production. This appears to be the most efficient therapy because GnRH stimulates release of endogenous gonadotropin, thus avoiding problems with species specificity and quality of the gonadotropin preparations. In addition, these decapeptides are commercially available, and GnRHa can be readily dissolved in saline and administered by intramuscular or intraperitoneal injection.

This technique has been used to successfully synchronize spawning in salmonid fish; however, there are disadvantages to this mode of administration. To be most effective, the GnRHa in saline must be given in two injections, requiring additional handling of broodstock. Because the treatment causes acute elevations in gonadotropins, it is most effective in synchronizing maturation in fish that are within 2 to 3 weeks of natural spawning time, but not very effective for a major advancement of spawning time. It is therefore advantageous to administer GnRHa in a vehicle that produces a more sustained delivery of the analog.

Technology for controlled-release GnRHa delivery systems has been developed for induction of ovulation and spermiation in salmonids. The advantages of this technique are that 1) the quantity of hormone administered and labor required for the treatment can be reduced, making the treatment more cost-effective, and 2) stress to the broodfish associated with protocols requiring multiple injections can be reduced.

We considered two types of delivery systems for GnRHa that have been developed and tested in salmonids: ethylene-vinyl acetate copolymer (EVAC) GnRHa implants and an injectable suspension of biodegradable microspheres, poly [fatty acid dimer-sebasic acid] (FADSA) . Both delivery systems have been used to induce ovulation in a variety of broodstock; however, none of these studies directly compared the two types of controlled-release devices, and the effects of the hormone on sperm production and gamete quality were not thoroughly tested. Therefore, in the present study, we compared the effectiveness of these two types of controlled-release devices containing GnRHa for induction of ovulation and spermiation in Lake Wenatchee sockeye salmon.

Adult Lake Wenatchee sockeye salmon (40 males and 40 females) were collected during their spawning migration during early August and transported to holding facilities at the Northwest Fisheries Science Center in Seattle, WA. Fish were maintained without feeding on 10°C recirculated fresh water in circular fiberglass tanks. On August 14, approximately one week after transport, fish were weighed, anesthetized, and individually tagged with passive-integrated transponder (PIT) tags.

We tested EVAC pellets containing either 25 or 75 μg of GnRHa. All fish treated with EVAC pellets received one pellet, which was injected into the dorsal muscle with an 11 -gauge needle and a modified syringe with a steel plunger. The two dosages of GnRHa-FADSA microspheres were administered at 25 and 75 μg GnRHa/kg body weight. Fish were injected intramuscularly with 0.1 mL microsphere suspension per kg body weight. The site of injection

was lateral to the dorsal fin. An 1%gauge needle and l-cc tuberculin syringe were used for the injections.

Fish were examined for maturity at 3, 7, 10, 14, 18, 21, 25, 28, 32, 34, 35, 42, 50, and 57 days after the treatment. Milt and eggs were collected and stored on ice until used for fertilization. Both spermatocrit and milt volumes were measured during the first 2 weeks of milt collection. The total weight of eggs and weight of 100 eggs from each female were recorded.

To determine if the hormone treatment had a negative effect on gamete quality, survival of embryos up to hatch was monitored. Only gametes from the highest dose of each treatment were compared to those of controls. Eggs from females (two per treatment) from the control, EVAC-75 and microsphere FADSA-75 treatments were fertilized by sperm from males (two per treatment) from the same treatments. Eggs from each female were divided into 6 lots containing 100 eggs each, and each lot was fertilized with milt from 6 males (2 per treatment). This matrix was triplicated.

Treatment of female fish with GnRHa significantly advanced and synchronized the time of ovulation in a dose-dependent manner. The average number of days to spawning was 36 in the controls, and 14 in the fish treated with EVAC-75. The 25-μg/kg body weight dose of the FADSA was less effective than either dose of EVAC or the 75-μg/kg dose of FADSA. All GnRHa treatments induced increases in plasma levels of LH in an approximately dose-dependent manner. Advancement of ovulation using the GnRHa treatments decreased average egg weight, but did not affect fecundity or the survival of embryos to hatch.

Treatment of male fish with GnRHa significantly advanced and synchronized spermiation, with the 25-μg/kg body weight dose of the FADSA being only slightly less effective than all other GnRHa treatments. The response to GnRHa was accompanied by increases in plasma levels of LH, again with the 25-μg/kg body weight dose of the FADSA being less effective. Spermatocrit generally increased in all fish from 6 to 18 days post-treatment, but with GnRHa-treated fish having higher spermatocrit than controls prior to 18 days.

The treatment of fish with GnRHa did not affect the number of ovulated eggs or survival of embryos to hatch. However, we observed a significant effect of the GnRHa treatment on egg weight at the time of spawning. Since egg weight may affect subsequent growth and survival of alevins, we were concerned about this result. Yet, we did not observe any difference among the groups in survival of the embryos to the eyed-stage or to hatch. It is possible that we would have seen a treatment effect if we had followed the development of fry to later stages, but this was not possible due to facilities limitations.

In summary, treatment of fish with GnRHa implants effectively advances and synchronizes ovulation and spermiation, without impairing gamete quality. The most effective implant appear to be GnRHa-EVAC at a dose of 25 μg/kg body weight for males and females. **It** is possible that lower doses could be used in males since the lowest dose of 25 μg was maximally effective in the present study. We recommend that this technology be applied to reduce loss of gametes due to prespawning mortality, and to advance or synchronize spawning of broodstock.

VIII: Research on Quantitative Genetic Consequences of Captive Broodstock Programs for Pacific Salmon Populations

We are focusing our research on the quantitative genetic consequences of captive broodstock programs in three areas related to three genetic risks of artificial propagation. These areas are directional genetic change, loss of genetic variability within a population, and loss of population distinctiveness.

Domestication selection, or adaptation to a protective culture environment, can produce directional genetic change. Inbreeding depression, or the reduction in fitness due to low heterozygosity or to unmasking of deleterious recessive alleles, can result from the loss of genetic variability or from nonrandom mating within a population and can further reduce this variability through the subsequent loss of genotypes from either genetic drift or selection. Outbreeding depression, which is a reduction in fitness due to loss of local adaptation or to the breakup of coadapted gene complexes, may result from interbreeding between distinct populations, and can further reduce population variability through the production of crossbred offspring.

These processes relate to three aspects of a captive broodstock program. First, domestication selection can be a direct consequence of variability in mortality and reproduction of cultured individuals that reflects differences between natural and protective culture environments. Second, inbreeding depression can result from the loss of genetic variability in a founder population or during the culture process. Third, outbreeding depression may be expressed when cultured individuals or their offspring are released to the wild to interbreed with wild individuals, if these groups are genetically distinct as a result of genetic change or loss of genetic variability.

Although we plan to conduct research on domestication selection and outbreeding depression, these studies were not scheduled to start during the current funding period. We plan to initiate experiments on domestication selection and outbreeding depression during 1997. Research and results to date from investigations on inbreeding depression is described below.

Inbreeding and Inbreeding Depression

The purposes of this study are to 1) determine the extent of inbreeding that results in a population of Puget Sound fall chinook salmon under two experimental mating schemes (full-sib and half-sib mating) for three generations; and 2) characterize the relationship between the degree of inbreeding and the inbreeding depression that occurs, if any, in several phenotypic characters that affect fitness.

Adult fall chinook salmon returning to Grovers Creek Hatchery were spawned in 1994 to establish a conventional half-sib/full-sib family breeding design. 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. It also provides a convenient means of establishing different levels of inbreeding in experimental groups within a population.

Grovers Creek Hatchery adults used to establish this experiment were taken from 992 males and 387 females returning to the hatchery and ready to spawn between 21 September and 3 1 October. The adults that were sampled from 541 males (54.5% of the returning males) and 225 females (58.1% of returning females) between 3 October and 2 1 October represented the central 56% of the run. Adults returning in 1994 ranged in age from 1 to 4 years for males and 3 to 5 years for females. Sampling of adults was made without regard to observed phenotypic characters through the use of a random numbers table.

To establish experimental groups of fish, milt from each of 30 males was mixed with unfertilized eggs from an average of 4 females, producing 30 half-sib and 120 full-sib families of offspring with 4 full-sib families per half-sib family. Each adult was identified with a unique number and photographed to provide information on variation in body morphometry and coloration. Each adult was measured and weighed before spawning, and three scales were placed on scale cards for confirmation of aging. In addition, tissues were sampled from the eye, heart, liver, and dorsal muscle for analysis of allozyme and DNA variation. For each female, total egg volume and average egg diameter and weight was estimated.

Of 120 full-sib families established at spawning, approximately 100 were large enough (i.e., produced > 1,000 fry) at the swim-up stage to maintain for experimental purposes. Because some half-sib families suffered substantially greater mortality or were already much smaller than others, several full-sib families were dropped from the experiment at the time of ponding to reduce imbalance in the experimental design. Thus the fry of only 97 full-sib and 30 half-sib families remained, with an average of 3.28 full-sib families per half-sib family.

Between 4 and 26 April 1995, 257,093 chinook salmon smolts, representing 96 full-sib and 30 half-sib families, were marked at Grovers Creek Hatchery by Northwest Indian Fisheries Commission personnel. Marking involved removing adipose fins and inserting family-specific coded-wire tags into each fish. The number of fish tagged in each of the 96 full-sib families ranged from 66 to 5,189.

Up to 410 fish from each of the 96 families were retained at the hatchery to be monitored for tag retention after 30 days. The remaining fish were allowed to outmigrate volitionally from a common pond throughout May. Fish that had not outmigrated from the pond by early June were forced to move into the outlet stream draining into Miller Bay in northwestern Puget Sound.

Of the 410 fish retained from each of the families, 150 fish from each of 84 full-sib families were transported during 18-19 May 1995 to the National Biological Service laboratory in Seattle to determine inheritance of resistance to infection by *Vibrio anguillarum.* Family variation in mortality induced by *V. anguillarum* cannot be determined until a coded-wire tag has been removed from each fish and decoded to determine its family origin. This process will require several months to complete, and we expect preliminary analyses of these data in summer 1996. The remaining 260-410 fish in each of the 96 full-sib families remained in separate family rearing tanks at Grovers Creek Hatchery until early June 1995.

From 6 to 8 June, 50 fish from each of these families were individually injected with passive integrated transponder (PIT) tags by NMFS personnel; the 4,850 fish were combined after marking into a circular concrete rearing pond at the hatchery, where they were held for

approximately 10 days before transfer to seawater net-pens. The remaining 19,720 fish (which had already been marked with family-specific coded-wire tags) were transferred into another, similar pond and held for an equivalent period.

During 19-20 June 1995, smolts were transported approximately 80 km from freshwater tanks at Grovers Creek Hatchery to seawater net-pens at the NMFS Marine Experimental Station at Manchester, Washington in southwestern Puget Sound. Fish were transferred as three groups: two groups of 9,860 fish marked solely with coded-wire tags, and one group of 4,850 fish marked with PIT tags as well as coded-wire tags.

These preliminary results do not address the primary objective of this experiment, and results for this objective will not be available until all first-generation offspring have returned from the release groups as adults or have matured in the captive groups. These fish will be available in fall 1999.

One of the three objectives of our captive broodstock genetics research is now being addressed experimentally; if sufficient funding is available, we plan to initiate the remaining experiments over the next 2 years. The past year's work has permitted us to set up a mating design that should allow a powerful determination of the nature of the relationship between inbreeding and inbreeding depression in Pacific salmon.

This design has been replicated in both captive and released groups, and will provide considerable novel information on the genetic basis of variation in several quantitative traits for these fish. These results will help both to guide management decisions on captive broodstock management and to shed light on the evolution of characters that affect fitness and performance of these animals in the wild.

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PART I

FISH HUSBANDRY

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BACKGROUND

Fish husbandry research goals for BPA Project 93-56 (Research on captive broodstocks for Pacific salmon) include monitoring individual growth, survival, and health of salmon reared to maturity in freshwater and seawater environments. Experiments rearing fish in fresh water are being conducted at the National Marine Fisheries Service (NMFS) hatchery at the University of Washington's Big Beef Creek Research Station near Seabeck, WA. Experiments rearing fish in seawater are being conducted at the NMFS Manchester Marine Experimental Station in Manchester, WA.

The research proposal for this project identified fish-husbandry research objectives for both sockeye salmon and chinook salmon. This research provides information on performance of individual fish of both species throughout the juvenile-to-adult rearing phase to determine if growth during the period of rearing influences health and reproductive performance. The following information summarizes progress in fish husbandry research to September 1995.

GROWTH, SURVIVAL, AND HEALTH OF SOCKEYE SALMON REARED IN FRESH WATER VS. SEAWATER

NMFS is currently conducting studies to compare reproductive performance and survival of Lake Wenatchee stock sockeye salmon reared with and without a period in seawater. Substantial information relating to BPA Project 93-56 has been obtained from BPA Project 92-40 through captive rearing studies of Lake Wenatchee sockeye salmon. During these studies, replicated groups of 1990- and 199 1 -brood Lake Wenatchee stock sockeye salmon were reared in the following conditions:

1) 4.1 -m fiberglass circular tanks supplied with pathogen-free fresh water;
2) 4.1 -m fiberglass circular tanks supplied with pumped, filtered, and UV-sterilized seawater; and
3) conventional seawater net-pens.

Growth, survival, and spawning information were collected for the treatments. Results were analyzed using ANOVA, student-t tests, Tukey's multiple comparison test, and regression analysis, as appropriate.

A summary of information from Project 92-40 regarding captive rearing of 1990-brood Lake Wenatchee sockeye salmon through age-4 spawning in fall 1994 is presented below (see Flagg and McAuley 1994, and Flagg et al. In prep. for complete details). Project 93-56 focuses on comparison of individual 199 1 -brood Lake Wenatchee sockeye salmon reared in freshwater tanks to those reared in tanks supplied with sterilized seawater. Results for these fish are presented through prespawning as 4-year-old fish in August 1995.

SURVIVAL, GROWTH, AND SPAWNING OF 1990-BROOD LAKE WENATCHEE SOCKEYE SALMON

Survival of 1990-brood Lake Wenatchee sockeye salmon during 28 months of rearing, from to hatch prespawning as 4-year-old fish at the end of August 1994, averaged about 32% for replicates held in circular tanks supplied with fresh (well) water; 35% for replicates in circular tanks supplied with pumped, filtered, and ultraviolet (UV) light-sterilized seawater; and 26% for replicates held in conventional seawater net-pens. There were no significant differences ($P > 0.05$) between percentages of fish remaining in the freshwater tank, seawater tank, and seawater net-pen treatments to prespawning in 1994 (Fig. 1).

In fall 1993, about 15% of the 1990-brood Lake Wenatchee sockeye salmon reared in fresh water matured at age-3. Male spawners averaged 42.7 cm and 1 .01 kg, and female spawners averaged 41.5 cm and 0.87 kg. Fecundity averaged 1,359 eggs/female, and egg viability averaged about 36% (Figs. 2 and 3). No fish from either seawater tank or seawater net-pen treatments matured in 1993.

In fall 1994, 79% of 1990-brood Lake Wenatchee sockeye salmon reared in the freshwater treatment and 70% of those reared in the seawater tank treatment matured as 4-year-old brood. In contrast, only 8% of 1990-brood fish reared in the net-pen treatment spawned in the same time frame. Growth differences were also noted between treatments. Prespawning fish size averaged 2.26 kg in replicates from the freshwater tanks, 1.57 kg in those from the seawater tanks, and 1.49 kg in replicates from the seawater net-pen (Fig. 4).

There were significant differences ($P < 0.05$) between average weights of fish in the three treatments: fish reared in freshwater tanks were about 44% larger than fish reared in seawater tanks and 52% larger than those reared in seawater net-pens. Average fish weight in the treatments was ranked as follows: freshwater tanks > seawater tanks = seawater net-pens ($P < 0.10$).

Average fecundity of female 1990-brood Lake Wenatchee sockeye salmon in the three rearing treatments varied as follows: 2,477 eggs/female with 49.6% eyed-egg viability for fish reared to maturity in the freshwater tanks; 1,899 eggs/female with 42.4% viability for fish from the seawater tank treatment; and 1,783 eggs/female with 45.8% viability for fish from the seawater net-pen treatment (Figs. 2 and 3). There were significance differences ($P < 0.01$) in fecundities of female spawners in the experiment, with the treatments ranked as follows: freshwater tanks > seawater tanks = seawater net-pens ($P < 0.02$). As expected, fecundity was positively correlated with spawner size ($P < 0.01$). There were no significant differences ($P > 0.10$) in eyed-egg survival (viability) of female spawners from the three treatments.

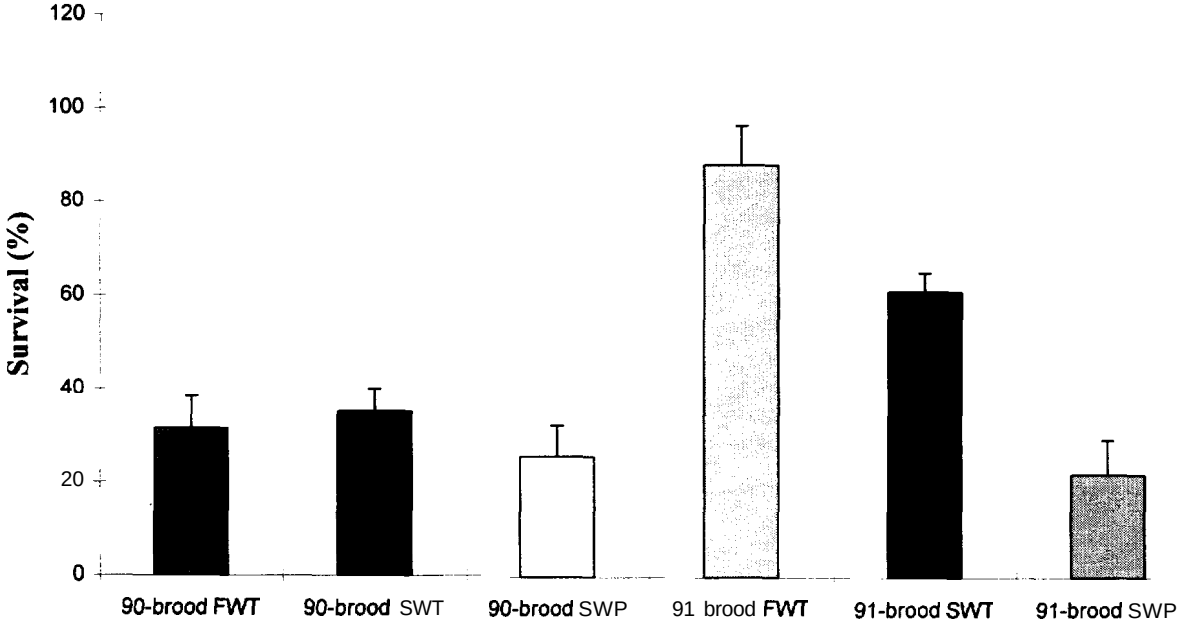

Figure 1. Comparison of prespawning survival for 1990- and 1991 -brood Lake Wenatchee sockeye salmon reared in freshwater tanks (FWT), seawater tanks (S WT), or seawater net-pens (SWP). Bars indicate standard deviation.

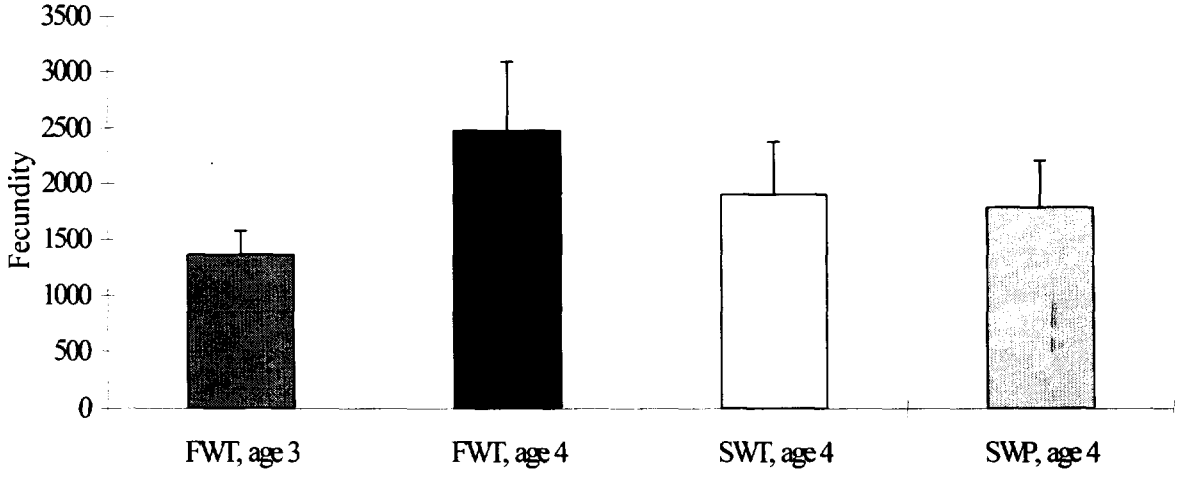

Figure 2. Comparison of fecundity of 1990-brood Lake Wenatchee female sockeye salmon reared in freshwater tanks (FWT), seawater tanks (SWT), or seawater net-pens (SWP). Age-3 fish spawned in fall 1993, age-4 fish spawned in fall 1994. Bars indicate standard deviation.

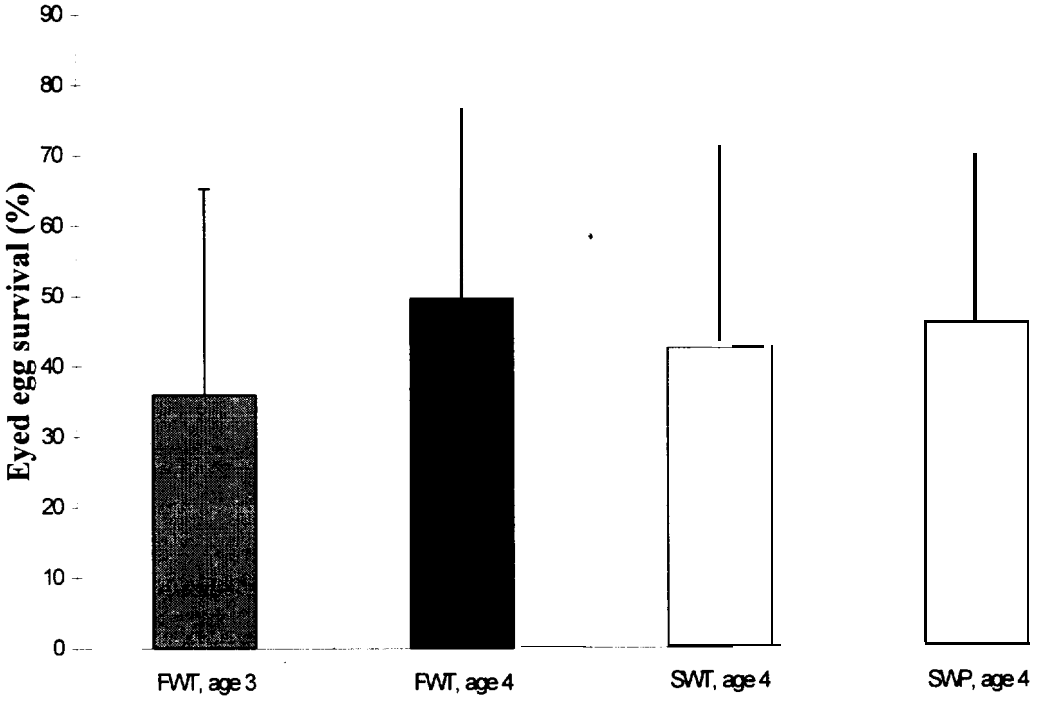

Figure 3. Comparison of egg viability of 1990-brood Lake Wenatchee sockeye salmon reared in freshwater tanks (FWT), seawater tanks (SWT), or seawater net-pens (SWP). Age-3 fish spawned in fall 1993, age-4 fish spawned in fall 1994. Bars indicate standard deviation.

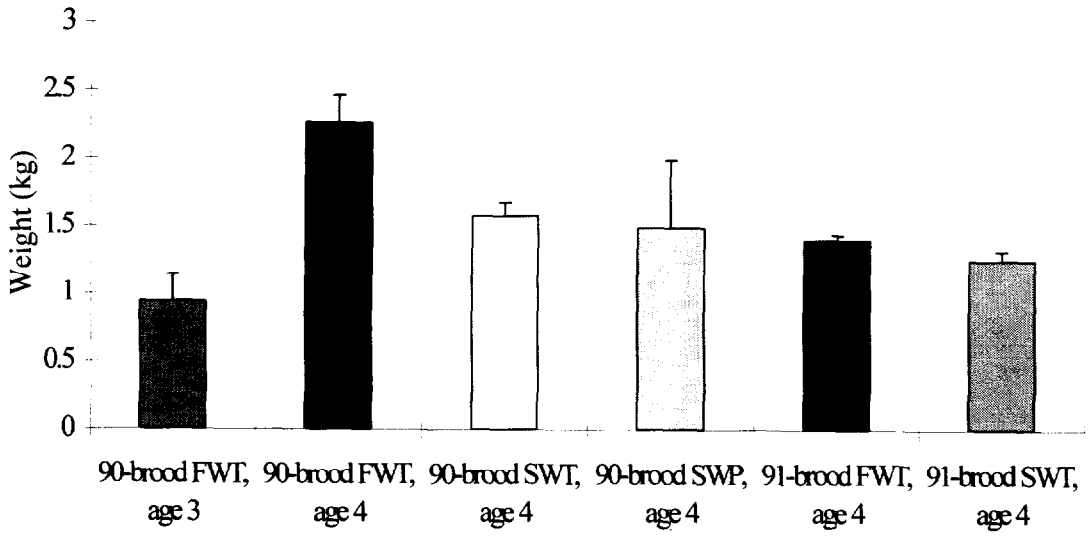

Figure 4. Comparison of prespawning weight of 1990- and 199 1 -brood Lake Wenatchee sockeye salmon reared in freshwater tanks (FWT), seawater tanks (SWT), or seawater net-pens (SWP). Bars indicate standard deviation.

The 36-50% eyed-egg survival rate documented by Flagg and McAuley (1994) and Flagg et al. (In prep.) is much lower than the 70 to 90% usually seen from sockeye salmon adults sourced from the wild (Mullan 1986, Flagg et al. 1991). However, this rate is similar to the 30-60% eyed-egg survival documented for NMFS and IDFG Redfish Lake sockeye salmon captive broodstock spawned in 1993 and 1994 (Flagg and McAuley 1994; Flagg et al. 1995; Flagg et al. In prep.; K. Johnson, IDFG, 1800 Trout Road, Eagle, ID. 83616. Pers. commun., December 1993, December 1994).

We are unsure of causes of these low egg viability rates from captive-reared fish. However, spawning techniques have been ruled out. Obviously, low fertilization rates will hamper recovery efforts using captive broodstocks. Several studies are underway under Project 93-56 that should aid in increasing spawning success of captively reared and spawned fish. These studies include development of species-specific broodstock diets (sockeye salmon are planktivorous whereas commercial brood diets are formulated for picivorous fish), refinement of husbandry technology, and implementation of environmental and hormonal manipulation of reproduction.

To determine timing and onset of maturation, 1990-brood Lake Wenatchee sockeye salmon in these experiments were periodically sampled for plasma hormone levels (sex steroids and pituitary gonadotropins) during about the last 6 months prior to maturation in fall 1994. Results of this research are presented in Part 2 of this report, *Endocrine Changes During Maturation of Sockeye Salmon Reared in Freshwater or Saltwater.*

SURVIVAL AND GROWTH TO PRESPAWNING FOR 1991-BROOD LAKE WENATCHEE SOCKEYE SALMON

Survival of 1991 -brood Lake Wenatchee sockeye salmon during 28 months of rearing under Project 92-40 (to prespawning as 4-year-old fish at the end of August 1995) averaged about 88% for replicates held in circular tanks supplied with fresh (well) water, 61% for replicates in circular tanks supplied with pumped, filtered, and ultraviolet (UV) light-sterilized seawater, and 22% for replicates held in conventional seawater net-pens (Fig. 1). There were significant differences ($P < 0.01$) between percentages of fish remaining in the experiment, with the treatments ranked as follows: freshwater tanks > seawater tanks > seawater net-pens treatments ($P < 0.05$).

In February 1995, three freshwater tank replicates (of approximately 150 to 160 fish each) of Lake Wenatchee sockeye salmon from BPA Project 92-40 were PIT tagged for BPA Project 93-56 and placed into 4.1-m fiberglass circular tanks supplied with pathogen-free fresh water. Additionally, two seawater tank replicates (of 110 to 115 fish each) of Lake Wenatchee sockeye salmon from BPA Project 92-40 were PIT tagged for BPA Project 93-56 and placed in 4.1 -m fiberglass circular tanks supplied with pumped, filtered, and UV-sterilized seawater. At tagging, the freshwater tank replicates averaged 0.79 kg and the seawater tank replicates averaged 0.69 kg. Survival was above 95% for both treatments during the 7+ months of rearing from February to the end of August 1995. At the end of August 1995, prespawning fish from the freshwater tank treatment averaged 1.41 kg and were significantly larger than fish from the seawater tank treatment, which averaged only 1.25 kg ($P < 0.05$) (Fig. 4).

The majority of fish in both freshwater and seawater tank rearing treatments will mature in fall 1995. Individual fish from both treatments have been sampled for growth parameters (length, weight, condition, etc.) on a monthly basis. Mortalities have been necropsied to determine cause of death. After spawning, information on individual fish will be examined to determine if traits such as pattern of growth rate influence reproductive performance.

Fish in these experiments are being sampled for plasma hormone levels (sex steroids and pituitary gonadotropins) to determine timing and onset of maturation. Results of this research are presented in Part 2 of this report, *Endocrine Changes During Maturation of Sockeye Salmon Reared in Freshwater or Saltwater.*

GROWTH, SURVIVAL, AND HEALTH OF SPRING CHINOOK SALMON REARED IN FRESHWATER VS. SEAWATER

In our literature review, we determined that the culture of chinook salmon to maturity has often ended in failure due to mortality from disease (e.g., bacterial kidney disease) and poor gamete quality (Flagg et al. 1995). Husbandry technology for chinook salmon captive broodstocks must be refined to the point where causes of mortality can be identified and corrected and reproductive performance maximized.

In 1994, we proposed a study of performance of individual fish throughout the juvenile-to-adult rearing phase to determine if growth traits influence health and reproductive performance. This experiment was intended to document individual growth and survival during the important smolt-to-adult period for chinook salmon. Expected results included correlation of individual growth parameters and fish survival and health. In addition, examination of individual fish growth rates would have provided information on the rate and timing of maturation. Unfortunately the group of spring chinook salmon targeted for research in the initial proposal (Objective 2.3. Task 1) was lost due to bacterial kidney disease (BKD) and precocious maturation in fall 1994.

A second stock of spring chinook was identified in 1995 for this research. In early June 1995, three replicates of about 250 PIT-tagged fish each were established in these treatments:

1) circular tanks supplied with pathogen-free freshwater;
2) circular tanks supplied with pumped, filtered, and UV-sterilized seawater; and
3) conventional seawater net-pens.

Survival during 3 months of rearing, from smolts at the beginning of the experiment through early September 1995, averaged 87% in the freshwater tank treatment, 46% in the seawater tank treatment, and 42% in the seawater net-pen treatment (Fig. 5). There were significant differences ($P < 0.01$) between percentages of fish remaining in the experiment, with the treatments ranked as follows: freshwater tanks > seawater tanks = seawater net-pens ($P < 0.01$).

All surviving fish in the experiment will be individually measured on a quarterly basis (beginning mid-September 1995). Mortalities are being necropsied for cause of death; however, cause of death for most fish appears related to BKD. Fish in all the treatments are expected to mature as 4- and 5-year-old fish in 1997 and 1998.

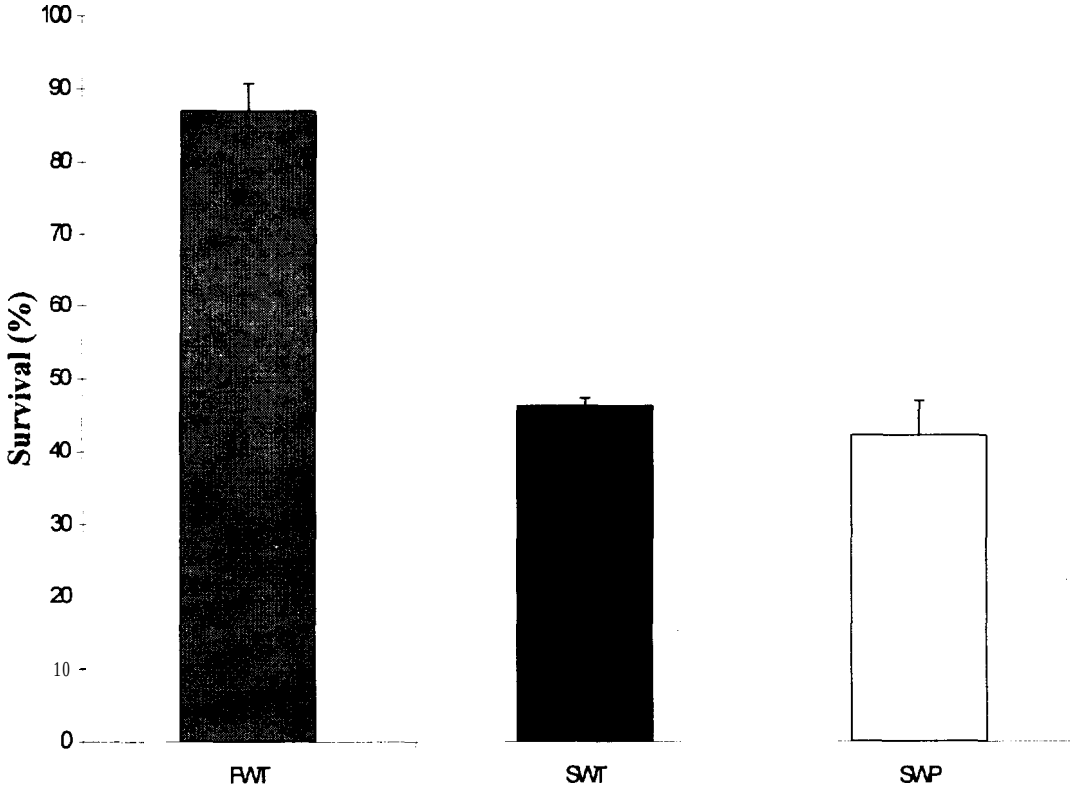

Figure 5. Comparison of survival for 1993-brood spring chinook salmon reared for 3 months post smolting in freshwater tanks (FWT), seawater tanks (SWT), or seawater net-pens (SWP). Bars indicate standard deviation.

RECOMMENDATIONS

1) Current data from our captive rearing experiments suggests the following ranking priority among methods for rearing salmonid captive broodstocks to maturity: circular tanks supplied with pathogen-free fresh water; circular tanks supplied with pumped, filtered, and UV-sterilized seawater; and 3) seawater net-pens. Pathogen-free water (e.g., well or treated water) is limited at most fish culture facilities. However, the data is also encouraging regarding culture to maturity in processed seawater, and it appears reasonable to consider this strategy for additional rearing of some captive broodstocks.

2) In many situations, eyed-egg viability of captive broodstocks has been shown to be 50% or less than that of wild-reared fish. Low egg viability from captively reared fish will hamper recovery efforts using captive broodstocks. Captive broodstock egg viability must be increased to be comparable to egg viability of wild fish. We recommend that research priorities be directed at development of husbandry and nutritional and physiological guidelines to increase reproductive success of captive broodstocks.

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PART II

ENDOCRINE CHANGES DURING MATURATION OF LAKE WENATCHEE SOCKEYE SALMON (BROOD YEAR 1990) REARED IN EITHER FRESH WATER OR SEAWATER

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INTRODUCTION

It is well established that Pacific salmon (*Oncorhynchus* sp.) can be reared to maturity in fresh water even though most species are anadromous (Smirnov et al. 1968, Stauffer 1976, Peterschmidt 1991). Very little work has been done to directly compare the effects of seawater versus freshwater rearing environments on spawning success of adult Pacific salmon broodstock. The most thorough study was done by Peterschmidt (199 1), who compared freshwater and seawater rearing environments on spawning success of coho salmon broodstock.

No significant effect of rearing environment on growth of adult fish or survival of embryos from fertilization to hatch was found, although there was higher mortality of fish reared in seawater net-pens. Peterschmidt (199 1) also found that development of ova in female broodstock reared in freshwater lagged slightly behind those in seawater, and females reared in freshwater spawned later than those reared in seawater. However, this developmental difference did not appear to have any negative effect on egg quality.

In captive broodstock programs for recovery of endangered or threatened stocks of Pacific salmon, both freshwater and seawater rearing environments are being employed; however, the freshwater rearing strategy is used preferentially because of higher smolt-to-adult survival (Flagg et al. 1994). To be assured that reproductive performance of broodstock is not compromised as a result of the freshwater rearing, we have conducted two separate studies (1990- and 199 1 - brood fish) comparing growth, survival and maturation of Lake Wenatchee sockeye salmon reared either in a continuous freshwater environment or in seawater from smoltification as 1+ fish to just prior to final maturation.

To monitor the timing and progress of maturation in fish reared in the two environments, we have measured a variety of reproductive hormones, including gonadotropins, and sex steroids and analyzed gonadal stage by histology. In this report, data for one study of 1990-brood Lake Wenatchee sockeye salmon are described.

MATERIALS AND METHODS

Fish and Sampling

Three-year-old Lake Wenatchee sockeye salmon (1990 brood) were reared as described by Flagg et al. (this report). Fish were reared in either of two environments: 1) 4.1 -m fiberglass tanks supplied with pathogen-free fresh water at the University of Washington Big Beef Creek Field Station, or 2) 4.1 -m fiberglass tanks supplied with filtered and UV-sterilized seawater pumped from Clam Bay at the NMFS Manchester Marine Experimental Station, Manchester, WA. There were three tanks per treatment. Fish were fed to satiation daily (l-l .25% body weight/day) Biodiet brood formulation (Bioproducts) through mid-September 1994. At that time, fish in the filtered seawater group were transferred to tanks containing fresh water at the Big Beef Creek Field Station. It was not possible to maintain a constant water temperature between the two groups. In the freshwater group, water temperature remained relatively constant at 8-9°C throughout the year. In the seawater group, water temperature ranged from 8-14°C.

To follow changes in gonadal development and reproductive hormones in fish reared under these conditions, we individually tagged some fish in each group (10 fish per tank) using PIT tags and external floy tags. During 1994, fish were measured (body weight and length) and blood was collected on May 12, July 2 1, September 1 (freshwater group) or 2 (seawater group), and at spawning on September 30 and October 4, 7, or 8. Nonmaturing fish in each group were also sampled on October 19, 1994. In addition, 10 fish per treatment were sacrificed during the May, July, and September samplings to collect samples of blood and gonads for histology. After anesthesia in tricaine methane sulfonate (MS-222, 0.02%) blood was collected from the caudal vein using heparinized syringes and 2 1 -gauge needles, and plasma was stored frozen at -80°C.

Spawning and Embryo Incubation

Fish were examined for maturity every 3-5 days from September 25 to October 19, 1994. Milt was collected into sterile plastic bags, oxygenated, and stored on an insulated layer of ice until used for fertilization. Eggs were collected from ovulated females into sterile bags and stored on an insulated layer of ice until fertilized. The total weight of eggs and weight of 100 eggs from each female were recorded. Survival of the embryos up to hatch was monitored. Eggs from eight females were fertilized by sperm from eight males from the same treatments. Eggs from each female were divided into 8 lots containing 100 eggs each, and each lot was fertilized with 0.1 mL milt from one of 8 males.

Eggs were incubated in individual cups within Heath trays with flow-through water at 8-9°C at the Big Beef Creek Field Station. Twenty-four hours after fertilization, damaged or unfertilized eggs were counted and removed. At the eyed stage, eggs were shocked and any dead eggs were counted and removed. Finally, embryos surviving to hatch were counted.

Hormone Analysis

Plasma levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were analyzed by radioimmunoassay (RIA) (Swanson et al. 1989). FSH and LH were previously called GTH I and GTH II, respectively. Plasma levels of the following steroid hormones were analyzed by RIA: estradiol- 17β (E) (Sower and Schreck 1982), 11 -ketotestosterone (11 -KT) (Schulz 1984) and 17α,20β-dihydroxy-4-pregnen-3-one (17,20-P) (Scott et al. 1982).

Gonad Histology

Small pieces of gonads were removed and fixed in Bouinis fixative for 24 hours, then stored in 70% ethanol until processed for histology. Tissue was dehydrated through a series of increasing concentrations of ethanol, imbedded in Paraplast, sectioned (6 μm) and stained with hematoxylin and eosin. Stages of spermatogenesis were determined by light microscopic observation according to Schulz (1984) and briefly described in Table 1. Staging of ovarian development was done according to Nagahama (1983) and is briefly described in Table 2.

Table 1. Criteria for stage of spermatogenesis determined according to Schulz (1984).

Stage	Cell Types Observed
I	spermatogonia and Sertoli cells as intratubular types
II	spermatogonia, primary spermatocytes
III	spermatogonia, primary and secondary spermatocytes, spermatids
IV	spermatogonia, primary and secondary spermatocytes, spermatids, spermatozoa; many spermatogenic cysts; no spermiation
V	no or few spermatogenic cysts; tubuli tilled with spermatozoa
Va	no spermatogenic cysts; tubuli filled with spermatozoa; spermiation

Table 2. Developmental stages of oogenesis according to Nagahama (1983).

Stage
oogonium stage
chromatin-nucleolus stage
early perinucleolus stage
late perinucleolus
yolk vesicle stage
oil drop stage
primary, secondary and tertiary yolk globule stages
migratory nucleus stage

Statistics

Statistical analyses were performed using either SuperANOVA or Statview TM (Abacus Concepts, Berkeley, CA, 1992). Multiple mean comparisons were made using the Fisher PLSD test. Data were analyzed by one- or two-way ANOVA using a significance level of $P < 0.05$.

RESULTS

By October, female fish reared in fresh water were larger (Figs. 1 and 2) than those reared in filtered seawater (Figs. 3 and 4), but males in the two groups did not differ significantly in size. The females reared in freshwater increased in size throughout the rearing period, while those in seawater maintained similar body weight and length from May through October. Fish that did not mature were smaller than maturing fish (Figs. 1 and 3) and either grew poorly (Fig. 1) or lost weight (Fig. 3).

At the time of spawning, females reared in fresh water were larger and produced more total eggs than fish reared in seawater (Fig. 5). However, females reared in seawater produced more eggs per unit body weight (Fig. 5). Ovulated and unfertilized eggs produced by females reared in fresh water were significantly larger in wet weight than those produced by females reared in seawater (Fig. 6). Although the eggs differed in size, the percent of offspring surviving to hatch did not differ between the two rearing regimes (Fig. 7): survival was near 50% for both groups.

The relative pattern of changes in sex steroids and gonadotropins in female (Figs. 8 and 9) and male (Figs. 10 and 11) were similar irrespective of rearing environment. In females, E and FSH were elevated during vitellogenesis and declined at spawning. In males, 1 l-KT increased throughout the sampling period, reaching highest levels at the time of spawning. FSH levels in males were elevated from May through September and declined slightly during October (Figs. 10 and 11). LH and 17,20-P levels in females were below the detection limits of the assays until the time of spawning when both increased (Figs. 8 and 9). In the freshwater group, LH and 17,20-P levels increased in parallel from September to October (Fig. 10).

In contrast, LH levels in males increased in September in the seawater group, and this was followed by increases in 17,20-P levels in October (Fig. 11). Immature males (Fig. 12) and females (Fig. 13) had significantly lower levels of both FSH and sex steroids than maturing fish of the same sex. Although FSH levels in immature fish were relatively static during the year, both 11 -KT (Fig. 12) and E (Fig. 13) increased significantly from June to July in immature male and female fish, respectively.

Changes in hormone levels during the study period were accompanied by developmental changes in the gonads. Because of the low number of samples it was not possible to analyze this data extensively. We assumed that the samples from maturing fish were representative of the populations in all replicate tanks within the treatment.

Maturing males (Table 3) from the two treatment groups were in similar stages of gametogenesis at each sampling time. In both groups, immature males remained in stage I of spermatogenesis throughout the study period. In maturing males of both treatments, spermatogenesis had progressed to stages II and III by the first sampling. By early September, the testes were filled with spermatozoa, but no fish were producing milt. Because fish were not checked frequently enough it was not possible to determine when spermiation first occurred; however, by the end of September some males in each group were producing low volumes of milt.

