GENETIC, ENVIRONMENTAL, AND PHYSIOLOGICAL FACTORS INVOLVED IN
THE PRECOCIOUS SEXUAL MATURATION OF CHINOOK SALMON
(Oncorhynchus tshawytscha)

by

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ABSTRACT

Male chinook salmon (*Oncorhynchus tshawytscha*), that mature sexually one year prior to females and after at least one summer in sea water, are known as jacks. A breeding experiment to test for genetic and environmental (temperature at early rearing) effects on the incidence of jacking in chinook salmon showed significant sire, dam, and environmental effects, as well as genotype-by-environment interactions. Heritability estimates for incidence of jacking based on sire-offspring regressions within dams were 0.48 (± 0.24) and 0.32 (± 0.14) for the accelerated and non-accelerated groups respectively.

DNA fingerprinting was used to detect differences in allele distribution between precocious males and randomly selected fish, such differences indicate genetic involvement. Two oligonucleotide DNA fingerprinting probes were developed, however the resulting banding patterns were judged unsuitable for this application. A novel extension of Random Amplification of Polymorphic DNA (RAPD) allowed the isolation of a single-locus DNA probe for chinook salmon. This probe and another developed for Atlantic salmon, were hybridized with DNA from 74 jacks and 94 females from farmed chinook salmon (Robertson Creek stock; RC), and with DNA from 45 precociously mature and 56 non-mature chinook salmon parr from the Nicola River (NR). The allele distributions of the jack and female RC adults differed significantly, however, there was no difference between the precocious and non-maturing NR parr due, in part, to the relatively low genetic variability of that stock.

The weight-frequency distributions for three year classes of chinook salmon became significantly bimodal in the May prior to maturation due to faster growth of the jacks, relative to the non-maturing fish, from April to June. Plasma cortisol, T3, and testosterone concentrations were measured for one of those year classes during the spring and summer. No significant difference between the jacks and non-maturing fish were found for cortisol; however, T3 levels were higher in the jacks in March, and testosterone levels were higher
in the jacks throughout the spring and summer. Only T3 levels were correlated (negatively) with growth in the jacks.

A correlation analysis using the full- and half-sib families in the breeding experiment showed that growth-related variables did not predict jacking rates, although resting plasma glucose concentration, dam weight, and weight difference between the jacks and non-mature fish at the final sample were significantly correlated with jacking rate.

The implications of these finding are discussed with respect to evolutionary theory, aquaculture, and chinook salmon physiology.
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GENERAL INTRODUCTION

Coastal chinook salmon (Oncorhynchus tshawytscha) stocks in southern British Columbia usually smolt and migrate to sea in the spring of their first year, although in some populations the fry may remain in the river for an additional year (Healey 1991). The adult salmon return to the spawning grounds after 1-5 years at sea (Healey 1991). The term "jacking" in chinook salmon is used to describe the precocious sexual maturation of the sea-run males after at least one year in sea water, usually one year prior to the first maturation of the females (Healey 1991). Male chinook salmon may also sexually mature before smolting as "precocious parr" (Taylor 1989, Foote et al. 1991), however this phenomenon is much more common in Atlantic salmon (Salmo salar). Precocious sexual maturation of females only rarely occurs; this sexual dimorphism may be due to the lower energy allocation necessary for sexual maturation in males compared to female fish (Ware 1980, Healey 1991). Jacking has been documented in chinook salmon (Healey 1991, Bocking & Nass 1992), coho salmon, Oncorhynchus kisutch (Bilton 1980, Sandercock 1991), and sockeye salmon Oncorhynchus nerka (Burgner 1991), while precocious parr have been observed in chinook salmon (Taylor 1989, Foote et al. 1991), coho salmon (Silverstein & Hershberger 1992), amago and masu salmon Oncorhynchus rhodurus & O. masou (Kato 1991), and sockeye salmon (Ricker 1972, Burgner 1991). Up to 90% of the males in a given chinook salmon stock may be jacks (Hard et al. 1985), although most chinook stocks exhibit jacking rates around 5-15% of returning fish (both sexes; Bocking & Nass 1992, Healey 1991, Ricker 1972).

Precocious maturation in male salmonids is an example of an alternative male strategy; the smaller, younger males gain access to females for reproduction by utilizing "sneaking" behavior which capitalizes on their smaller size, while the larger, older males guard females and fight to defend their territory (Gross 1984, 1985, Leonardsson & Lundberg 1986, Bohlin et al. 1990, among others). Jacking in coho salmon and precocious maturation of Atlantic salmon parr have been successfully modelled using game
theory, as alternate Evolutionary Stable Strategies, or ESS (Gross 1984, 1985, Leonardsson & Lundberg 1986, Bohlin et al. 1986). Over 64 species of fish have been shown to exhibit alternate reproductive strategies (Chan & Ribbink 1990), and size-dependent alternate mating behavior has been reported for mammals (Leboeuf 1974), reptiles (Trivers 1976), amphibians (Arak 1988), and insects (Cade 1981). Precocious maturation in Pacific salmon is particularly useful for life history analysis, since maturation generally occurs only once in their lifespan (but see Bernier et al. 1992, Appendix B) and thus the reproductive strategies are discrete (Gross 1985). A key question in these studies of ESS theory is the inheritance of precocious maturation. If there is no genetic component to precocious maturation, then selection cannot lead to ESS, and jacking might best be modelled based on phenotypic plasticity (Stearns & Koella 1986). However if a genetic component exists, then ESS theory can be applied.

There is strong evidence that there are both genetic and environmental components to the incidence of precocious sexual maturation of coho and Atlantic salmon parr (Glebe & Saunders 1986, Thorpe et al. 1983, Iwamoto et al. 1984, Silverstein & Hershberger 1992, see Chapter 2.1). There is, however, very little published information on precocious maturation of Pacific salmon after smolting and migration. What is published is based on returns of released smolts, usually with very low survival rates (Bilton 1984, Hard et al. 1985). The genetic basis of jacking in Pacific salmon species has not been studied extensively, perhaps due to the logistical difficulties of rearing large numbers of fish to sexual maturation in salt water.

Figure I.1 shows a schematic representation of the most widely accepted factors that contribute to the determination of precocious sexual maturation in salmonids. It is important to note that there are also interactions among the three broad categories (i.e. genetic effects, environmental effects and physiological "triggers"). For example, the hormonal processes that trigger maturation are probably under some form of genetic control. Furthermore, the timing of the non-genetic factors may be critical, thus a
Figure I.1: A schematic diagram of factors hypothesized to affect the incidence of precocious sexual maturation in male salmonids. The horizontal line represents the male fishes' life cycle, starting with the fertilization of the egg and proceeding through the freshwater rearing, smolting, and seawater rearing. The genetic effects are passed on at fertilization, while the environmental effects act throughout the life cycle. The physiological "triggers" are generally thought to act during the 6-9 months prior to maturation (see text).
minimum level of energy stores could be required for sexual maturation, but only during the spring prior to maturation. The purpose of Fig. I.1 is to present the possible areas of study for researchers interested in precocious sexual maturation.

This thesis is primarily concerned with the investigation of the genetic and environmental contributions to precocious sexual maturation of male chinook salmon. To this end, an extensive breeding experiment was designed and executed. Furthermore, an additional approach, based on the relatively new DNA "fingerprinting" technology, was also used. During the course of these experiments, a number of physical and physiological characteristics of precociously maturing male salmon were also examined in an effort to better understand the physiological events that accompany precocious sexual maturation. However, the primary goal of this work remained the analysis of the genetic and environmental contributions to an alternate life history strategy in male chinook salmon, that is precocious sexual maturation.

This thesis has been organized into three sections that address different factors that potentially affect jacking in chinook salmon (as identified in Fig. I.1): The first section reports on initial observations (i.e. incidence and growth) on jacking in farmed chinook salmon. The second section comprises an analysis of the genetic and environmental contributions to jacking in chinook salmon based on an extensive breeding program as well as on allele frequencies at two minisatellite DNA loci. The third section describes selected physiological characteristics which accompany jacking in chinook salmon as potential triggers.