Oogenesis in both seawater and freshwater-reared groups (Table 4) appeared to proceed similarly. Secondary oocyte growth was initiated before May, when the early yolk globule stage was observed in both groups. From July to September, when plasma E levels increased to peak levels (Figs. 8 and 9), the oocytes progressed from the primary and secondary yolk globule stage to the tertiary yolk globule stage. Oocytes from females that did not mature remained in the oil drop stage.

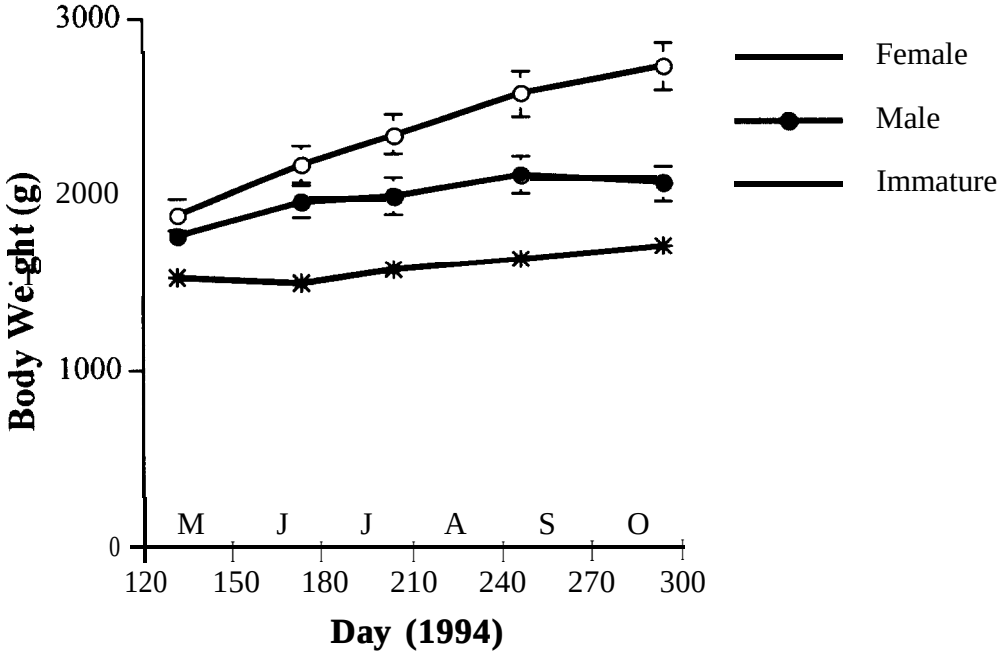

Figure 1. Body weight of tagged Lake Wenatchee sockeye salmon (1990 brood) reared in tanks containing fresh water. Data are mean +/- standard errors, n = 2-5 (immature), n = IO-22 (females), n = 10-21 (males).

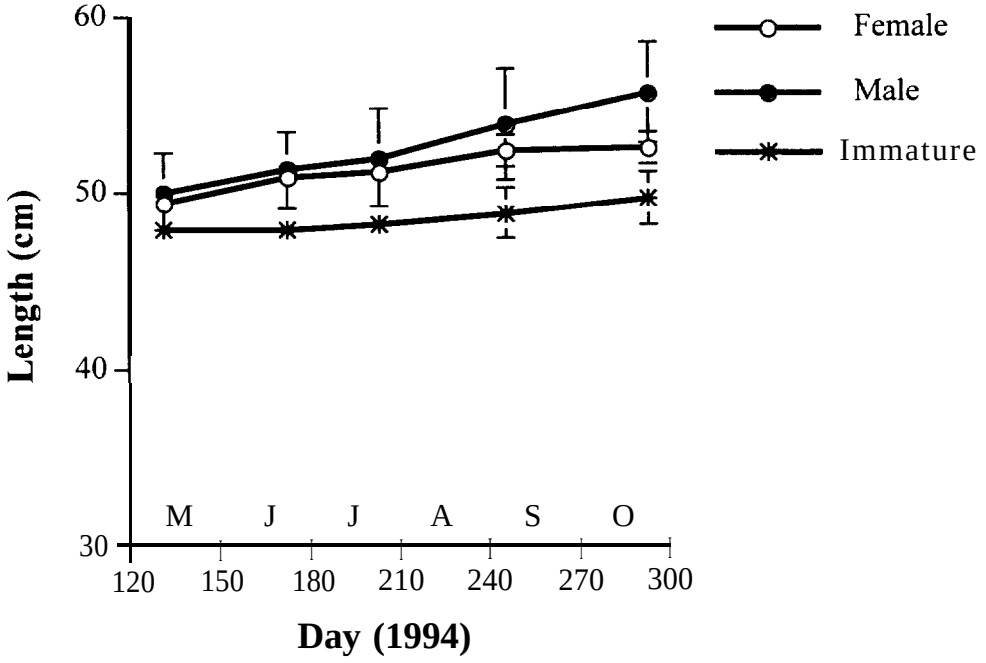

Figure 2. Body length of tagged Lake Wenatchee sockeye salmon (1990 brood) reared in tanks containing fresh water. Data are mean +/- standard errors, n = 2-5 (immature), n = 10-22 (females), n = 10-2 1 (males).

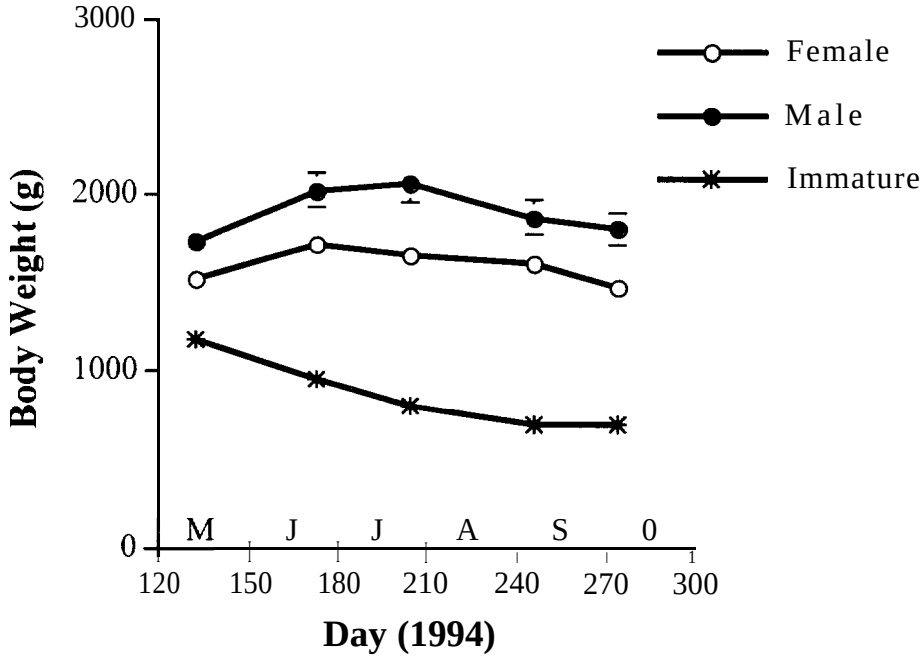

Figure 3. Body weight of tagged Lake Wenatchee sockeye salmon (1990 brood) reared in tanks containing filtered seawater until September 10, when fish were transferred to tanks containing fresh water. Data are mean +/- standard errors. n = 2 (immature), n = 9-l 6 (females), n = 5-l 8 (males).

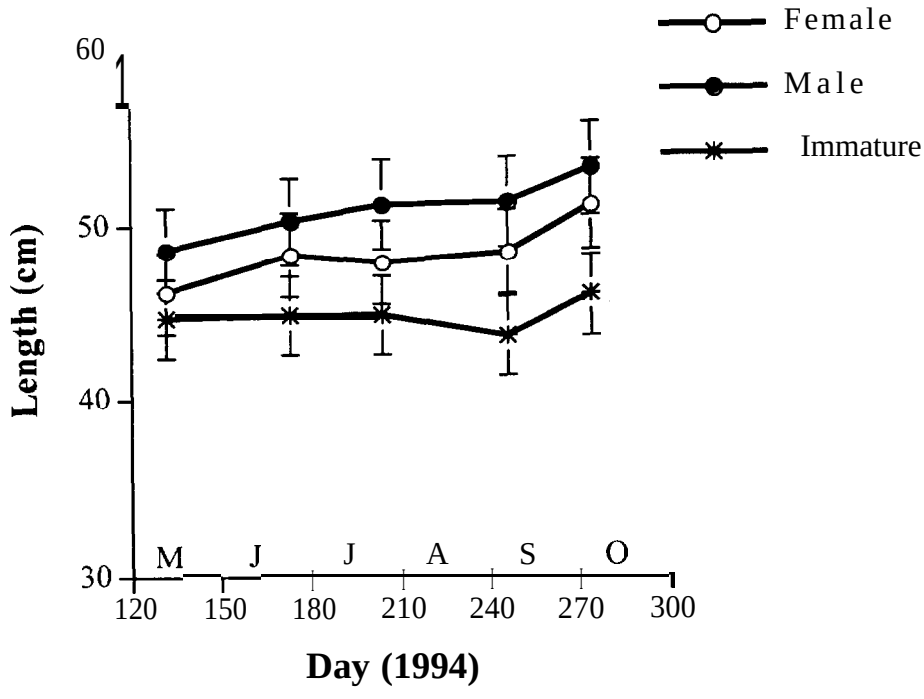

Figure 4. Body length of tagged Lake Wenatchee sockeye salmon (1990 brood) reared in tanks containing filtered seawater until September 10, when fish were transferred to tanks containing fresh water. Data are mean +/- standard errors, n = 2 (immature), n = 9-16 (females), n = 5-l 8 (males).

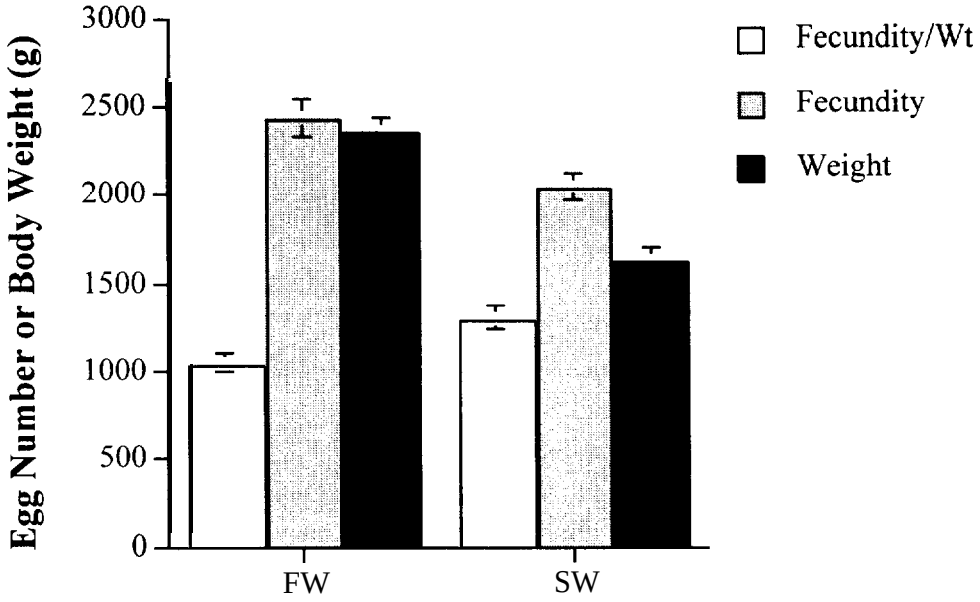

Figure 5. Fecundity, body weight, and fecundity/body weight of Lake Wenatchee sockeye salmon (1990 brood) reared in tanks containing either seawater (SW) or fresh water (FW). Data are mean +/- standard errors, n = 8 per treatment.

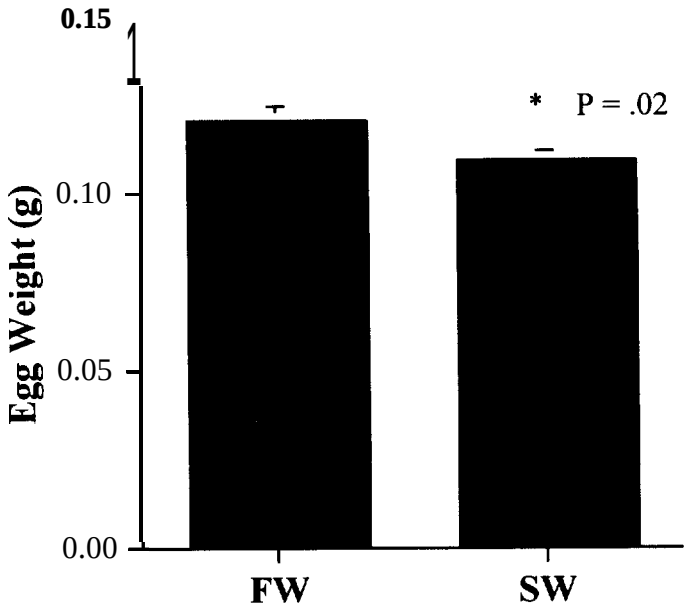

Figure 6. Average egg weights (unfertilized) from Lake Wenatchee sockeye salmon (1990 brood) reared in tanks containing either seawater (SW) or fresh water (FW). Data are mean +/- standard errors, n = 8 per treatment.

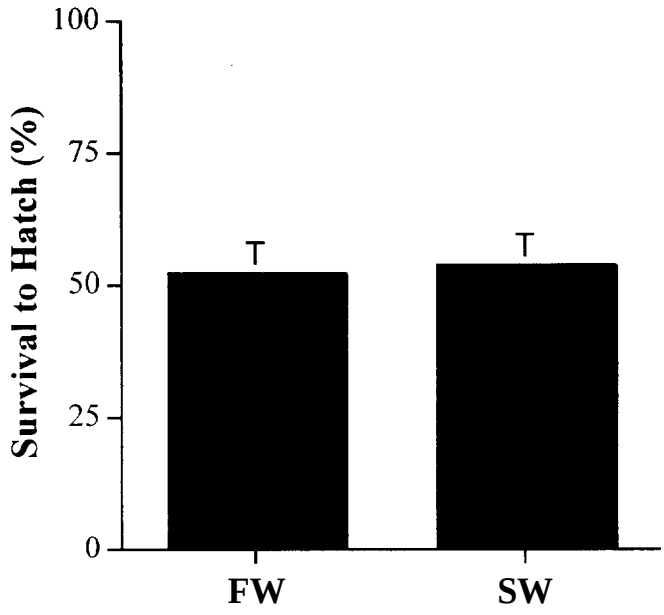

Figure 7. Survival of offspring from Lake Wenatchee sockeye salmon (1990 brood) reared in tanks containing either seawater (SW) or fresh water (FW). Data are mean +/- standard errors, n = 8 per treatment.

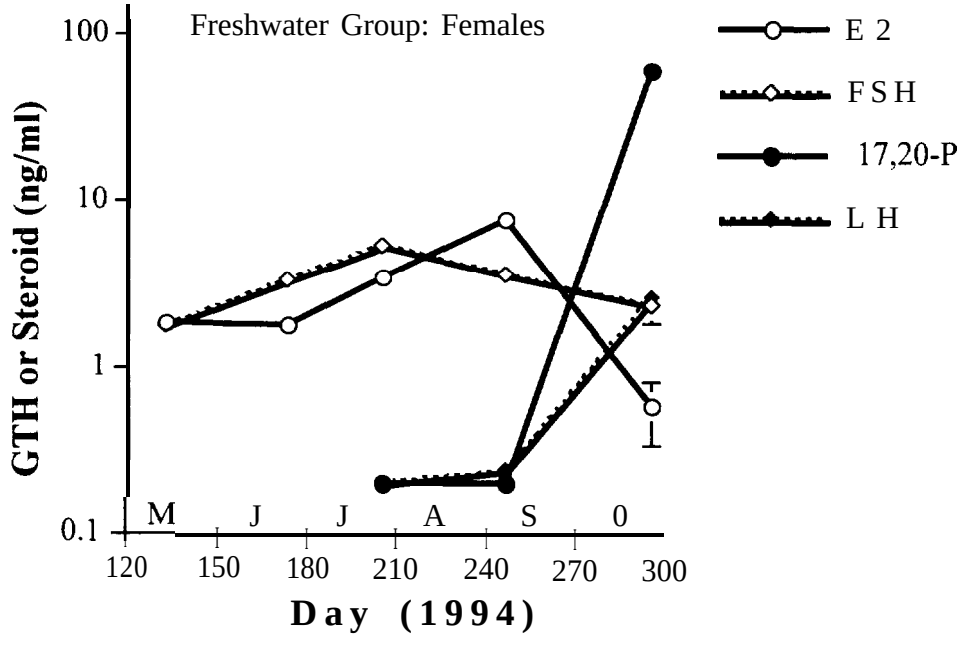

Figure 8. Plasma levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol- 17β (E), and 17α,20β-dihydroxy-4-pregnen-3-one (17,20-P) in female Lake Wenatchee sockeye salmon (1990 brood) reared in tanks containing fresh water. Data are mean +/- standard errors; n = 10-22.

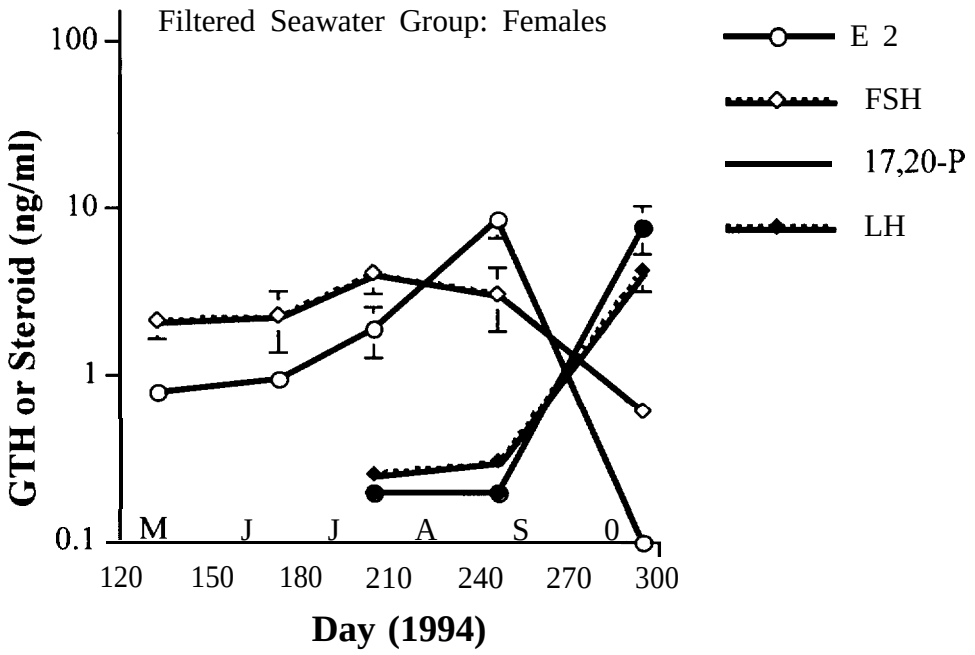

Figure 9. Plasma levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol-17β (E), and 17α,20β-dihydroxy-4-pregnen-3-one (17,20-P) in female Lake Wenatchee sockeye salmon (1990 brood) reared in tanks containing filtered seawater until September 10 when fish were transferred to tanks containing fresh water. Data are mean +/- standard errors; n = 9- 16.

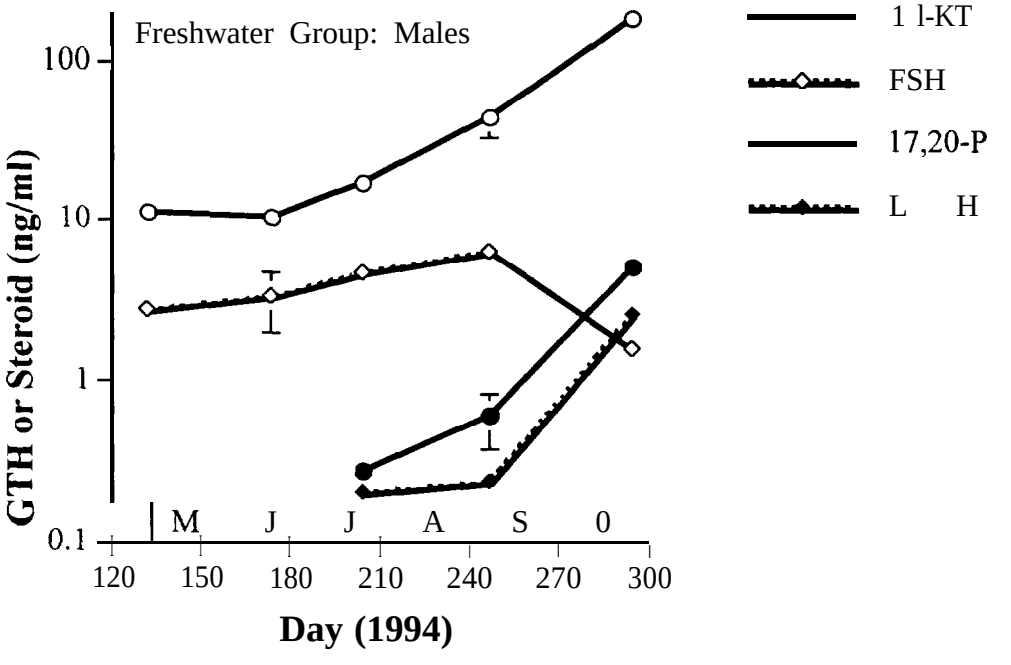

Figure 10. Plasma levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), 11 -ketotestosterone (11 -KT), and 17α,20β-dihydroxy-4-pregnen-3-one (17,20-P) in male Lake Wenatchee sockeye salmon (1990 brood) reared in tanks containing fresh water. Data are mean +/- standard errors; n = 1 O-2 1.

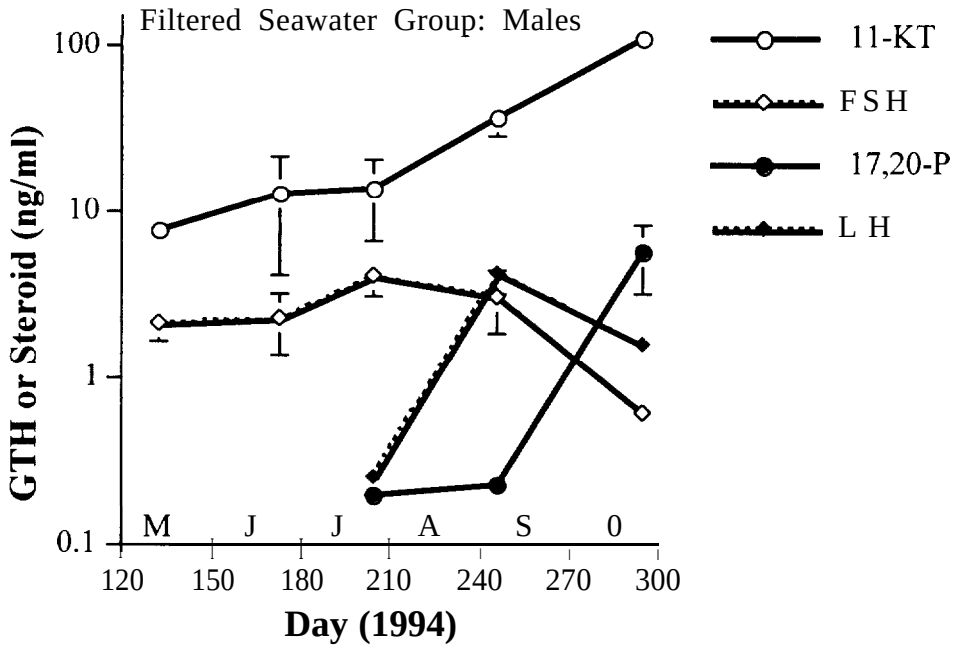

Figure 11. Plasma levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), 11 -ketotestosterone (11 -KT), and 17α,20β-dihydroxy-4-pregnen-3-one (17,20-P) in male Lake Wenatchee sockeye salmon (1990 brood) reared in tanks containing filtered seawater until September 10, when fish were transferred to tanks containing fresh water. Data are mean +/- standard errors; n = 5-18.

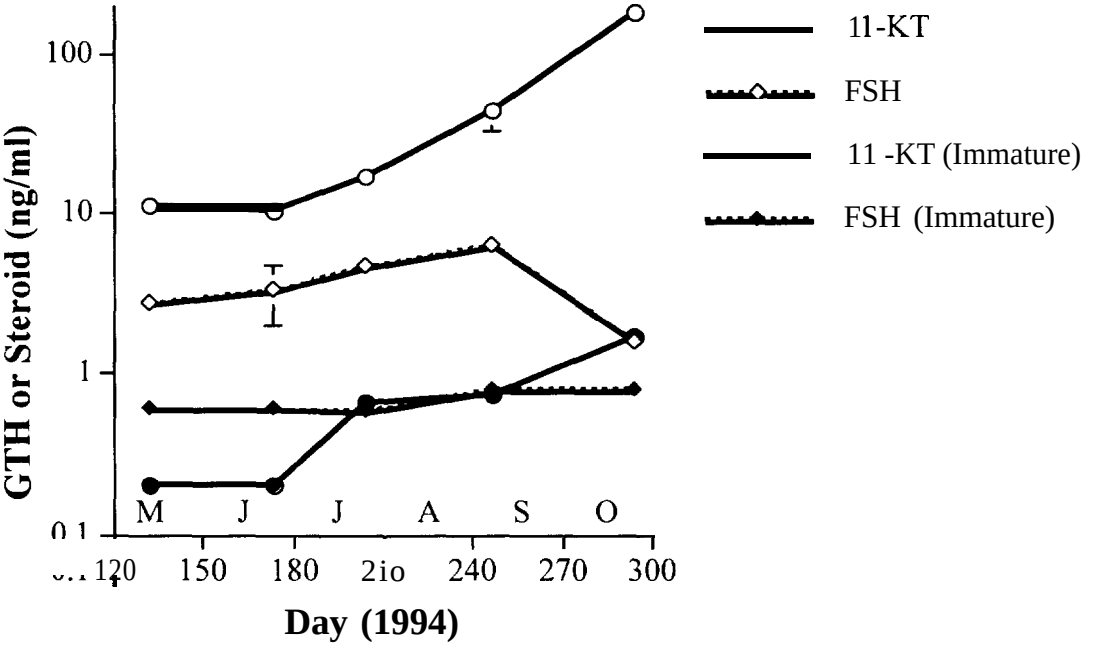

Figure 12. Plasma levels of follicle-stimulating hormone (FSH) and 1 l-ketotestosterone (11 -KT) in Lake Wenatchee sockeye salmon (1990 brood) reared in tanks containing fresh water. Data are mean +/- standard errors, n = 2-3 (immature), n = 1 O-2 1 (maies).

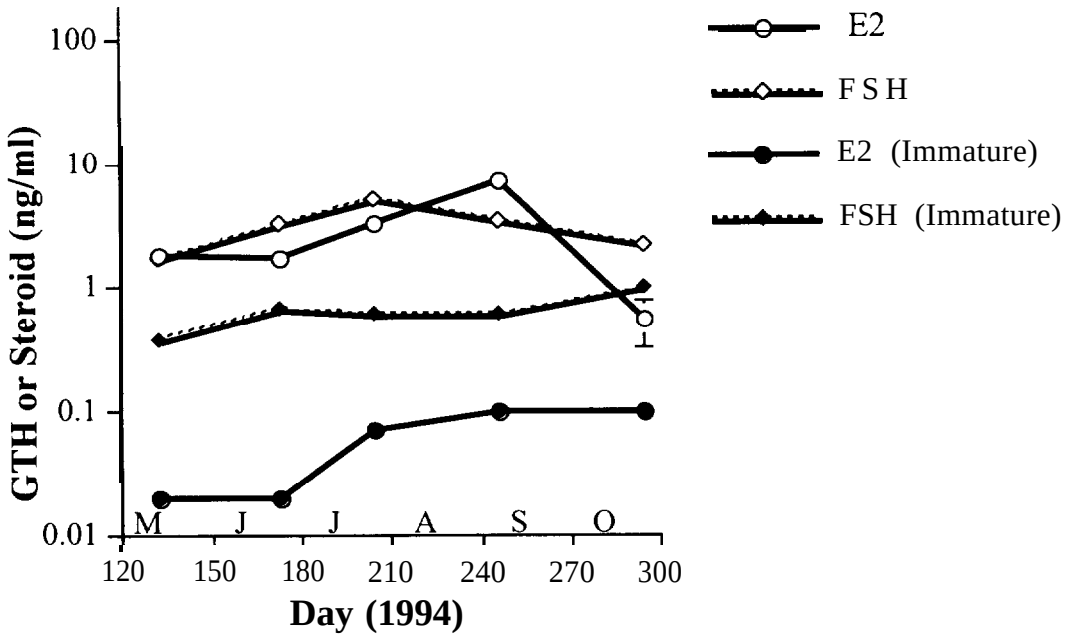

Figure 13. Plasma levels of follicle-stimulating hormone (FSH) and estradiol-17β (E) in female Lake Wenatchee sockeye salmon (1990 brood) reared in tanks containing fresh water. Data are mean +/- standard errors, n = 2 (immature), n = 10-22 (females).

Table 3. Stages of spermatogenesis observed during 1994 in 1990-brood Lake Wenatchee sockeye salmon reared in either fresh or seawater.

Date	Treatment	Maturity	Stages Observed*
May 12	Freshwater	Maturing	III (3), IV (1)
		nonmaturing	I (1)
May 12	Filtered Seawater	Maturing	III (3)
		nonmaturing	I (2)
July 21	Freshwater	Maturing	IV (4)
July 21	Filtered Seawater	Maturing	IV (4)
		nonmaturing	I (1)
September 2	Freshwater	Maturing	V (4)
September 1	Filtered Seawater	Maturing	V (2)
		nonmaturing	I (2)
October 19	Freshwater	Nonmaturing	I (5)
October 19	Filtered Seawater	Nonmaturing	I (5)

* number in parentheses indicates number of samples at this stage

Table 4. Stages of oogenesis observed during 1994 in 1990-brood Lake Wenatchee sockeye salmon reared in either fresh or seawater.

Date	Treatment	Maturity	Stages Observed*
May 12	Freshwater	Maturing	Oil drop (1), primary yolk globule (3)
May 12	Filtered Seawater	Maturing	Oil drop (2), primary yolk globule (2)
July 21	Freshwater	Maturing	Primary (3) and secondary (1) yolk globule
July 21	Filtered Seawater	Maturing	Oil drop (I), primary (2) and secondary (3) yolk globule
September 2	Freshwater	Maturing	Tertiary yolk globule (3)
September 1	Filtered Seawater	Maturing	Tertiary yolk globule (4)
October 19	Freshwater	Nonmaturing	Oil drop (1)
October 19	Filtered Seawater	Nonmaturing	Oil drop (2)

* number in parentheses indicates number of samples at this stage

DISCUSSION

There were three major findings from this study. First, endocrine changes associated with sexual maturation in sockeye salmon are similar to what has been reported for other salmonids, and did not differ between fish reared in seawater and fresh water. Second, initiation of spermatogenesis and secondary oocyte growth occurred prior to May in the Lake Wenatchee stock of sockeye salmon. Third, rearing environment did not affect gametes as assessed by the survival of offspring to hatch; however, eggs from seawater-reared females were smaller.

We initiated this study to determine whether gametogenesis in sockeye salmon proceeds similarly in fish reared for a period in seawater compared to those reared continuously in a freshwater environment. Because both oogenesis and spermatogenesis are regulated via the reproductive endocrine system, we monitored circulating levels of pituitary gonadotropins and sex steroids as indices of the maturation process.

It is well established that salmon produce two types of gonadotropins, which were initially called GTH I and GTH II (Suzuki et al. 1988, Kawauch et al. 1989. Swanson et al. 1991). Because of their structural homology to vertebrate gonadotropins, the classical terminology of FSH and LH has been adopted for GTH I and GTH II, respectively. As in other vertebrates, gametogenesis is regulated by gonadotropins, primarily via their affects on gonadal steroid biosynthesis.

Studies of other salmonids, including coho salmon (Peterschmidt 199 1, Swanson 1991), rainbow trout (Pratt et al. 1996), chinook salmon (Slater et al. 1994), and Atlantic salmon (Oppen-Bernsten et al. 1994) have shown that plasma levels of FSH are elevated during secondary oocyte growth and spermatogenesis, while LH levels are non-detectable until final oocyte maturation, ovulation, and spermiation, when levels increase. Results from our study of sockeye salmon also show that FSH levels are elevated during secondary oocyte growth in females, and throughout spermatogenesis in males, while LH levels increase only at the time of spawning.

Furthermore, there was no difference in the seasonal pattern of changes in gonadotropin levels due to rearing environment as has been shown for coho salmon (Peterschmidt 1991). However, FSH levels in sockeye salmon from our study were 1 0-fold lower than those reported for other salmonids, and the changes associated with oogenesis were less dynamic.

We know of at least two possible explanations for the low levels of plasma FSH in Lake Wenatchee sockeye salmon. First, the FSH RIA was developed using coho salmon FSH standards, antisera, and tracer. Serial dilutions of sockeye plasma were parallel to the FSH standard curve, indicating that the assay measures something antigenically similar to the FSH standards. Sockeye salmon FSH may have weak affinity to this antisera; thus we may be underestimating levels of FSH in sockeye salmon. However, we do not think this is likely, because the FSH RIA has been used to measure FSH in migrating Fraser River sockeye salmon, and FSH levels in this stock are comparable to previous measurements in coho and chinook salmon (E.M. Donaldson, Dep. Fish. and Oceans, Vancouver, B.C. Pers. Commun. August 1996).

Second, it is possible that there are stock or year-class differences in levels of FSH, as has been observed for LH in rainbow trout (Sumpter and Scott 1989.). In the study of Willamette River chinook salmon by Slater et al. (1994), plasma levels of FSH differed drastically between two different years of the same stock. Thus, there appear to be variables affecting levels of FSH in the circulation that we do not yet fully understand. Despite low FSH levels in the fish, both oogenesis and spermatogenesis proceeded, and fish produced mature gametes.

The pattern of changes in sex steroids in male and female sockeye salmon were similar to what has previously been reported for other salmonid fish (Fostier et al. 1978, 1983; Scott et al. 1982; Schulz 1984; Fitzpatrick et al. 1986; Slater et al. 1994) and was not affected by rearing environment. In females, plasma E levels increased during secondary oocyte growth and declined during final ooctye maturation when 17,20-P levels increased. In males, plasma 11 -KT levels increased throughout spermatogenesis and 17,20-P levels were low until spermiation.

Plasma sex steroid levels in nonmaturing fish were 10 to 100 times lower than those of maturing fish in May, indicating that levels of these steroids could be used to distinguish maturing and nonmaturing fish at this time. The increase in both E and 1 1-KT that occurred in nonmaturing fish from June to late July may indicate that the fish are initiating maturation for the subsequent year. However, no change in development was noted in the gonads at this time.

Oogenesis and spermatogenesis proceeded similarly in maturing sockeye salmon reared in either seawater or freshwater. This result is somewhat consistent with that of Peterschmidt (199 1) who found only a slight difference in oogenesis between female coho salmon reared in freshwater or seawater. During July sampling, the oocytes from coho salmon reared in freshwater appeared to be at earlier stages of vitellogenesis than those from females reared in seawater, and the females reared in freshwater spawned later than those reared in seawater.

At all other samplings, oocytes from female coho salmon reared in the two environments were at similar stages of development. In our study of sockeye salmon, we did not detect differences in oogenesis between the two treatment groups; however, this may be due to the small sample size. We were limited in the number of fish that could be sacrificed for collection of gonads. Females reared in fresh water tended to spawn about a week later than those reared in seawater.

Gamate quality (as assessed by survival of offspring to hatch) produced by fish reared in the two environments did not differ. However, females from the seawater group were smaller and produced smaller eggs, but more eggs per unit body weight than females reared continuously in freshwater. This difference in egg number and size may be due to the growth differences observed in the two groups of females. Studies in rainbow trout have shown that ration, and consequently growth at critical stages of oogenesis, affects maturation, egg number, and egg size (Scott 1976, Bromage et al. 1992).

Reduced egg size may ultimately affect survival of offspring. Previous studies have shown that egg size affects the size of fry at hatch, as well as growth and short-term survival (Springate and Bromage 1985), and these qualities may affect long-term survival in nature. However, we monitored survival of offspring only to hatch in a captive environment.

The survival of offspring to hatch that we observed in this study was low compared to anadromous fish of the same stock when spawned in a hatchery. Our results indicated that this was not due to rearing fish in fresh water or seawater. There are three major factors which may be reducing the quality of eggs produced by captive broodstock: nutritional effects on egg composition, stress and timing of egg collection (Bromage et al. 1992, Campbell et al. 1992, Pickering et al. 1987, Sumpter et al. 1987).

We noted during the spawning period that substantial numbers of females had what appeared to be over-ripe eggs. Numerous studies in rainbow trout have indicated that timing of egg collection is the most critical variable affecting fertilizability of eggs and survival of offspring (Springate and Bromage 1985, Springate et al. 1984)). Based on this observation and information from the literature, we plan to check females earlier in the season and more frequently in our study of 1991-brood Lake Wenatchee sockeye salmon. It is not known to what degree stress of the captive rearing environment or the frequent monthly handling of fish could have contributed to the low survival of offspring that we observed.

CONCLUSION

We did not find a significant difference in endocrine changes associated with maturation in sockeye salmon reared in either fresh water or seawater. Growth appeared to differ between the two groups, particularly in females, and this resulted in differences in egg size.

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PART III

EFFECTS OF REARING TEMPERATURE ON GROWTH, REPRODUCTIVE PERFORMANCE, AND IMMUNE FUNCTION IN CAPTIVELY-REARED SOCKEYE SALMON

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EFFECTS OF REARING TEMPERATURE ON GROWTH AND MATURATION OF SOCKEYE SALMON

Background

The seasonal timing of reproduction in salmonids is regulated primarily by photoperiod and secondarily by temperature. Although there is substantial information on the effects of temperature on embryonic development, the effects of rearing temperature on seasonal timing or age of sexual maturation, gamete quality, and fish health in Pacific salmon have not been rigorously tested through controlled experiments. The effects of temperature on timing of maturation may vary considerably among species and strains. Response to environmental temperature most likely reflects differences in adaptations to thermal regimes in the native environment of the strain. Therefore, data from rainbow trout (0. *mykiss*) and Atlantic salmon (*Salmo* ***salar)*** studies cannot be used to reliably predict the responses of other Pacific salmon to water temperature changes.

Rearing temperature influences fundamental physiological processes that control growth, development, reproduction, and general fish health. In captive culture of broodfish, as with the culture of all fish, there are practical limitations to controlling water temperature and duplicating temperatures experienced by fish of all life-history stages in the wild. Thus, there is a critical need to establish optimal ranges of rearing temperatures which ensure: 1) appropriate rates of growth, development, and timing of reproduction for the target species; 2) production of viable gametes and offspring; and 3) high rates of survival to spawning.

One of the primary causes of prespawning mortality in captive fish is disease. There is substantial information on the incidence of a variety of bacterial and viral diseases at specific life-cycle stages (e.g., smoltitication) and at a variety of water temperatures in fresh water. In many cases, prophylactic measures are taken to minimize outbreaks during these periods. However, the underlying causes of poor disease resistance during these periods are not well understood, and the effects of life-cycle stage vs. season have not been separated experimentally. To develop better fish health management strategies, a basic understanding of the physiological basis for how a variety of factors (temperature, life history stage, and season) affect immune function is needed. We are convinced that the best strategy to limit mortality due to disease is through preventative measures such as maintaining appropriate fish husbandry practices that limit transmission and by rearing fish with a healthy immune system. In the present study, we are investigating the effects of three rearing temperatures on sockeye salmon growth rate, development, immune function, gamete and embryo viability, and timing and age of sexual maturation.

Introduction

Photoperiod is regarded as the most important environmental factor controlling gonadal development and spawning time in salmonids; however, it does not act alone in controlling reproductive function. The extent to which temperature acts in concert with photoperiod to regulate the timing and rate of gametogenesis is poorly understood. Few experimental studies have been conducted on the effects of rearing temperature on reproduction in salmonids (Henderson 1963; Breton and Billard 1977; Morrison and Smith 1986; Beacham and Murray 1988; Bromage and Cumaranatunga 1988; Nakari et al. 1987, 1988; Johnston et al. 1987, 1990, 1992; Taranger and Hansen 1993).

In rainbow trout, studies have shown that temperatures ranging from 8 to 16°C have little effect on timing of the reproductive cycle, but higher temperatures adversely affect the quality and quantity of gametes (Billard 1985). The approximate upper limit for successful reproduction of salmonids in the wild is considered to be 13 °C (MacCrimmon 1971, Scott 1990). However, this upper limit may be lower for salmonids, which naturally spawn in the northernmost latitudes or high altitudes (Taranger and Hansen 1993).

Because fish are poikilothermic animals, temperature directly affects their gonadal physiology by changing the rate of yolk sequestration, steroid biosynthesis, and other general metabolic reactions. In salmonids, low temperatures (< 8 “C) reduce the rate of maturation, causing vitellogenesis (Johnston et al. 1987, Korsgaard et al. 1986) and oocyte growth (Crim et al. 1983b) to proceed slowly. At very low temperatures (< 3 “C), the final stages of oocyte maturation and ovulation are completely inhibited (Billard 1985). Testicular steroid production is also reduced at low temperatures (Manning and Kime 1985).

Spawning time in autumn-spawning trout can be delayed until spring by adverse cold weather conditions, and conversely, spring-spawning trout can be made to spawn in winter or autumn by movement into temperate water conditions (Morrison and Smith 1986, Nakari et al. 1987, Titarev 1975). In Atlantic salmon, high water temperatures (increases from 10 to 13-14°C) during the spawning season inhibit ovulation and have a detrimental effect on gamete quality (Taranger and Hansen 1993). Heggeberget (1988) found that in this species, peak spawning in Norwegian rivers occurred when water temperatures were decreasing. This suggests that among salmonid species and/or strains, temperature may affect reproduction differently. Recent studies have shown that thermal preferences of Pacific salmon vary considerably, with sockeye salmon preferring 8.9 °C (Welch et al. 1995).

Here we report results to date on the effects of three rearing temperatures on growth rate, development, immune function, timing and age of sexual maturation, and gamete and embryo viability.

Materials and Methods

Fish and Experimental Design

Sockeye salmon of the Lake Wenatchee stock (1994 brood) are being reared in fresh water throughout the life cycle (from fertilized eggs to mature adults) under three water temperature regimes: 1) constant 12 °C, 2) constant 8 °C, and 3) constant 12 °C for 8 months (March through October) and 8 °C for 4 months (November through February). Gametes for the three groups were collected from 10 mature adult fish at the Big Beef Creek Field Station during fall 1994 and incubated at 8 °C in isobuckets. Only gametes from adult fish that were negative for tests of bacterial kidney disease (BKD) and infectious hematopoietic necrosis (IHN) virus were used for the experiment.

Fry were ponded into 1.9-meter (diameter) circular tanks and reared at 8°C until April 1995, when fish were distributed into 6 tanks (1,600 fish/tank, 2 tanks/treatment) and gradually adapted to rearing temperatures. Fish will be maintained through the smolt stage (spring 1996) in 1.9-meter circular tanks (two replicate tanks per treatment) at the research facilities at the National Biological Service (NBS), Pacific Northwest Natural Science Center in Seattle, Washington. This facility utilizes filtered and UV-sterilized water from Lake Washington. During summer 1996, fish will be transferred to 3.9-meter circular tanks (two replicate tanks per treatment). Prior to spawning of the 12 °C group (fall 1997 or 1998), a subgroup of maturing fish will be transferred to tanks where temperature will be gradually declined over a 3-week period to 6-8 “C. Fish are being fed to satiation twice daily, 5 days per week.

Monitoring Growth and Sexual Maturation

The initial experimental plan included sampling of fish monthly throughout the period of rearing; however, insufficient numbers of fish were available for monthly sampling over the 4-year period. In addition, to monitor smolt development during spring 1996, biweekly sampling will be necessary. Therefore, sampling of fish will begin during January 1996. At this time, fish (1 O/tank) will be sacrificed every 2 weeks through June 1996 and their whole body weights and lengths will be recorded. Gill tissue will be collected for measurement of sodium-potassium ATPase levels, and plasma will be collected for thyroxine and insulin-like growth factor I levels. During August 1996, we will begin monthly sampling (10 fish/tank) to determine body weight, length, and maturity status. Gonads will be collected for histological determination of stage of spermatogenesis or vitellogenesis. Pituitary glands and plasma will be collected for gonadotropin measurements. Analyses of these samples will aid in determining when secondary oocyte growth and spermatogenesis are initiated, and if temperature affects the age or seasonal timing of maturation. Monthly sampling will continue through fall 1998, when it is expected that the majority of fish will mature and be spawned.

DEVELOPMENT OF ASSAYS OF CELL-MEDIATED IMMUNE FUNCTION

Introduction

To measure the effect of rearing temperature on the immune system of sockeye salmon, a number of immunological assays are being developed by the National Biological Service at the Pacific Northwest Natural Science Center in Seattle, Washington. These assays will provide measures of nonspecific and specific immune functions of fish from each of three temperature groups at various points during rearing. Aspects of both the humoral and cellular immune functions will be measured in a panel of assays selected to provide a general index of the status of nonspecific defense mechanisms (Table 1). Progress was made during the current reporting period in modifying and optimizing those assays to meet the requirements of this project to measure immune functions in large numbers of fish.

Materials and Methods

Fish

Unless specified otherwise, broodyear 1992 spring chinook salmon (*Oncorhynchus tshawytscha)* from Carson National Fish Hatchery in Carson, Washington, or broodyear 1993 sockeye salmon (0. *nerka*) from Lake Wenatchee were used for the experiments completed during this reporting period. All fish were raised at the Northwest Biological Science Center in sand-filtered, UV-treated Lake Washington water and fed a pelleted moist diet *ad libitum 5* days per week.

Production of Oxygen Radicals by Neutrophils and Monocytes

The measurement of superoxide anions released by neutrophils and monocytes during an oxidative burst was determined using nitroblue tetrazolium (NBT) reduction dye. When the oxidative burst occurred, the NBT was reduced to formazan and solubilized for quantification.

Chinook salmon were anesthetized with 150-200 ppm tricane methanesulfonate (MS-222; Argent Chemical Laboratories). Blood was taken from caudal vessels and placed into heparin-coated glass test tubes on ice. The glass test tubes were rinsed with 300 units of ammonium heparin, then dried at 60°C for 48 hours. A 0.2% (w/v) NBT (Sigma Chemical Co.) solution was prepared in 0.01 M phosphate buffered saline (PBS) at pH 7.3 with 0.01% (w/v) thimerosal (Sigma Chemical Co.). Dimethyl sulfoxide (DMSO) and 2 M potassium hydroxide (KOH) were used to solubilize formazan. Fixed cells were treated with 1% (w/v) paraformaldehyde in PBS. Cells were stimulated with a commercial NBT stimulant (Sigma Chemical Co.).

After blood was taken, 25-μL samples were placed into each of three microcentrifuge tubes on ice. One tube was centrifuged at 645 x g for 5 minutes at room temperature. Serum was then removed and the pellet was resuspended with 25 μL of 1% (w/v) paraformaldehyde. This sample was incubated for 1 hour at room temperature and served as the fixed cell sample. Cells in a second tube received 25 μL of NBT stimulant and served as the stimulated sample, and cells in the remaining tube served as the unstimulated sample. The appropriate reagent controls were included in each analysis. Each sample was then incubated for 1 hour at 15 "C with 25 μL of 0.2% (w/v) NBT solution. All samples were washed by the addition of 500 μL of PBS and centrifuged at 645 x g for 5 minutes at room temperature. Approximately 500 μL of the supernatant was discarded, and the pellet was resuspended in the remaining buffer (- 25 μL). All samples received 480 μL of 2M KOH and 560 μL of DMSO and were centrifuged at 7,172 x g for 15 minutes. After centrifugation, 100 μL of supernatant from each sample was placed in each of six wells of a 96-well microtiter plate. The absorbances were measured at eight wavelengths between 405 and 690 nm.

Phagocytic Activity

The ability of macrophages to phagocytize foreign particles was assessed by determining the percentage of glass-adherent anterior kidney cells that ingested fluorescently labeled *Staphylococcus aureus*.

To prepare salmonid anterior kidney leukocytes, chinook salmon were killed by an overdose of MS-222 and a blow to the head. The entire surface of the fish was disinfected with 70% (v/v) ethanol, and the anterior kidney was removed aseptically. Kidney tissue fragments from individual fish were processed separately, with each placed into 25 mL of ice-cold leukocyte isolation medium, pH 7.4. The leukocyte isolation medium contained Eagles essential minimal medium (Sigma Chemical Co.) supplemented with $NaHCO_3$ (1.6 g/L), glucose (1.65 g/L), ammonium heparin (5000 units/L), and 10% (v/v) fetal calf serum (HyClone Laboratories, Inc.).

A single-cell suspension was prepared by passing the kidney material through sterile, stainless steel wire mesh with a syringe plunger. After the suspension was filtered through glass wool, it was placed over 5 mL ice-cold Histopaque 1077 (Sigma Chemical Co.) and centrifuged at 1000 x g for 15 minutes at 4 "C. Leukocytes at the interface of the media and Histopaque were removed with a sterile Pasteur pipette and suspended in 15 mL Leibovitz's L- 15 media (Gibco BRL) containing 20 units/mL ammonium heparin (L- 15/heparin). After centrifugation at 1000 x g for 10 minutes at 4 °C, the pelleted cells were resuspended in 2 mL L- 1 5/heparin. A sample of the cell suspension was diluted in trypan blue (Sigma Chemical Co.), and the concentration of glass-adherent cells, assumed to be macrophages, was calculated based on standard cell-counting

Table 1. Hematological and serological assays being developed for measurement of humoral and cellular immune functions in sockeye salmon (*Oncorhynchus nerka*).

Assay	Principle
Hematocrit level	Changes in the percent volume of erythrocytes may indicate stress, dietary deficiencies, presence of contaminants, or infection.
Leukocrit level	Changes in the percent volume of leukocytes may indicate dietary stress, the presence of contaminants, or different stages of infection.
Differential cell count	The relative proportions of erythrocytes and the various types of leukocytes can change due to stress, or acute and chronic infections
Nitroblue tetrazolium (NBT) test	Phagocytes can reduce NBT to dark blue, insoluble crystals within the cell by generation of superoxides. Activated phagocytes reacting to pathogens will have higher readings. Other adverse conditions may result in decreased NBT activity.
Phagocytic activity	The phagocytic index is a measurement of the ability of neutrophils, monocytes, and macrophages to remove foreign particles.
Myeloperoxidase	Myeloperoxidase is an enzyme present in neutrophils. In conjunction with hydrogen peroxidase and halide, it has a germicidal effect.
Total serum protein	Changes in the total serum protein may indicate a dietary problem, or an immune response to a pathogenic microorganism or vaccine.
Total serum immunoglobulin	Changes in the total serum immunoglobulin suggest a response to a pathogenic microorganism. Those changes may reflect an active response (increase) or immunosuppression (decrease).
Lysozyme	Lysozyme is an enzyme that acts on the cell wall of bacteria, resulting in osmotic lysis. Principally active against gram-positive organisms, it may also contribute to the action of complement and antibodies on gram-negative microorganisms.
Complement fixation	Represents a system of proteins that facilitate numerous biological activities, including bacteriolysis, opsonization, chemotaxis, phagocytosis, and hemolysis.

procedures. The leukocyte suspension was then adjusted to 2.5 x 106 macrophage cells/mL in L-15/heparin.

Staphylococcus aureus cells were labeled with fluorescene isothiocyanate (FITC) as described by Cantinieaux *et al.* (1989). This method was developed for S. *aureus* according to the method described by Gelfand *et al.* (1976). For the phagocytosis assay, each well of a 16-chambered microscope slide (Nunc, Inc.) received 200 µL of the kidney cell suspension. The slide was incubated at 15 °C for 1 hour in a humid chamber. The non-adherent cells were removed by repeated, gentle filling and dumping of the wells with 200µL Leibovitz's L-l 5 media prewarmed to 15 "C. Individual chambers received 200 µL of a 1: 1 mixture of Leibovitz's L-l 5 media and Macrophage-SFM Media (Gibco BRL) containing 10 µM CellTracker™ Orange CMTMR (Molecular Probes, Inc.) and one of three concentrations of FITC-labeled S. *aureus.*

Bacterial concentrations were 1 x 10^9, 1 x 10^8, and 1 x 107 bacteria/ml, respectively. The slide was incubated for 1 hour at 15 °C in a humid chamber before the non-phagocytized bacteria were washed out with L-l 5 media as described above. The remaining cells were fixed by the addition of 200 µL of PBS, pH 7.4, containing 1% (w/v) paraformaldehyde for 15 minutes at room temperature. Each chamber was then washed with PBS containing 0.01% (w/v) thimerosal (PBS/thimerosal). To each chamber, 200 µL PBS/thimerosal containing 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma Chemical Co.) was added. The slide was incubated for 15 minutes at room temperature before washing the chambers with PBS/thimerosal. The plastic wells and the silicone gasket were removed. Several drops of prewarmed mounting media (Glycergel; Dakopatts, Denmark) were placed on the slide followed by cover slips.

Cells were observed by fluorescent microscopy using a Zeiss Axiophot microscope and a mercury lamp. A triple wavelength filter (Molecular Probes, Inc.) was used to simultaneously view the fluorescent light generated by the FITC, DAPI, and CellTracker™ Orange CMTMR. In some experiments the cell perimeters were visualized by the simultaneous use of low-light phase contrast microscopy and epifluorescence. Leukocytes with horseshoe-shaped nuclei were considered macrophages. Those with two or more FITC-labeled *S. aureus* within the periphery of the orange-stained cytoplasm were considered phagocytic. Results were reported in percentage of macrophage cells with phagocytized bacteria.

Myeloperoxidase Activity

Presence of myeloperoxidase in salmonid blood and anterior kidney leukocytes was determined through a staining procedure that positively stained azurophil granules containing the enzyme. Sockeye salmon were anesthetized with 150-200 ppm MS-222. Blood was taken from caudal vessels and placed into heparin-coated, glass test tubes on ice. A suspension of anterior kidney leukocytes was prepared in the L-l 5/heparin medium as described above for the preparation of salmonid anterior kidney leukocytes. 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 Chemical Co.). Ethanol-formaldehyde fixative solution was prepared by combining one part 37% formaldehyde with nine parts 95% (v/v) ethanol.

For the myeloperoxidase activity determinations, blood and anterior kidney leukocyte smears were made on glass microscope slides with 25 μL of either blood or anterior kidney leukocytes and allowed to air dry at room temperature. Two blood smears and two anterior kidney leukocyte smears were made from each fish. Each smear was fixed in ethanol-formaldehyde for 30 seconds at room temperature, then washed by submerging in gently running tap water for 2 minutes. Washed slides were air-dried at room temperature in the dark. Each smear was assigned to either a test group that was stained for myeloperoxidase activity, or to a control group.

To stain for myeloperoxidase activity in the test group smears, Tris-maleate buffer was warmed to 37 °C in a water bath, and hydrogen peroxide and phenylenediamine hydrochloride-catachol solutions were added and mixed thoroughly. Immediately after mixing, one blood smear and one anterior kidney leukocyte smear from each fish were placed into the test jar for 30 minutes in the dark. Smears in the control group were treated identically, except Tris-maleate buffer was substituted for the hydrogen peroxide and phenylenediamine hydrochloride-catachol solutions. All slides were washed for 15-30 seconds in gently running tap water and allowed to air dry at room temperature. Slides were examined by light microscopy at 630X magnification to determine the best preparation for ease of reading and scoring. Cells in the test and control groups were rated on the cell morphology and degree of myeloperoxidase staining.

Total Serum Immunoglobulin

The relative amounts of total serum immunoglobulin will be assessed by a double-antibody enzyme-linked immunosorbent assay for fish immunoglobulin (Fish IgM-ELISA). Three mouse hybridoma cultures that produce antibody specific to salmonid immunoglobulin have been selected for evaluation as sources of anti-fish immunoglobulin for development of the Fish IgM-ELISA (Table 2). Immunoglobulin from each hybridoma will be evaluated either as culture supernatants or mouse ascitic fluid.

Hybridoma cultures were maintained at 37 °C in RPMI medium supplemented with 10% (v/v) fetal bovine serum. The culture fluid was removed and centrifuged at 2000 x g for 20 minutes at 4°C to remove cellular debris. The cell-free supernatant was stored at -20°C. For production of ascitic fluid, cells from the A3 and Warr 1.15 hybridomas were prepared and injected into BALB/c mice according to the method described by Harlow and Lane (1988).

Mouse immunoglobulin in untreated and purified preparations of hybridoma supernatant or ascites fluid was quantified by radial immunodiffusion. Control immunoglobulin preparations and sample buffers were used according to the manufacturers' instructions. Each hybridoma preparation was tested at 1: 10 (v/v) or 1:30 (v/v) in the sample buffer. Following sample application, plates were incubated at 22°C for at least 72 hours. The diameter of the precipitin rings was measured to the nearest 0.1 mm with a calibrated optical magnifier. The concentration of immunoglobulin in the hybridoma samples was calculated on the basis of a linear regression from a standard curve.

Isolation of mouse immunoglobulin from culture supematant of the CsA3 hybridoma was performed at 4°C using the Econo-system equipped with a protein A column (BioRad Laboratories, Richmond, CA). Commercially prepared binding and elution buffers (BioRad Laboratories) were used according to the manufacturer's instructions. Each CsA3 supematant was adjusted for optimal binding to protein A by the addition of the recommended amount of binding buffer salts. The adjusted solutions were then passed over the protein A column at a flow rate of 1 mL per minute. Following extensive washing of the column with binding buffer, the bound immunoglobulins were removed by the addition of elution buffer to shift the pH. Fractions containing the eluted mouse immunoglobulin were returned to neutral pH by addition of 2M NaOH. The purified immunoglobulin was dialyzed against PBS, pH 7.4 for 24 hours at 4°C. Following dialysis, the samples were stored at -20 "C.

To estimate the proportion of the total immunoglobulin removed from the culture supematant by a single passage through the protein A column, the unbound fraction was passed over the column a second time. Equivalent volumes of the unbound fraction and the original supematant were used so that the relative amounts of immunoglobulin isolated from each solution could be directly compared.

Ten microtiter plates from four manufacturers were evaluated in an ELISA that measured their relative abilities to bind different concentrations of bovine serum albumin (BSA) conjugated to DNP (BSA-DNP; Calbiochem). The amount of bound BSA-DNP was quantified by ELISA using purified rabbit immunoglobulin against DNP conjugated with horseradish peroxidase (rabbit IgG-HRP; Dakopatts, Denmark). Commercially prepared wash buffer, ABTS peroxidase substrate system, and ABTS stop solution (Kirkegaard and Perry Laboratories, Inc.) were used according to the instructions of the manufacturer. All volumes are 200 μL unless otherwise stated.

Wells were coated with BSA-DNP at 1000, 100, 50, 25, 10, 5, 2.5, and 1 ng per mL in carbonate-bicarbonate coating buffer, pH 9.6. The rabbit IgG-HRP conjugate control wells and the substrate-chromogen control wells received coating buffer without BSA-DNP. Protein conjugate was allowed to coat the well surfaces for 16 hours at 4°C, then unbound conjugate was removed by rinsing each well five times with wash buffer. The rabbit IgG-HRP conjugate was

diluted to 1:3,000 (v/v) in Tris-buffered saline. pH 8.0 containing 1% (v/v) Tween-20 and 3% (w/v) **BSA,** then added to the appropriate wells. Substrate-chromogen control wells received conjugate diluent without the rabbit IgG-HRP conjugate. Plates were incubated at 37°C for 1 hour, then unbound rabbit IgG-HRP conjugate was removed by rinsing each well five times with wash buffer. The ABTS substrate was incubated for 15 minutes at 37 °C and then stopped by the addition of 50 µL of 4X strength stop solution. Absorbance was measured at 40.5 nm. The signal-to-noise ratio (S/N) was calculated for each concentration (χ) **of BSA-DNP as** the ratio of the specific **(BSA-DNP)** and nonspecific (Rabbit IgG-HRP conjugate control) protein binding:

$$S/N = \frac{\text{Mean Absorbance [BSA-DNP]}\chi}{\text{Mean Absorbance Rabbit IgG-HRP}}$$

A signal-to-noise ratio equal to, or greater than, 2.0 was considered to be a positive reaction.

Lysozyme Activity

Methods to measure lysozyme activity include the turbidimetric assay, agar plate diffusion (lysoplate assay; Ellis 1990; Lie et al. 1986, 1989), ELISA (Taylor et al. 1992), and rocket electrophoresis (lysorocket electrophoresis; Lie et al. 1989). Each measures the relative activity of lysozyme in test and control samples to lyse the Gram positive bacterium, *Micrococcus Zysodeikticus.* Preliminary investigations suggested that the turbidimetric assay for serum lysozyme may be unsuitable for this study because the required sample volume is difficult to obtain from juvenile sockeye salmon. This report describes preliminary results from evaluating the lysoplate assay as a method to accurately measure lysozyme levels in serum samples. A complete comparison of the turbidimetric and lysoplate assays will be included in a later report.

Fish and serum samples--Chinook salmon were injected with approximately 1 x 10^6 or 1 *x* 10^8 live *Renibacterium saimoninarum* cells to monitor the progression of infection by testing tissues in the BKD-ELISA; these challenges are described fully below. Fourteen days after injection, 12 fish from the group injected with 1 x 108 bacteria were anesthetized with 150-200 ppm MS-222 and blood was taken from the caudal vessels and placed into heparin-coated glass test tubes. The blood was centrifuged at 1,000 x g for 15 minutes at 4 °C, and the serum was removed and stored at -70°C. The serum from one fish was tested undiluted, and at 1:2 (v/v) and 1:4 (v/v) dilutions in PBS, pH 7.4, in the temperature experiment.

***Micrococcus lysodeikticus* in agarose--A** stock solution of *M. lysodeikticus* **(ATCC** 4698; Sigma) at 750 µg/ml was prepared in 0.5 M PBS, pH 6.2 and stored at -70°C. A sufficient volume of the stock bacteria was added to 1% (w/v) low temperature agarose (FMC) in 0.05 M PBS, pH 6.2 such that the final concentration of bacteria was 50 µg/mL. The suspension

Table 2. Mouse hybridomas being evaluated as sources of immunoglobulin against fish IgM.

Hybridoma designation	Immunoglobulin isotype	Source of fish IgM for immunization of mice	Reference
A3	IgG1	Rainbow trout (*O. mykiss)*	Bartholomew et al. 1991
4C10	Not Reported	Rainbow trout (*O. mykiss)*	Thuvander et al. 1990.
Warr 1.14	IgG1	Rainbow trout (*O. mykiss)*	DeLuca et al. 1983

was held at 45 “C to insure that it remained above the gel temperature of the agarose. Clean glass plates were precoated with 0.064 ml of 0.5% (w/v) low temperature agarose per mm^2 of glass. Each coated slide was then dried at 100 “C. The agarose with *M. lysodeikticus* was applied to the precoated slides (0.015 mL per mm2 of glass) and allowed to gel at room temperature. Wells of 3-mm diameter were punched in the agarose for application of the test and control samples.

Hen egg white lysozyme (HEWL) controls--A stock solution of HEWL at 1 mg/mL was prepared in 0.05 M PBS, pH 6.2 supplemented with 1% (w/v) BSA (PBS-BSA). This solution was diluted further in PBS-BSA to the appropriate concentrations (between 0.125 µg/mL and 500 µg/mL) to serve as standards.

Lysoplate assay--Individual wells received 9 µL of either fish serum or different concentrations of the HEWL standards. Plates were incubated in a humid chamber for approximately 16 hours at 15, 25, or 37°C. The diameter of the cleared zones was measured with a calibrated optical magnifier and used to calculate the concentration of lysozyme (Ellis 1990).

Results

Production of Oxygen Radicals by Neutrophils and Monocytes

Other investigators have specified optimal wavelengths for the measurement of formazan. Pick *et* al. (198 1) measured unsolubilized formazan in an aqueous medium at 550 nm. Rook *et al.* (1985) used 630 nm for determining the amount of formazan solubilized with DMSOKOH. In our studies we solubilized the formazan in salmonid leukocytes with DMSO/KOH, which produced a blue-green solution. The absorbance of this solution was found to be higher at 414 nm than at the suggested wavelengths (Fig. 1).

During the NBT assay, salmonid leukocytes were incubated in PBS containing NBT. Stimulation of the oxidative burst was achieved by concomitant incubation with a commercially prepared NBT stimulant. Fixed cells were incubated with paraformaldehyde before the addition of the PBS-NBT solution. A difference in NBT reduction was not apparent between the unstimulated and stimulated leukocytes (Fig. 1). In contrast, fixation of the leukocytes appeared to reduce the oxidative burst when read at 540 and 4 14 nm, but not at 620 nm.

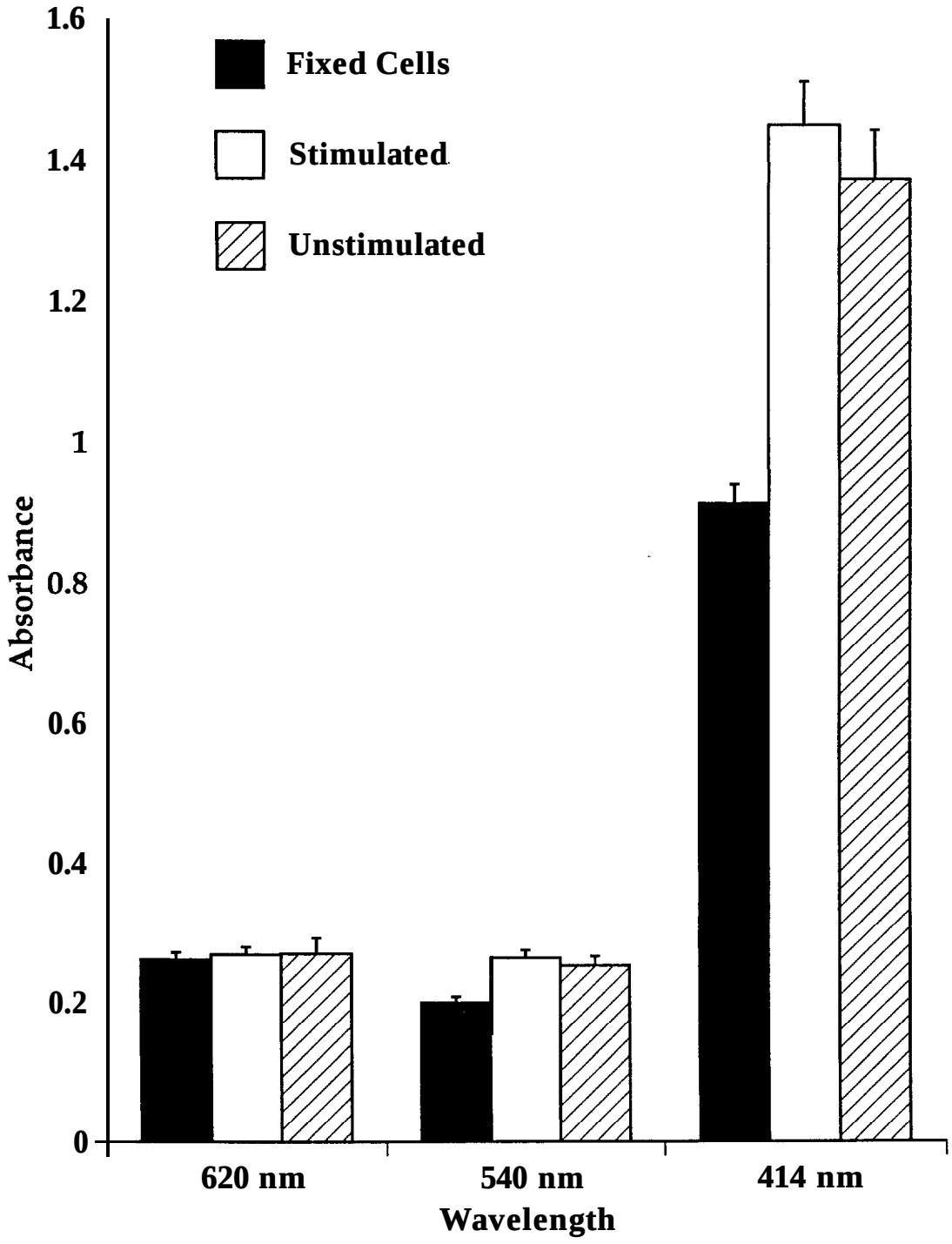

Figure 1. Effect of the wavelength of visible light on absorbances produced by blood leukocytes in the NBT assay. Cells in the unstimulated group were analyzed without other treatments. Stimulated cells were treated with a commercially prepared NBT stimulant during the assay. Fixed cells were treated with 1% (w/v) paraformaldehyde before the addition of NBT.

Phagocytic Activity

Fluorescence microscopy with the triple filter permitted the observer to visualize the FITC-labeled S. *aureus* and the morphological features of glass adherent kidney cells; the size and shape of the cell's nucleus and the cytoplasmic boundary were highlighted by the staining of nucleic acids by DAPI and the action of cytoplasmic enzymes on the CellTracker dye, respectively. Whereas the three fluorescent dyes could be viewed simultaneously with the triple filter, the use of that filter also reduced the fluorescence intensity of the nuclei stained by the DAPI. When the triple filter was replaced with the filter specific for DAPI, the nuclear morphology was readily apparent, allowing macrophage identification.

Although the CellTracker dye was no longer visible with the DAPI filter, recent experiments (data not shown) have suggested better visualization of the cytoplasmic boundary is possible through the concomitant use of the DAPI filter and phase contrast microscopy. This combination allowed the viewer to readily distinguish the morphological characteristics important in the identification of macrophages, while also being able to visualize the FITC-labeled S. *aureus.*

When the salmon cells were presented with FITC-labeled *S. aureus,* bacteria were seen within the boundary of some cells, suggesting phagocytosis had occurred. The number of phagocytic cells detected increased when the concentration of FITC-labeled S. *aureus* was increased (Table 3). Our recent attempt to quantify phagocytosis among leukocytes from sockeye salmon macrophages was confounded by unexplained lysis of the glass-adherent cells.

Myeloperoxidase Activity

Leukocytes containing darkly stained azurphilic granules were observed by light microscopy in both the blood and anterior kidney preparations. When the myeloperoxidase activity of leukocytes from the blood and anterior kidney of sockeye salmon were compared, stained cells were more difficult to locate in blood preparations because of the high percentage of erythrocytes. Although stained cells were readily located in the anterior kidney preparation, further analysis was confounded by large amounts of precipitated dye that often masked the presence of the cells, or their internal morphology. We started photographic reference log of cells with various levels of staining to standardize future analyses.

Total Serum Immunoglobulin

Immunoglobulin from mouse hybridoma cultures--Ascitic fluid containing high levels of anti-fish immunoglobulin from both groups of mice has been harvested and stored at -70°C for later purification. The levels of mouse immunoglobulin in these fluids was found to range between 1 and 15 mg/ml when measured by radial immunodiffusion. Preliminary estimates of the ability of the protein-A system to purify the immunoglobulin from supernatants of the A3 hybridoma have been encouraging; 300-400 mL of culture supematant can be processed during a normal work day. Although the purity of immunoglobulin from these fractionizations appears acceptable, reapplication of the unbound fraction to a protein A column yielded additional immunoglobulin.

Selection of a microtiter plate for the Fish-IgM ELISA--The differences in adsorption of the BSA-DNP conjugate among the various types of microtiter plates could generally be divided into two categories: 1) low-binding microtiter plates, such as the Dynatech Immulon 1 and 3 and the Nunc 475094 microtiter plates, which had poor adsorption of the BSA-DNP to the well surfaces and resulted in low or very low mean absorbances, regardless of the BSA-DNP concentration; and 2) moderate-to-high binding microtiter plates, where the mean absorbances increased at higher concentrations of the BSA-DNP (Table 4). Only microtiter plates in the latter category were considered for use in the Fish-Ig ELISA.

The Coming 25801 and Costar 3590 microtiter plates had the desired S/N ratio of at least 10 when the BSA-DNP was 1000 ng/ml. Although the Costar 3590 microtiter plate also produced positive S/N ratios (very near or greater than 2) throughout the range of BSA-DNP concentrations, the Coming 25801 failed to produce a positive reaction when the BSA-DNP concentration was 2.5 ng/ml. Four other varieties of microtiter plates produced higher mean absorbances than the Costar 3590 at the lowest BSA-DNP concentration, but three of those varieties (the Dynatech Immulon 4, and the Nunc 442404 and 439454) had lower S/N ratios because of high background binding by the rabbit IgG-HRP. The fourth microtiter plate. the Dynatech Immulon 1, had a higher S/N ratio than the Costar 3590 at the lowest BSA-DNP concentration, but was not considered further because of erratic mean absorbances at the other BSA-DNP concentrations. Based on these results, the Costar 3590 microtiter plate was selected for development of the Fish-Ig ELISA.

Lysozyme Activity

A positive correlation (R^2 10.97) was observed between incubation temperature and the diameter of cleared zones produced by the HEWL (Fig. 2). However, the slope of the standard curve was similar at each incubation temperature. The diameter of the cleared zones produced by each dilution of the chinook salmon serum was greater than that produced by the highest HEWL concentration (data not shown).

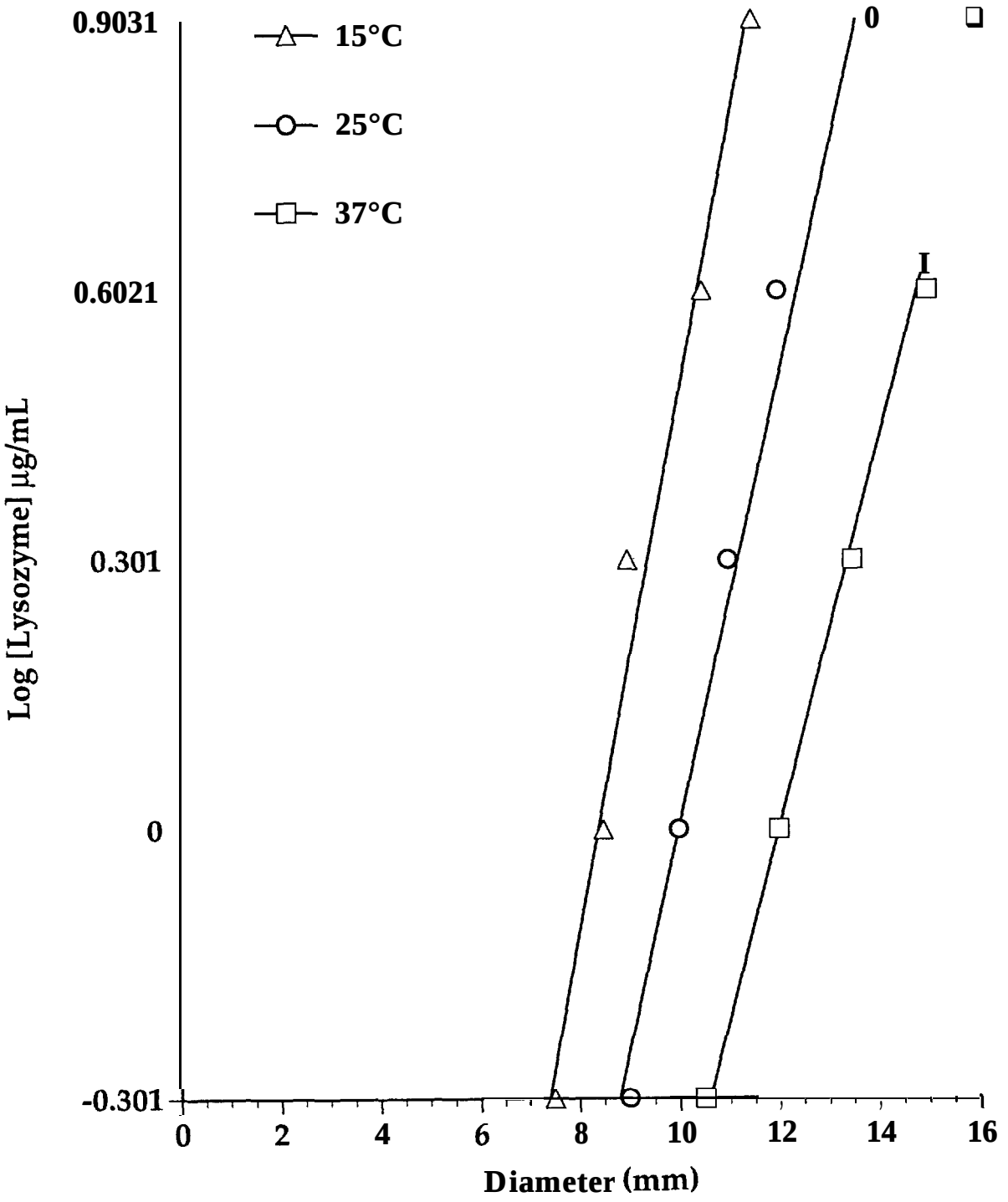

Figure 2. Effect of temperature on diameter of cleared zones produced by hen egg white lysozyme (HEWL) in the lysoplate assay. The log value for HEWL concentrations of 0.5, 1.0, 2.0, 4.0, and 8.0 µg/mL are shown on the x-axis.

Table 3. Microscopic examination of adherent leukocytes from the anterior kidney of a chinook salmon. Glass adherent cell cultures were incubated 1 h with different concentrations of FITC labeled-S. *aureus*. Cell layers were washed to remove unassociated bacteria and the number of phagocytic cells tabulated. A cell was categorized as phagocytic if at least 2 fluorescing bacteria were observed within the cell perimeter.

Concentration of *S. aureus* (bacteria/mL)	Adherent cells	
	Total	Phagocytic (%)
1 x107	140	11 (8)
	128	19 (15)
1x108	114	52 (46)
	121	54 (45)
1×10^9	111	63 (57)
	106	52 (49)

Discussion

During this reporting period, a panel of assays to assess nonspecific immune parameters of sockeye salmon was developed. Other investigators have recommended the use of assays for nonspecific host defenses to evaluate fish health (Fletcher 1986, Anderson and Siwicki 1993), or the effect of immunostimulants (Jeney and Anderson 1993). One of the objectives of this study is to determine if the defense systems of sockeye salmon are affected by immunomodulations related to rearing temperature.

The assays were intended to measure humoral and cellular functions from a single blood sample. However, the small number of phagocytic cells found in the blood of healthy sockeye and chinook salmon made it difficult to efficiently quantify phagocytic functions. Results from subsequent testing have suggested that it may be possible to substitute the anterior kidney as a source of cells for measuring different aspects of phagocytic cell function. The kidney cells would be used in the NBT, phagocytosis, and myeloperoxidase assays.

Table 4. Comparison of the relative adsorption of a bovine serum albumin-dinitrophenyl conjugate (BSA-DNP) to ten types of microtiter plates for the ELISA. Bound conjugate was detected with rabbit immunoglobulin to DNP conjugated with horseradish peroxidase (rabbit IgG-HRP), and a 15-minute substrate-chromogen (ABTS-H_2O_2) incubation at 37°C.

	BSA - DNP concentration [a]							
Microplate[b]	1000	100	50	25	10	5	2.5	0
Corning	1.064 10.86	0.936 9.55	0.804 8.20	0.642 6.55	0.408 4.16	0.257 2.62	0.164 1.67	0.098
Corning 25805-	0.713 6.42	0.892 8.04	0.798 7.19	0.712 6.41	0.388 3.50	0.192 1.73	0.151 1.36	0.111
Costar	1.283 II.88	I.091 IO.10	0.894 8.28	0.669 6.19	0.423 3.92	0.319 2.95	0.215 1.99	0.108
Dynatech Immulon	0.061 1.11	0.064 1.16	0.582 10.58	0.062 1.13	0.241 4.38	0.063 1.15	0.231 4.20	0.055
Dynatech Immulon	1.089 9.64	0.912 8.07	1.003 8.88	0.629 5.57	0.415 3.67	0.306 2.71	0.191 1.69	0.113
Dynatech Immulon	0.269 2.20	0.161 1.32	0.190 1.56	0.069 0.57	0.092 0.75	0.059 0.48	0.058 0.48	0.122
Dynatech Immulon	I.412 8.99	I.140 1.26	0.918 5.85	0.716 4.56	0.474 3.02	0.343 2.18	0.236 I.50	0.157
Nunc	1.346 9.68	1.043 7.50	0.896 6.45	0.728 5.24	0.452 3.25	0.339 2.44	0.237 1.71	0.139
Nunc	1.408 7.61	1.132 6.12	0.956 5.17	0.743 4.02	0.491 2.65	0.376 2.03	0.273 1.48	0.185
Nunc	0.213 4.02	0.177 3.34	0.152 2.87	0.125 2.36	0.094 1.77	0.074 1.40	0.059 1.11	0.053

[a] Mean absorbance and standard deviation (in parentheses) at 405 nm for six replicate wells; signal-to-noise ratio below.

[b] Corning Glass Works, Corning, NY; Costar@, Cambridge, MA; Dynatech Laboratories, Inc., Alexandria, VA; Nunc, Inc., Naperville, IL.

Numbers of phagocytic cells recovered from the kidneys of individual fish of either species were large enough to supply cells for an accurate analysis in the NBT and phagocytosis assays. A tissue imprint from the anterior kidney is also being evaluated in the myeloperoxidase assay to eliminate variability in the quality of test samples obtained from a blood or kidney smear. We will continue to use blood to measure the percent volumes of erythrocytes (hematocrit level) and leukocytes (leukocrit level).

Serum will also be separated from blood samples for complement fixation and lysozyme assays and for measurement of total or specific immunoglobulin. Previous studies have shown that the level of lysozyme varies among tissue types within a species and also among fish species. Lie et al. (1989) reported that rainbow trout had the highest kidney lysozyme activity among 15 fish species tested with the turbidimetric assay, but sockeye salmon were not included in the comparison. Among the tissues and organs of a rainbow trout, Lie et al. (1989) found that the kidney had the highest levels of lysozyme and muscle the lowest; serum ranked fifth among the eight sample types tested. Mock and Peters (1990) recommended serum as the sample of choice because the kidney is difficult to remove and the variability of lysozyme activity in kidney is greater than that in serum. We will continue to evaluate serum in the lysoplate assay rather than kidney tissue because serum can be stored frozen prior to testing and has less variability in the assay than kidney tissue.

Serum will also be used in Fish-Ig ELISAs to measure total immunoglobulin levels and changes in the levels of immunoglobulin specific to the p57 protein antigen of R. *salmoninarum*. An important consideration in developing the Fish-Ig ELISAs is the choice of microtiter plate. Variation in the adsorption of proteins to the wells of microtiter plates from different manufacturers has been reported (Kenny and Dunsmoor 1983, Shekarchi et al. 1984, Pascho and Mulcahy 1987). The results of our testing agree with those of Pascho and Mulcahy (1987) who reported that the use of the Costar 3590 provided the greatest sensitivity among seven varieties of microtiter plates in an ELISA for a soluble antigen fraction of *R. salmoninarum*.

Pascho and Mulcahy (1987) coated the wells with rabbit immunoglobulin, which may have different binding characteristics than the BSA-DNP conjugate we used: binding to the wells by the BSA-DNP conjugate was primarily facilitated by adsorption of the BSA moiety. Kenny and Dunsmoor (1983) compared the binding of BSA and human immunoglobulin to microtiter plates from several manufacturers and found that the Costar 3590 microtiter plate adsorbed both BSA and human immunoglobulin well. In contrast to results of the current study, those authors also reported high background binding of a peroxidase-conjugated goat immunoglobulin. The background binding was reduced by increasing the level of Tween 20 in the conjugate diluent from 0.05 to 1.6%. A direct comparison of the results is difficult because the conjugate diluent used in this study contained 1% Tween 20 and 3% BSA. These data suggest that the protein adsorption characteristics of the Costar 3590 microtiter plate will be acceptable for coating wells with either mouse immunoglobulin or the p57 antigen.

EFFECTS OF REARING TEMPERATURE ON IMMUNE FUNCTION IN SOCKEYE SALMON

Introduction

Experiments to examine the effects of rearing temperature on immune function are scheduled to begin during 1996. During the spring (April-May) and late summer (August-September) of years 1996 through 1998, the immunological competence of fish in each temperature group will be assessed by measuring selected factors related to specific and nonspecific host defense mechanisms. Results from these measurements will provide relative estimates of the general immune status of fish from the temperature groups and of their ability to resist infection by *R. salmoninarum.* Testing will be done annually during these two periods to separate effects of season from those of developmental stage.

Testing of the susceptibility of fish to infectious disease is scheduled to begin during 1996. During the spring (April-May) and late summer (August-September) of 1996 through 1998, subgroups of fish from each temperature group will be tested for their susceptibility to infection by either of these two fish pathogenic microorganisms: 1) *R. salmoninarum,* the causative agent of bacterial kidney disease (Fryer and Sanders 198 1); or 2) infectious hematopoietic necrosis virus (IHNV), which causes infectious hematopoietic necrosis (IHN). The principal challenge method for the *R. salmoninarum* and IHNV challenges will be a waterborne exposure.

To evaluate susceptibility to infection by *R. salmoninarum,* the relative levels of bacterial antigen in live fish from the challenge and control subgroups will be compared 100 days after exposure to the kidney disease bacterium. For the IHNV challenges, the relative susceptibility of fish in the various subgroups will be based on mortality during a 21-day period following exposure to the virus.

During this reporting period, an injection challenge for *R. salmoninarum* was evaluated as an alternative to the waterborne challenge. In addition, fish injected with the *R. salmoninarum* were evaluated as a source of infected fish that could be combined with healthy sockeye salmon in a cohabitation challenge. The relative production of bacterial antigen was compared among subgroups of chinook salmon following intraperitoneal injection of either of two levels of *R. salmoninarum.* Chinook salmon were used in this challenge because of the availability of fish large enough to permit testing of tissues from individual fish in the BKD-ELISA (described below).

Materials and Methods

Fish

Spring chinook salmon from Carson National Fish Hatchery in Carson, Washington were used for this injection challenge. Several egg lots were selected from females that tested negative for *R. salmoninarum* infection by the BKD-ELISA. The eggs. approximately 2,000 from each female, were delivered to the Northwest Biological Science Center in Seattle (NBSC) as eyed eggs during October 1993. The fish were raised in sand-filtered, UV-treated Lake Washington water and fed a pelleted moist diet *ad libitum* 5 days per week.

Preparation of Bacterial Isolate and Enumeration of Bacteria

Renihacteriurn sulmoninarum (stock isolate DWK-90) was cultured on a selective medium (Austin et al. 1983) from the kidney of a chinook salmon smolt collected from Dworshak NFH during March 1990. A pure culture of DWK-90 was grown at 15 "C for 8 days in KDM 2 broth medium (Evelyn 1977) modified to contain one-half strength cysteine (0.5 g/L) and 10% (v/v) fetal bovine serum (FBS). Bacteria were then centrifuged at 4,923 x g for 20 minutes at 4°C and the supernatant was discarded. The *R. salmoninarum* pellet was resuspended in a freezing medium made by supplementing KDM 2 broth medium with 10% (v/v) FBS and 2% (w/v) gelatin. and aliquots of the suspension were stored at -70°C.

To prepare an experimental culture, a frozen aliquot of this stock isolate was thawed and 1 mL was added to 1 liter of the modified broth medium supplemented with 1.5% (v/v) of the cell-free supernatant of KDM 2 broth which had been used to grow *R. sulmoninurum* at 15 °C (Nurse medium; Evelyn et al. 1990). The nurse medium consisted of the supematant of a 16-day broth culture, centrifuged at 5,000 x g for 20 minutes and stored at -70°C. The experimental cultures were incubated at 15 °C for 10 days. The challenge bacteria were harvested by centrifugation at 4,923 x *g* for 20 minutes at 4°C and resuspended in 0.01 M phosphate buffered saline (PBS pH 7.4) containing 0.1% (w/v) peptone (PBS-peptone).

To determine the number of viable *R. sulmoninurum* in the challenge suspension, a sample was cultured for 40 days at 15°C on charcoal agar medium (Daly and Stevenson 1985) with 1.5% (v/v) nurse medium added. The concentration of *R. sulmoninurum* in the final challenge suspension was also determined by the membrane-filtration fluorescent antibody test (MF-FAT) (Elliott and Barila 1987). The levels of *R. sulmoninurum* antigen in the kidney-spleen homogenates were measured in an enzyme-linked immunosorbent assay (ELISA) for *R. salmoninarum* antigen (BKD-ELISA that detects a soluble fraction of the bacterium (Pascho et al. 1991; ELISA II).