The goal of the work described in the first section was to test the hypothesis that jacking chinook salmon could be identified based on their size relative to the non-maturing fish as early as the spring prior to maturation. This work also provided some background data on the incidence of jacking in the Robertson Creek chinook stock reared at Yellow Island Aquaculture Ltd., where the breeding experiment was performed.
The purpose of the work described in the second section was twofold; primarily to determine if there were genetic and environmental components to jacking in Robertson Creek chinook stock reared at Yellow Island Aquaculture Ltd., and secondarily, to evaluate the utility of relatedness estimates, based on DNA "fingerprinting" (Burke et al. 1991), in identifying family effects for specific phenotypes within a random mating population. In order to test the DNA "fingerprinting" approach it was necessary to develop and evaluate minisatellite DNA probes for use in chinook salmon.

The goal of the third section was to identify physiological characteristics that distinguish jacks from non-jacks (i.e. normally maturing fish) within the family groups in the breeding program. The third section tested some of the published observations and hypotheses on the physiological "causes" of precocious sexual maturation in male salmonids for jacking in chinook salmon.

Although the principal goal of the work described here was to investigate the genetic and environmental contributions to jacking in chinook salmon, the phenomenon is complex and generally poorly understood. I therefore expanded the scope of my work to include ancillary projects. I feel that the physiological descriptions gathered in these ancillary projects were valuable for understanding the role of jacking in chinook salmon, not only for aquaculture, but also in evolutionary and ecological contexts.

Besides the research detailed in this thesis, two other projects were carried out by the author in collaboration with Nicholas Bernier. The first was an analysis of the genetic, environmental, and interaction effects on the stress response and growth of chinook salmon fry. This work is in press (Canadian Journal of Fisheries and Aquatic Sciences) and the manuscript is presented in Appendix A. The objective of these experiments was to study the correlations between the measured physiological parameters and the observed jacking rates. The second project was a study of precocious sexual maturation in the under-yearling Nicola River chinook parr at the Spius Creek hatchery. These fish were sampled for DNA fingerprinting analysis, and the hatchery staff later informed us that some of the
precociously mature parr had survived maturation, and had resumed feeding. Surviving mature parr and non-mature controls were collected and held in fresh and salt water until the following breeding season. This work has been completed and is in press (Canadian Journal of Zoology); the abstract is presented in Appendix B.
1.1 MATURATION IN CHINOOK SALMON: EARLY IDENTIFICATION BASED ON THE DEVELOPMENT OF A BIMODAL WEIGHT-FREQUENCY DISTRIBUTION

1.1.1 INTRODUCTION

Although the phenomenon of jacking, or precocious maturation of male Pacific salmon (Oncorhynchus spp.) is well documented (Ricker 1972, Hagar & Noble 1976, Scott & Crossman 1979, Bilton 1980, Hard et al. 1985), most information on jacking is based on fish returning to Federal hatcheries, or taken in the sport and commercial fisheries (but see Iwamoto et al. 1984). Typically, survivals (egg to adult) in these stocks have been five percent or less (Bilton 1980, 1984). Since there is little known concerning the sources of mortality during the seawater phase of the salmon’s life, conclusions concerning jacking require the assumption of uniform mortality (see Ricker 1972, Bilton 1978, 1980). In aquaculture, jacks are either sorted out and sold at little or no profit, or die in the pens. Sorting or removal of the dead fish is costly, therefore monosex female salmon stocks (Hunter et al. 1983) have been developed to avoid the problems of jacking in chinook salmon, while diet restriction (and consequent slowed growth) has been effective in reducing the number of precocious parr and grilse in Atlantic salmon, Salmo salar L., (Rowe & Thorpe 1990b, Thorpe et al. 1990). Since jacks are reported as being the most hardy (Pers. comm. J. Brackett, Syndel Laboratories Ltd., Nanaimo, B. C.) and rapidly growing (Lamont 1990) individuals during the first 18 months of ocean rearing (the faster growth may be due to the rise in plasma anabolic androgen levels of maturing male salmon - Dye et al. 1986, Higgs et al. 1982) they represent a potential asset to salmon farmers.