Challenge Procedure

Chinook salmon were assigned randomly to two challenge levels and one control group. There were 200 fish in each group, and each group of fish was placed in a single tank (1.9-m diameter) supplied with 12°C treated Lake Washington water. Two stock suspensions of *R. salmoninarum* were prepared in peptone-saline from the cells and maintained on a stirrer at 4°C. On the basis of MF-FAT bacteria counts, the high and low concentrations of challenge bacteria were adjusted to approximately 5 x 10^8 (High-BKD) and 5 x 106 bacteria/ml (Low-BKD), respectively. The *R. salmoninarum* suspension for the Low-BKD group was made by diluting the high-BKD group bacteria 1: 100 (v/v) in PBS-peptone.

To begin the experiment. fish were netted into buckets (1.56 L) of clean water supplied with oxygen, and anesthetized with 50-100 ppm MS-222. Each fish was then injected intraperitoneally with 200 µL of the appropriate bacterial challenge level; injections were done with an Eppendorf Repeater 4780 pipettor. The control group was injected with 200 µL of PBS-peptone. The order in which the groups were challenged was PBS-peptone control, low-BKD, and high-BKD.

Fish were maintained in filtered and UV-treated Lake Washington water at 12°C and fed a commercial, pelleted diet *ad libitum* 3 days per week. Twenty live fish were removed from each group every other day for analysis by the BKD-ELISA. All fish were frozen at -70°C until completion of the experiment. Any mortalities that occurred during the holding period were collected and frozen at -70°C for later processing and analysis by the BKD-ELISA. The experiment was terminated 23 days post-challenge.

For analysis by the BKD-ELISA, each fish was thawed and the kidney and spleen were removed aseptically and pooled in a 2 mL screw-cap tube (Sarstedt Inc.). Each sample was diluted 1:8 (w/v) in PBS-T20 and processed as described by Pascho et al. (1991). Twenty uninjected fish were also processed for the BKD-ELISA in an identical manner and served as prechallenge controls. Samples with a mean BKD-ELISA absorbance less than 0.074 were considered negative for *R. sulmoninarum* infection. Each fish positive for *R. salmoninarum* infection was assigned to an infection-level category as follows: a fish producing a mean BKD-ELISA absorbance in the range 0.074-o. 199 was considered to have a low infection level, a fish producing mean absorbance in the range 0.200-0.999 was considered to have a medium infection level, and one producing a mean absorbance ≥ 1 .OOO a high infection level. Mean BKD-ELISA absorbances were compared among the control and treatment groups at each sample date by the analysis of variance.

Results

The concentration of viable *R. salmoninarum* in the suspension used to inject fish in the high-BKD group was 5.5 x 10^8 bacteria/ml bacteriological culture, indicating that each fish in that group received 1.1 *x* 10^8 *R. salmoninarum.* Because this bacterial suspension was diluted 1: 100 (v/v) for injection into fish in the low-BKD group, it is presumed that those fish received *1.1 x* 10^6 *R. salmoninarum.*

Analysis of tissues by the BKD-ELISA showed that the prevalence of *R. salmoninarum* infection among fish in the control group ranged from 5 (1/20) to 20% (4/20) during the challenge. All of the fish positive for BKD infection produced BKD-ELISA absorbances that placed them in the low infection level category (Fig. 3).

Among fish tested from the low-BKD group, the prevalence of *R. salmoninarum* infection was 95% (19/20) on days 5 and 9, and 100% (20/20) on days 14, 19, and 23. Of infected fish in the low-BKD group, the progression of *R. salmoninarum* infection resulted in an increasingly higher proportion of fish with medium infection levels: 10% (2/20) on day 5. 45% (9/20) on day 9, 60% (12/20) on day 14, and 85% (17/20) on days 19 and 23. Only a single fish, sampled on day 19, had a high level of *R. salmoninarum* infection. Mean BKD-ELISA absorbances on days 19 and 23 were higher ($P \leq 0.05$) than those of the corresponding controls (Fig. 3).

The prevalence of *R. salmoninarum* infection among fish tested from the high-BKD group was 100% (20/20) on each sample date. All of these fish had medium or high infection levels, and the proportion of fish with high *R. salmoninarum* infection levels exceeded 90% on days 19 and 23. At each day of sampling, the mean BKD-ELISA absorbance for fish tested from the high-BKD group was higher ($P \leq 0.05$) than for the corresponding control group (Fig. 3).

Discussion

The injection of chinook salmon with *R. salmoninarum* demonstrated that an infection by a normally slow-growing organism may progress rapidly when the natural defense mechanisms of a fish are circumvented. Elliott and Pascho (1993, 1994) reported similar results when they injected brook trout and chinook salmon with different levels of *R. salmoninarum.* By use of the BKD-ELISA, these authors detected moderately severe infections 14 days after challenge in some fish that had been injected with as few as 6,000 *R. salmoninarum* cells.

Evaluation of the susceptibility of fish from the temperature groups to infection by IHNV or *R. salmoninarum* will require a disease challenge that better approximates a natural route of infection. There is evidence that IHNV or *R. salmoninarum* may be vertically transmitted (Mulcahy and Pascho 1984, Evelyn et al. 1986), but the mechanism by which vertical

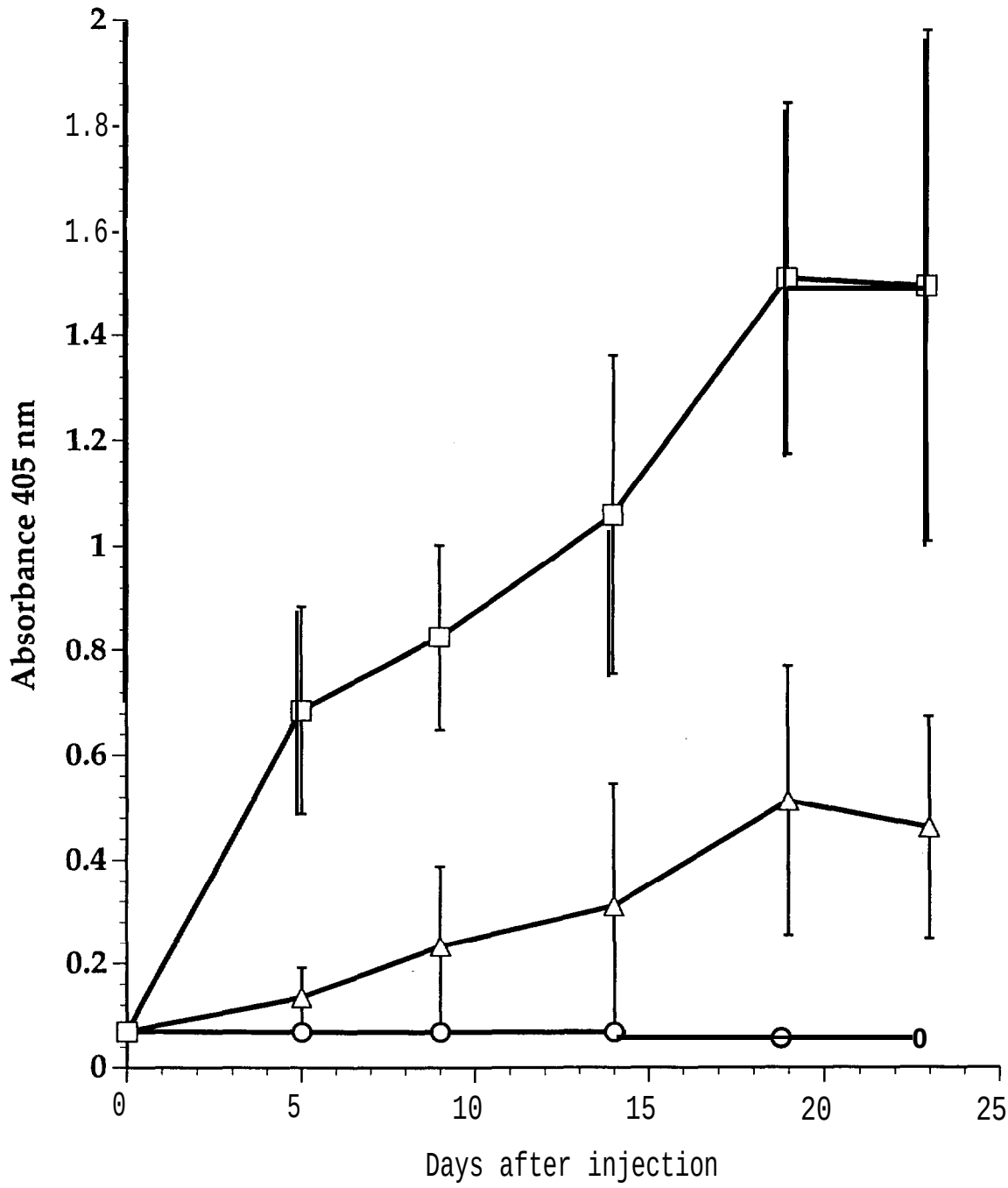

Figure 3. BKD-ELISA absorbance values for kidney/spleen homogenates from spring chinook salmon challenged with *R. salmoninarum.* Fish were injected intraperitoneally with either PBS-peptone (0 Control), 1.1 *x* 1 0^8 *R. salmoninarum* cells (□ High-BKD), or 1.1 *x 106 R. salmoninarum* cells (A Low-BKD). The mean BKD-ELISA absorbance value and one standard deviation for 20 fish is given at each time point. The lowest BKD-ELISA absorbance value considered positive for *R. salmoninarum* infection was 0.074.

transmission of these organisms occurs is poorly understood, making it difficult to develop reproducible laboratory challenge methods.

Horizontal transmission is a second route by which these microorganisms can infect susceptible fish (Elliott et al. 1989, Winton 1991), and is presumably the result of virus or bacteria being shed from infected fish residing in the same water column. Laboratory methods for the waterborne transmission of IHNV and *R. salmoninarum* have been developed (LaPatra et al. 1993, Elliott and Pascho 1994), and waterborne exposure of sockeye salmon in our temperature groups may be more suitable for evaluating their nonspecific defense mechanisms. Other investigators have reported that fish may recover from *R. salmoninarum* infections established by a waterborne exposure to the bacterium (Elliott and Pascho 1994), suggesting that a fish's defenses are not circumvented by this exposure route.

The source of a pathogenic microorganism in a laboratory waterborne exposure may be microorganisms grown in the laboratory and placed in the challenge water, or microorganisms shed from infected fish cohabitating with the healthy test fish. The fish from the high-BKD injection group in this experiment may have developed sufficient levels of infection to serve as a source of *R. salmoninarum* for a cohabitation challenge with the sockeye salmon in the temperature groups.

Elliott and Pascho (1993, 1994) reported the transmission of *R. sulmoninurum* to healthy chinook salmon and brook trout in a cohabitation challenge with other chinook salmon previously injected with the kidney disease bacterium. They were also able to detect *R. sulmoninurum* cells shed from injected fish into the water during the challenge. Antigen levels in injected fish in that study had mean BKD-ELISA absorbances similar to those produced by fish in the high-BKD group about 15 days after injection. These data have provided a quantitative analysis of the progression of *R. salmoninarum* infections among injected fish, and suggest that a cohabitation challenge may be a suitable alternative for *R. sulmoninurum* waterborne challenges scheduled during 1996.

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PART IV

PROGRESS IN COMPARING THE EFFICACY OF AZITHROMYCIN AND ERYTHROMYCIN AS THERAPEUTIC FEED ADDITIVES TO CONTROL BACTERIAL KIDNEY DISEASE IN CHINOOK SALMON

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INTRODUCTION

In 1987, the Pacific Northwest Fish Health Protection Committee ranked bacterial kidney disease (BKD) as the largest deterrent to successful culture of salmonids in the Pacific Northwest. In 1993- 1994 this disease was responsible for catastrophic losses of endangered Redfish Lake sockeye salmon being held as captive broodstock, and many other cases of severe mortality due to BKD have been reported (Bullock and Herman 1988, Murray et al. 1992).

Erythromycin has been the primary antibiotic used by fish culturists in attempts to prevent and control BKD in salmonids; however, this drug has not been an effective chemotherapeutant against this disease. Azithromycin is a new macrolide antibiotic that concentrates in polymorphonuclear leukocytes (Wildfeuer et al. 1994), macrophages, and fibrocytes (Physician's Desk Reference 1995). These cellular elements have been reported to sequester and protect *Renihacterium salmoninarum (Rs)* , the causative bacterium of BKD (Young and Chapman 1978).

Most antibiotics, including erythromycin, do not penetrate tissues well, but after administration orally or parenterally, they are bound to serum proteins and remain in extracellular spaces (Harvey 1975). Azithromycin is rapidly absorbed in tissues and is widely distributed at higher concentrations in cells than in plasma or in serum (Physician's Desk Reference 1995).

Numerous studies have reported greatly increased intracellular uptake and superior antibacterial activity of azithromycin over erythromycin in in vitro and in vivo studies with animals other than fish (Fass 1993, Strachunskii et al. 1993, Hof 1994). Rakita et al. (1994) measured the intracellular activity of azithromycin and erythromycin against several enteric pathogens that had been phagocytosed by neutrophils. Azithromycin was effective in reducing the intracellular viabilities of nearly all strains tested; however, erythromycin was not as effective as azithromycin.

The authors suggested that the concentration of azithromycin in neutrophils may be particularly useful in treating infections caused by intracellular pathogens. Blais et al. (1994) compared the intracellular activity of azithromycin and erythromycin against an intracellular protozoan parasite, *Toxoplasma gondii*, and reported superior performance of azithromycin. Azithromycin accumulated readily and remained inside macrophages infected with the protozoan, interfering with growth of the parasite.

Niki et al. (1994) compared the protection of azithromycin to erythromycin in mice infected with *Chlamydia pneumoniae* and observed 100% survival in mice given azithromycin; however, 100% of the erythromycin-treated mice died within 10 days post-infection. Pajukanta et al. (1992) reported that the in vitro antibacterial activity of azithromycin against *Actinobacillus actinomycetemcomitans* was highly effective, inhibiting 100% of the strain tested, while erythromycin activity was poor.

There are no publications or current studies using azithromycin in fish to our knowledge. Therefore, the objective of this experiment was to compare the effectiveness of azithromycin and erythromycin in reducing mortality due to BKD infection of chinook salmon *(Oncorhynchus tshawytscha*).

MATERIALS AND METHODS

Fish

Approximately 2,000 1994-brood Minter Creek fall chinook salmon were transported to the NMFS Manchester Marine Experimental Station on 10 April 1995. Fish (approx. 3.4 g body weight) were placed in a 4.9-m^3 rectangular holding tank and were maintained on a 1.5-mm semi-moist salmon grower feed (BioDiet) at a ration of 2.0% body weight per day for 17 days. Water from an adjacent stream, Beaver Creek, was supplied to the holding tanks.

Preparation and Feeding Medicated Diets

Test feeds were prepared at the Northwest Fisheries Science Center in Seattle, Washington, during April 1995. We milled two nonmedicated control feeds comprising treatments A and B, and three medicated feeds comprising treatments C, D and E (Table 1). We added powdered azithromycin dihydrate (Zithromax®; Phizer Laboratories Inc., NY., NY.) to treatments C and D and erythromycin phosphate to treatment E. Target concentrations in fish (Table 1) were based on the following: 6.5 fish per tank. 4.9 g mean weight, 2.9% body weight/day ration, 15% added weight (10% water plus 5% krill), 5 replicates/treatment, 14 days of medication, and 8 10 μg/mg active ingredient of erythromycin phosphate.

The quantities of antibiotics shown in Table 1 were dissolved in 50 mL of 95% ethanol (5.0% of each feed, w:w) and were brought up to a final volume of 150 mL by adding water (10% of feed, w:w). The 150 mL of antibiotic solution was then sprayed and mixed into 1 kg portions of #3 BioDiet starter mash for each feed after milling nonmedicated control feeds. Separate spray bottles were used for each test feed. Control feeds, A and B, received 10% (w:w) water only. Antibiotics were sprayed and feeds were pelletized in ascending order (A > B > C > D > E) to prevent cross-contamination.

Approximately 2 kg of starter mash was run through the pellet machine after each test feed was pelleted. The pellet machine was then cleaned, and the first 100 g of the following test feed was discarded to reduce the possibility of contamination. Finished pellets were placed in a drying oven for 1 hour to evaporate residual ethyl alcohol. No residual alcohol odor could be detected on the dried pellets. Pellets were then frozen at -20°C overnight. For palatability, all finished feeds were top-dressed with 5.0% (w:w) homogenized krill (*Euphausia pacifica*), which was poured and mixed evenly into the test feeds. Top-dressed feeds were held at -20°C before removal daily for feeding.

Table 1. Test feeds.

Treatment	No. Tanks	Antibiotic	Concentration in Fish (ppm)	Concentration in Diet (ppm)	Concentration Fed to Fish (ppm)
A	5	Nonchallenged-Control	0	0	0
B	5	Challenged-Control	0	0	0
C	5	Azithromycin	20	690	20
D	5	Azithromycin	50	1,724	50
E	5	Erythromycin	90	3.106	92

Twenty-five covered, circular (24.1-cm radius X 26.7 cm-height; 0.05 m^3) tanks were filled to a volume of 40 liters and supplied uniformly with approximately 7.5 liters per minute (2 gpm) of Beaver Creek water. Each tank was stocked with 65 randomly selected fish on 27 April 1995 after euthanizing 75 from the stocking population to measure any baseline levels of *Rs* present in kidney tissues. Euthanized fish and all subsequent mortalities were measured for fork length and weighed, and spleen and kidney tissues were streaked onto trypticase soy agar (TSA) plates. Wet mounts of gill and skin scrapings were observed microscopically, and kidney tissue imprints were made onto microscope slides, heat-fixed, methanol-fixed, and stored at 4°C for fluorescent antibody technique (FAT) analysis. Remaining kidney tissues were bagged, labeled, and frozen at -70°C for potential analysis of *Rs* absorbance levels by enzyme-linked immunosorbent assay (ELISA).

The five treatments (five replicates per treatment) were randomly assigned to the 25 tanks. Test fish were acclimated to the tanks for 5 days on 1.5-mm semi-moist BioDiet pellets fed at a ration of 2.0% body weight/day. No mortality occurred before or after stocking, prior to medication.

Feeding of the test diets was initiated on 3 May 1995 at rations of 2.9% body weight/day for 14 days, ending on 16 May 1995. The day after medication on 17 May 1995, all fish were weighed and tank populations were enumerated. Mean weights, ranging from 6.80 g to 6.96 g, were very similar and were not significantly different (ANOVA; P = 0.6612) demonstrating that all fish had consumed the test feeds as visually observed.

Renibacterium salmoninarum Challenge

On 1 June 1995, fish from the 20 tanks assigned to treatments B, C, D, and E were challenged by immersion for 24 hours in tanks containing 40 liters of static water supplied with oxygen. Stock *Rs* was collected, cultured, and quantified by microbiologists at the National Biological Service, Northwest Biological Service Center in Seattle, WA. The isolate, ATCC-33209 (fourth passage). was cultured in duplicate 1 -liter flasks containing KDM 2 broth growth medium incubated for 9 days at 15°C prior to the challenge (23 May to 1 June 1995).

Duplicate samples of the log-phase cultures were preliminarily quantified by FAT at serial dilutions (v:v) of $1\ 0^{-5}$, $1\ 0^{-6}$, and 10^{-7} in phosphate-buffered-saline (PBS) containing peptone saline (PBS-peptone). Simultaneously, quadruplicate aliquots (0.1 mL) of stock *Rs* were inoculated onto KDM 2 plates (100 X 15 mm) to determine final bacteria concentrations at a later date.

Based on the FAT counts, we projected final tank concentrations to be 3.0×10^6 *Rs* cells/ml. Upon quantifying the KDM 2 plates after a 6-week incubation period at 15°C (1 June to 13 July 1995), we determined that the stock *Rs* concentration was 3.87×10^9 *Rs* cells/mL resulting in final tank challenge concentrations of $2.90 \times 1\ 0^6$ *Rs* cells/ml. We added 30 mL of stock *Rs* to 300 mL of PBS-peptone in 500-mL Erlenmeyer flasks to each of the challenge tanks. The five control flasks. destined for the non-challenged control group replicates (treatment "A"), received 300 mL of PBS-peptone only. Control flasks were filled before handling bacteria and placed in a separate cooler, and all flasks were transported on ice to the hatchery facility at Manchester.

The PBS-only control flasks were poured into the five control tanks before adding bacteria to challenge tanks. Control tanks were covered with plastic during the challenge. The fish were carefully observed overnight, and oxygen cylinders were changed as necessary during the 24 hour, static challenge. Water temperatures in tanks ranged from 13 to 15°C during the challenge. No mortalities occurred during the challenge; however, several fish that jumped through small cracks in tank lids during and after the challenge were discarded from the analysis.

Transfer of Fish to Seawater

On 9 June 1995, fresh water was turned off and raw, unfiltered seawater (7.5 liters/minute) was introduced to the tanks. Mortalities caused by osmoregulatory stress began occurring in all tanks 4 days later (13 June), peaked on the second day, and ended 9 days later on 21 June. Mortalities after transfer to seawater ranged from 15.1 to 20.0% among treatments and were not significantly different (ANOVA; $P = 0.9098$). No disease organisms or gross pathological signs were observed in saltwater transition mortalities (BKD analyses pending). Mortality caused by osmoregulatory dysfunction during saltwater transfers in this study closely followed that observed in multiple stocks of chinook salmon in other studies. Mortality due to osmoregulatory stress typically stops within 10 days and rarely continues past 15 days post-saltwater transfer (Mahnken and Waknitz 1979, Prentice et al. 1980).

Before this study began, we discussed the possibility of vaccinating against *Vibrio anguillarum* or treating with antibiotics in the event that a vibriosis epizootic were to occur during the study. We elected to not vaccinate or treat fish with antibiotics to prevent confounding of the independent variable being studied. After a period of 7 days with no mortalities, we detected *V. anguillarum* in wet mounts and in TSA agar cultures from mortalities examined on 29 June 1995. The diagnosis was confirmed by bacterial growth inhibition around 6.4 mm concentration disks, which were permeated with vibrio-specific 2,4-diamino-6,7-di-iso-propyl pteridine phosphate.

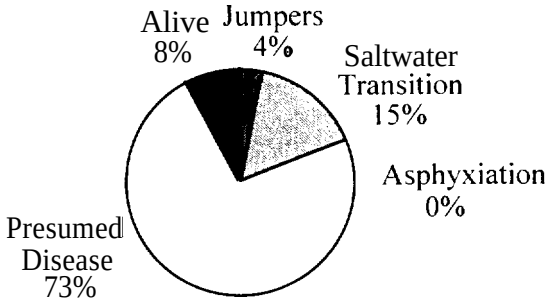
Unfortunately, vibriosis mortality has continued since the original episode, with cumulative mortality in all treatments currently converging at approximately 85%. Kidney tissue samples from all vibriosis mortalities not severely decayed are being prepared for BKD analyses. In addition to vibriosis mortality, four incidents of mortality due to asphyxiation occurred due to mussel shells clogging raw saltwater inflow pipes. The tanks involved (Fig. 1) were discarded from mortality analyses as they occurred.

RESULTS

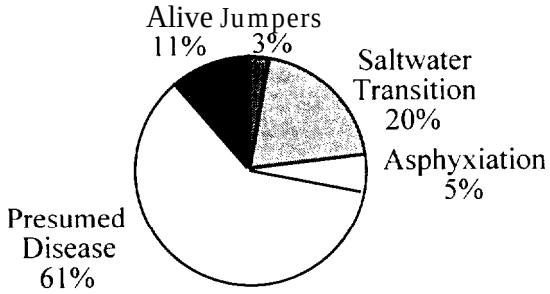
Mortality in all treatments rose uniformly to approximately 20% (due to saltwater transition and fish jumping from the tank), stopped completely for 7 days, and then began to diverge among the treatments due to vibriosis 64 days post medication (Fig. 2). By excluding the non-disease factors contributing to mortality, a clearer picture of disease-related mortality can be observed (Fig. 3). As can be seen, disease mortality in fish fed azithromycin, particularly the high dose treatment ("D"), was delayed as much as 3 months post medication.

We also compared the time to reach 20, 50, and 80% mortality (days post-medication) among the treatments (Fig. 4). The time required to reach 20% mortality was significantly ($P < 0.01$; Fisher's PLSD) longer for chinook salmon fed the high dose of azithromycin (86 days) than the time to 20% mortality of fish in all other treatments (70 to 75 days). The time required to reach 50% mortality was also significantly longer for chinook salmon fed the high dose of azithromycin (93 days $P < 0.05$; Fisher's PLSD) than for non-medicated control fish (treatments A and B) and fish fed erythromycin (72 to 80 days), but was not significantly different than for fish fed 20 ppm azithromycin (83 days). No significant differences ($P > 0.10$) were detected in the time to 80% mortality among treatments; however. the time required for fish fed the highest dose of azithromycin to reach 80% mortality (102 days post-medication) was longer than that of all other groups (83 to 99 days).

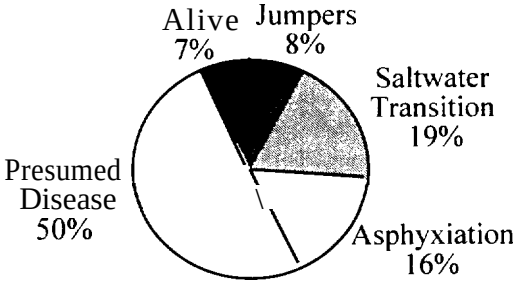
Nonchallenged, Nonmedicated Control Treatment "A"



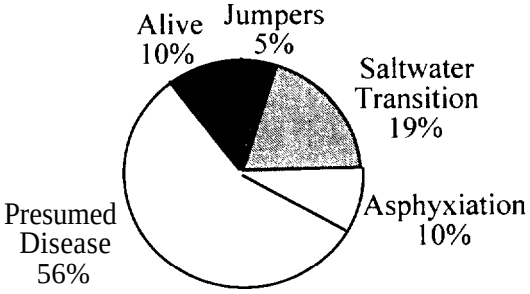
Challenged Nonmedicated Control Treatment "B"



20 ppm Azithromycin Treatment "C"



50 ppm Azithromycin Treatment "D"



90 ppm Erythromycin Treatment "E"

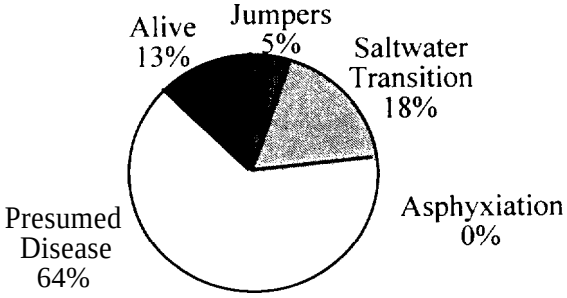


Figure 1. Factors contributing to overall mortality in the experiment

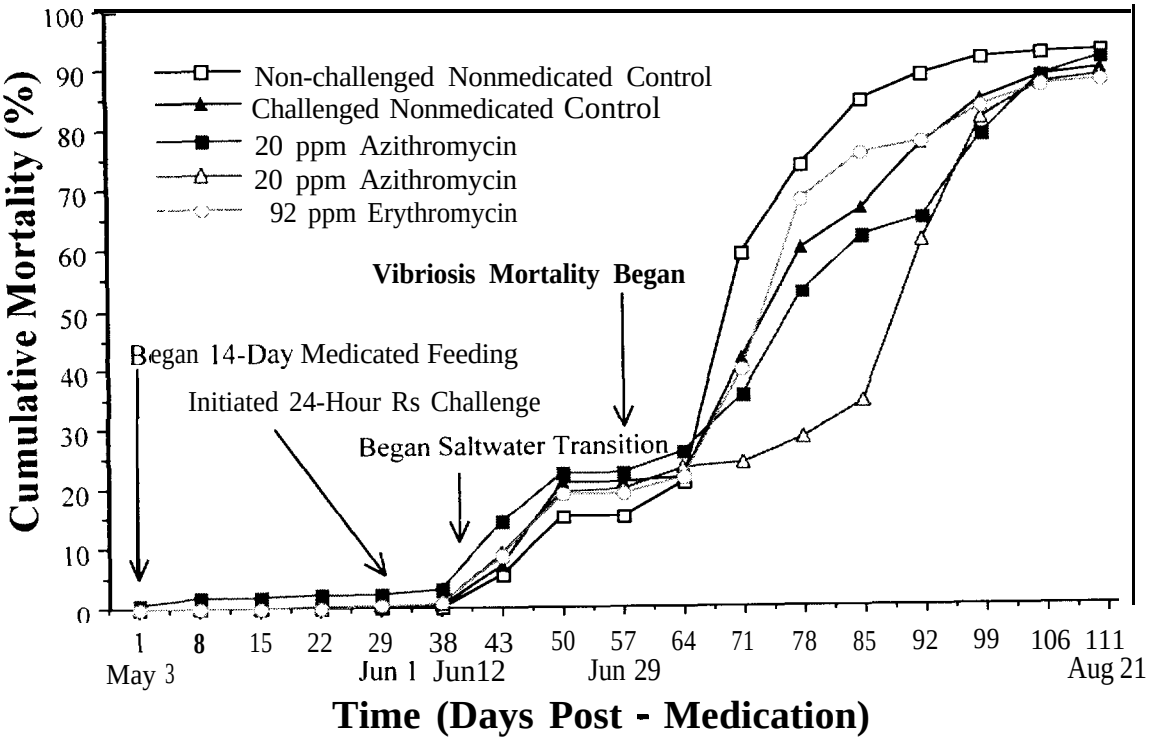


Figure 2. Cumulative mortality of fish among all treatments. Mortalities due to asphyxia were excluded.

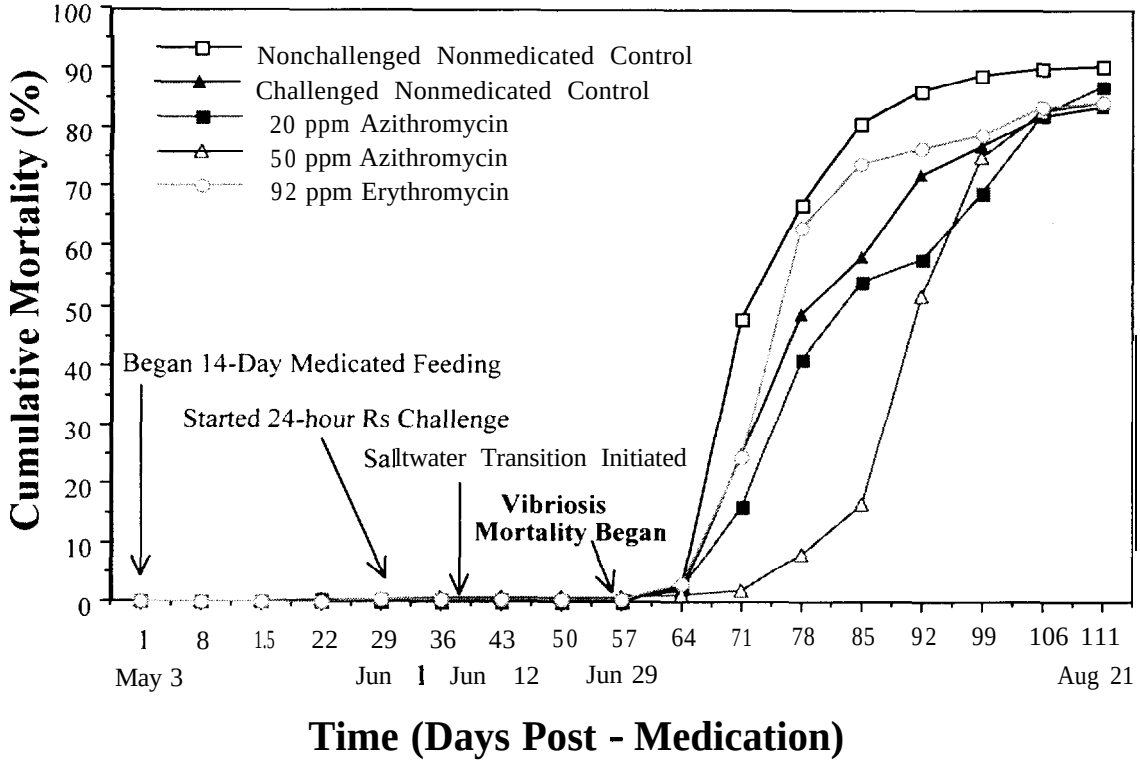


Figure 3. Cumulative mortality due to *Vibrio anguillarum* infection in all treatments

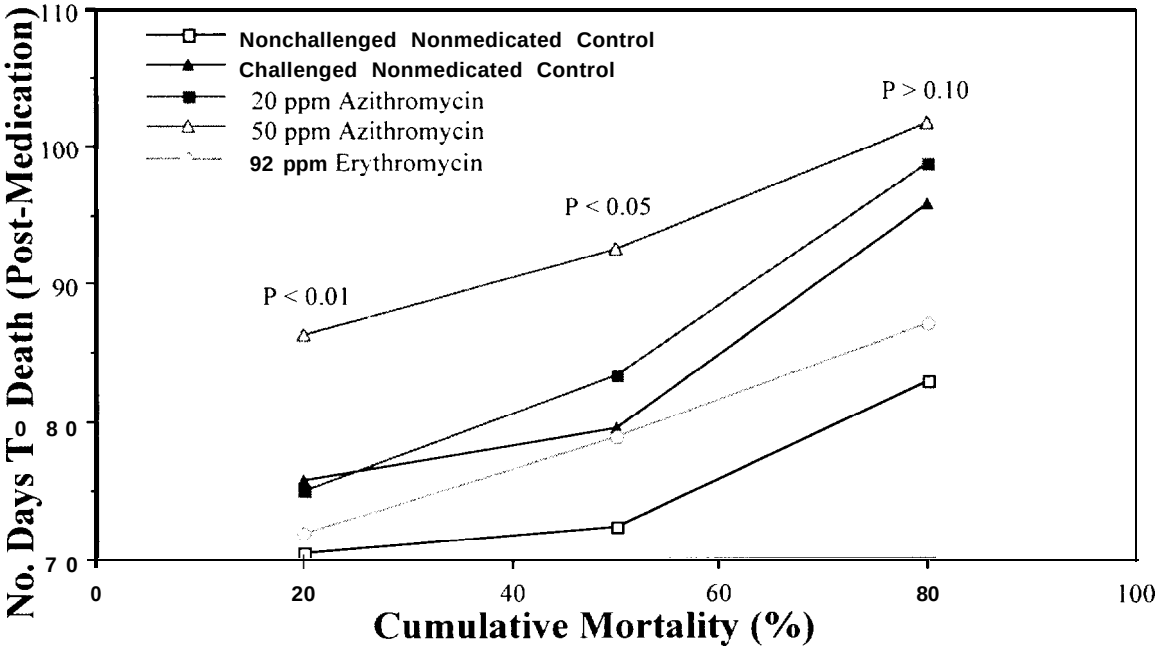


Figure 4. Days to 20, 50, and 80% mortality among all treatments. Time to 20% mortality for fish treated with the highest dose of azithromycin was significantly longer than that of all other treatments. Time to 50% mortality for fish treated with the high dose of azithromycin was significantly longer than for control or erythromycin-treated fish, but shorter than for fish treated with the low dose of azithromycin.

DISCUSSION

Although mortality due to vibriosis has hindered our ability to discern efficacy of azithromycin against BKD, the prolonged (nearly 3 .5 months post-medication) period of anti-vibrio activity in fish fed 50 mg/kg for 2 weeks was particularly encouraging and strongly suggests that further investigation of this drug is warranted. This is the first investigation of azithromycin's antibacterial activity in fish to our knowledge, and the duration of antibacterial protection observed from its use for a single feeding period was considerably longer than that described in any published or unpublished literature. The comparatively high intracellular activity of azithromycin to other antibacterial drugs, combined with our observations on the prolonged duration of antibacterial activity, are two necessary prerequisites for effective chemotherapy to control BKD.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Ron Pascho of the National Biological Service for quantification of *Renibacterium salmoninarum* in cultures used to challenge fish for this study.

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PART V

CAPTIVE SALMON BROODSTOCK NUTRITION RESEARCH

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EFFECTS OF FEEDING A NUTRITIONALLY ENHANCED DIET DURING THE YEAR BEFORE SPAWNING ON SURVIVAL AND REPRODUCTIVE PERFORMANCE OF SOCKEYE SALMON

Introduction

In our literature review, we found evidence suggesting that the natural diet of sockeye salmon differs substantially from that of coho or chinook salmon during the adult, marine phase of the life cycle of the fish. Presumably, this diet results in different intake of a number of essential nutrients, including carotenoid pigments. This evidence is supported by anecdotal reports of beneficial results with Pacific salmon when krill or squid is included in the diet fed to adults.

Although the causative dietary components have not been identified, we believe that the first phase in developing a sockeye salmon broodstock feed is to compare reproductive performance between fish fed conventional and enhanced diets. The enhanced diet will be fortified with all of the nutrients and ingredients suspected of being beneficial to the fish. If reproductive performance of the fish is increased, a second phase of work can begin to identify specific components of the enhanced diet responsible for increased reproductive performance. In this experiment, we formulated and are testing an enhanced diet containing elevated levels of vitamins, a dietary protein and energy ratio that mimics the natural diet of the fish, and a significant proportion of unprocessed crustacea, mainly frozen krill.

Methods and Results

Fish and Rearing

Sockeye salmon of the Lake Wenatchee stock (199 1 brood year) were reared in fresh water at the University of Washington Seward Park Hatchery from March of 1995 to the end of the experiment. Prior to being transferred, the fish were raised at the University of Washington Big Beef Creek Research Station near Seabeck, Washington. All fish were implanted with PIT tags and allowed to recover from this procedure for several weeks before transfer. After arriving at the Seward Park Hatchery, the fish were placed in circular concrete ponds (13-m diameter), each with untreated Lake Washington water (400 L/min).

The lower water intake for the Seward Park Hatchery was used to deliver cool water (11-12°C) to the ponds. Each pond was divided into four pie-shaped sections separated by Vexar screen (6.35-mm mesh) attached to PVC pipe frames. Water was delivered to each section through a manifold system along the outside wall of each pond, with water flow directed from the outside wall toward a center standpipe drain. The directed flow prevented feed from drifting between sections.

Each section was considered a replicate, in a Latin-Square Design, with the replicates moving from section to section through time. The fish were individually weighed upon transfer (Table 1), and triplicate rearing groups were assigned to the two experimental diets. Because fewer fish were available for the study than originally planned, monthly sampling was dropped from the experimental design. Thus, only initial and final sampling will be done. A disease outbreak occurred in late May, causing some mortality. The bacterial pathogen responsible for the mortality was identified as *Aeromonas hydrophilus,* and after the fish were promptly treated with Medadyne (oxytetracycline hydrochloride) by intraperitoneal injection, mortality stopped.

Experimental Diets

Two diet formulations were developed for this study, with each based upon the Abernathy salmon diet formulation 19-2. However, each formula was slightly modified to reflect feed-ingredient availability and new information concerning recommended levels of essential nutrients (Table 2). The ingredients, minus the added fish oil, were ground, mixed, and pelleted into 6.35-mm diameter pellets using a laboratory California Pellet Mill operated without steam. After drying without heat overnight, the pellets were top-dressed with fish oil to achieve the final level specified in the formulation. Feeds were made monthly and stored at room temperature until use. Fish were fed to apparent satiation twice per day, 5 days per week.

Sampling and Analysis

Our experimental plan called for monthly sampling to measure growth and changes in somatic and gonadal composition. However, fewer fish were available for the study than originally planned. Therefore, only a final sampling will be done in October 1995, when nine female fish from each dietary treatment group will be removed for tissue and whole body sampling. Frequent handling is thought to be stressful on the fish; therefore, less handling may reduce mortality over the course of the experiment. Because the central goal of this experiment is to examine the effect of diet on sexual maturation and gamete quality, it was necessary to modify the sampling schedule to increase the chance that sufficient numbers of fish would survive to the spawning stage.

Fish from each dietary group will be spawned in a replicated matrix design (Split Plot Design) so that eggs from each female are fertilized with milt from three males from each dietary treatment, and vice versa (Table 3). It is anticipated that this robust design will allow detection of main effects (e.g. diet, or male or female parent) and interactions (e.g. diet x female, diet x male, male x female, etc.). A total of 18 males and 18 females (9 from each dietary treatment group) will be spawned, and survival of offspring to swim-up (end of endogenous feeding, beginning of exogenous feeding) will be enumerated.

At spawning in early October 1995, all fish in the tank will be identified by PIT-tag number and weighed to determine growth and survival to spawning. The first nine mature males and females removed from each dietary treatment group will be used in the spawning matrix. The remaining fish will be sacrificed and spawned. If the results of this experiment suggest a positive

effect of diet on survival of adults to spawning, and on quality and hatchability of offspring, a new study will be designed to begin in fall 1995. This new study will involve fortification of the vitamin and mineral supplements used in the previous years' study.

Table 1. Mean weight of sockeye salmon at initial stocking (April 1995) for experiment designed to test the effects of nutritionally enhanced diets on reproductive performance.

Tank Section	Diet[a]	Number of fish	Mean wt. (g) ± S.D.
1	1	35	874.2 ± 189.8
2	2	35	935.2 ± 187.8
3	1	35	962.5 ± 200.0
4	2	35	928.1 ± 141.6
5	1	35	923.8 ± 197.8
6	2	35	945.7 ± 186.8

[a] Diet 1 = enhanced Abernathy 19-2 Diet 2 = standard Abernathy 19-2

Table 2. Composition of experimental diets used to examine the effects of nutritionally enhanced diets on reproductive performance in sockeye salmon broodstock.

Ingredient	Diet 1 (%)	Diet 2 (%)
Herring meal	50.00	50.00
Blood meal	5.00	5.00
Wheat gluten	0.00	4.00
Wheat mids	13.22	16.22
Fish oil	9.00	9.00
Vitamin C	0.50	0.10
Choline	0.58	0.58
TM salt	0.10	0.10
Vitamin premix	3.00	1.50
Poultry BP meal	2.50	6.50
Permapel	2.00	2.00
Krill (dry meal)	0.00	0.00
Krill (Frozen)	10.00	0.00
Whey	4.00	5.00
Selenium yeast	0.01	0.00

Table 3. Split Plot Design for determining differences in egg and sperm quality related to dietary treatment. The XX denotes the mating of the female (left) with the male (top). Each cross is composed of 300 eggs.

Females	Males					
Treatment	Diet 1 Ml	Diet 1 M2	Diet 1 M3	Diet 2 Ml	Diet 2 M2	Diet 2 M3
Diet 1 F1	x x	x x	x x	x x	x x	x x
Diet 1 F2	x x	x x	x x	x x	x x	x x
Diet 1 F3	x x	x x	x x	x x	x x	x x
Diet 2 F1	x x	x x	x x	x x	x x	x x
Diet 2 F2	x x	x x	x x	x x	x x	x x
Diet 2 F3	x x	x x	x x	x x	x x	x x

ARTEMIA AS FOOD FOR FIRST FEEDING SOCKEYE *SALMON (ONCORHYNCHUS NERKA)*

Introduction

The use of live prey organisms, primarily zooplankton, as food for larva1 fish has generally been limited to fish species which cannot be reared on prepared feeds. Brine shrimp (Artemia sp.) and rotifers (*Brachionus plicatilis*) are the two most commonly cultured prey organisms for feeding fish larvae without developed digestive tracts (Watanabe et al. 1983, Bengtson et al. 1991). These prey organisms are generally fed to the fish larvae until they can be weaned on to less labor-intensive prepared feeds.

Salmonids, unlike fish species with larval stages, have a fully developed digestive system at the first feeding stage and are normally fed prepared diets immediately at the swim-up stage. However, several advantages may exist in using zooplankton as a food source for salmonids, for example:

1) Growth rates of first-feeding rainbow trout fed *Artemia* nauplii have been reported to be greater than those fed commercially available salmonid diets (Greg Hudson, Domsea Broodstock, Inc., Pers. commun., August 1994) and first-feeding coho salmon fed adult *Artemia* also had higher growth rates than fish fed commercially prepared feeds (Kim et al. 1996).

2) Fish learn to feed on live food. This may be advantageous for fish that are later released to the wild for enhancement purposes. Salmon raised in hatcheries have a higher survival rate from egg to smolt (or to release) than do wild fish, but high mortality occurs after hatchery release. This high mortality rate may be partially related to hatchery rearing and feeding practices, which do not prepare the fish for survival in the natural environment after hatchery release. Thus, by feeding zooplankton or other live prey organisms, hatcheries may be able to produce fish which are better adapted to the natural environment and more likely to survive than fish fed solely on pelletized feed.

3) There may be increased nutritional benefits for the fish. Carotenoids have been reported to have a positive effect on the growth rate of first feeding Atlantic salmon (Torrissen 1984) and are found in high *levels* in *Artemia* (Gallagher and Brown 1975, Bengtson et al. 1991). *Artemia* also have high contents of free amino acids (Dabrowski and Rusiecki 1983) and proteolytic enzymes (Bengtson et al. 1991) which may be beneficial to first feeding fish. However, *Artemia* are also known to be deficient in certain essential amino acids and fatty acids (Watanabe et al. 1983, Bengtson et al. 1991) as well as certain vitamins (Bengtson et al. 1991). Fortification of these nutrients in the *Artemia* may be necessary for maximum fish growth.

The purpose of this study was to evaluate the use of *Artemia* as a live food source for sockeye salmon, with weight and length gain, and survival over a period of 8 weeks as the criteria for evaluation.

Methods

Experimental Diets

Live *Artemia* adults were obtained from San Francisco Bay (Bayou Aquatic Foods and Supplies, Ontario, CA). Adult *Artemiu* were reared in refrigerated (4-6°C) water at 35‰ salinity. Adult *Artemia* were fed 200,000 cells/mL of microalgae *(Nanochloropsus* sp.) to maintain nutritional value for 24 hours prior to feeding to sockeye fry. *Artemia eggs* were also of San Francisco Bay origin and were obtained from the same commercial supplier who supplied the adult *Artemia* (Bayou). The eggs were hatched at 28°C and 35‰ salinity under continuous aeration and illumination. The *Artemia* nauplii were fed to the fish as first instars (24-hour incubation) and as third instars (24-hours post hatch).

Since *Artemia* nauplii do not begin to feed until the second instar stage (12-hours post hatch), only third-instar nauplii were fed microalgae (*Nanochloropsus* sp.) to maintain nutritional value prior to feeding to sockeye fry. Biodiet starter (Bioproducts Inc., Warrenton, OR) is the standard practical diet most commonly fed to first-feeding salmon in enhancement hatcheries and was used as the standard in this study. Both Biodiet starter #2 and #3 were used. All diets were fed on an equal, dry-weight basis at a feeding rate in excess of what the fish could eat (6% body weight /day). This feeding rate varied slightly due to *Artemia* availability (poor hatch rate on some days reduced the amount that could be fed to all tanks).

Fish and Feeding

On 15 February 1995, approximately 1,000 sockeye salmon fry of the Lake Wenatchee stock (1994) were obtained from the National Biological Service hatchery facility in Seattle, WA. The yolk-sac fry were from a single parental cross and averaged 0.18 g (± 0.005 SD) in weight at the start of the study.

On 2 1 February 1995, the fry reached the first feeding stage and were counted, weighed, and randomly distributed (50 fish/tank) into 12 12-L tanks. Each tank was supplied with 1 .O L/min of dechlorinated city water that increased in temperature from 8 to 11°C over the experimental period (8 weeks). Fish were fed the experimental diets (3 replicates/diet) to excess daily, 6 days per week. The fish receiving the live diets were fed once per day, since the *Artemia* remain alive and active for at least 8 hours, while the Biodiet starter was fed 16 times per day. At the end of the day, the center standpipes in each tank were replaced with standpipes with 3-mm mesh, and the tanks were drained to remove residual food. At the beginning of the study and at weekly intervals,

each group of fish was counted and bulk-weighed. Individual fork lengths were measured at the start and end of the feeding trial (8 weeks).

Fish and Feed Sampling

At the start and after 8 weeks, 10 fish were removed for determination of whole body proximate and elemental composition. Feed samples from each treatment were taken weekly for proximate and elemental analysis. Moisture, protein, crude lipid, and ash were measured using Association of Official Analytical Chemists (AOAC 1990) procedures: moisture by drying samples at 105°C to a constant weight. protein by measuring Kjeldahl nitrogen, crude lipid by Soxhlet extraction using methylene chloride, and ash by heating for 5 hours at 550°C in a muffle furnace. Mineral composition for fish and diet samples were determined by plasma emission spectrophotometry (Jarrel-Ash Plasma Atom Comp, Waltham, MA) according to methods described by Hardy and Shearer (1984).

Data Analysis

Average weight, instantaneous growth rates, and survival were determined weekly for the duration of the feeding trial. Data was subjected to ANOVA, and significant differences ($P < 0.05$) among mean values were determined using the Student-Newman-Keuls (SNK) multiple mean comparison test (Zar 1984). ANOVA and the SNK test were performed using SuperANOVA[a] software (Abacus Concepts, Inc., Berkeley, CA).

Results and Discussion

Sockeye salmon fed adult *Artemia* were significantly larger than fish fed other test diets at the end of 8 weeks (Table 4). Fish fed Biodiet starter were significantly higher in average weight than fish fed third-instar *Artemia* nauplii, but not than fish fed first instar *Artemia* nauplii. While significant differences were found among dietary treatments at the end of 8 weeks, fish grew well on all diets for most of the study (Fig. 1). The fish fed third-instar nauplii were significantly smaller than the other treatment groups at the end of 8 weeks, but were approximately the same weight as fish fed the other live diets up to the fourth week of the study. Conversely, fish fed Biodiet were the smallest in average weight up to the fifth week of the study.

Compared to an earlier study in which coho salmon fry were fed *Artemia-based* diets (Kim et al. 1996), the sockeye fry receiving adult *Artemia* did not show improvement in length and weight after 2 weeks of feeding. It was only after the sockeye, which were approximately one-half the weight of the coho fry at first feeding, reached a weight of 0.35-0.4 g that differences in average weight among dietary treatment groups were apparent. This suggests that adult *Artemia,* which are 10- to 15-mm in length, may be too large for first-feeding sockeye to efficiently catch and consume.

Growth rates, expressed as percent body weight gain per day (%BWG/day), increased with time in all treatment groups through week 6 and then leveled off through week 8 (Table 5). At 2 weeks, the growth rates were significantly higher in all groups fed *Artemia* than in the group fed Biodiet. By the fourth week, the growth rates were significantly higher in groups fed either adult *Artemia* or Biodiet than in groups fed *Artemia* nauplii.

The diets varied in proximate composition (Table 6), as in our previous study (Kim et al. 1996). Percent moisture was similar in the adult *Artemia* (90.6%) and third-instar *Artemia* (91.4%) but was lower in the first-instar *Artemia* group (83.7%). The moisture content of the Biodiet starter (2 1.5%) was much lower than that of the live feeds. Protein content also varied among treatment diets. Adult *Artemia* had the highest percent protein on a dry weight basis at 57.0%, while first- and third-instar *Artemia* had a slightly lower protein composition at 54.4 and 55.5%, respectively, than observed previously.

The minimum commercially guaranteed value for protein in Biodiet starter diet is much lower (45%) than that found in live *Artemia.* Percent lipid in the diets was also measured and was found to be lower in the adult *Artemia* (10.1%) than in the other diets. Lipid levels in the other diets were 18.2, 18.6 and 16.0% for first instar *Artemia,* third instar *Artemia* and Biodiet, respectively. Ash and elemental analysis is in progress and will be completed fall 1995.

The proximate composition of fish after 8 weeks of feeding showed similar trends to those seen in the diets, with the exception of moisture (Table 7). Fish fed the adult *Artemia* had a higher percent body protein and a lower percent body lipid than fish fed the other dietary treatments.

The results of this study show that sockeye fry thrive when fed *Artemia,* regardless of the stage of growth of the *Artemia.* First-feeding fry grew equally well when fed *Artemia* or Biodiet until they reached a size at which they could effectively prey on adult *Artemia,* approximately 0.4 g. The results are sufficiently encouraging for further trials to be conducted, and we plan both to refine the *Artemia* feeding strategies and to use adult *Artemia* as a carrier for therapeutic compounds used to treat or prevent BKD.

Table 4. Average weight", fork length[b], and percent body weight gain' per day (% BWG/day) of sockeye salmon fed the various dietary treatments after 8 weeks. Data are means ± standard deviation (n = 3). Mean weights with different subscripts are significantly different (P < 0.05).

Diet	Mean wt. (g) (± SD)	Mean lg. (mm) (± SD).	% BWG/day[b]
Artemia adults	1.25 ± 0.06"	50.7 ± 1.5	3.76
Biodiet starter	1.15 ± 0.02[b]	49.0 ± 4.0	3.60
Artemia 1st instar	1.13 ± 0.06[b]	49.5 ± 1.4	3.43
Artemia 3rd instar	1.03 ± 0.03'	48.4 ± 1.6	3.39

[a] Initial weights 0.18g ± 0.0 1.
[b] Initial fork lengths 28.8 mm ± 0.1.
[c] %BWG/day = eGW-1 x 100, where GW = instantaneous growth rate [(In final wt.-ln initial wt./time in days.]

Table 5. Percent body weight gain per day (% BWG/day)* of sockeye salmon at two-week intervals over an eight-week period. Data are mean ± standard deviation (n = 3). Mean % BWG/day with different subscripts are significantly different (P < 0.05).

Diet	%BWG/day O-2 weeks	%BWG/day 3-4 weeks	%BWG/day 5-6 weeks	%BWG/day 7-8 weeks
Artemia adults	2.49 ± 0.22a	3.40 ± 0.21a	4.39 ± 0.39a	3.89 ± 0.21ab
Biodiet starter	1.60 ± 0.08b	3.54 ± 0.07a	4.29 ± 0.08a	4.21 ± 0.17a
Artemia 1st instar	2.53 ± 0.20a	3.12 ± 0.07b	4.23 ± 0.31a	3.65 ± 0.1 lb
Artemia 3rd instar	2.49 ± 0.22a	2.91 ± 0.03c	3.78 ± 0.16a	3.70 ± 0.23b

* %BWG/day = eGW-1 x 100, where GW = instantaneous growth rate [(In final wt-ln initial wt)/ days].

Table 6. Proximate composition (%) of the dietary treatments fed to first-feeding sockeye salmon. Data are mean values (± S.D.).

Treatment	Moisture[a]	Protein	Lipid	Ash
1 st instar Artemia	83.7 ± 1.5	53.5 ± 1.1	18.2 ± 1.4	NA
3rd Instar *Artemia*	91.4 ± 1.6	55.5 ± 1.1	18.6 ± 3.4	NA
Biodiet[b]	21.5	45.0	16.0	8.5
Adult *Artemia*	90.6 ± 2.8	57.0 ± 2.1	10.1 ± 0 8	NA

[a] Values except moisture are expressed on a dry weight basis.
[b] Values are minimum guaranteed levels.

Table 7. Proximate composition (%) of sockeye salmon fry after 8 weeks of feeding. Data are mean values (± S.D.).

Treatment	Moisture*	Protein	Lipid	Ash
1 st instar *Artemia*	77.2 ± 0.1	61.2 ± 2.6	29.0 ± 1.1	NA
3rd instar *Artemia*	78.2 ± 0.3	65.4 ± 0.8	26.9 ± 1.2	NA
Biodiet	78.3 ± 0.3	61.7 ± 1.4	29.7 ± 0.5	NA
Adult *Artemia*	79.7 ± 0.5	70.4 ± 1.4	18.6 ± 1.3	NA

* Values except moisture are expressed on a dry weight basis.

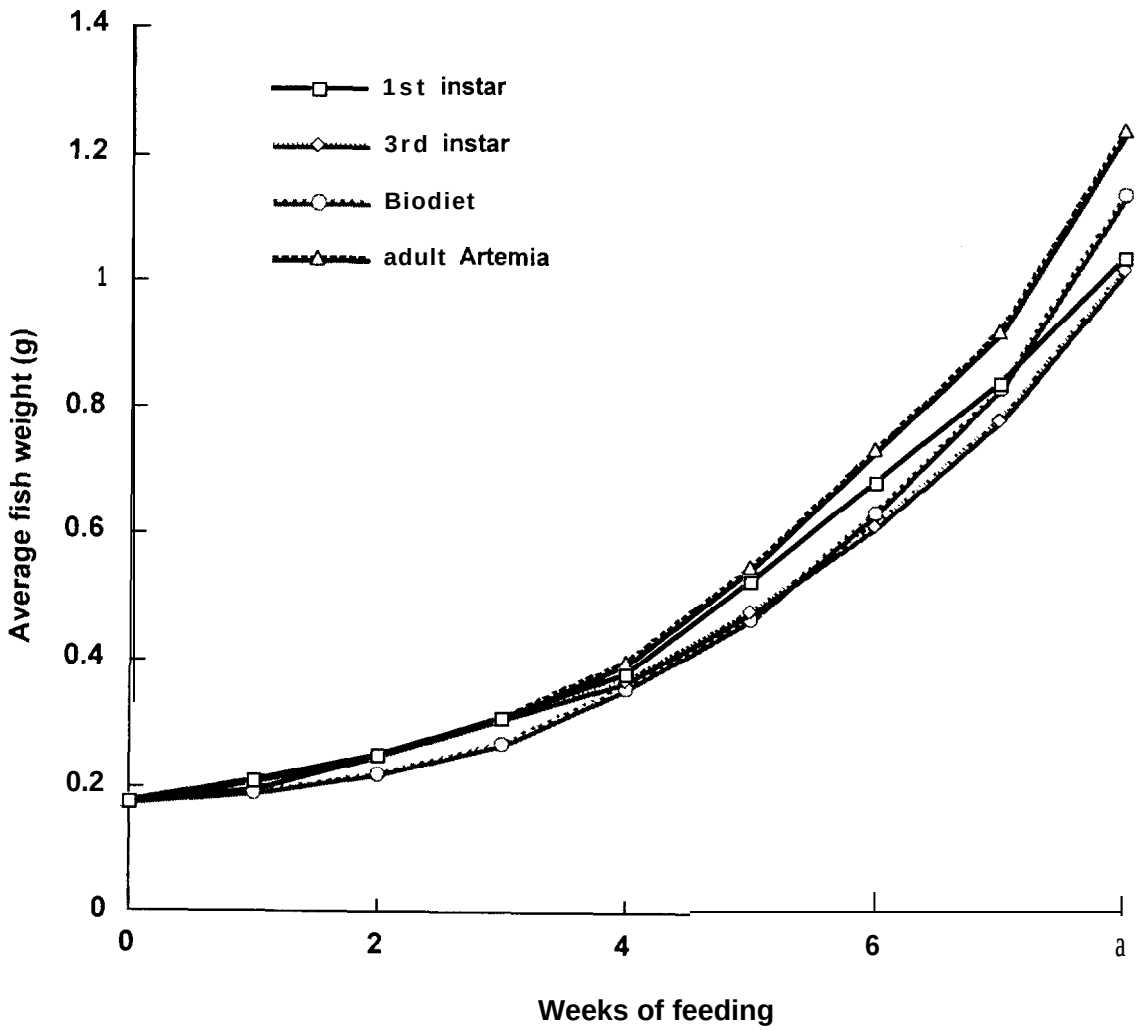

Figure 1. Average weight of first-feeding sockeye salmon fry fed *Artemia* and Biodiet for 8 weeks.

EFFECT OF DIETARY FAT CONTENT AND GROWTH REGIME ON AGE OF SEXUAL MATURATION OF SOCKEYE SALMON REARED IN CAPTIVITY

Introduction

One objective of captive broodstock programs for recovery of depleted salmon stocks is to get a high yield of viable offspring from what is usually a small number of founding stock. A major obstacle to meeting this objective is the inappropriate timing of sexual maturation. Sockeye salmon typically mature at 3, 4, or 5 years of age. Age and seasonal timing of maturation is influenced by genetic, physiological. nutritional, and environmental factors. In cultured broodstock it may be possible to influence the age of maturation by modified rearing practices, mainly by alterations in feeding level and/or diet formulation. However, nothing is known about the effects of these alterations on maturation timing in sockeye salmon.

The trend in commercial salmon feeds is toward high-fat, extruded feeds, even for fingerlings. Dietary fat levels of some commercial feeds are 22% or greater, compared to 12- 15% in commercial feeds a decade ago. As described in our review of literature (Hardy and Forster 1995), dietary energy level is known to control body fat reserves, which in turn is one of the factors affecting maturation time. Related problems concerning captive rearing of sockeye salmon are death of fish before spawning, uncertainty about whether fish will mature after 3 or 4 years, and asynchronous maturation of males and females. The effects of feeding rate and dietary energy levels on these issues in sockeye salmon are unknown.

Methods

Fertilized eggs from Lake Wenatchee sockeye salmon (1994 brood) were reared at the NWFSC hatchery facility to approximately 10 g average weight (summer 1995). In fall 1995, they will be divided into eight dietary treatment groups, with three replicate tanks devoted to each dietary treatment. A 2 x 3 factorial design will be used to allow detection of main effects and interactions of dietary treatments. The dietary treatments will be composed of two feeding levels: restricted (pair-fed) and apparent satiation, and four diets: three experimental and one commercial (Biodiet, Bioproducts, Inc., Warrenton, OR). The three experimental diets will be formulated to contain similar protein levels but different fat levels: 10, 15. and 20%.

The commercial diet treatments will be included as controls to permit comparison of the results with those previously obtained in connection with efforts to rear Redfish Lake sockeye salmon at both NWFSC and Idaho Fish and Game facilities. These fish are expected to mature and spawn in fall 1998, with signs of precocious maturation expected in summer and fall of 1996. The fish will be reared at the NWFSC Utilization Research Division hatchery facilities. Although the fish will be reared indoors, water temperature and day length will be controlled to approximate normal seasonal changes.

EFFECTS OF SUPPLEMENTATION OF COMMERCIAL DIETS WITH NATURAL CAROTENOIDS ON REPRODUCTIVE PERFORMANCE OF SOCKEYE SALMON IN CAPTIVE PROPAGATION

Introduction

Most pelleted rations developed for salmon are produced for carnivorous species (e.g., rainbow trout, coho, chinook and Atlantic salmon) and may not be adequate for species such as sockeye salmon that feed primarily on carotenoid-rich invertebrates. We could find no published study that demonstrates a significant correlation between egg carotenoid content and survival of juvenile fish. However. there is general consensus among fish farmers and biologists that superior quality and viability are synonymous with high pigmentation in eggs (Smith 1990).

In dairy cattle, positive correlations exist between β-carotenoid and reproductive performance (Tacon 1981). Craik (1985) suggested that rainbow trout eggs with less than a critical level of carotenoid (l-3 μg carotenoid/gram eggs wet weight) experienced reduced survival to hatch. Other possible functions of egg carotenoid are to stimulate and attract spermatozoa, to supplement respiration. to protect from photodynamic damage, to aid in resistance to heat and ammonia. and to act as a precursor to vitamin A. In addition, egg carotenoid provides pigmentation in newly hatched salmonids. Because of the possibility that deficiencies in carotenoids lead to poor egg quality and offspring survival, we propose to determine if diets with elevated carotenoid levels affect egg quality and survival of offspring in sockeye salmon.

Methods and Results

One hundred Lake Wenatchee sockeye salmon adults (1991 brood, ca 800-g body wt) were individually PIT tagged, measured, weighed, and randomly divided into two groups of 50 fish each (Table 8). Groups were placed into replicate circular tanks (2.3-m diameter, 25 fish/tank) receiving pumped, untreated seawater from Clam Bay (NMFS Manchester Marine Experimental Station). Treatment groups were fed an open-formula salmon diet (Abernathy 19-2) modified by replacing 10% of the fish-meal component of the diet with whole frozen krill (Euphausia pacifica) or by adding synthetic astaxanthin (Carophyll pink", Hoffman-LaRoche Ltd., Basel, Switzerland) to achieve approximately 50 ppm supplemental carotenoid in the diet (Table 9). Fish were fed test diets twice per day to apparent satiation starting on 12 April 1995.

Starting in late April, low-level fish mortality was recorded in all tanks. The mortalities were inspected for pathogens, but no single pathogen was identified as the main cause of death. However, many of the mortalities showed signs of bloating, which suggested an osmoregulatory problem. Subsequently, blood samples were taken from live fish that exhibited bloating, and the samples were analyzed for calcium, magnesium, sodium, and phosphorus content. No evidence of excessive serum calcium or sodium, which would have suggested osmoregulatory failure, was found (Table 10). Chronic mortality persisted throughout the study until the numbers of remaining fish were below the number needed to continue the experiment. We terminated the experiment on 23 June 1995.

Table 8. Mean weight and length of sockeye salmon at initial stocking for the study on the effects of diets supplemented with carotenoid on reproductive performance.

Tank	Diet	Number of fish	Mean weight (g) ± S.D.	Mean length (mm) ± S.D.
7	1	25	827.72 ± 229.07	388.4 ± 41.44
8	2	25	820.04 ± 195.91	386.46 ± 32.36
9	1	25	806.25 ± 2 10.77	384.21 ± 36.09
10	2	25	843.92 ± 200.42	387.24 ± 39.12

Table 9. Composition of two experimental diets for the study on the effects of supplementation with carotenoid on reproductive performance.

Ingredient	Diet 1 (%)	Diet 2 (%)
Herring meal	47.72	51.40
Blood meal	0.00	5.00
Wheat gluten	4.00	4.00
Wheat mids	13.00	17.82
Fish oil	9.00	10.40
Vitamin C	0.10	0.10
Choline	0.58	0.58
TM salt	0.10	0.10
Vitamin premix	1.50	1.50
Poultry BP meal	2.00	2.00
Permapel	2.00	2.00
Krill (dry meal)	5.00	0.00
Krill (Frozen)	10.00	0.00
Whey	5.00	5.00
Astaxanthin (Carophyll pink)	0.00	0.10

Table 10. Concentration (μg/ml) of selected ions in sera of seawater-reared sockeye salmon exhibiting signs of bloating at the NMFS Manchester Marine Experimental Station (April 1995).

Fish	Calcium	Magnesium	Sodium	Phosphorus
1	128	26.1	3830	566
2	136	29.7	3730	536
3	146	31.4	4640	579
4	148	39.3	3790	540
5	135	27.9	3730	570
6	138	28.2	3950	513
7	149	25.6	3790	672
8	156	26.1	3660	869
9	149	30.5	3830	513
10	141	44.7	3810	706
Mean ± SD	143 ± 8.4	31.0 ± 6.3	3880 ± 279	606 ± 112

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PART VI

THE EFFECT OF WHOLE BODY LIPID STORES ON EARLY MATURATION OF MALE SPRING CHINOOK SALMON *(ONCORHYNCHUS TSHAWYTSCHA)*

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SUMMARY

An experiment was designed to examine the relationship between the level of whole-body lipid and early maturation in 1^+ spring chinook salmon (*Oncorhynchus tshawytscha*). Fertilized eggs were obtained from a wild stock of Yakima River chinook salmon and were hatched in December 1992. Fry were fed a commercial diet from February until August 1993 and then the fish (5.6 g) were divided into groups of 320 and fed one of five experimental diets (2 replicate groups/diet) containing 4, 9, 14, 18 or 22% lipid and 82, 77. 73, 69, or 65% protein for 13 months. Fish were reared on natural photoperiod and ambient temperature (6 to 16°C). and pair-fed to a level based on the tank with the lowest feed consumption. Fish were weighed monthly and subsamples were collected to determine body composition, pituitary follicle-stimulating hormone (FSH) levels, plasma insulin-like growth factor I (IGF-I) levels, and stage of gonadal development.

Throughout the experimental period the mean fish weight was similar among treatment groups. However, from December 1993 through the end of the experiment (September 1994), maturing males were significantly larger than nonmaturing fish. Initial lipid levels in experimental fish were near 6%, which is similar to wild fish of the same stock captured in the Yakima River during August 1993. Fish fed diets containing more than 4% lipid increased in whole-body lipid content during the first 2 months of feeding and maintained relatively constant levels during the course of the experiment. Whole-body lipid levels for the dietary treatment groups averaged 5.6, 7.1, 8.2, 9.4, and 9.6%.

Based on histological examination of the testes of experimental fish, primary spermatocytes were first observed in maturing males during November 1993, indicating that maturation was initiated at this time. In addition, pituitary FSH levels were significantly higher in maturing than nonmaturing males at this time and throughout the study period, and levels increased as spermatogenesis proceeded. Plasma IGF-I levels were significantly higher in maturing males than nonmaturing fish from May 1994 through the end of experiment. The difference in IGF-I levels does not appear to be related to growth rate, since we observed differences in size between maturing and nonmaturing males as early as December when there was no difference in IGF-I levels.

The percentage of maturing males was significantly influenced by whole-body lipid, increasing from 34% in fish fed the 4% lipid diet to 45% in fish fed the 22% lipid diet. These data suggest that whole-body lipid levels influence the rate of maturation of male spring chinook salmon. In addition, both endocrine and histological indicators suggest that maturation was initiated almost a full year prior to the time the fish will spawn.

INTRODUCTION

The salmonids have been described as phenotypically plastic opportunistic generalists because of the variation observed in the length of their life cycle stages (Thorpe 1989). Two forms of spring chinook salmon (*Oncorhynchus tshawytscha*) are recognized: the ocean type, which normally migrate to sea during their first year and return in late summer just prior to spawning. and a stream or spring type. which spends one or more years in fresh water and returns in spring or early summer before spawning in the fall (Mullan et al. 1992). Early male maturation occurs in wild fish but appears to be much more common in fish reared in hatcheries (Thorpe 1991. Foote et al. 1991).

Jonsson and Jonsson (1993) and Thorpe (1994) have discussed the ecological advantages of early male maturation and have, along with others, discussed possible factors that influence this life cycle strategy. Evidence has been presented which demonstrates that genetic (Thorpe and Morgan 1980; Hard et al. 1985; Gjerde 1984a, b; Heath 1992; Silverstein and Hershberger 1992). abiotic (Saunders et al. 1982, Taylor 1989) and biotic (Thorpe 1986) factors play a part in life cycle strategy. Rowe and Thorpe (1990 a, b) have shown that growth rate during specific periods of the year influence the rate of early male maturation. Rowe et al. (1991) and Thorpe (1994) have more recently suggested that fish may assess body energy stores and that critical thresholds for size and energy storage must be met at a specific time of year or maturation is inhibited.

The relative importance of body energy stores, growth rate, and size in permitting maturation to occur are not well understood. Therefore, we designed a study to assess the effect of whole-body lipid stores on the incidence of maturation in 1^+ male spring chinook salmon that had similar rates of body growth. We also monitored levels of selected reproductive and metabolic hormones. and examined some of the testes histologically to establish the period when spermatogenesis was initiated in this stock of fish.

MATERIALS AND METHODS

Fish and Rearing Conditions

Spring chinook salmon gametes were obtained from wild adults returning to the Yakima River near Cle Elum, WA in December 1992. Fertilized eggs were incubated in Heath trays and maintained on dechlorinated municipal water at 8°C until hatching in February 1993. At the swim-up stage, fry were ponded into circular fiberglass tanks (1.5-m diameter) supplied with flow-through dechlorinated municipal water (8-l 5°C) and were fed a commercial diet (Bioproducts Inc., Warrenton, OR) according to the manufacturer's specifications from first feeding until the start of the experiment in August 1993.

Examination of a number of fish prior to the start of the experiment revealed that some fish were infected with bacterial kidney disease (BKD). Therefore, fish were fed a commercial diet containing erythromycin (100 mg/kg fish/day) for 3 weeks prior to the initiation of the experiment. In August 1993 fish were graded to remove the largest and smallest fish and were then randomly distributed into 10 fiberglass tanks (130 L; 200 fish per tank) supplied with dechlorinated municipal water (4 L/min). Initial mean fish weight was 5.8 ± 1.2 g. During the experimental period fish were reared on natural photoperiod and ambient water temperature, except during summer months when water was chilled so that the maximum temperature would not exceed 16°C. Water temperature varied seasonally and ranged from a maximum of 16°C (July and August) to a minimum of 8°C (January). A failure in the chilling system in August 1994 caused the temperature to briefly rise to 20°C, which necessitated moving the fish to a new location and larger tanks (720 L). During September 1994 the experiment was terminated and all fish were sacrificed.

Diets and Feeding

The objective of this study was to determine whether whole-body lipid levels, not size, affected the percentage of male spring chinook salmon which sexually matured at 2 years of age. Therefore, to create fish with graded levels of whole-body lipid, fish were reared on diets containing excess protein (more than required for maximum growth) and 5 different levels of lipid. Ration was controlled to maintain similar size among the various dietary treatments. Samples were collected at monthly intervals to monitor seasonal changes in whole-body lipid levels, gonad development, and levels of pituitary gonadotropins as indices of sexual maturation.

Five experimental diets were prepared (Table 1) every 4 months and were stored at -20°C. The composition of experimental diets is shown in Table 2. The diets were formulated to supply protein in excess (> 55%, Delong et al. 1958). The diets differed in the levels of protein (82, 77, 73, 69, 65%) and lipid (4, 9, 14, 18, 22%). The amount of feed fed to each tank of fish each day was determined using the method reported earlier (Shearer et al. 1997a). On day one of the experiment, the average amount of feed consumed by the fish in each tank was noted. For the remainder of the first month, fish in each tank were fed 90% of the amount of feed consumed

by the group that ate the least feed on day one. Fish were fed slightly below satiation to ensure that all the feed could be consumed. The day after each monthly sampling all groups were fed to satiation. The amount of feed to be fed to each tank of fish for the remainder of the month was then calculated as follows:

$$(C \times 0.9 \times N) + \frac{\left(\frac{(W_L - W) \times N}{F}\right)}{d}$$

where:

C	= the average amount of feed consumed/fish in the tank that ate the least feed the first day after sampling
0.9	= a factor to help insure that all feed fed was consumed
N	= the number of fish in the tank
W_L	= the largest mean fish weight of all the groups
W	= the mean weight of the fish in the tank
F	= the feed efficiency in the tank the previous month
d	= the number of feeding days before the next sampling

The fish were normally fed 5 days per week, but if they failed to consume their allotted ration in 5 days they were fed on days 6 and 7. Feed was withheld for 2 days prior to sampling. During weeks l-4, 9-12, and 17-20, erythromycin phosphate was added at the rate of 100 mg/kg fish/day to all diets to control BKD. No antibiotics were fed after week 20. Feed consumption and mortality were recorded daily.

Table 1. Formulation of the experimental diets.

Ingredient	Diet				
	1	3	3	4	5
	g/kg diet				
Dried fish muscle’	680	640	606	576	548
Gelatin	120	114	108	102	97
Wheat gluten	60	57	54	51	49
Ascorbic acid	10	10	10	10	10
Arginine	6	6	5	5	5
Herring oil	36	91	140	184	223
Choline Cl	12	11	11	10	10
Trace mineral mix’	1	1	1	1	1
Carboxymethyl cellulose	24	23	22	20	19
Binder’	24	23	22	20	19
CaH_2PO_4	18	17	16	15	15
Vitamin mix’	18	17	16	15	15

[1] Supplied by NMFS. Kodiak, AK 94% protein, 2% lipid, 4% ash.
[2] USFWS, No. 3 (Hardy 1989).
[3] Algea Produkter A/S. Lier, Norway.
[4] USFWS. Abernathy (Hardy 1989).

Table 2. Composition of experimental diets. Protein and lipid determined by analysis. Energy calculations based on: protein, 23.6; lipid, 36.2 and carbohydrate, 17.2 KJ/g (Brafield 1985).

Component	Diet				
	1	2	3	4	5
Protein (%)	82	77	73	69	65
Lipid (%)	4	9	14	18	22
Protein/Energy (mg/KJ)	40	36	33	30	28
Energy KJ/g	28.0	21.5	22.3	22.8	23.3
Energy (% from protein)	93	84	77	71	66

Sampling

Fish were bulk weighed and counted at approximately monthly intervals. Prior to each monthly weighing, 10 fish from each tank were randomly removed for determination of length. weight, sex, state of gonadal development, and whole-body lipid levels. From November 1993 through September 1994, pituitary glands, blood and gonads were collected from the sacrificed fish. Blood was collected from the tail vein in heparinized hematocrit tubes after severing the caudal peduncle. Blood was centrifuged at 1000 x g and plasma was stored at -70°C. Pituitaries were removed and frozen in liquid nitrogen, then stored at -70°C. Gonads were removed and fixed in Bouin's fixative for 24 hours, then stored in 70% ethanol. Carcasses were frozen at -20°C for whole-body lipid determination.

At the end of the experiment, September 1994, all fish (approx. 1 00/tank) were sacrificed and measured (fork length and weight). Additional sampling of blood: pituitaries, gonads and carcasses was performed on the first five females, five immature males, and five maturing males collected randomly from each tank. In addition, gonad and liver weights were recorded. All remaining fish were sexed and each fish was classified as nonmaturing or maturing based on gross morphology of the gonad and gonadosomatic index (GSI).

Whole-body Lipid Analysis

Whole-body lipid levels were determined on pooled samples of 10 fish from each tank throughout the experiment and on individual fish from the sampling at the end of the experiment (September 1994). Fish were ground and dried to constant weight at 105°C and a sub-sample was extracted with dimethyl chloride using the soxlet method. Dietary lipid was determined for each new batch of feed, and percent lipid between batches of any particular diet varied less than 1%. Condition factor (CF) was calculated as CF = w/l', where w = weight in g and 1 = length in cm. Data from wild chinook salmon of the same stock and age as those used in the experiment were kindly provided by Dr. Walton Dickhoff (Dickhoff et al. 1996).

Hormone Analyses

Plasma insulin-like growth factor I levels were determined by radioimmunoassay (Moriyama et al. 1994). Pituitary levels of gonadotropins (FSH and LH) were analyzed by radioimmunoassay (Swanson et al. 1989). Briefly, pituitaries were sonicated in 0.5-mL barbital buffer (0.75 M sodium barbital, 10 mm EDTA, 1 mM PMSF, pH 8.6), centrifuged at 10,000 g for 10 minutes. Pituitary extracts were diluted in assay buffer (0.75 M sodium barbital, 1 .O% bovine serum albumin, pH 8.6) prior to assay.

Gonad Histology

Testes were removed and fixed in Bouin's fixative for 24 hours. then stored in 70% ethanol until processed for histology. Tissue was dehydrated through a series of increasing concentrations of ethanol, imbedded in Paraplast, sectioned (6 μm) and stained with hematoxylin and eosin. Stage of spermatogenesis was determined by light microscopic observation according to Schulz (1984) and briefly described in Table 3.

Table 3. Criteria for stage of spermatogenesis according to Schulz (1984).

Stage	Cell Types Observed
I	spermatogonia and Sertoli cells as intratubular types
II	spermatogonia, primary spermatocytes
III	spermatogonia, primary and secondary spermatocytes, spermatids
IV	spermatogonia, primary and secondary spermatocytes, spermatids, spermatozoa; many spermatogenic cysts; no spermiation
V	no or few spermatogenic cysts; tubuli filled with spermatozoa
Va	no spermatogenic cysts; tubuli filled with spermatozoa; spermiation

Statistics

Statistical analysis were performed using Statview™ (Abacus Concepts, Berkeley, CA, 1992), and multiple mean comparisons were made using the Fisher PLSD test. Percentage data were arcsine transformed prior to analysis. Analyses were performed using mean tank values as the unit of observation and $P < 0.05$ was accepted as significant unless otherwise stated.

RESULTS

Gonadal Development

From September through December 1993, no gross morphological signs of maturation in male fish were observed. Histological analyses of testes collected from fish during this period indicated that the majority of males remained in Stage I of spermatogenesis, and these fish were classified as nonmaturing. However, testes from some individuals collected during November and December 1993 were in Stage II of spermatogenesis, and these fish were classified as maturing.

The first gross morphological signs of maturation of male fish were observed during January 1994, when a slight enlargement of the anterior portion of the testes was seen in some males fed more than 4% lipid. Histological examination confirmed that these testes were in Stage II of spermatogenesis. From that point on, males with testicular enlargement were designated as maturing males.

A random sample (20/month) of males classified as maturing was collected from January through March, and analyses revealed that the testes were in Stage II of spermatogenesis. By April, individuals were found in Stages II to IV. From May through July, Stages IV and V were observed. Finally, during September the majority of maturing males were in Stage V or Va, with a few fish producing milt with gentle abdominal pressure.

Nonmaturing fish throughout the year remained in Stage I. Stages of spermatogenesis observed at each sampling point are summarized in Table 4. Since we did not analyze all testes collected. we could not quantify the proportion of fish at each stage. The proportion of maturing males among the treatment groups varied, but did not differ significantly among the treatment groups at any sampling time. However, a significant correlation was found between average whole-body lipid and percentage of males maturing (see below).

Growth and Whole-body lipid Levels

Growth of fish in body weight was similar among the five treatment groups, with body weight gradually increasing from August 1993 to March 1994, at which time the rate of growth increased in all treatments. However, mean fish weights did not differ significantly among the treatment groups except at the final sampling (Fig. la), when fish fed the lowest fat diet were significantly smaller than all other fish. Comparison of the weights of fish sampled from all treatment groups indicated that nonmaturing males and females grew at equivalent rates (Fig. lb). However, males that were classified as maturing were significantly larger in body weight that nonmaturing males or females. The larger size of maturing males was also evident in weight frequency distributions of all fish sampled monthly (Fig. 2).

Table 4. Stages of spermatogenesis observed during experimental period.

Date	Nonmaturing	Maturing
September 27, 1993	I	
October 25, 1993	I	
November 29, 1993	I	II
December 27, 1993	I	II
January 31, 1994	I	II
February 28, 1994	I	II
March 25, 1994	I	II
May 2, 1994	I	II, III, IV
May 31, 1994	I	III, IV
July 5, 1994	I	IV
August 4, 1994	I	IV, v
September 12, 1994	I	IV, V, Va

Table 5. Final weight[1], feed efficiency[2], and whole-body lipid[3], of fish at the end of the experiment.

Treatment	Final weight (g)	Feed fed (g)	Feed efficiency	Mortality (%)	Whole-body lipid (%)
1	48.8±0.4	46.0	0.94	4.5	5.9±0.13
2	52.5±1.0	46.4	1.01	1.3	7.1±0.35
3	53.3±0.1	47.2	1.01	2.8	8.6kO.45
4	52.9±1.6	46.4	1.02	3.8	9.9±0.82
5	50.6±2.8	46.4	0.97	10.4	9.7±0.51

[1] Based on mean tank weight.
[2] Wet weight gain/dry feed fed.
[3] Based on 5 males, 5 females and 5 mature males.

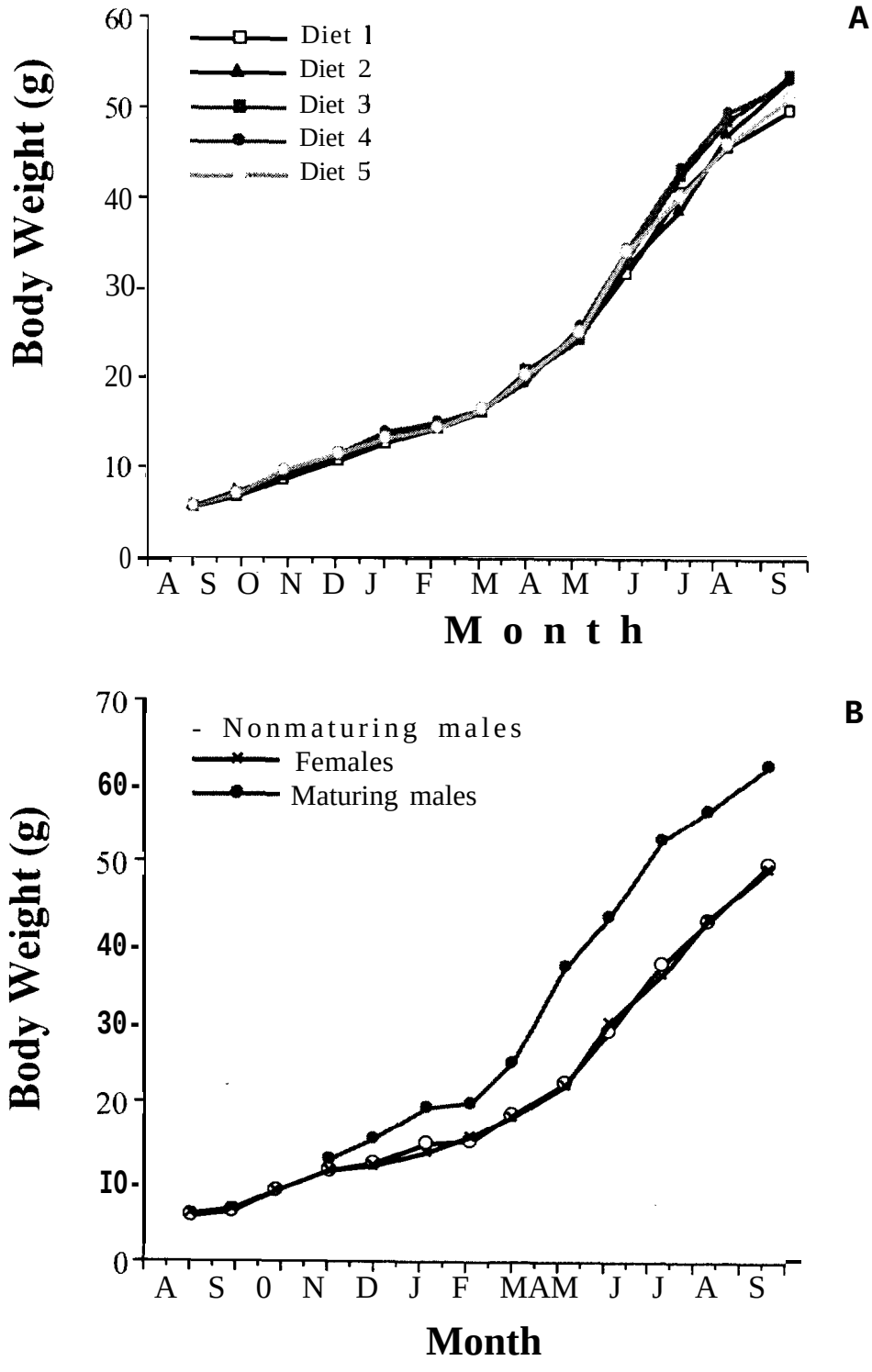

Figure 1. Mean weight (n = 2 tanks/treatment) of 1+ spring chinook salmon fed experimental diets containing varying levels of protein and lipid (A). Mean weight of female, nonmaturing and maturing male fish sampled at monthly intervals during the experimental period (B). Maturing males were significantly ($P < 0.05$) larger than nonmaturing fish from December through the end of the experiment.

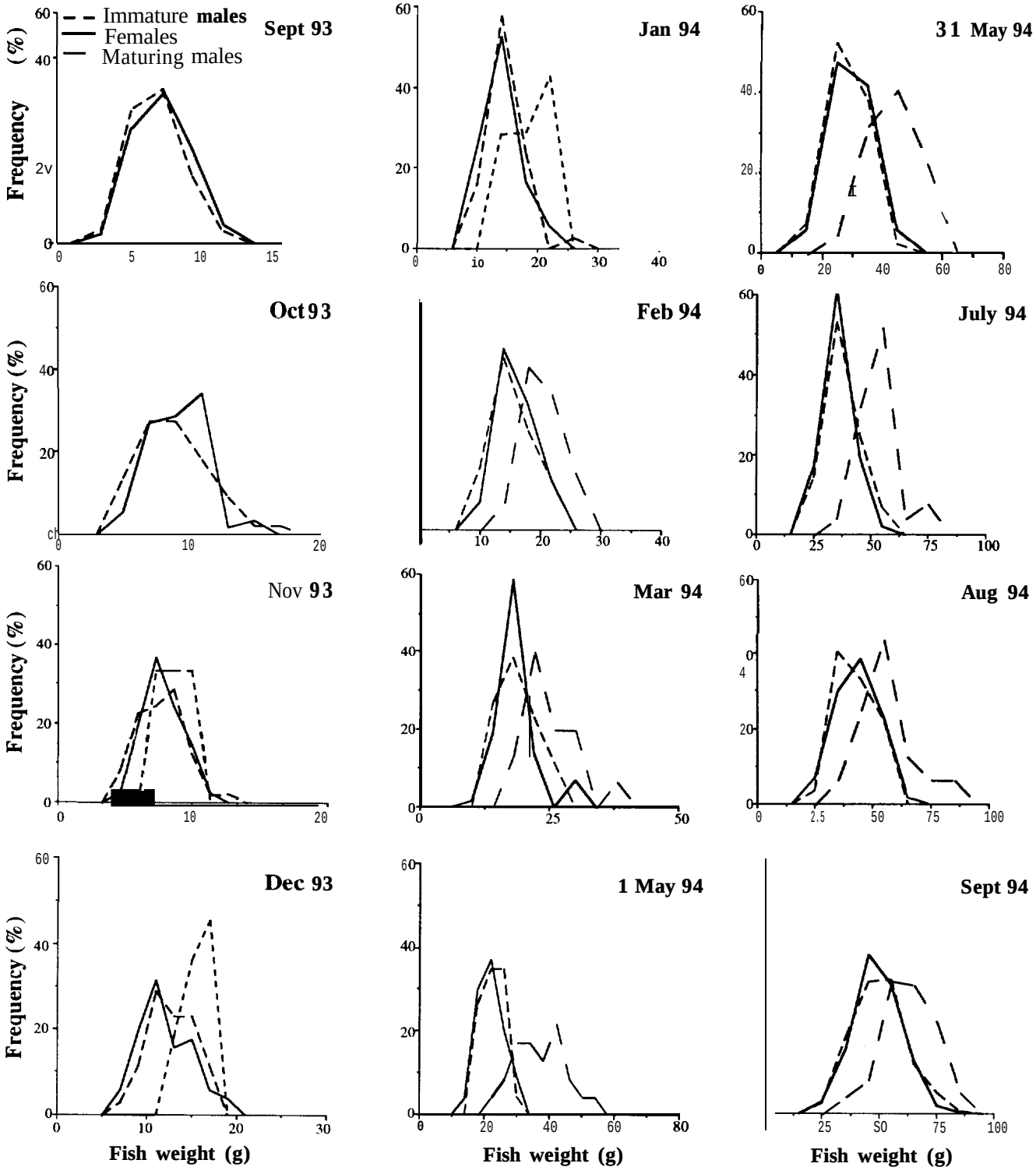

Figure 2. Weight frequency distribution of immature males and females and maturing male chinook salmon at each sampling; n = 100 fish per sampling period. The sex ratio and number of maturing males varied at each sampling.

Overall mortality (Table 5) was 4.6% and the majority of this occurred in one replicate of treatment 5 (18.5%). Postmortem examination revealed the presence of bacterial kidney disease. Feed efficiency for all treatments averaged 99 ± 4.8% (weight gain/feed fed) and did not differ between treatments (Table 4).

In all groups, whole-body lipid increased during the first 2 months of feeding experimental diets (September and October 1993) and then decreased slightly throughout the winter and early spring, reaching their lowest level in April 1994 (Fig. 3a). There was a significant effect of diet on whole-body lipid levels, and relative differences in whole-body lipid among the groups were generally maintained throughout the study. Condition factor did not differ among treatment groups at any sampling (Fig. 3b). Condition factors remained constant from August to November 1993 (approximately 1.5) but declined markedly, to approximately 1.1, between the November and December samplings (Fig. 3b). There appeared to be no relationship between condition factor and body lipid levels at the end of the experiment (Fig. 4).

Since gonadal histology indicated that the maturation process appeared to begin between the November and December 1993 samplings, regression analysis was performed to determine if there was a relationship between the mean levels of body lipid in each tank of fish in December 1993 and the incidence of maturation in September 1994 (Fig. 5). The percentage of males maturing at 2 years of age was positively correlated with average whole-body lipid levels during October, November and December ($P < 0.05$).

Hormone Levels

Pituitary FSH and LH levels were measured in all male fish sampled during the experimental period, and from females collected during September 1993 and 1994. Pituitary FSH levels were significantly higher in maturing males than nonmaturing fish of either sex (Fig. 6a). Levels of pituitary FSH increased during the year, but most dramatically from July to August in maturing males when the transition to later stages of spermatogenesis was observed in the testes samples (Table 4). Pituitary LH levels were not detectable in nonmaturing fish. In maturing males, pituitary LH (Fig. 6b) levels increased slightly from May to July 1994, and increased more than a hundred-fold from August to September 1994. It was not possible to measure plasma levels of either FSH or LH due to insufficient plasma for the analysis.

Plasma levels of IGF-I (Fig. 7) were relatively constant from September 1993 to May 1994 and did not differ significantly among the treatments. However, from July 1994 through September 1994, maturing males had significantly higher plasma IGF-I levels than nonmaturing fish, and levels increased during this period. In both maturing and nonmaturing fish, significant increases in plasma IGF-I levels were observed from August to September 1994. In September 1994, there was a significant treatment effect on IGF-I levels, with both maturing and nonmaturing fish fed Diets 1 and 2 having higher IGF-I levels than those fed Diets 3, 4, and 5 (Fig. 8). Among the diets fed in this experiment, Diets 1 and 2 were lowest in fat and highest in protein:energy ratio (Table 2).

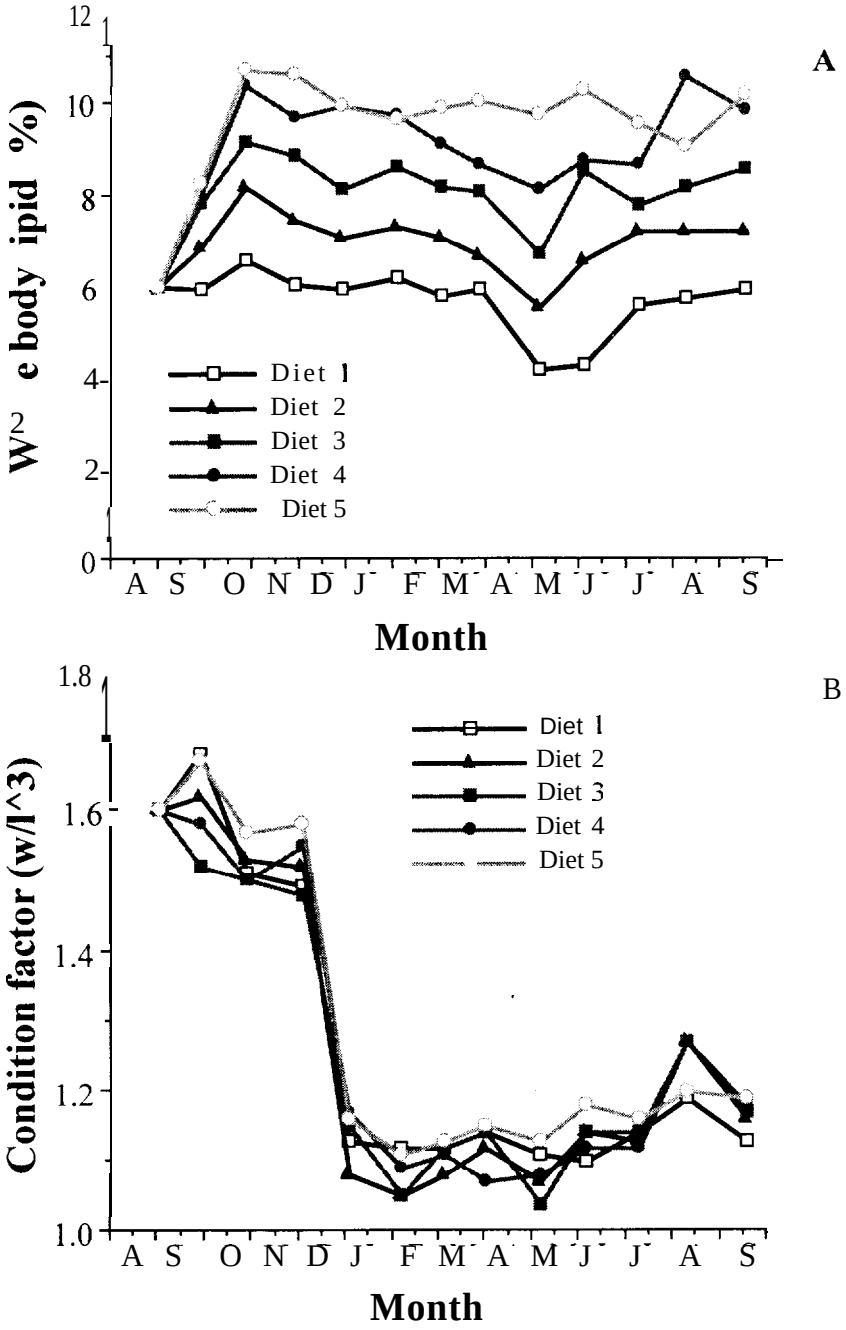

Figure 3. Whole-body lipid levels (A) and condition factor (B) of 1+ chinook salmon fed experimental diets containing varying levels of dietary lipid and protein. See Table 1 for description of diets. Data are mean of replicate tanks. Whole-body lipid levels were determine on pooled carcasses of 10 fish per tank.

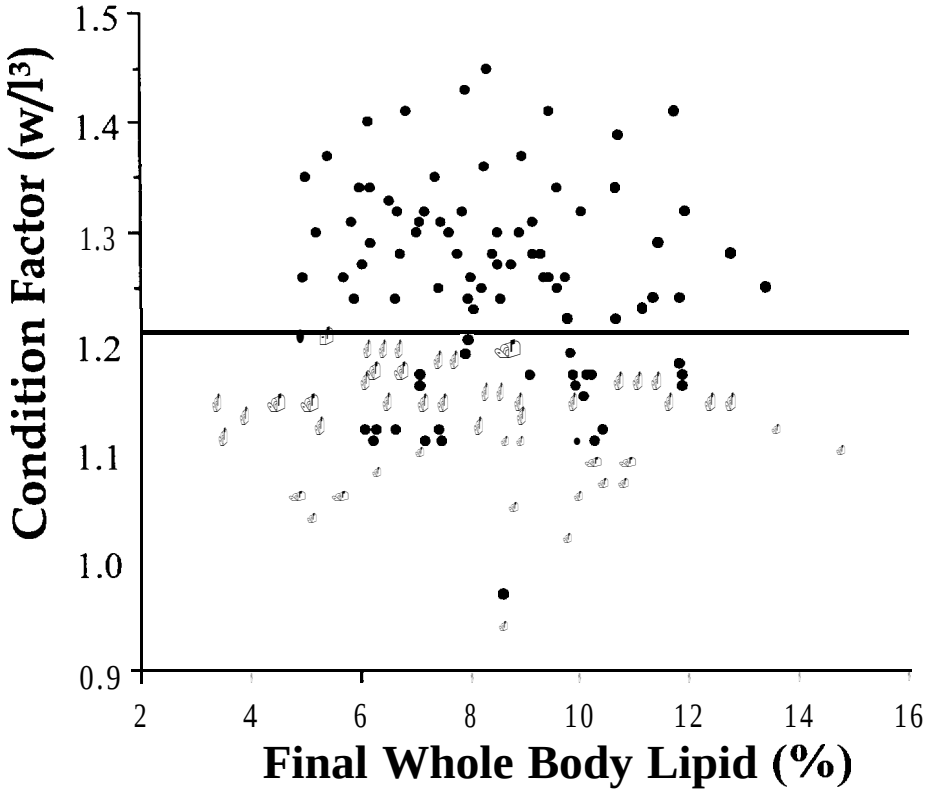

Figure 4. Relationship between individual whole-body lipid level and condition factor in September 1994. Measurements were made on 5 immature males, 5 females and 5 maturing males from each replicate tank (n = 150).

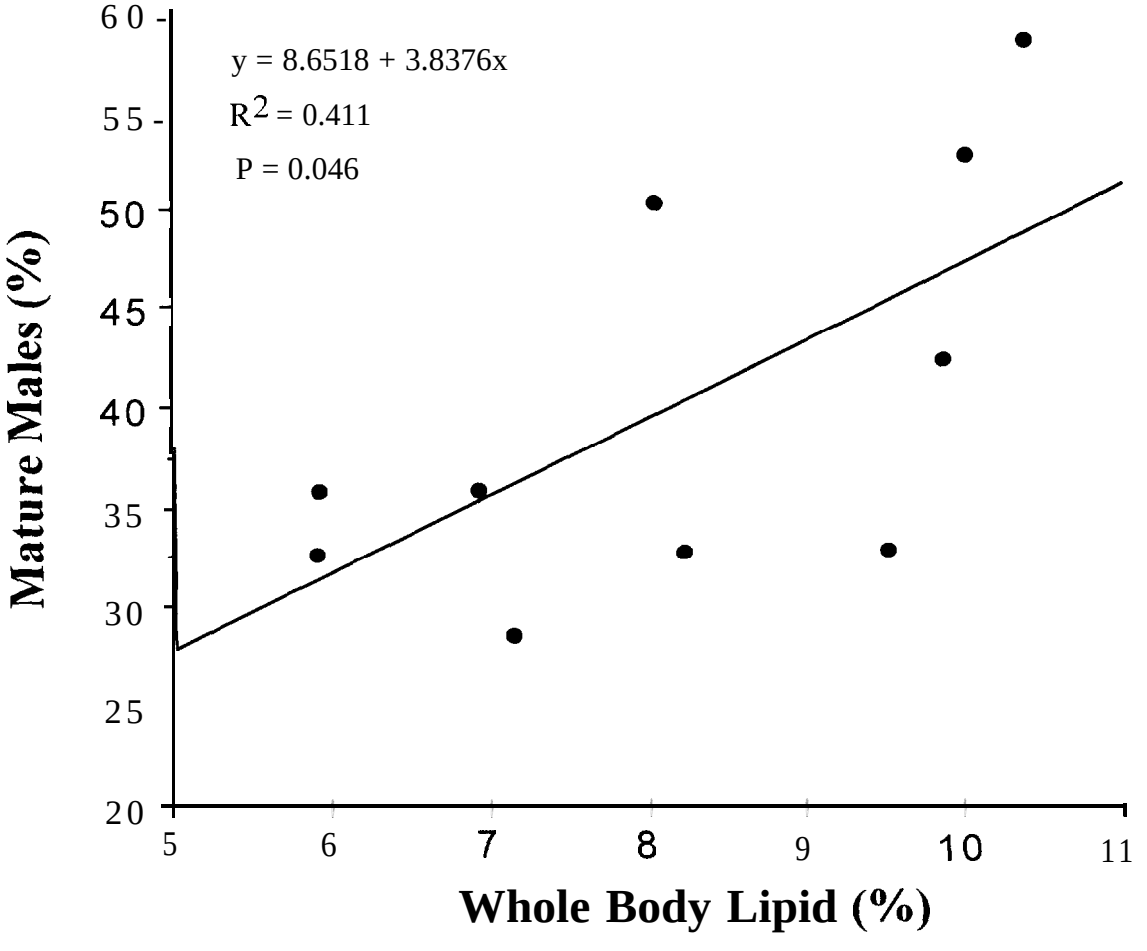

Figure 5. Percent maturing 1-year-old male spring chinook salmon in each tank at the end of the experiment (September 1994) in relation to mean whole-body lipid levels in December (1993).

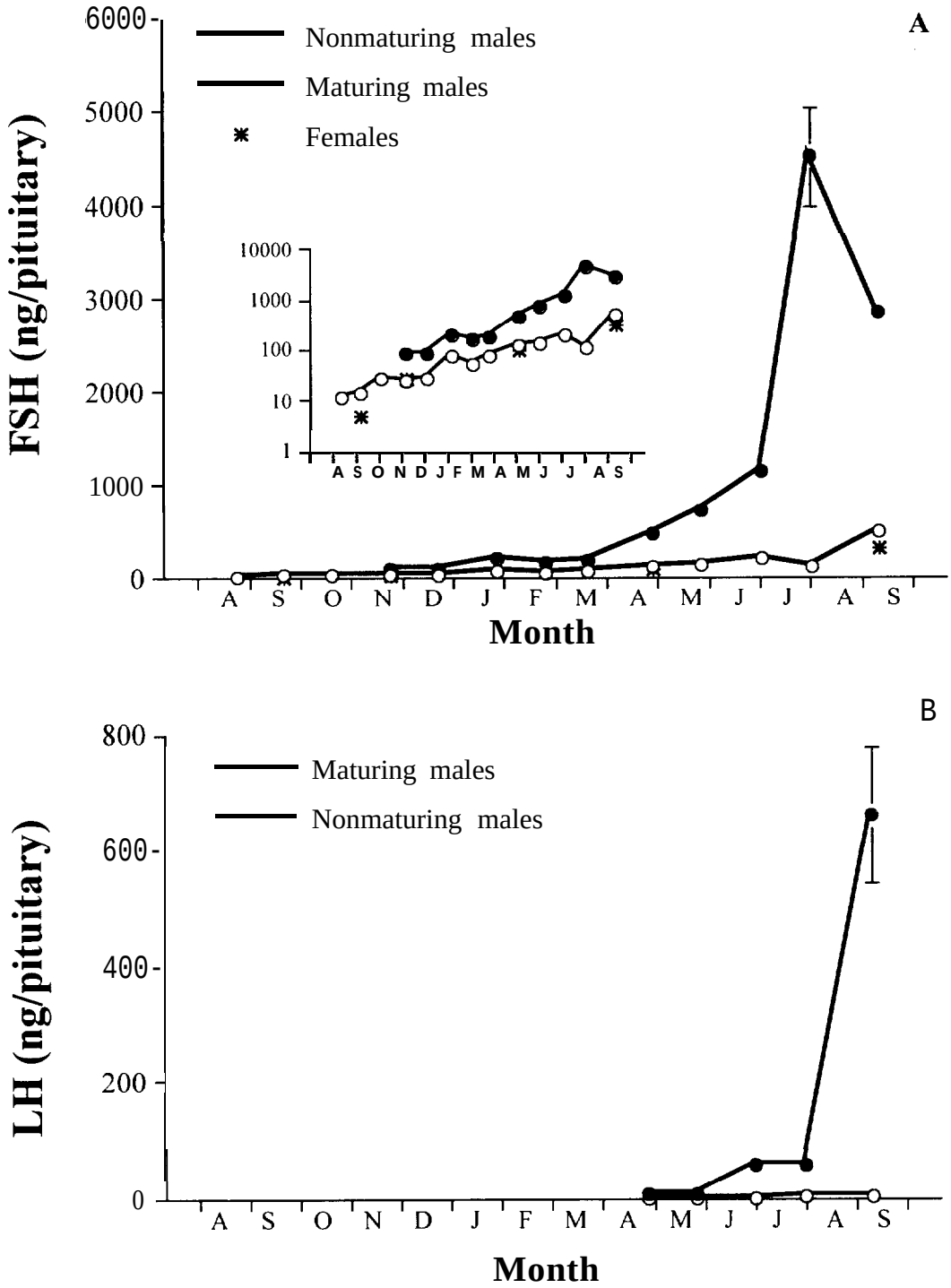

Figure 6. Pituitary levels of FSH (A) and LH (B) in nonmaturing male and female, and maturing male 1+ spring chinook salmon sampled during the experimental period. Insert in (A) represents log-transformed data. Data are means +/- standard errors of 10-60 replicates. There was no effect of diet on pituitary FSH or LH levels, but significant differences due to maturity and date of sampling.

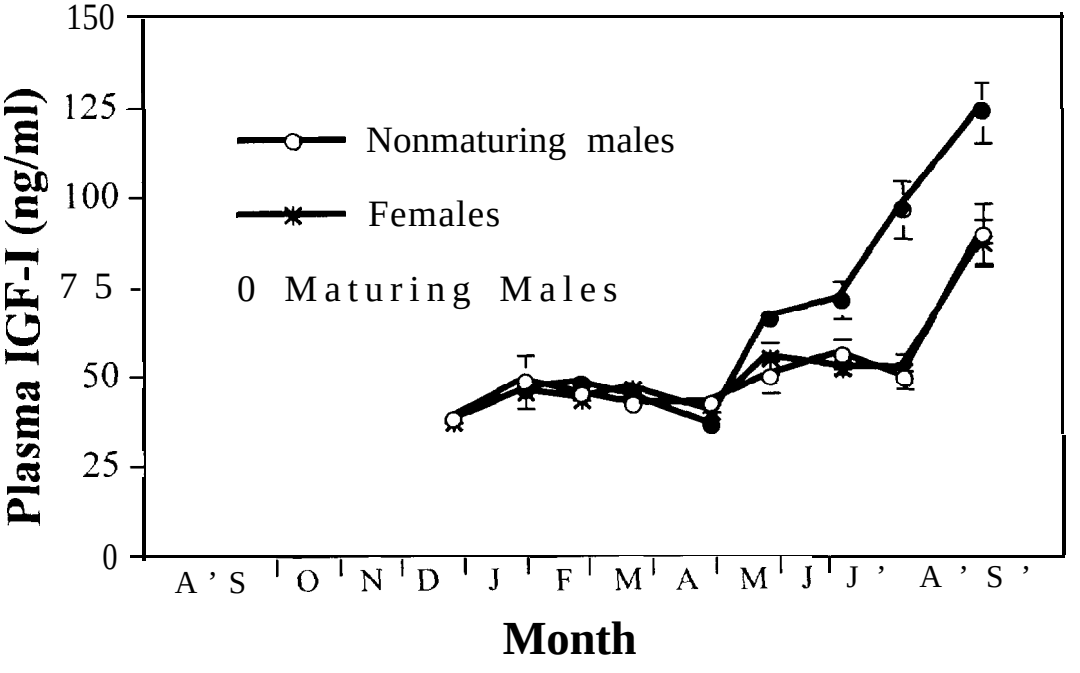

Figure 7. Plasma IGF-I levels in nonmaturing male and female, and maturing male 1+ spring chinook salmon sampled during the experimental period. Data are means +/- standard errors of 1 O-60 replicates.

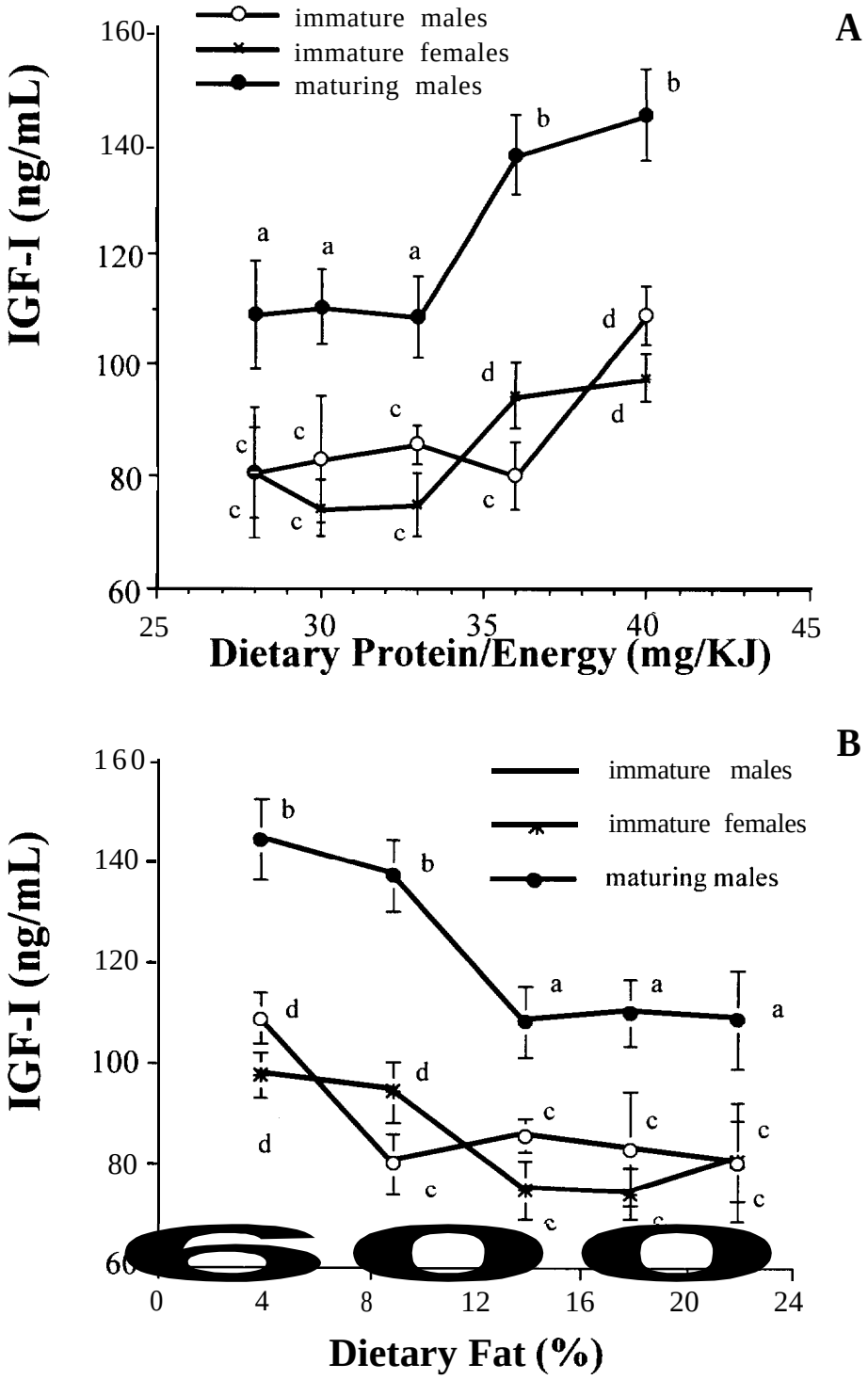

Figure 8. Plasma IGF-I levels in nonmaturing male and female, and maturing male l+spring chinook salmon sampled during September 1994 in relation to dietary protein/energy ratio (A) and dietary fat (B). Data are mean +/- standard errors, n = 10 per data point.

DISCUSSION

There were three major findings that resulted from this study. First, we were able to successfully manipulate whole-body lipid levels and control size of juvenile spring chinook salmon with a diet and feeding regime. Second, maturation of 2-year-old male chinook salmon was influenced by whole-body lipid levels during autumn, when maturation was initiated. Third, we confirmed that a critical period when maturation is initiated in male spring chinook salmon is a full year prior to spawning.

Growth and Body Fat Levels

The diets and feeding regime used in the present study successfully produced groups of fish with similar growth rates, but different levels of body fat. Daily growth was approximately 0.6%, which is less than that normally observed under hatchery conditions (about 2.0%; Hardy 1991). This may be due to the relatively low food consumption of fish fed the diet containing the highest level of lipid. Since the food consumption of this group was used to calculate the ration for all of the other groups, ration was consequently reduced across all treatments.

In another study, we found that feed intake is lower in fat compared to lean fish (Shearer et al. 1997b). Therefore, it is most likely that the low feed intake observed in fish fed the highest level of dietary lipid was due to the high energy density of this diet or the high level of body lipid in these fish (Shearer et al. 1997b). The maximum body fat level observed at the end of the experiment was 10%. which is higher than that observed in hatchery fish of similar size (8%) by Higgs et al. (1985). This may be due to the high energy density (23.3 to 28.0 KJ/g) of the diets used in our study, which were higher than diets normally fed to juvenile chinook salmon (Silver et al. 1993). Despite the relatively low growth, we were able to maintain graded levels of body fat among the treatment groups throughout the duration of the experiment.

Sexual Development and Growth

In November 1993 the males classified as maturing were similar in weight to the nonmaturing males and females, but by December, the mature males were significantly larger than nonmaturing fish. Males showing testicular development remained larger than nondeveloping males and females for the remainder of the experiment. A weight increase associated with male sexual maturation has previously been reported (Aksnes et al. 1986, Taylor 1989, Kadri et al. 1996, Tveiten et al. 1996).

Since our results showed no difference in size between maturing and nonmaturing males during November 1993, our results support the suggestion by Foote et al. (1991) that maturation is initiated prior to the increase in growth that is observed in maturing fish. However, it should be noted that although no statistical difference in size was observed during November, it was

apparent from weight frequency distributions that there was a trend of higher body weights in maturing males than nonmaturing fish of either sex. Therefore, our data are insufficient to determine whether the physiological commitment to initiate maturation occurs first, and that enhanced growth occurs as a result of this process (i.e. anabolic reproductive hormones), or whether higher growth causes the fish to initiate maturation.

Recent studies by Shearer et al. (1997a) and Silverstein et al. (1997a,b) have shown that both growth and body adiposity affect the percentage of male chinook salmon that mature at 2 years of age. It would be difficult to conclude that growth does not affect the onset of maturation, since maturation rates were clearly altered by growth.

Condition Factor and Adiposity

Condition factors were similar in all groups throughout the study, irrespective of body-fat levels. Condition factor is an index of body shape, and the decrease observed between November and December 1993 is probably due to a morphological change associated with smoltification (Folmar and Dickhoff 1980). Recent studies by Beckman and Dickhoff (Unpubl. manuscr.) have shown that juvenile spring chinook salmon show physiological and morphological changes associated with smoltification during the fall as yearling fish and again in the subsequent spring. Based on the whole-body lipid determinations made at the end of the experiment, there appears to be no relationship between whole-body lipid and condition factor . These results are in contrast with those of Herbinger and Friars (1991) and Simpson (1992). It is possible that condition factor may be an appropriate index of body fat levels in some species during part of the life-cycle; however, it should not be used universally for this purpose.

Effect of Whole-body lipid on the Incidence of Male Maturation

One of the main objectives of the experiment was to determine if there was a relationship between whole-body lipid level and the incidence of sexual maturation. In our study, regression analysis of mean tank whole-body lipid levels in December, and the incidence of male maturation in September 1994, indicated that a higher incidence of maturation was associated with higher whole-body lipid the previous winter. From the start of the experiment in August 1993 until August 1994, only pooled lipid samples were analyzed. It was therefore impossible to determine if the males that initiated maturation had higher whole-body lipid levels than nonmaturing males; thus we could not confirm the hypothesis put forth by Thorpe (1994) and Silverstein et al. (1997b) that males making the " decision" to mature were those that exceeded a threshold lipid level the fall before final maturation occurred. However, we were able to confirm with both endocrine and histological data that maturation was initiated in some fish during the autumn, a full year in advance of the spawning period.

At the end of the experiment, when lipid analyses were performed on individual fish, whole-body lipid levels of maturing males were lower than nonmaturing males. Whole-body lipid analyses were performed on all nonmaturing males and females fed diet 3 (n = 119) and the lipid levels were found to have a mean of 7.9%, a range from 5.5 to 11.9%, and to be normally

distributed. The lipid levels of nonmaturing fish analyzed in the other treatments (n = 20 fish/treatment; data not presented), also appeared to be normally distributed. The lower lipid level in mature males is most likely due to mobilization of stored energy for gonadal growth (Ware 1980, Roff 1983).

Endocrine Correlates of the Initiation of Maturation and Growth

Sexual maturation in male fish includes the production of mature sperm, development of secondary sex characteristics, and appropriate mating behavior. In this study we confined our measurements of maturation to histological analysis of testicular development. Spermatogenesis involves mitotic divisions of germ cells (spermatogonial proliferation). two meiotic divisions (formation of primary and secondary spermatocytes), spermiogenesis (formation of the sperm from secondary spermatocytes), and spermiation (release of sperm into the sperm duct). Numerous studies have implicated the role of the pituitary in controlling sexual maturation (Billard et al. 1990, Billard 1993). The formation of spermatogonia does not appear to be under the control of the pituitary gland; however, both pituitary gonadotropins and androgens are involved in regulating spermatogenesis (Miura et al. 199 1, 1992).

In this study, the transition from mitotic to meitotic divisions (appearance of spermatocytes) was correlated with elevations in pituitary-FSH levels, suggesting an activation of the pituitary-gonad axis. We first observed this transition during November and December, 11 months before fish produced mature sperm. We concluded from this data that a critical period for initiation of spermatogenesis is a full year prior to spawning, as has been previously suggested by others (Thorpe 199 1, Thorpe et al. 1992, Mange1 1994, Bergland 1995, Silverstein et al. 1997b). The level of FSH in the pituitary of maturing fish increased drastically during the spring, reaching a peak level in August. The spring rise in pituitary FSH occurred when more advanced stages of spermatogenesis were observed in cysts within the tubules in the testis. This also corresponded to a time when LH could be detected in the pituitary of maturing males.

In previous studies we have shown that plasma levels of FSH increase in adult coho salmon during early stages of spermatogenesis and then decline at spawning, whereas LH levels increase at the time of spermiation (Swanson 1991). Similar results have been obtained in recent studies of rainbow trout (Prat et al. 1996) and adult spring chinook salmon (Slater et al. 1996). Pituitary levels of FSH are also elevated in precocious masu salmon (Amano et al. 1993, 1994). These results, combined with studies of steroidogenic activity of FSH (Planas and Swanson 1994) strongly indicate that FSH, not LH, plays a critical role in the initiation of spermatogenesis in fish.

At the outset of this study, we were interested in measuring levels of a metabolic hormone that regulates growth and to determine whether levels of this hormone could be used to distinguish growth differences among individual fish during a period when maturation was initiated. Thorpe and others have suggested that fish perceive their “growth opportunity,” which in turn influences the “decision” or physiological commitment to mature. The endocrine mechanism by which this occurs is not known. We speculated that a metabolic hormone may be a critical signal to the reproductive system that there are sufficient energy reserves or that growth rate is sufficient to initiate maturation for the subsequent year.

In fish, as well as other vertebrates. growth is regulated by a complex suite of hormones including pituitary growth hormone (GH), thyroid hormones, pancreatic hormones and a variety of growth factors. GH stimulates hepatic production of IGF-I, which in turn acts peripherally to stimulate tissue growth. The GH-IGF-I axis is influenced by photoperiod, temperature (Bjornsson et al. 1995, McCormick et al. 1995), and nutrition (Perez-Sanchez et al. 1995).

Beckman and Dickhoff (submitted) have suggested that the GH-IGF-I axis provides the animal with an integrated signal for season, temperature, and food supply. Recent studies have also shown that IGF-I is produced in the testis, stimulates spermatogonial cell division (Loir and Le Gac 1994) and testicular steroid production in rainbow trout (Le Gac and Loir 1995). Because of the role of IGF-I in both growth and reproduction, we were interested in measuring IGF-I during the critical period when growth may be influencing the physiological commitment to maturation.

In our study, we found that maturing fish had higher IGF-I levels than nonmaturing fish from the spring growth period (May) to the end of the experiment (September). However, because the maturing fish were also larger we cannot determine whether the increase in IGF-I was related to growth and/or maturation. Interestingly, there was no differencein IGF-I levels when significant size and growth differences were noted between maturing and nonmaturing males from December through May. This was surprising in view of recent studies by Beckman et al. (1997) who demonstrated a significant correlation between plasma levels of IGF-I and growth rate, but not body size.

In addition to maturation effects on IGF-I levels, we found that IGF-I levels during September were higher in fish fed Diets 1 and 2, which had the lowest fat content but highest protein/energy ratios. This result is consistent with observations of Perez-Sanchez et al. (1995) who found that increases in protein intake influence the GH-IGF-I axis in a marine fish by increasing plasma IGF-I and liver GH receptors. At the end of the experiment, we analyzed individual body-fat levels and plasma IGF-I. We did not find a significant correlation of IGF-I with body-fat levels as reported by Silverstein et al. (1997b).

The seasonal increases in IGF-I levels that we observed in both maturing and nonmaturing fish were similar to those reported by Beckman et al. (1997) and Silverstein et al. (1997b), with peak levels occurring in late summer and early autumn. Previous studies have also shown smoltification-associated increases in IGF-I levels (Lindahl et al. 1985, Duguay et al. 1994, Beckman et al. 1997). However, the autumn increases in IGF-I observed in the fish in our study were not associated with smoltification because the fish would have smolted during the previous spring or autumn.

CONCLUSIONS

Previous studies have suggested that there are two critical periods during the process of maturation (Thorpe 1991, Thorpe et al. 1992, Mange1 1994, Berglund, 1995). First, there is a critical period during autumn, 1 year prior to maturity, when sexual maturation (puberty) is initiated. Second, there is a period in the spring, when maturation is permitted to continue if environmental conditions and threshold size or energy status are sufficient to support gonadal development. The important distinction between these two periods is that the second is "permissive." In other words, the maturation process starts in the autumn, and can either be permitted to continue in the spring or inhibited. There is considerable evidence that size, growth rate, and energy storage are important factors that influence the physiological commitment to sexual maturation (Alm 1959, Rowe and Thorpe 1990b, Rowe et al. 199 1. Berglund 1995). In most studies to date, the relative importance of these factors could not be discerned.

In our study, where growth in terms of body size was controlled and body fat levels were altered, we found that higher body-fat levels were associated with higher rates of male maturity. In studies conducted subsequently, we have found an interactive effect of body size and whole-body adiposity on maturation of male spring chinook salmon (Shearer et al. 1997a, Silverstein et al. 1997b). Together, these studies indicate that the rate of maturation of male salmonids can be manipulated by controlling growth and adiposity. Since we have confirmed in our present study that the autumn is a critical period for the initiation of spermatogenesis, it is clear that manipulations of growth and adiposity during this season are critical to affect maturation rates of males in the subsequent year.

These data have important implications for controlling age of maturity in captively-reared male salmonids. It may be desirable to reduce the number of males maturing at 1 and 2 years of age to avoid asynchronous age of maturity of male and female fish. Data from the present study and others indicate that it may be possible to reduce the number of males maturing at 2 years of age by reducing growth and fat levels during the autumn, a full year prior to maturation. Because reduced ration 8 to 12 months prior to spawning also affects maturation rates and fecundity of female rainbow trout (Bromage et al. 1992), the effects of manipulations of both ration and fat levels that we have studied in male chinook salmon need to be examined in females.

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PART VII

INDUCTION OF OVULATION AND SPERMIATION IN SOCKEYE SALMON USING GONADOTROPIN-RELEASING HORMONE ANALOG (GNRH-A) IN CONTROLLED-RELEASE DEVICES

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INTRODUCTION

Reproduction in captive broodfish can be artificially controlled at two levels: through manipulation of environmental cues and through alterations in the reproductive endocrine system. Environmental influences on reproduction, after perception and integration by the central nervous system. alter release of hormones by the hypothalamus (primarily gonadotropin-releasing hormone, GnRH). These hormones in turn regulate secretion of pituitary gonadotropins that act on the gonad to regulate gametogenesis. Techniques for the control of reproduction through exogenous administration of hormones have been developed which intervene at each level of the brain (GnRH)-pituitary (gonadotropin)-gonad (steroid) axis (reviewed by Donaldson and Hunter 1983).

Although manipulation of gametogenesis (gonadal growth) has been achieved in some species through chronic treatment of fish with various preparations of gonadotropins and/or steroids (Billard 1992, 1993; Billard et al. 1986, 1990), this technology is not presently used on cultured salmonids. However, hormonal induction of final oocyte maturation, ovulation, and spermiation is widely used on salmon broodstock to prevent losses due to prespawning mortality and to advance or synchronize spawning time (Donaldson and Hunter 1983; Donaldson 1986; Zohar 1988. 1989).

Technology for hormonal induction of ovulation and spermiation has several important applications to captive broodstock programs for endangered or threatened salmonid fish. This technology can be used to 1) prevent loss of gametes due to prespawning mortality. 2) synchronize spawning in wild and captive fish, 3) extend the period of spermiation and yield of sperm, and 4) to synchronize and/or advance spawning in male and female broodfish. Hatchery managers may not need to routinely induce spawning in broodstock, but this technology is a tool that should be available to managers who may need to produce a predictable spawning period or advance spawning if there is a risk of prespawning mortality or risk of poor egg quality due to delayed maturation.

GnRH or superactive analogues of GnRH (GnRHa) are most commonly used for induction of ovulation and sperm production. This appears to be the most efficient therapy because GnRH stimulates release of endogenous gonadotropin, thus avoiding problems with species specificity and quality of the gonadotropin preparations. In addition, these decapeptides are commercially available, and GnRHa can be readily dissolved in saline and administered by intramuscular or intraperitoneal injection.

This technique has been used to successfully synchronize spawning in salmonid fish (Donaldson et al. 198 1, 1984; Van der Kraak et al. 1983, 1984, 1985; Crim et al. 198 1; Sower et al. 1982, 1984; Fitzpatrick et al. 1984, 1987); however, there are disadvantages to this mode of

administration. To be most effective, the GnRHa in saline must be given in two injections, requiring additional handling of broodstock. Because the treatment causes acute elevations in gonadotropins, it is most effective in synchronizing maturation in fish that are within 2 to 3 weeks of natural spawning time, but not very effective for a major advancement of spawning time. It is therefore advantageous to administer GnRHa in a vehicle that produces a more sustained delivery of the analog.

Technology for controlled-release GnRHa delivery systems has been developed for induction of ovulation and spermiation in salmonids (Crim et al. 1983b, 1988a; Crim and Glebe 1984; Zohar et al. 1990b; Breton et al., 1990). The advantages of this technique are that 1) the quantity of hormone administered and labor required for the treatment can be reduced, making the treatment more cost-effective, and 2) stress to the broodfish associated with protocols requiring multiple injections can be reduced. Several types of delivery systems for GnRHa have been developed and tested in salmonids.

Crim and colleagues (Crim et al. 1983a, b; Crim and Glebe 1984) developed cholesterol pellet GnRHa implants. Although the pellets were effective for synchronizing and advancing ovulation, they required a relatively high amount of GnRHa. Zohar and colleagues (Zohar 1988; Zohar et al. 1989; 1990b; Mylonas et al. 1995) have developed two types of GnRHa delivery systems: nonbiodegradable and biodegradable. The nonbiodegradable delivery system is a pellet (approximately OS-mm diameter) composed of an ethylene vinyl acetate copolymer (EVAC).

The second type of delivery system developed by Zohar and colleagues is a preparation of biodegradable microspheres consisting of a poly [fatty acid dimer-sebasic acid] (Mylonas et al. 1995). Both delivery systems have been used to induce ovulation in a variety of broodstock (Breton et al. 1990, Swanson 1994, Mylonas et al. 1995); however, none of these studies directly compared the two types of controlled-release devices, and the effects of the hormone on sperm production and gamete quality were not thoroughly tested.

Before this technology is widely used to control spawning time in captive broodstock of endangered or threatened fish, further testing is necessary to ensure that the hormone treatment does not impair gamete quality or embryo survival. Therefore, in the present study, we compared the effectiveness of these two types of controlled-release devices containing GnRHa for induction of ovulation and spermiation in Lake Wenatchee sockeye salmon.

MATERIALS AND METHODS

Fish

Adult Lake Wenatchee sockeye salmon (40 males and 40 females) were collected during their spawning migration during early August 1995 at the Tumwater Dam and transported to the fish holding facilities at the Northwest Fisheries Science Center, Seattle, WA. Fish were maintained without feeding on 10°C recirculated fresh water in circular fiberglass tanks (2.7 m diameter). On August 14, approximately 1 week after transport, fish were weighed, anesthetized and individually tagged with passive-integrated transponder (PIT) tags. Average body weight of males and females were 1,336 ± 46 g and 1,579 ± 65 g, respectively.

Hormone Treatments and Sampling

Two types of controlled-release devices were compared: 1) a nonbiodegradable delivery system composed of pellet of an ethylene vinyl acetate copolymer (EVAC), and 2) a preparation of injectable biodegradable microspheres consisting of a poly [fatty acid dimer-sebasic acid] (FADSA). Each delivery system contained des-Gly^{10}[D-Ala^{6}]-mammalian GnRH ethylamide (GnRHa). The GnRHa-EVAC and GnRHa-FADSA preparations were purchased from Aquapharm Technologies (Columbia, Maryland). Two doses of each delivery system were compared.

We tested EVAC pellets containing either 25 or 75 µg of GnRHa. All fish treated with EVAC pellets received one pellet, which was injected into the dorsal muscle with an 1 l-gauge needle and a modified syringe with a steel plunger. The two dosages of GnRHa-FADSA microspheres were administered at 25 and 75 µg GnRHa/kg body weight. Microspheres (125 mg) were swollen in 5.0-mL vehicle provided by the manufacturer for 1 hour prior to injection. The final concentration was 75-µg GnRHa/2.5-mg microspheres/0. 1 -mL vehicle.

This solution was diluted threefold in vehicle to obtain a second stock of 25 µg GnRHa/2.5 mg microspheres/0. 1 mL vehicle. Fish were injected intramuscularly with 0.1 mL microsphere suspension per kg body weight. The site of injection was lateral to the dorsal fin. An 1 S-gauge needle and 1 -cc tuberculin syringe were used for the injections.

Fish were treated with either EVAC pellets or microspheres containing GnRHa on 28 August 1995. At the time of treatment, neither male nor female fish exhibited secondary sex characteristics or spawning coloration. In addition, none of the males produced milt with gentle abdominal pressure, and females had not ovulated. To monitor the kinetics of the response to GnRHa, plasma levels of luteinizing hormone (LH) were measured by radioimmunoassay (Swanson et al. 1989). Fish were anesthetized in tricaine methane sulfonate (MS-222, 0.02%), and blood was collected from the caudal vein using heparinized syringes and 21-gauge needles. Blood was sampled from females prior to treatment and 3, 7, 10, and 14 days after treatment and

at the time of egg collection. Blood was sampled from males 3, 7, 10, 14, 25, and 32 days post-treatment.

Body Coloration Measurements

The onset of coloration in the skin of males was quantified using a Minolta CR-1 00 color meter (Minolta Corp., Ramsey, NJ) set for type 'C' illuminant (6774K) with the L*a*b* chromaticity coordinates. This setting quantifies color along these three axes: 1) L* represents a white (negative values) to black (positive values) axis; 2) a* represents a green (negative values) to red (positive values) axis; and 3) b* represents a blue (negative values) to yellow (positive values) axis. Skin color was measured beneath the posterior insertion of the dorsal fin midway between the bottom of the dorsal fin and the lateral line. Values presented as "Red color index" represent the a* axis values.

Spawning and Embryo Incubation

Fish were examined for maturity at 3, 7, 10, 14, 18, 21, 25, 28, 32, 34, 35, 42, 50, and 57 days after the treatment. Milt was collected into sterile plastic bags, oxygenated, and stored on an insulated layer of ice until used for fertilization. Both spermatocrit and milt volume were measured during the first 2 weeks of milt collection. Males were completely stripped at each collection during the first 2 weeks. Eggs were collected from ovulated females into sterile bags and stored on an insulated layer of ice until fertilized. The total weight of eggs and weight of 100 eggs from each female were recorded.

To determine if the hormone treatment had a negative effect on gamete quality, survival of embryos up to hatch was monitored. Only gametes from the highest dose of each treatment were compared to those of controls. Eggs from females (two per treatment) from the control, EVAC-75 and microsphere FADSA-75 treatments were fertilized by sperm from males (two per treatment) from the same treatments. Eggs from each female were divided into 6 lots containing 100 eggs each, and each lot was fertilized with 0.1 mL milt from 6 males (2 per treatment). This matrix was triplicated.

Eggs were incubated in individual cups within Heath trays with flow-through dechlorinated municipal water at 10°C. Twenty-four hours after fertilization, damaged or unfertilized eggs were counted and removed. At the eyed stage, eggs were shocked and any dead eggs were counted and removed. Finally, embryos surviving to hatch were counted.

Statistics

Statistical analyses were performed using either SuperANOVA or Statview™ (Abacus Concepts, Berkeley, CA, 1992). Multiple mean comparisons were made using the Fisher PLSD test. Data were analyzed by one- or two-way ANOVA using a significance level of $P < 0.05$.

RESULTS

Maturation of Females

Treatment of female fish with GnRHa significantly advanced and synchronized the time of ovulation in a dose-dependent manner (Fig. 1). The average number of days to spawning was 36 in the controls. and 14 in the fish treated with EVAC-75 (Fig. 2). The 25-μg/kg body weight dose of the FADSA was less effective than either dose of EVAC or the 75-μg/kg dose of FADSA. All GnRHa treatments induced increases in plasma levels of LH in an approximately dose-dependent manner (Fig. 3). Advancement of ovulation using the GnRHa treatments decreased average egg weight (Fig. 4), but did not affect fecundity (Fig. 5) or the survival of embryos to hatch (Fig. 6).

Maturation of Males

Treatment of male fish with GnRHa significantly advanced and synchronized spermiation, with the 25-μg/kg body weight dose of the FADSA being only slightly less effective than all other GnRHa treatments (Fig. 7). The response to GnRHa was accompanied by increases in plasma levels of LH (Fig. 8), again with the 25-μg/kg body weight dose of the FADSA being less effective. Spermatocrit generally increased in all fish from 6 to 18 days post-treatment, but with GnRHa-treated fish having higher spermatocrit than controls prior to 18 days (Fig. 9).

The average total milt volume collected from spermiating fish within the first 14 days was significantly higher in GnRHa-treated fish than controls (Fig. 10). The increase in milt volume was roughly dose-dependent when comparing the same mode of administration. However, the fish treated with EVAC-GnRHa pellets produced more milt for a given dose compared to those treated with FADSA-GnRHa.

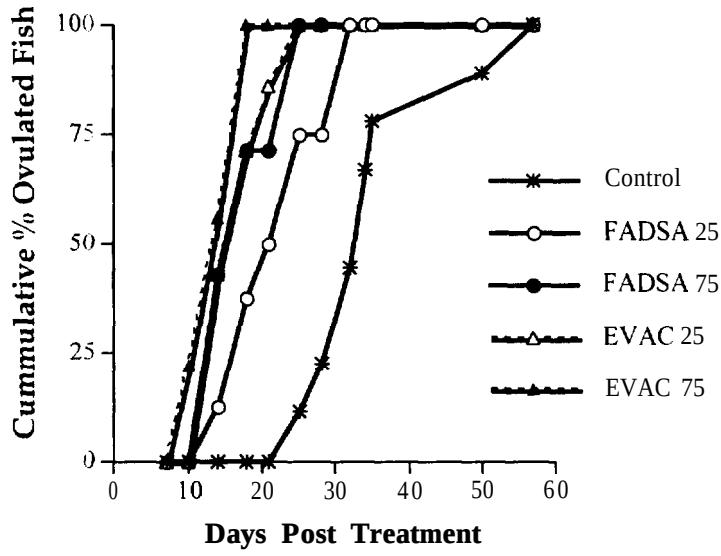

Figure 1. Timing of ovulation after treatment of fish with controlled-release devices containing GnRHa. Fish were treated on day 0 (August 28) with either ethylene vinyl acetate copolymer (EVAC) pellets containing 25 or 75 μg GnRHa, or microspheres (FADSA) at a dose of 25 or 75 μg GnRHa/kg body weight; n = 10 females per treatment.

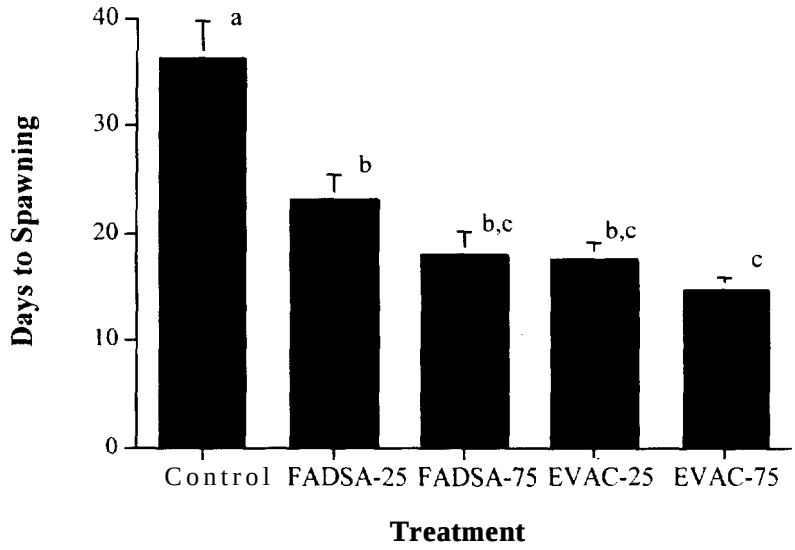

Figure 2. Average number of days to ovulation after treatment of fish with controlled-release devices containing GnRHa. Fish were treated on day 0 (August 28) with either ethylene vinyl acetate copolymer (EVAC) pellets containing 25 or 75 μg GnRHa, or microspheres (FADSA) at a dose of 25 or 75 μg GnRHa/kg body weight. Control fisl received injection vehicle only; n = 10 females per treatment. Data are mean ± standard errors.

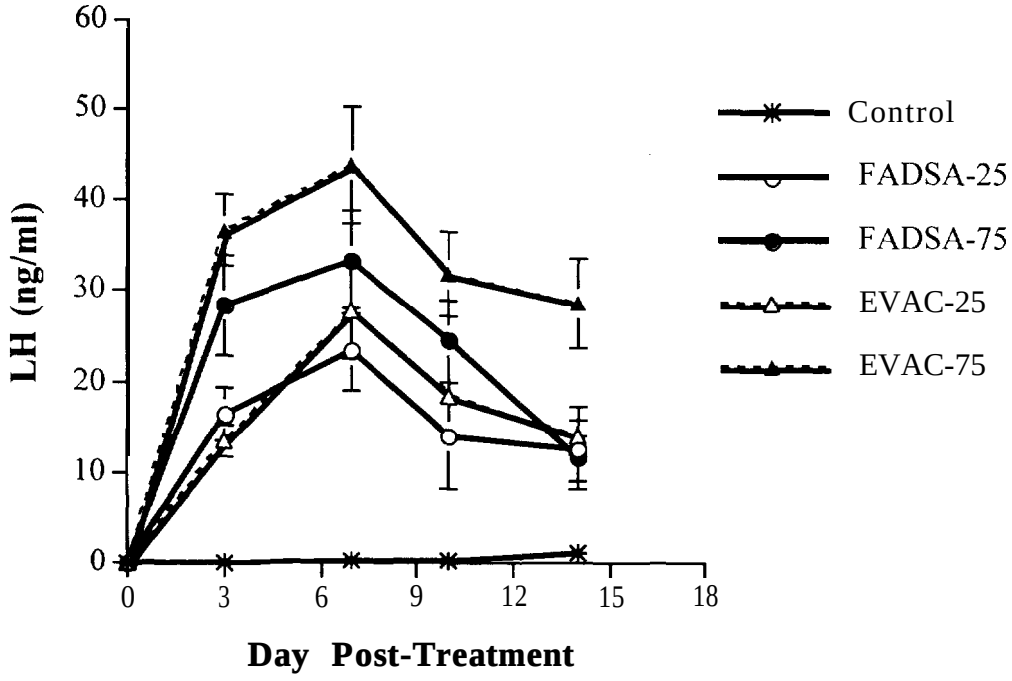

Figure 3. Plasma levels of luteinizing hormone (LH) after treatment of fish with controlled-release devices containing GnRHa. Fish were treated on day 0 (August 28) with either ethylene vinyl acetate copolymer (EVAC) pellets containing 25 or 75 μg GnRHa, or microspheres (FADSA) at a dose of 25 or 75 μg GnRHa/kg body weight. Control fish received injection vehicle only, n = 10 females per treatment. Data are mean ± standard errors. Data from all GnRHa-treated fish are significantly different ($P<0.05$) from controls at all time points.

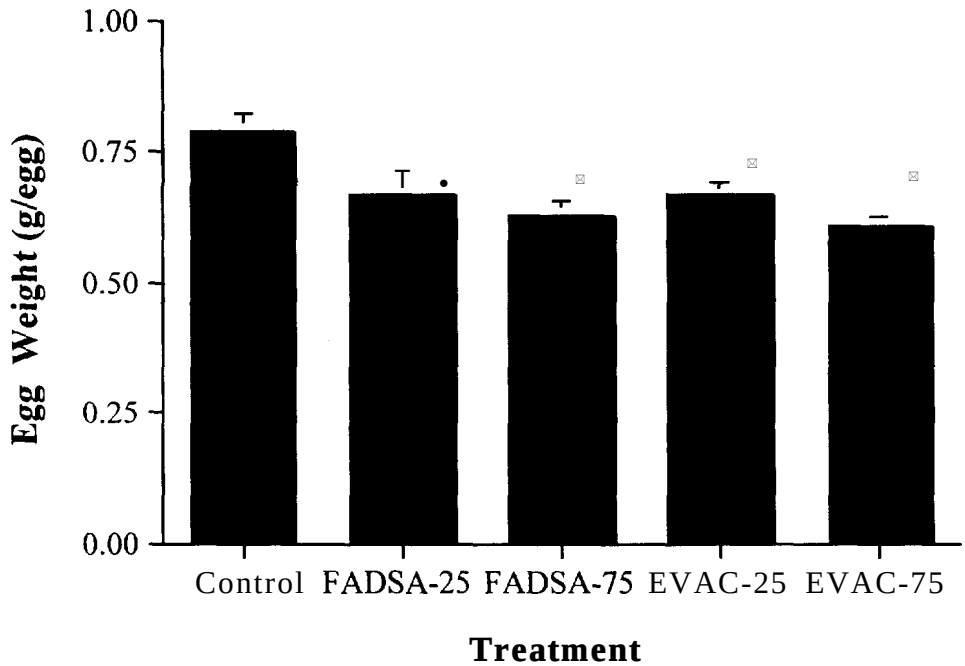

Figure 4. Average weight of ovulated eggs produced by females that had been treated fish controlled-release devices containing GnRHa. Fish were treated on day 0 (August 28) with either ethylene vinyl acetate copolymer (EVAC) pellets containing 25 or 75 μg GnRHa, or microspheres (FADSA) at a dose of 25 or 75 μg GnRHa/kg body weight. Control fish received injection vehicle only, n = 10 females per treatment. Data are mean ± standard errors; * indicates significantly different from controls (P<0.05).

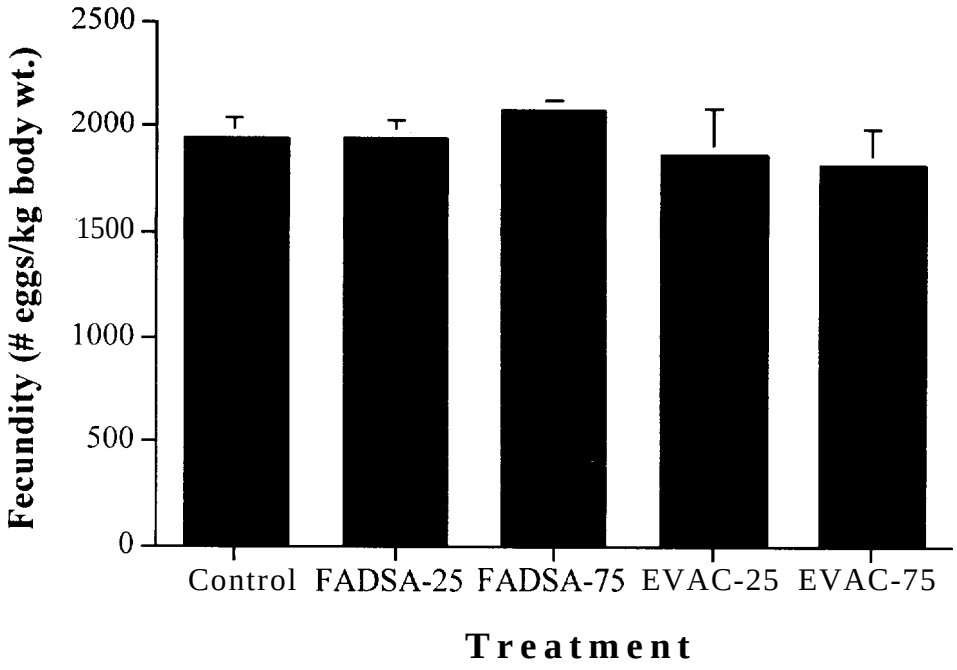

Figure 5. Average number of ovulated eggs per kg body weight produced by females that had been treated fish controlled-release devices containing GnRHa. Fish were treated on day 0 (August 28) with either ethylene vinyl acetate copolymer (EVAC) pellets containing 25 or 75 μg GnRHa, or microspheres (FADSA) at a dose of 25 or 75 μg GnRHa/kg body weight. Control fish received injection vehicle only, n = 10 females per treatment. Data are mean ± standard errors. Fecundity is not corrected for body weight of the females.

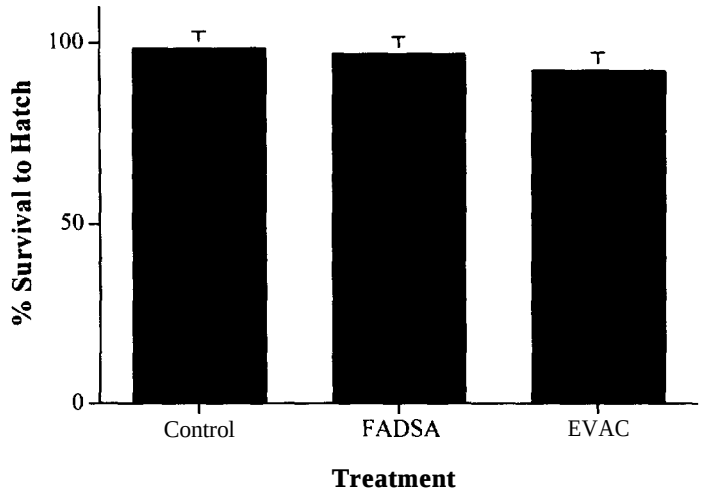

Figure 6. Average survival to hatch of embryos from control females or those females treated with controlled-release devices containing GnRHa. Fish were treated on day 0 (August 28) with either ethylene vinyl acetate copolymer (EVAC) pellets containing 75 μg GnRHa, or microspheres (FADSA) at a dose of 75 μg GnRHa/kg body weight. Control fish received injection vehicle only. Data are mean ± standard errors.

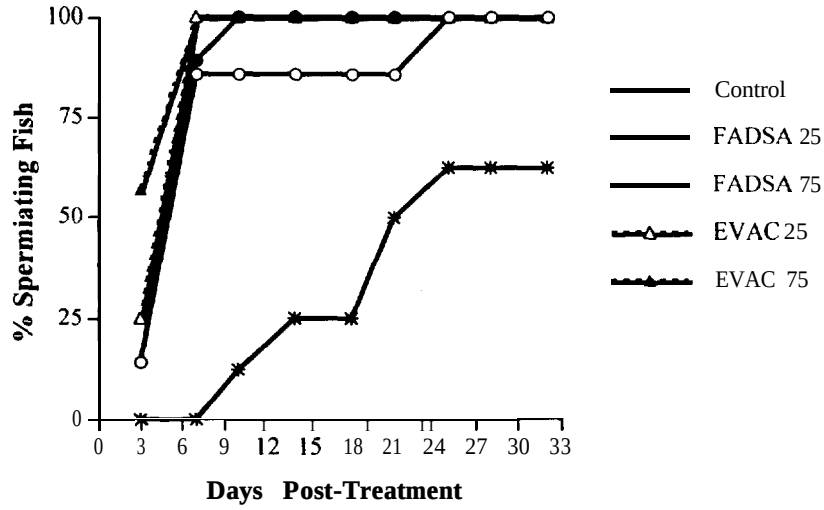

Figure 7. Timing of spermiation after treatment of fish with controlled-release devices containing GnRHa. Fish were treated on day 0 (August 28) with either ethylene vinyl acetate copolymer (EVAC) pellets containing 25 or 75 μg GnRHa, or microspheres (FADSA) at a dose of 25 or 75 μg GnRHa/kg body weight; n = 10 males per treatment.

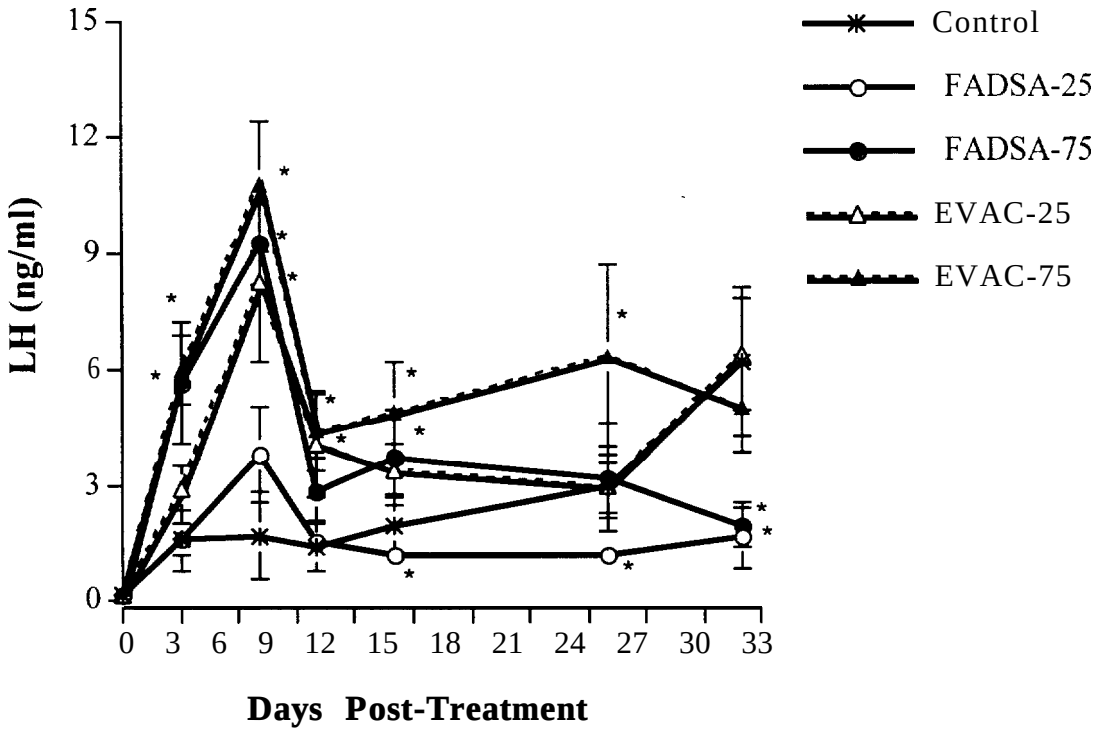

Figure 8. Plasma levels of luteinizing hormone (LH) after treatment of fish with controlled-release devices containing GnRHa. Fish were treated on day 0 (August 28) with either ethylene vinyl acetate copolymer (EVAC) pellets containing 25 or 75 μg GnRHa, or microspheres (FADSA) at a dose of 25 or 75 μg GnRHa/kg body weight. Control fish received injection vehicle only, n = 10 females per treatment. Data are mean ± standard errors; * indicates significantly different from controls at that time point ($P<0.05$).

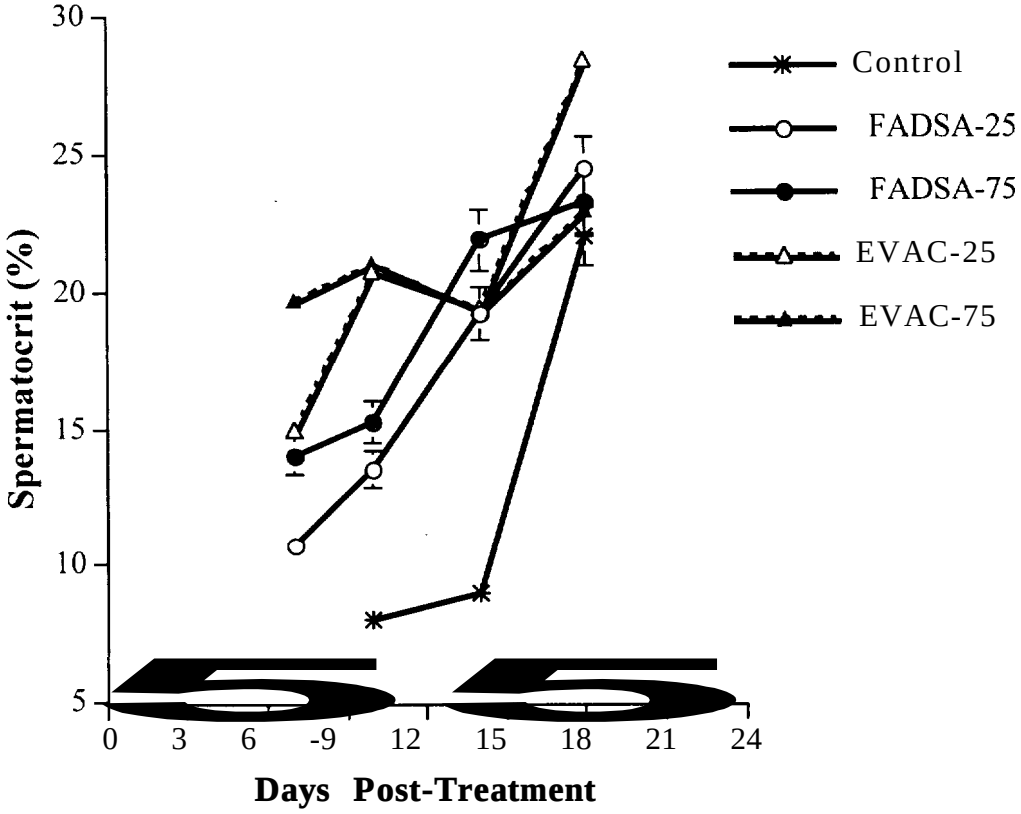

Figure 9. Average spermatocrit of milt produced by males that had been treated with controlled-release devices containing GnRHa. Fish were treated on day 0 (August 28) with either ethylene vinyl acetate copolymer (EVAC) pellets containing 25 or 75 μg GnRHa, or microspheres (FADSA) at a dose of 25 or 75 μg GnRHa/kg body weight. Control fish received injection vehicle only, n = 10 males per treatment, data are mean ± standard errors. Data from all GnRHa-treated fish are significantly different ($P<0.05$) from controls at 10 and 14 days post-treatment but not at 18 days.

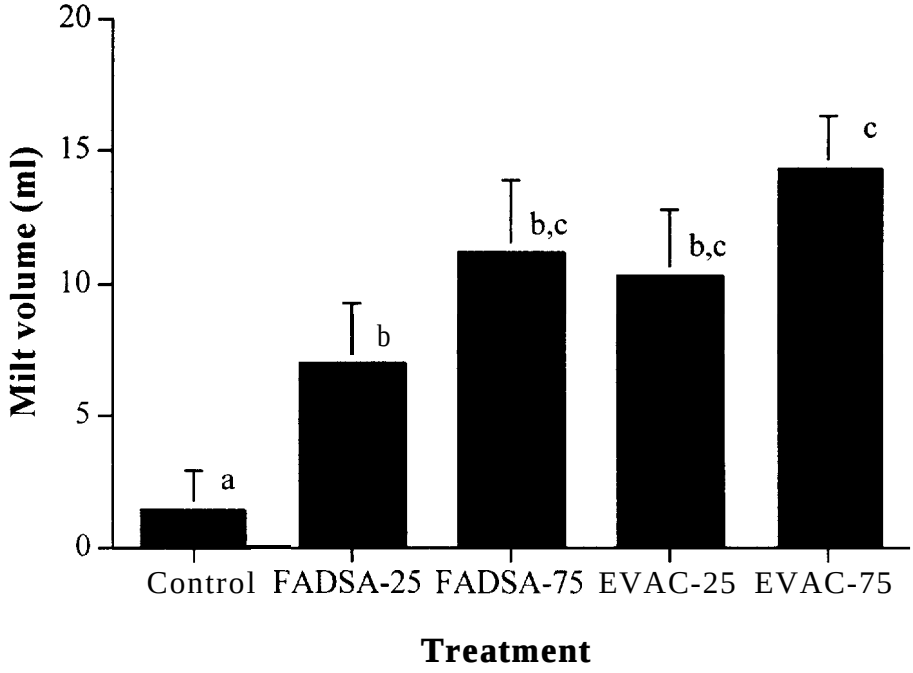

Figure 10. Average volume of milt produced by males that had been treated with controlled-release devices containing GnRHa. Fish were treated on day 0 (August 28) with either ethylene vinyl acetate copolymer (EVAC) pellets containing 25 or 75 μg GnRHa, or microspheres (FADSA) at a dose of 25 or 75 μg GnRHa/kg body weight. Control fish received injection vehicle only, n = 10 males per treatment. Data are mean ± standard errors. Common letters indicate data are not significantly different ($P<0.05$).

Body Color Development

The development of red coloration in the skin of males was monitored throughout the study in control and EVAC-G&Ha-treated fish (Fig. 11) and at a few time points in FADSA-GnRHa treated fish (Fig. 12). Within 7 days of treatment, G&Ha-treated fish were significantly more red than controls. The highest red color index occurred in EVAC-GnRHa-treated fish 10 days after the treatment and declined thereafter. Both doses of GnRH-EVAC were equally effective in inducing red coloration in the skin; however, the FADSA-GnRH treatments were relatively ineffective. Slight, but significant, differences in red coloration between control and FADSA-GnRH-treated fish were observed 10 days after the treatment, but not at other times.

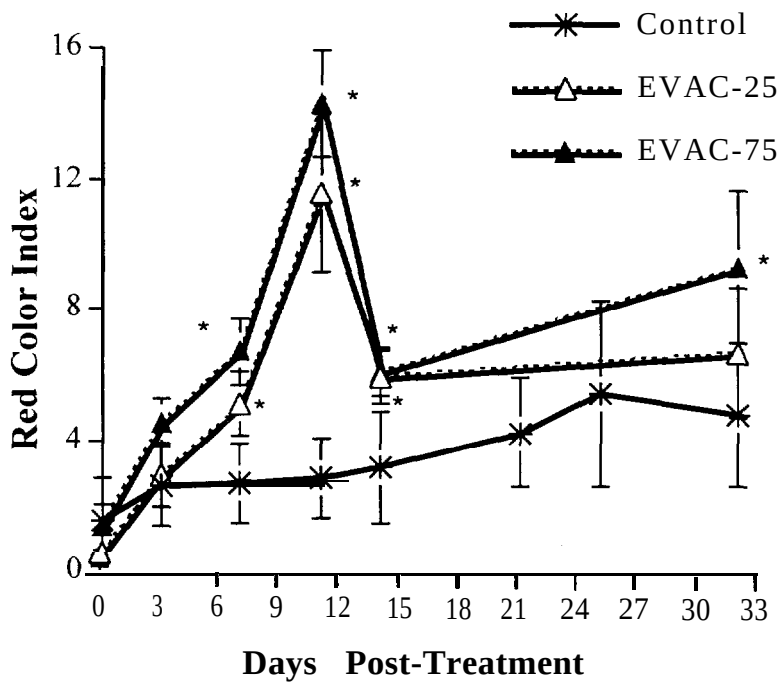

Figure 11. Red coloration in the skin of fish after treatment with controlled-release devices containing GnRHa. Fish were treated on day 0 (August 28) with ethylene vinyl acetate copolymer (EVAC) pellets containing 25 or 75 μg GnRHa. Control fish received injection vehicle only, n = 10 males per treatment. Data are mean ± standard errors; * indicates significantly different from controls at that time point (P<0.05).

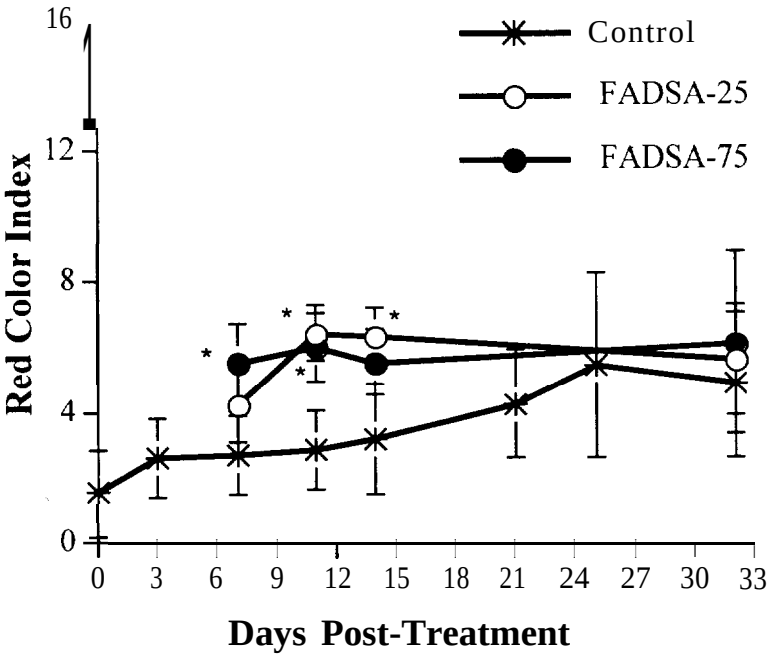

Figure 12. Red coloration in the skin of fish after treatment with controlled-release devices containing GnRHa. Fish were treated on day 0 (August 28) with microspheres (FADSA) containing GnRHa at a dose of 25 or 75 μg GnRHa/kg body weight. Control fish received injection vehicle only, n = 10 males per treatment. Data are mean ± standard errors; * indicates significantly different from controls at that time point ($P<0.05$).

DISCUSSION

In this study we have shown that the timing of ovulation and spermiation were significantly advanced and synchronized in sockeye salmon using sustained delivery of GnRHa with either biodegradable or nonbiodegradable polymers. These results are similar to what has been previously reported for female salmonids (Breton et al. 1990, Zohar et al. 1990b, Haraldsson et al. 1993), but provide new information on a direct comparison between the two types of delivery devices in both sexes of sockeye salmon.

Females

The mean days to spawning were reduced from 38 in control fish to 14 in EVAC-7.5 treated fish. Both EVAC pellets and FADSA microspheres were effective in advancing ovulation and in increasing plasma levels of LH in a manner somewhat related to the dose of GnRHa. Using GnRHa injected in saline, Slater et al. (1995) significantly advanced ovulation sockeye salmon by 1 O-l 2 days. Previous studies in Atlantic salmon rainbow trout, brown trout and coho salmon (Donaldson et al. 198 1, Van der Kraak et al. 1983, Crim et al. 198 1, Fitzpatrick et al. 1984, Mylonas et al. 1992) have shown similar advancement and synchronization of broodstock using two injections of GnRHa dissolved in saline when fish were treated within 2 to 3 weeks of their natural spawning period.

In our study, the fish were treated 4 weeks in advance of when they historically would have been spawned in captivity. The use of controlled-release devices for administration of GnRHa appeared to cause a more sustained elevation in plasma LH levels than shown by other investigators (Breton et al. 1990), and also resulted in a greater advancement of ovulation than GnRHa-saline treated fish (Breton et al. 1990, Zohar et al. 1990b).

The treatment of fish with GnRHa did not affect the number of ovulated eggs or survival of embryos to hatch. However, we observed a significant effect of the GnRHa treatment on egg weight at the time of spawning. Since egg weight may affect subsequent growth and survival of alevins (Wallace and Aaasjord 1984) we were concerned about this result. Yet, we did not observe any difference among the groups in survival of the embryos to the eyed-stage or to hatch. It is possible that we would have seen a treatment effect if we had followed the development of fry to later stages, but this was not possible due to facilities limitations.

We do not know if the differences in egg weight were due to moisture content of the eggs. Since significant hydration of the egg occurs during final oocyte maturation and ovulation, it is possible that accelerating this process using GnRHa reduces the amount of hydration that can occur. Previous studies have not reported a significant effect of GnRHa on egg size, egg

composition, egg moisture, or viability of embryos (Haraldsson et al. 1993), but there has been a trend of smaller eggs being produced by fish with the most advanced ovulation period. Again, no negative effect of the treatment on fertilization, hatching time, or embryo length was observed in the study by Haraldsson et al. (1993).

Mylonas et al. 1995 have reported a negative effect of GnRHa treatment on quality of eggs produced by brown trout. Reduction in egg quality was correlated with decreased time to ovulation in both control and GnRHa-treated fish (Mylonas et al. 1995). In our experience, as in the reports of others (Springate et al. 1984, Bromage et al. 1992), one of the key factors affecting egg quality is the timing of egg collection relative to ovulation. Poor fertility has been observed when eggs are collected from partially ovulated females. Thus, caution must be taken in judging when females are ready to be spawned.

Males

Although it is well established that GnRH or gonadotropins can be used to induce spermiation in male fish (Donaldson and Hunter 1983), few studies have examined the effects of GnRHa on sperm production (Weil and Crim 1983, Mylonas et al. 1995), and no studies have examined the fertility of males treated with GnRHa. In our study, we demonstrated that both EVAC pellets and FADSA microspheres were effective in advancing spermiation, increasing milt volume, and increasing plasma levels of LH in a manner somewhat related to the dose of GnRHa. The lowest dose of FADSA-GnRHa seemed to be the least effective in causing increases in plasma and was slightly less effective than the other treatments in advancing spermiation and increasing the total volume of milt collected during the first 2 weeks of the study.

Within 1 week of treatment, greater than 90% of GnRHa-treated fish were producing milt, whereas none of the controls produced milt at this time. Spermatocrit generally increased over time in all groups, with the GnRHa treatment advancing the timing of this increase. No effect of the hormone treatment on the fertility of males was observed. Together, these data indicate that GnRHa administered by either vehicle can be used to greatly accelerate sperm production in male salmon; however, we do not yet know whether GnRHa administered using saline as a vehicle would be equally effective.

Coloration

The rate of development of red coloration and intensity of red color in the skin of males were enhanced with the EVAC-GnRHa treatments, while the FADSA-GnRHa treatments had little effect on coloration. However, by the end of the sampling period, GnRHa-treated fish had lost some red coloration and were not different from controls. This may be due to the stress-induced changes in body color because fish were handled frequently. It was noted that all fish darkened during the period of study, and some pigment loss was noticed as fish health appeared to decline. The change in color due to the GnRHa treatment is most likely due to the subsequent effects of GnRHa on gonadotropin and androgen levels in the males, and not due to a direct effect of GnRHa on skin color. Previous studies have shown that androgens affect the development of secondary sex characteristics and spawning coloration in fish (Borg et al. 1994).

Summary

In summary, administration of GnRHa using either FADSA microspheres or EVAC pellets is effective in advancing ovulation and spermiation in sockeye salmon, without impairing gamete quality. The EVAC devices appeared to be most effective in inducing maturation in both sexes. The lowest dose of GnRHa tested (25 μg) was sufficient; however, it is possible that even lower doses could be used, particularly in males.

ACKNOWLEDGMENTS

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PART VIII

RESEARCH ON QUANTITATIVE GENETIC CONSEQUENCES OF CAPTIVE BROODSTOCK PROGRAMS FOR PACIFIC SALMON POPULATIONS

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BACKGROUND

We are focusing our research on the quantitative genetic consequences of captive broodstock programs in three areas. These areas relate to three genetic risks of artificial propagation (see Busack and Currens 1995): directional genetic change, loss of genetic variability within a population, and loss of population distinctiveness. Domestication selection, or adaptation to a protective culture environment, can produce directional genetic change. Inbreeding depression, or the reduction in fitness due to low heterozygosity or to unmasking of deleterious recessive alleles, can result from the loss of genetic variability or from nonrandom mating within a population and can further reduce this variability through the subsequent loss of genotypes from either genetic drift or selection. Outbreeding depression, which is a reduction in fitness due to loss of local adaptation or to the breakup of coadapted gene complexes, may result from interbreeding between distinct populations, and can further reduce population variability through the production of crossbred offspring.

These processes relate to three aspects of a captive broodstock program. First, domestication selection can be a direct consequence of variability in mortality and reproduction of cultured individuals that reflects differences between natural and protective culture environments. Second, inbreeding depression can result from the loss of genetic variability in a founder population or during the culture process. Third, outbreeding depression may be expressed when cultured individuals or their offspring are released to the wild to interbreed with wild individuals, if these groups are genetically distinct as a result of genetic change or loss of genetic variability. The following sections discuss these topics in more detail and, in the case of experimental work initiated to examine consequences of inbreeding and inbreeding depression, summarize some preliminary analyses of characteristics of the parental population used to establish the experimental design.

DOMESTICATION SELECTION

Introduction

Domestication selection, or rapid adaptation to the protective captive environment, is a concern in captive broodstock programs because the differences between the captive and wild environment are seldom expected to be trivial. In captive broodstock programs, the objective must be to minimize genetic and phenotypic divergence of cultured fish from the natural fish they are intended to supplement. The opportunity for selection to produce this divergence in a captive broodstock program is large because fish are cultured entirely in captivity for one or more generations. Although this opportunity is constrained by the time spent in captivity, its consequences for genetic variation depend on the degree to which the captive environment differs from that of the wild. This research is part of a comprehensive attempt to determine the major evolutionary consequences of captive broodstock programs for Pacific salmon.

Intentional artificial selection is not required in order to domesticate animals, as domestication can occur through the "inadvertent" process of adaptation to a change in environment (Doyle 1983, Kohane and Parsons 1988). In captive broodstock programs designed for reintroduction of animals to the wild, domestication is not desirable. Domestication is a process of adaptation to a novel, usually controlled environment, and this process is only partially independent of experimental design: the environment may be artificial, but the adaptive process is a natural one. The bulk of evidence for domestication in wild animals comes from animal breeding experience (Spurway 1952, Hale 1969, Price 1984, Fredeen 1986, Kohane and Parsons 1988) and from experimental work on other organisms (Doyle and Hunte 198 1a,b; Frankham et al. 1986; Parsons 1986; Holloway et al. 1990; Briscoe et al. 1992).

A population adapted to one environment, upon exposure to a novel environment with a different selective regime, can face a formidable evolutionary challenge. Different selective pressures will favor different allelic combinations. Depending on the structure of the population genetic variability, the process of adaptation to new environmental characteristics may result in rapid changes in allele frequencies corresponding to the newly favored alleles. During this process, prior to selective equilibrium, genetic correlations between fitness characters should become more positive, and the additive genetic variance for these characters should increase (Service and Rose 1985, Holloway et al. 1990). The expected result is a rapid reorganization in the genetic architecture of life history, including a phase characterized by positive genetic correlations among life history traits.

Empirical results generally appear to support the notion of rapid adaptation to novel environments. Experimental evidence supports a prominent role for antagonistic pleiotropy in the genetic control of life-history structure and the ability of rapid adaptation in novel environments to disrupt this structure. However, more work is needed on fishes and other

organisms to confirm the generality of these results. Research on the consequences of hatchery culture for genetically based change in salmonid life history is required to fully evaluate the genetic risks of captive broodstock programs for these fish. The large apparent differences in rearing environment between traditional salmon hatcheries and natural rearing habitats suggest that the types of selection experienced by fish developing in these environments may differ substantially (Hynes et al. 198 1, Doyle 1983). Waples (1991) argued that sharp differences exist in mortality profiles between hatchery and natural salmon, and that these differences transcend metamorphosis (smoltification) and preclude almost any chance that genetic differentiation between these two groups can be avoided.

Unfortunately, the empirical evidence for domestication in artificially propagated salmonids that are not subjected to artificial selection is equivocal. Most of this evidence is based on reduced performance of hatchery fish in the wild, including reduced juvenile survival (Schuck 1948, Reisenbichler and McIntyre 1977, Leider et al. 1990), reduced stamina (Green 1964, Leon 1986), altered behavior (Vincent 1960, Moyle 1969, Swain and Riddell 1990, Riddell and Swain 1991, Fleming and Gross 1992), and reduced reproductive success (Fleming and Gross 1993). Skaala et al. (1990) summarized genetic changes connected with salmon culture, although their survey clearly included other genetic problems such as inbreeding and inbreeding depression.

Indirect evidence for domestication selection comes from changes in quantitative genetic parameters in artificial selection experiments, and these changes are difficult to account for on the basis of the applied selection differential alone. For example, Gjedrem (1979) observed a larger response to selection for increased growth rate in Atlantic salmon than he could ascribe to the amount of artificial selection that he applied. Kinghorn (1988) presented evidence from farmed Norwegian Atlantic salmon compared to wild fish that was consistent with a response within four generations to domestication selection for growth. In an experiment to determine response to selection for high 8-month weight in coho salmon, Hershberger et al. (1990) detected an increase in weight in unselected (control) populations reared initially in a hatchery and then transferred to marine net-pens.

Kapuscinski et al. (1993) argued that the following four conditions must be met for domestication selection to result in genetic change during artificial propagation:

1) selection must occur “at the time *of reproduction* but [be] due to prior non-random mortality that...occurred during rearing”

2) selection must occur on “quantitative traits with sufficiently high heritabilities”

3) “the product of the selection differential and trait heritability must be high enough to yield a response...in the progeny generation”

4) "natural selection on the same trait must not substantially interfere with...the artificially induced selection differential."

However, the first three conditions are not very restrictive, and the last condition is irrelevant if no artificial selection is being practiced. The evolutionary genetic literature contains several examples of genetic change occurring over a few generations in quantitative traits with relatively low heritabilities (but potentially high additive genetic variances) that are difficult to attribute to genetic drift. The point is not that genetic change is difficult to produce, but that its direction and magnitude are difficult to predict (e.g., Sheridan 1988).

The purpose of this study is to document the nature and extent of domestication in a population of Pacific salmon undergoing captive culture. We are currently refining the conceptual approach to the study objective, but it is likely to require monitoring of variation in family size and to require integration of some molecular and quantitative genetic methods.

Materials and Methods

We have not yet initiated a study that addresses domestication selection. We intend to discuss the proposed research with the NMFS Alaska Fisheries Science Center Auke Bay Laboratory, the Washington Department of Fish and Wildlife, the Northwest Indian Fisheries Commission, and Long Live the Kings (a non-profit salmonid restoration organization) to determine appropriate facilities and salmon populations with which to conduct the experimental work. We plan to initiate experiments under this objective as early as 1997.

INBREEDING AND INBREEDING DEPRESSION

Introduction

Inbreeding and inbreeding depression are topics of considerable concern to fishery geneticists. Inbreeding has been defined as the mating of individuals more closely related to each other than to individuals chosen at random from the population (Wright 1978, Gall 1987). Related individuals have one or more common ancestors, and therefore may have received one or more identical genes. The measure of inbreeding, designated the inbreeding coefficient (F), is the probability that two alleles at a common locus are identical copies of an ancestral allele (Falconer 1989). Inbreeding can occur in populations either as a directed or a random process, and it can occur in large or small groups of animals. The basic impact of inbreeding on the genetic constitution of a population is an altered distribution of genotypes, with fewer heterozygotes and more homozygotes. This distribution can lead to the "unmasking" of deleterious recessive alleles and reduce the frequency of genotypes expressing codominance and overdominance.

Often, the result of increased inbreeding is a decrease in mean phenotypic value of one or more traits with respect to fitness, a phenomenon known as inbreeding depression. Inbreeding depression is commonly measured by comparing the average phenotypic values between an inbred population and the base population from which it was derived (Gall 1987). The traits most strongly affected by inbreeding are often those connected with reproductive capacity (e.g., fecundity, egg size, hatchability) or physiological efficiency (e.g., growth rate, feed conversion efficiency, survival) (Falconer 1989).

In general, inbreeding depression is thought to increase in proportion to the inbreeding coefficient during the early stages of inbreeding. However, as Kincaid (1983) pointed out, when the impacts of inbreeding on viability and survival traits become severe enough to result in the actual loss of inbred lines, the relationship between inbreeding depression and the inbreeding coefficient is often unpredictable.

Geneticists generally consider inbreeding depression to be the one of the most serious threats to the viability of small captive populations (Ralls and Ballou 1983, Lande and Barrowclough 1987, Ralls et al. 1988, Simberloff 1988, Hedrick 1992, Hedrick and Miller 1992). Inbreeding depression is an important concern in captive broodstock programs for threatened or endangered species, because it measures the additional risk of extinction that results when related individuals are mated. In populations that have existed at low numbers for any appreciable length of time, this risk can be high (Templeton and Read 1984).

Inbreeding depression has been documented repeatedly in many plant and animal taxa (Hedrick et al. 1986, Charlesworth and Charlesworth 1987, Hedrick 1992). To our knowledge, however, in Pacific salmon inbreeding depression has not been documented quantitatively in

hatchery populations, and its prevalence in wild salmon populations is unknown (Allendorf and Ryman 1987, Gall 1987). Inbreeding depression is often a laborious quantitative genetic problem to investigate because, unlike estimation of inbreeding alone, estimation of inbreeding depression requires the assessment of some measure of fitness, as well as knowledge of the relationship among the individuals under consideration. Such assessment requires a level of manipulation that is not typically practiced (and indeed, would be difficult to practice) in most production situations. Nevertheless, further research is needed to characterize the relationship between inbreeding and fitness in salmon populations.

In addition to examining the extent of inbreeding and inbreeding depression in both hatchery and natural populations, three research topics are particularly worthy of attention: 1) effective mating schemes to minimize further inbreeding, 2) estimation of critical levels of inbreeding that lead to substantial reductions in fitness, and 3) conditions necessary to avoid or recover from inbreeding depression (i.e., amount and type of outbreeding).

The purposes of this study are to 1) determine the extent of inbreeding that results in a population of Puget Sound fall chinook salmon under two experimental mating schemes (full-sib and half-sib mating) for three generations; and 2) characterize the relationship between the degree of inbreeding and the inbreeding depression that occurs, if any, in several phenotypic characters that affect fitness.

Materials and Methods

Sampling and Spawning of Adults

Adult fall chinook salmon returning to the Suquamish Tribe's Grovers Creek Hatchery were spawned in 1994 to establish a conventional half-sib/full-sib family breeding design. 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). It also provides a convenient means of establishing different levels of inbreeding in experimental groups within a population. Grovers Creek Hatchery fall chinook salmon have been a self-sustaining hatchery stock since about 1980; they were originally sourced from Washington Department of Fish and Wildlife's Green River Hatchery in eastern Puget Sound. Adults used to establish this experiment were taken from 992 males and 387 females returning to the hatchery and ready to spawn between 21 September and 31 October. The adults that were sampled from 541 males (54.5% of the returning males) and 225 females (58.1% of returning females) between 3 October and 21 October represented the central 56% of the run. Adults returning in 1994 ranged in age from 1 to 4 years for males and 3 to 5 years for females (Fig. 1). Sampling of adults was made without regard to observed phenotypic characters through the use of a random numbers table.

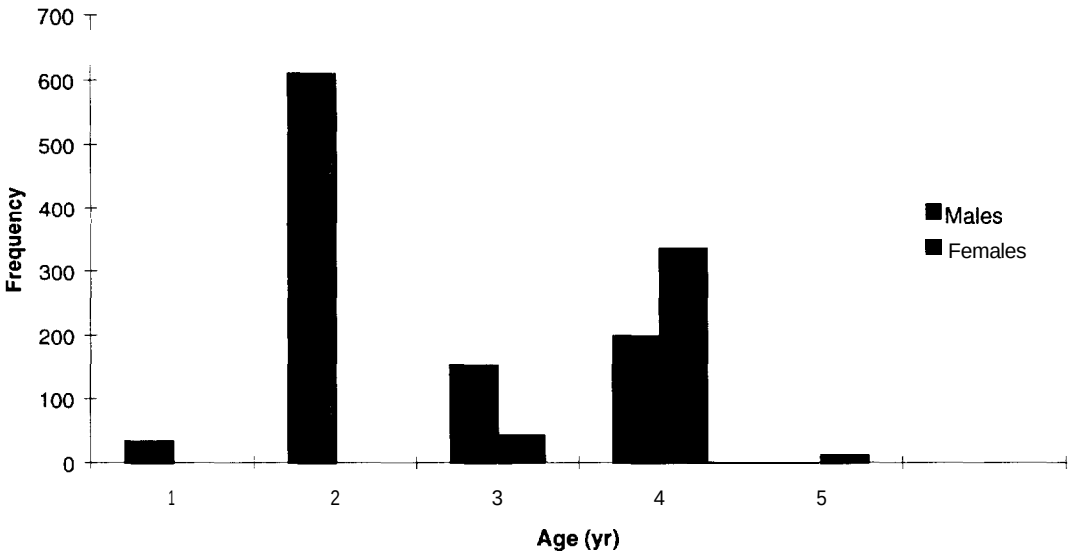

Figure 1. Age composition of fall-run chinook salmon returning to Grovers Creek Hatchery in 1994.

To establish experimental groups of fish, milt from each of 30 males was mixed with green (unfertilized and not water-hardened) eggs from an average of 4 females, producing 30 half-sib and 120 full-sib families of offspring with 4 full-sib families per half-sib family. Each adult was identified with a unique number and photographed to provide information on variation in body morphometry and coloration. Each adult was measured (snout-to-fork length to nearest mm and postorbital-to-hypural plate length to the nearest 3mm) and weighed to the nearest 220 g before spawning. For each adult, three scales were placed on scale cards for confirmation of aging, and tissues were sampled from the eye, heart, liver, and dorsal muscle for analysis of allozyme and DNA variation. For each female, total green egg volume was estimated to the nearest 50 mL, and approximately 20 green eggs were removed and preserved in buffered formaiin for estimation of average egg diameter and weight.

Thirty 100-mL aliquots of green eggs were collected from throughout the spawning run and preserved in buffered formalin for estimation of each female's fecundity from the product of average green egg number per 100 mL and each female's green egg volume.

Culture and Marking of Offspring

Eggs were picked after eyeing to estimate embryonic mortality and reduce opportunities for fungal growth in the incubators. Embryonic mortality due to fungal and bacterial infection was higher than expected for the 1994-brood offspring, probably because of insufficient frequency of prophylactic formalin treatment of incubating eggs. Of the 120 full-sib families established at spawning, approximately 100 families were large enough (i.e., produced > 1,000 fry) at the swim-up stage to maintain for experimental purposes. Because some half-sib families

suffered substantially greater mortality or were already much smaller than others, several full-sib families were dropped from the experiment at the time of ponding to reduce imbalance in the experimental design. Thus the fry of only 97 full-sib and 30 half-sib families remained, with an average of 3.28 full-sib families per half-sib family.

Release of Offspring and Establishment of Captive Populations

Between 4 and 26 April 1995, 257,093 chinook salmon smolts, representing 96 full-sib and 30 half-sib families, were marked at Grovers Creek Hatchery by Northwest Indian Fisheries Commission personnel. Marking involved removing adipose fins and inserting family-specific coded-wire tags into each fish. Average fingerling weight in each family during marking varied from 1.8 to 3.6 g (mean, 2.3 g). The number of fish tagged in each of the 96 full-sib families ranged from 66 to 5,189.

Up to 410 fish from each of the 96 families were retained at the hatchery to be monitored for tag retention after 30 days. The remaining fish were allowed to outmigrate volitionally from a common pond throughout May. Fish that had not outmigrated from the pond by early June were forced to move into the outlet stream draining into Miller Bay in northwestern Puget Sound.

During the 3-week tagging process, 20 fish were sampled arbitrarily from each of 97 full-sib families and 30 half-sib families for examination of body morphometry. Each fish was anesthetized briefly, and digital 8-bit grayscale images of the dorsal, lateral, and ventral sides were captured from each anesthetized fish for genetic analysis of three-dimensional fish shape. A ruler (in mm) was included in each image to provide a baseline scale, and in this sample (as in all juvenile samples) each fish was weighed to the nearest 0.1 g. To calculate truss network distances, 16 lateral, 7 dorsal, and 9 ventral landmarks were used in each image.

Of the 410 fish retained from each of the families, 150 fish from each of 84 full-sib families were transported during 18- 19 May 1995 to the National Biological Service laboratory in Seattle to determine inheritance of resistance to infection by *Vibrio anguillarum*. The fish (mean weight, 8.3 g) were acclimated from an initial temperature of 10°C to 14°C between 18 and 28 May. For each family, 150 fish were divided equally among 10 treatments: 2 replicate solutions each of 10^4, 10^5, 10^6, and 10^7 *V. anguillarum* cells/ml, and 2 replicate control solutions without *V. anguillarum*. Assignment of fish to treatments was made at random. A total of 1,260 fish were exposed to each treatment. All fish were exposed to the bacterial or control solutions for 30 minutes in static water at 14°C with supplemental oxygen. After water flows to the tanks were resumed, fish mortality in each tank was measured at about the same time daily for 21 days, after which the experiment was terminated.

Temperatures in each treatment were increased 1°C from 14°C for each of the first 5 days after exposure, after which time they were held at 18°C until the end of the experiment. Dead fish were removed at each sampling and frozen until coded-wire tags could be removed from

each fish and the tag information inspected and recorded. A primary advantage of this aspect of the experiment is that genetic variation in the response to *V. anguillarum* is unknown until after the experiment has been terminated. All fish remaining alive at the end of the experiment were killed and stored frozen for later tag decoding.

The remaining 260-410 fish in each of the 96 full-sib families remained in separate family rearing tanks at Grovers Creek Hatchery until early June 1995. From 6 to 8 June, 50 fish from each of these families were individually injected with passive integrated transponder (PIT) tags by NMFS personnel; the 4,850 fish were combined after marking into a circular concrete rearing pond at the hatchery, where they were held for approximately 10 days before transfer to seawater net-pens. The remaining 19,720 fish (which had already been marked with family-specific coded-wire tags) were transferred into another, similar pond and held for an equivalent period. Average fish size (± 1 SD) at marking was 99.4 ± 4.8 mm; weight was not measured.

During 19-20 June 1995, smolts were transported approximately 80 km from freshwater tanks at Grovers Creek Hatchery to seawater net-pens at the NMFS Marine Experimental Station at Manchester, Washington in southwestern Puget Sound. Fish were transferred as three groups: two groups of 9,860 fish marked solely with coded-wire tags, and one group of 4,850 fish marked with PIT tags as well as coded-wire tags.

Average fish weight at transfer (w_t) was estimated from average fish weight on 28 May (w,, 8.3 g) and the exponential growth equation $w_t = w_0 e^{rt}$, where r is the estimated growth rate of 1.5% per day and t is 22 days. Estimated average weight at transfer was 11.5 g. Between 25 July and 1 August 1995, fish in the PIT-tagged population were measured and weighed to determine individual lengths and weights as well as family averages. Average fork length (± 1 SD) was 123.13 ± 7.97 mm (n = 4,481), average wet weight was 18.57 ± 3.71 g (n = 3,639), and the average condition factor was 0.98 ± 0.07 (n = 3,639). Estimates of family variation in length and weight will be available after each PIT-tag code has been identified by full- and half-sib family.

Results and Discussion

Preliminary results described in this report do not address the primary objective of this experiment, and results for this objective will not be available until all first-generation offspring have returned from the release groups as adults or have matured in the captive groups. These fish will be available in fall 1999.

Summary of Parental Characteristics

A total of 30 adult males and 120 females were spawned on 9 dates during the 1994 Grovers Creek return. The frequency distribution of spawned males and females is shown in Table 1, and sizes of spawned fish are shown in Table 2.

Mean fecundity (±1 S.E.) was 4384 ± 106 cm; mean egg weight was 0.30 ± 0.001 g. Distribution of both traits was approximately normal (Figs. 2 and 3). For 29 egg samples collected during the run (one outlier was discarded from the analysis), mean egg number per 100 mL of green eggs was 301.2 ± 7.1 eggs. Mean egg diameter, calculated from the mean of 3 perpendicular diameter estimates for each of 20 eggs measured in each of the 29 samples, was 7.78 ± 0.06 mm. The correlation between egg diameter and number of eggs/100 ml was 0.82 ($P < 0.0001$); variation in egg diameter explained about 68% of the variation in number of eggs/100 mL.

Table 1. Frequency distribution of spawned males and females.

	Females spawned		Males spawned	
1994 Date	No.	%	No.	%
3 October	20	16.7	5	16.7
5 October	8	6.6	2	6.6
7 October	24	20.0	6	20.0
10 October	20	16.7	5	16.7
12 October	12	10.0	3	10.0
14 October	12	10.0	3	10.0
17 October	12	10.0	3	10.0
19 October	3	2.5	1	3.4
21 October	9	7.5	2	6.6

Table 2. Lengths (snout to fork length, SFL; postorbital-hypural plate, POH) and weights (round weight, RWT) of the adults spawned.

	Females			Males		
	Mean	S.D.	n	Mean	S.D.	n
SFL (cm)	79.1	6.1	124	78.1	14.0	27
POH (cm)	66.6	5.1	118	62.4	11.6	29
RWT (kg)	6.2	1.4	124	6.0	1.5	25

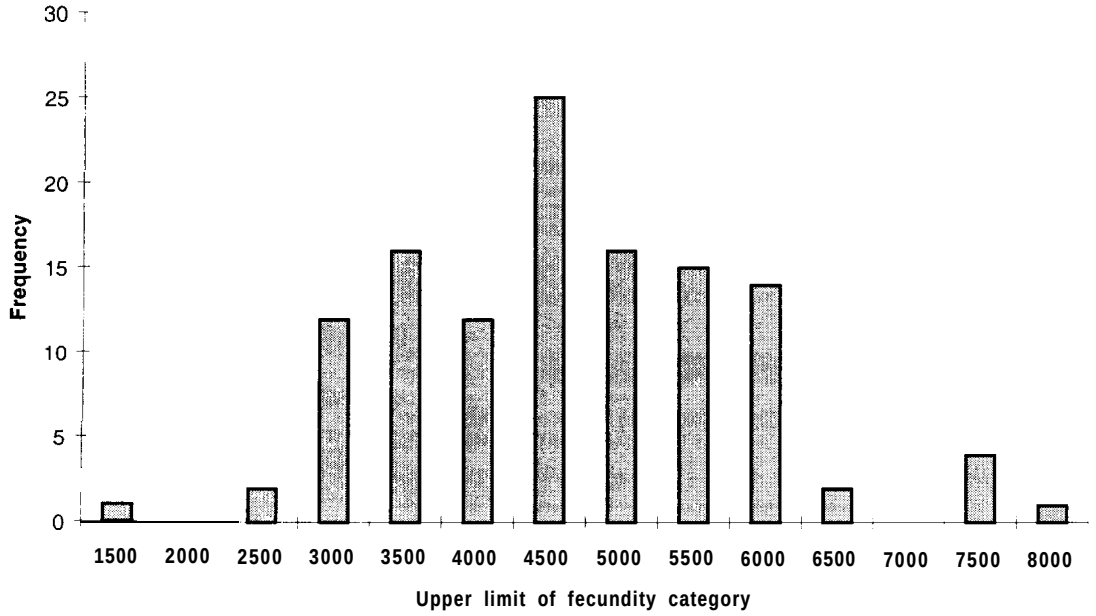

Figure 2. Frequency distribution of fecundity.

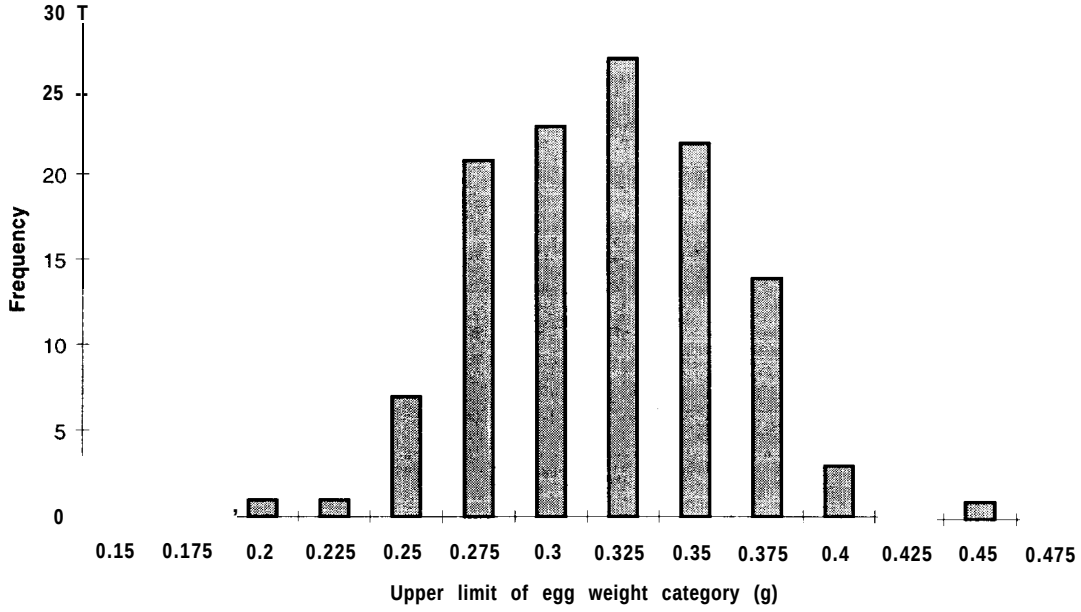

Figure 3. Frequency distribution of egg weight.

The relationship between egg size and egg number for females can potentially provide information on the nature of reproductive allocation in these fish. One form of this relationship, for the individual females used in the experiment, is shown in Figure 4. To generate this relationship, ln (egg weight) and ln (fecundity) were first each regressed on ln (female weight).

Each regression showed evidence of a significant ($P < 0.05$) allometric relationship with female size (i.e., the slope of the regression was significantly different from 1.0), indicating that both egg size and fecundity increased with female weight, but that the rate of increase declined with female weight. The residuals from each of these regressions were then regressed on each other (Fig. 4).

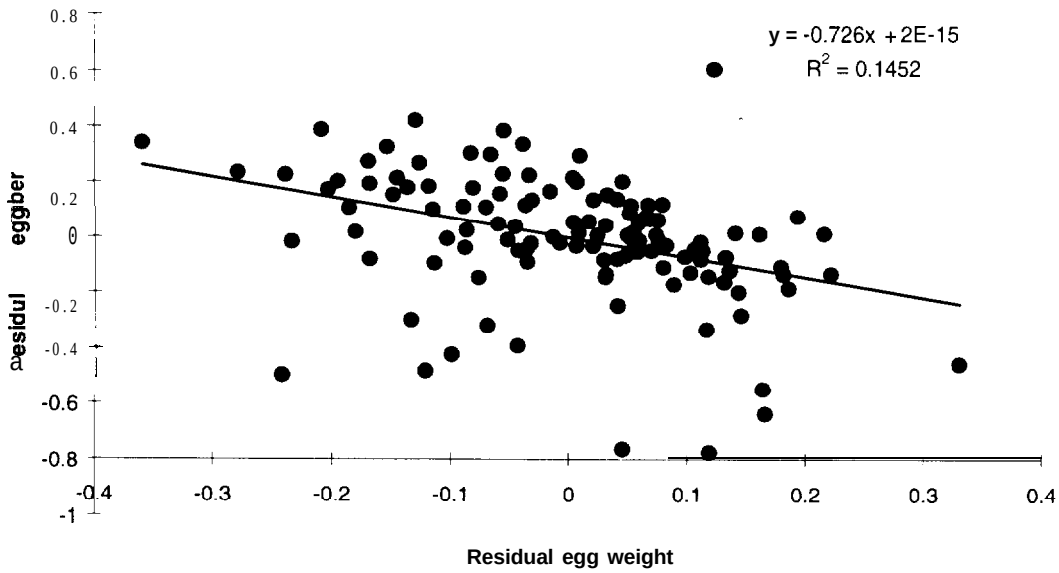

Figure 4. Relationship between egg number and egg size, estimated by linear regression of residuals from regression of egg number on female body weight on residuals from regression of egg weight on female body weight.

This approach took into account, as much as possible, the variation among females due to size-related variation. The approach permits one to detect whether females that devote more resources to individual eggs than expected, based on their size, exhibit a physiological tradeoff in the form of a reduction in expected egg number. The plot in Figure 4 suggests that this may be the case. However, there is a considerable amount of scatter in the relationship between residual egg number and residual egg weight, and the rate of decline in fecundity with egg size is not large (- 1.5 eggs per mg increase in average egg weight). Nevertheless, the data indicate that females experience a slight but significant (P c 0.0001) tradeoff between egg size and number. This outcome would be expected if limited resources available for reproduction constrained the resources available to each egg.

Analyses of body morphometry for individual male and female parents are currently being conducted and should be available in 1996.

Variation in Family Size and Preliminary Estimates of Mortality

Distributions of half- and full-sib family sizes at spawning, eyeing, and marking are depicted in Figures 5 and 6. Family sizes at marking are the most reliable of these data as they are exact counts. Estimates of family size at spawning are based on an estimated 3.01 green eggs/mL egg volume, as calculated for the 30 100-mL aliquots sampled from female parents. These estimates should be considered preliminary; they will be refined based on individual estimates of mean egg size for each female parent when these data become available. The estimates of family size at eyeing are based on V-tray estimates of egg number and are rough approximations.

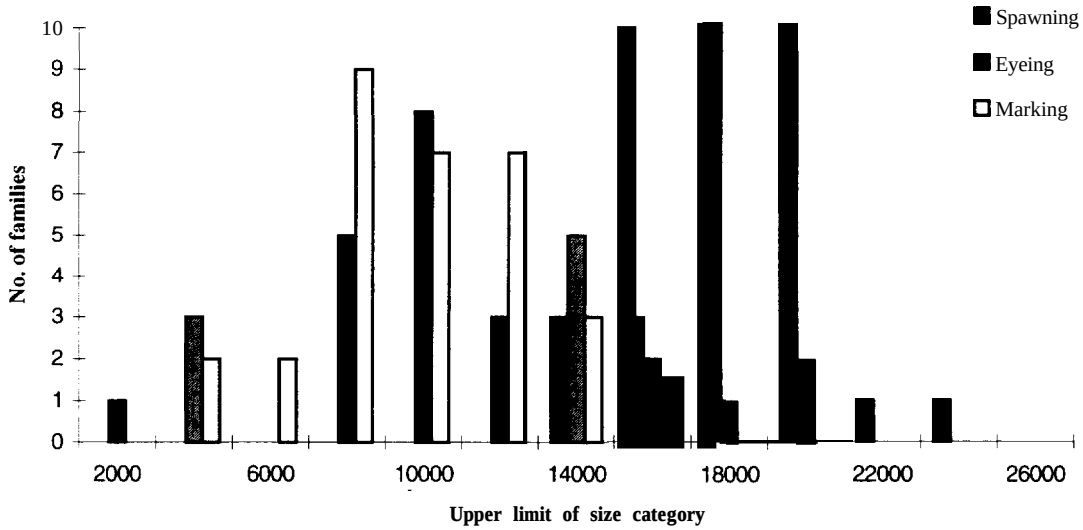

Figure 5. Frequency distributions of half-sib family size (total number of offspring sired by a single male, across all females available to him) at three life stages: spawning (fertilization), eyeing (the stage of embryonic development where eyes are readily visible through the chorion; approximately one month of age), and fingerling marking (approximately six months of age). The values along the abscissa represent the upper limit of the family-size category in increments of 2000 fish.

At marking, the number of females representing each male ranged from 1 to 4, with an average of 3.20 full-sib families per half-sib family. Average half-sib family size at marking was 8,570 ± 506. Based on the estimate of 3.01 green eggs/mL, average half-sib family size at spawning was estimated at approximately 18,06 1 ± 57 1. Average full-sib family size at marking was 2,521 ± 138. Average full-sib family size at spawning was approximately 4,635 ± 113. Average mortality from spawning to marking was 0.52 ± 0.03 among half-sib families and 0.55

± 0.03 among full-sib families. These data and those shown in Figures 5 and 6 suggest that there were no substantial shifts in distribution of half- or full-sib family sizes as a result of mortality that occurred in the hatchery.

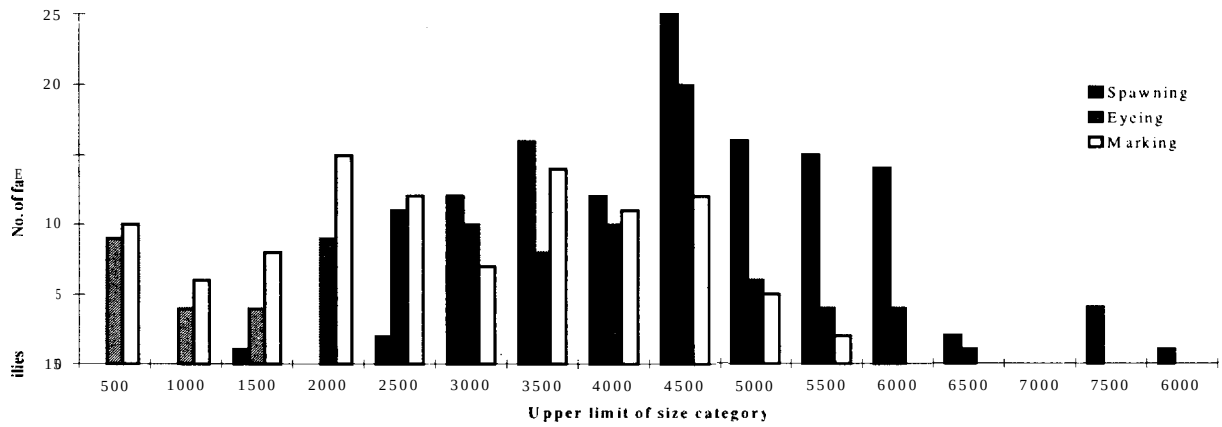

Figure 6. Frequency distributions of full-sib family size (total number of offspring produced by a single female) at three life stages: spawning, eyeing, and fingerling marking. The values along the abscissa represent the upper limit of the family-size category in increments of 500 fish.

The coefficient of variation (CV) is a more reliable way of looking at changes in the variability of family size because it accounts for mean differences among the sampling periods. The CV is a standardized measure of variability that takes into account variation in mean family size. Figure 7 suggests that the coefficient of variation in family size has increased for both full- and half-sib families from spawning to the marked fingerling stage. This apparent increase in CV suggests that variation in the contribution of different families to the next generation differs from the potential variation that existed at spawning, even at an early life-history stage in captive culture. However, a more informative comparison would be a test of trends in family size between released and captive fish after the first generation is complete. This comparison should provide considerable insight into the nature of selective differences between these two regimes and their consequences for artificially propagated salmon.

Since the captive fish were transferred to seawater on 20 June, total mortality as of 3 1 August in the three groups of marked fish held in captivity has been 2,553, or 9.2 % of the initial number transferred. This mortality resulted from a combination of transport stress, inability to osmoregulate in seawater, and infection by *V. anguillarum.*

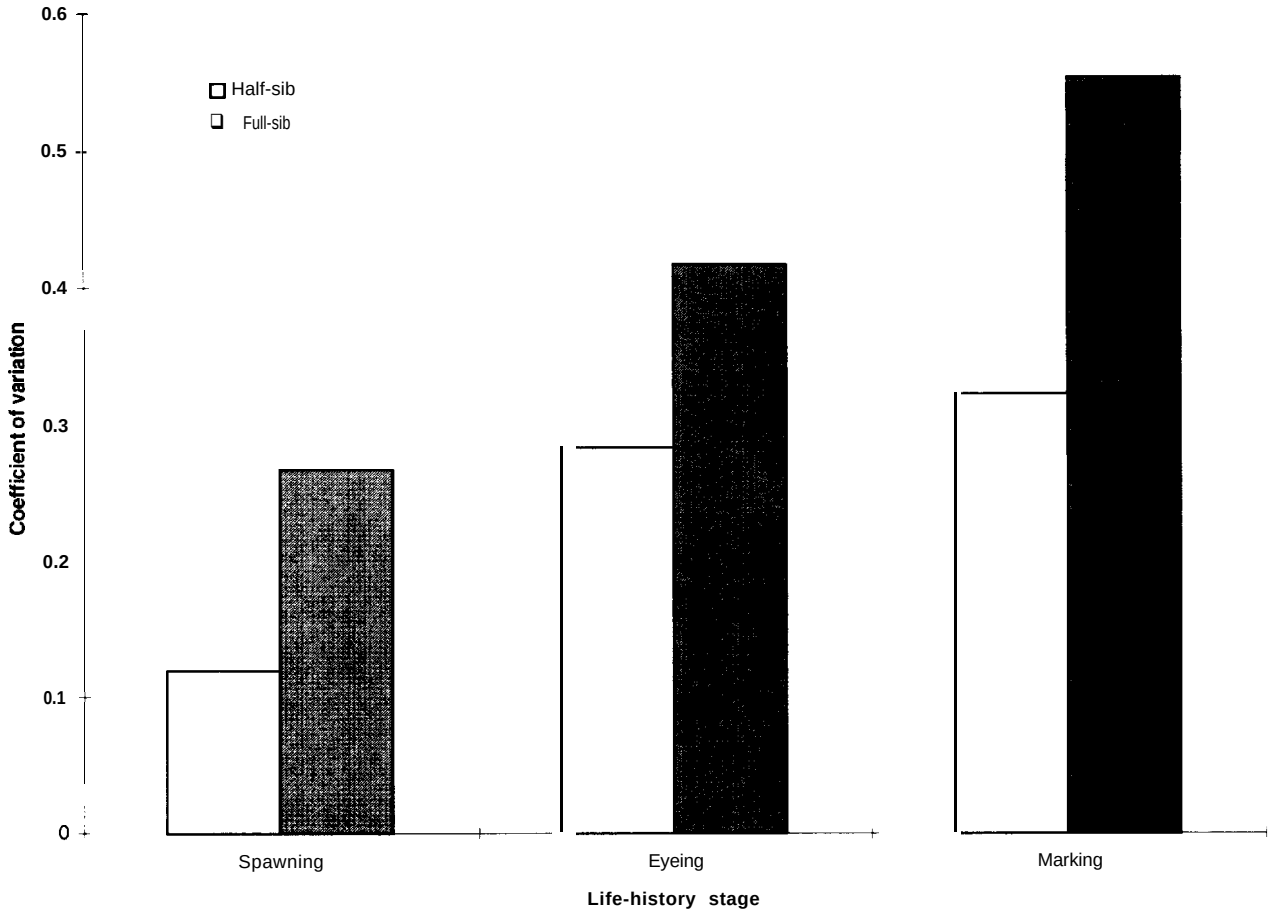

Figure 7. Changes in the estimated coefficient of variation (CV, the ratio of the phenotypic standard deviation to the phenotypic mean) of half-sib and full-sib family size at three life stages: spawning, eyeing, and fingerling marking.

Results from Experimental Work

Three-dimensional truss networks for estimation of morphometric parameters have been digitized for all 1,940 fingerlings sampled during coded-wire tagging in April 1995. These data have not yet been summarized by half- and full-sib family. We are presently calculating inter-landmark distances for principal components analysis. The PCA scores will be used to conduct genetic analyses of morphometry using either conventional estimation of quantitative genetic parameters from a nested analysis of variance (Falconer 1989) or estimation using maximum-likelihood techniques (e.g., Shaw 1987).

Initial results of the experimental challenge of the juvenile chinook salmon to infection by *Vibrio anguillarum* are presented in Figure 8. Fish mortality generally increased with the concentration of *V. anguillarum* in the treatment, but variation among replicate treatments confounds a reliable interpretation of these results. The average and range of mortality in each of the treatments at 14 and 21 days is summarized in Table 3.

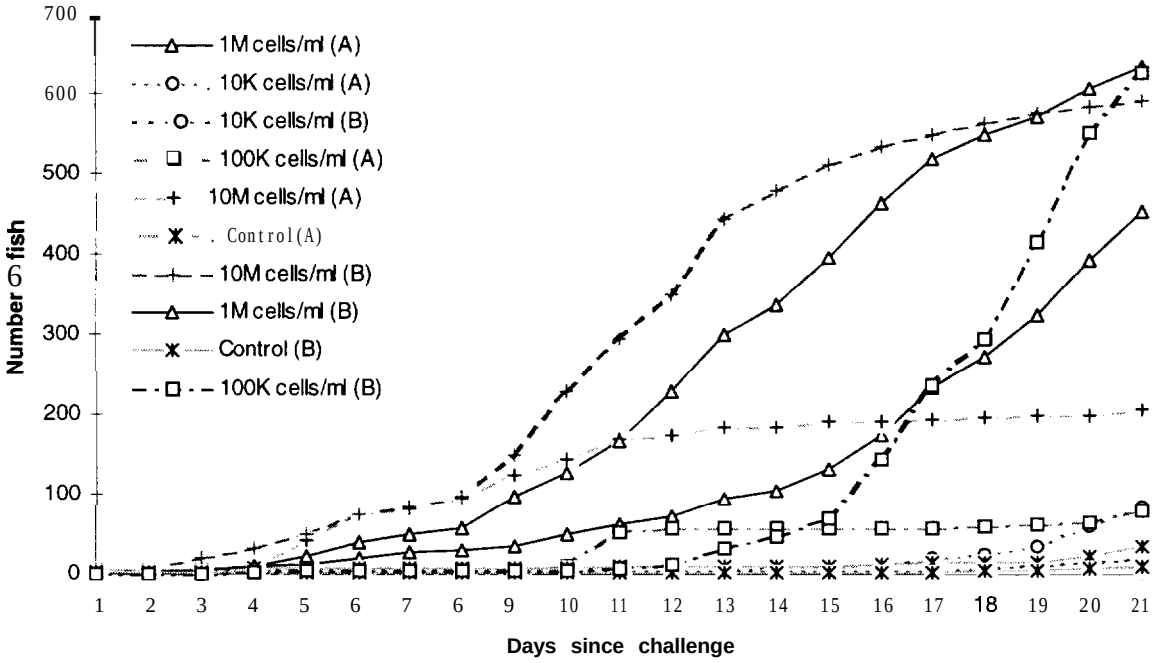

Figure 8. Cumulative mortality of juvenile chinook salmon exposed to various doses of Vibrio anguillarum. Pathogen concentration codes: 10M = ten million, 1M = one million; 100K = one hundred thousand; 10K = ten thousand. Letters in parentheses are replicate IDs.

Table 3. Mortality in experimental challenge of juvenile chinook salmon to infection by *V. anguillarum*. at 14 and 21 days (d) after challenge.

Treatment Numbers	Initial *Vibrio* (cells/mL)	Mortality - 14 d Mean (%)	Low	High	Mortalitv - 21 d Mean (%)	Low	High
6, 9 (controls)	0	0.4	0.2	0.8	1.8	0.8	2.7
2, 3	10^4	0.6	0.5	0.7	4.1	1.6	6.8
4, 10	10^5	2.1	3.7	4.6	28.1	6.4	49.8
1, 8	10^6	17.6	8.4	26.8	43.3	36.1	50.5
5, 7	10^7	25.0	14.8	35.3	31.7	16.4	47.0

These results suggest that most of the mortality observed occurred during the latter phase of the experiment except at the highest bacterial doses, and that the considerable variation among replicate doses arose largely during this latter phase. Rising mortality in the control lines after 14 days suggests that secondary infections or deaths from other causes may have contributed to the

cumulative mortality after 2 1 days. Whether genetic analysis of these results can determine the genetic basis of resistance to mortality from the environmental variation present in this experiment depends on the degree of family variation in resistance that exists in this population and on the relationship of family variation on the observed scale (died or survived) to that of the underlying continuous scale of liability to vibriosis (Falconer 1989).

Family variation in mortality induced by *V. anguillarum* cannot be determined until a coded-wire tag has been removed from eacn fish and decoded to determine its family origin. This process will require several months to complete, and we expect preliminary analyses of these data in summer 1996.

POPULATION DIFFERENTIATION AND INTERBREEDING

Introduction

The genetic mechanisms and consequences of population differentiation are closely tied to the issue of genetic change occurring in captive broodstock programs. Population differentiation may occur through dispersive (e.g., mutation, genetic drift) or systematic (e.g., selection, migration) means. Its consequences are important because they determine whether a captive broodstock program used to rebuild a declining wild population has maintained the genetic integrity of that population *in traits that are important to local adaptation* (i.e., life-history traits). Thus, change in the life-history structure of a supplemented population is a direct measure of the genetic success of captive broodstock or other supplementation programs. Assessment of adaptive differentiation requires quantitative genetic methods: controlled breeding, phenotypic evaluation, and tests of quantitative genetic models. Other genetic techniques simply do not address adaptation and life-history variation directly.

Outbreeding depression is the reduction in fitness that results from mating between unrelated or distantly related individuals. Outbreeding depression may result from loss of local adaptation (Templeton 1986, Emlen 199 1) or from the breakup of favorable gene combinations (Dobzhansky 1948). Therefore, like inbreeding depression, it often results from nonadditive expression of constituent genes (Lynch 1991).

Generally, outbreeding depression that results from the breakup of coadapted gene complexes is expected to manifest itself after segregation (i.e., in the F_2 or later generations) through reduced trait means and increased trait variances with respect to fitness. When two interbreeding populations are so distantly related that their genomes have diverged considerably, the resulting genetic interactions may be so strong that outbreeding depression is expressed in the F_1. However, outbreeding depression may also be expressed in the F, if hybrid offspring are poorly adapted to the habitat they occupy, regardless of the mode of gene interaction.

Although current interest in outbreeding depression is high, its extent and consequences in natural salmon populations or between hatchery and wild populations is unknown. Evidence exists for outbreeding depression in other organisms, but the quality of this evidence varies and it relies primarily on a few invertebrate species. The contentious nature of this issue for salmon supplementation warrants a detailed examination of the evidence.

The evolutionary consequences of outbreeding among salmonid populations are not clear. Virtually all the studies surveyed have examined only first-generation hybrids, and consequently most of these studies were not designed to detect outbreeding depression. Nevertheless, relatively few studies have found evidence for heterosis in first-generation hybrids, suggesting

little directional dominance (or epistasis involving directional dominance) for fitness among populations. Some crosses have shown reductions in fitness in the F_1, which could portend severe outbreeding depression in subsequent generations through the breakup of coadapted gene complexes if epistasis has contributed to population divergence.

In the only empirical study designed specifically to detect outbreeding depression in salmonids beyond first-generation hybrids, Gharrett and Smoker (1991) examined marine survival, return date, body size, and bilateral asymmetry in two generations of crosses between even- and odd-year populations of Auke Creek (Alaska) pink salmon. They observed substantially lower survival and increased asymmetry in the F_2 but not the F, hybrids, a result consistent with outbreeding depression by breakdown of coadapted genes.

However, it is important to recognize that even- and odd-year populations of pink salmon from the same stream may have been reproductively isolated for thousands of generations. Indeed, such populations are genetically more distinct from each other than from populations spawning in the same year in different streams (Aspinwall 1974, Beacham et al. 1988, Shaklee et al. 1991). Consequently, the results found by Gharrett and Smoker (1991) may not be representative of those expected between hatchery and natural populations with a greater natural opportunity for gene flow.

The purpose of the study proposed here is to determine the nature and extent of outbreeding depression among hatchery and natural populations of Washington coho salmon. The experiment will involve three populations of coho salmon reared at a Puget Sound hatchery. We intend to conduct this experiment with coho salmon for three reasons: 1) this species is more practical for an initial experiment of this complexity because of the almost complete lack of variable age structure in southern populations of this species; 2) coho salmon have higher rates of smolt-to-adult survival than do most other species of Pacific salmon in the region; and 3) an experiment spanning two salmon generations will require fewer years to complete than one involving most other species of Pacific salmon (e.g., coho salmon would require 6 years, whereas chinook salmon would require about 10 years).

Materials and Methods

We plan to initiate this research in 1996 or 1997 with the assistance of the Washington Department of Fish and Wildlife. Nevertheless, our conceptual approach to this objective is now nearly complete. The approach is to initiate crosses between three populations whose suitability is determined by their stock histories, phenotypic variation, and estimates of interpopulation genetic distance. Present plans are to use the following populations of western Washington coho salmon: a North Sound natural population (Skykomish River), a North Sound hatchery population (Wallace Hatchery), and a South Sound hatchery population (Soos Creek Hatchery).

The rationale for choosing coho salmon from outside the Columbia River Basin for the experimental model is twofold. First, the genetic differentiation among lower Columbia River coho salmon populations is weak. Repeated stock transfers of coho salmon among facilities and drainages and straying of hatchery fish in this region are believed to have contributed to widespread genetic introgression of hatchery genes into lower Columbia River coho salmon populations and consequent loss of genetic distinctiveness among them. Puget Sound/Olympic Peninsula coho salmon show greater genetic and phenotypic distinctiveness than do lower Columbia River coho salmon, which facilitates the choice of distinctiveness between stocks and allows greater opportunity for outbreeding depression to be detected if it exists.

Second, the success of the outbreeding depression study depends on its power to detect differences among experimental groups, which is determined in large part by smolt-to-adult survival in these groups. Puget Sound/Olympic Peninsula coho salmon survival rates are substantially higher than those of lower Columbia River coho salmon, at least in hatchery populations. These rates also appear to fluctuate less widely, which reduces the risk posed to the experiment by unpredictably poor environmental conditions.

The experiment will involve generating first- and second-generation hybrids and their reciprocals between these populations and comparing observed phenotypic characters to the expectations of various genetic models. Theory predicts that outbreeding depression is likely if the evolutionary differentiation of populations has occurred through interactions between different loci (sites in the genome). Our design should permit us to evaluate genetic models that distinguish these interactions. Characters of particular interest to us in this experiment are those that are apt to be under quantitative genetic control: stage-specific survival rates, body morphometry and meristic variation, fecundity, egg size, male age structure, disease resistance, and possibly some behaviors. The experiment is designed to determine 1) whether outbreeding depression occurs upon interbreeding of these stocks, and if so, at what approximate level of divergence; and 2) the genetic mechanism responsible for this depression.

We intend to generate 6 reciprocal crosses among the 3 populations. All crosses will involve approximately 45 parents of each sex, and about 36,000 smolts, marked with cross-specific coded-wire tags, will be generated from each cross in spring 1998. Adults maturing in these groups in 1999 will be used to generate 39 new “lines” (which include the 3 original populations and 6 first-generation hybrids, as well as 6 reciprocal second-generation hybrids and 24 reciprocal backcrosses). Fish in all lines (even those of different generations) will be cultured simultaneously by regenerating the first-generation groups to avoid the confounding effects of environmental variation between generations. All fish will be marked with cross-specific coded-wire tags, but if feasible, a portion of each cross will be cultured to maturity in captivity for a comparison with the released groups.

Because we do not plan to initiate this research until at least 1996, results are not yet available for this objective. However, the analysis of fish variation within and among the 39 groups should provide a means of determining whether outbreeding depression can be expected among relatively closely related populations of Pacific salmon, such as wild fish and the hatchery fish that are derived from them.

SUMMARY

One of the three objectives of our captive broodstock research is now being addressed experimentally; if sufficient funding is available, we plan to initiate the remaining experiments over the next 2 years. The past year's work has permitted us to set up a mating design that should allow a powerful determination of the nature of the relationship between inbreeding and inbreeding depression in Pacific salmon. This design has been replicated in both captive and released groups, and will provide considerable novel information on the genetic basis of variation in several quantitative traits for these fish. These results will help both to guide management decisions on captive broodstock management and to shed light on the evolution of characters that affect fitness and performance of these animals in the wild.

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