A reliable method of identifying jacks before secondary sexual characteristics express themselves would allow them to be harvested while they still have commercial value. In this study, growth and the weight-frequency distribution over time was measured for two year classes of chinook salmon reared on a commercial salmon farm in British
Columbia (B.C.), Canada. Our hypothesis was that jacks could be identified by their high body weight relative to the general population, before any secondary sexual characteristics were expressed.

1.1.2 MATERIALS AND METHODS

First generation domestic chinook salmon stock originally from the Robertson Creek Salmon Enhancement Facility (Department of Fisheries and Oceans, Canada), Vancouver Island, were reared from eggs at Yellow Island Aquaculture Ltd., a commercial salmon farm on Quadra Island, B.C. Fish were transferred to progressively larger seawater cages as they grew; the first cages were 4.5m X 9.0m X 9.0m deep, the next were 9.0m X 9.0m X 9.0m deep, and the last cages were 19m X 19m X 12m deep grow-out pens. The water temperature varied from about 7° C in the winter to approximately 13° C in the summer and salinity varied at 26-29 ppt. The fish were fed a varying ration of a commercial dry pelleted diet based on water temperature and fish weight, following the recommendations of the feed supplier (White Crest Mills, Campbell River, B.C.) at 10 to 20 minute intervals during daylight hours using automatic feeders.

A series of weight measurements was made on cohorts of chinook salmon spawned in the fall of both 1985, and 1986. The 1985 cohort was transferred to swim-up tanks on February 22, 1986 (avg. wt. =0.66g) and to seawater on June 14, 1986 (avg. wt. =8.96g). The 1986 cohort was transferred to swim-up tanks on February 21, 1987 (avg. wt. =0.74g) and to sea water on July 11, 1987 (avg. wt. =14.60g). The 1985 cohort was sampled from February to December, 1987 and the 1986 cohort from March to October, 1988. In both years the fish were approximately 16 months old (from fertilization) at the start of the sampling and approximately 25 months old at the final sampling. Survivals from transfer to seawater to age 19 months were; 81% for the 1985 cohort and 85% for the 1986 cohort (Table 1.1.1).
Table 1.1.1: A summary of the numbers of chinook salmon from the two year classes that were found to be jacks and silvers (non-jacks). The percentages of the total population, when the fish were sorted, are given in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>TOTAL</th>
<th>HIGH GRADE</th>
<th>LOW GRADE</th>
</tr>
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<tbody>
<tr>
<td><strong>1987</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of Smolts</td>
<td>36 000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Number of Fish at Sort</td>
<td>29 000 (80.5%)</td>
<td>3 475 (12.0%)</td>
<td>25 525 (88.0%)</td>
</tr>
<tr>
<td>Number of Jacks</td>
<td>4 997 (17.2%)</td>
<td>3 317 (11.4%)</td>
<td>1 680 (5.8%)</td>
</tr>
<tr>
<td><strong>1988</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of Smolts</td>
<td>13 450</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Number of Fish at Sort</td>
<td>11 391 (84.7%)</td>
<td>1 724 (15.1%)</td>
<td>9 667 (84.9%)</td>
</tr>
<tr>
<td>Number of Jacks</td>
<td>2 543 (22.3%)</td>
<td>1 685 (14.8%)</td>
<td>854 (7.5%)</td>
</tr>
</tbody>
</table>
Fish were sampled using a 13.0m X 10.0m deep seine, and from each set a random sample was removed with dip nets. These fish were anesthetized (0.15 ml•L\(^{-1}\) 2-phenoxethanol), weighed on a double beam balance (OHAUS) to the nearest 10g, and returned to the pens.

In late May of 1987, the 1985 cohort was sorted by size into two separate groups according to size such that the upper 10-15% size class (high-grade) was removed and kept in a separate netcage. In early June 1988, the 1986 cohort was similarly sorted. The sorting was done by estimating the wet weight of the live fish by comparing them to dead fish of known weight. Since every fish was handled and no anaesthetic was used, the fish were treated, as a prophylactic measure, with oxytetracycline before sorting.

All the high-graded fish of both year classes were harvested during the fall following sorting, while the low-graded fish were harvested during the winter. Some of the average weights reported between the sorting and the harvesting were estimated from the weights of mortalities. Due to logistic constraints some weight measurements were not made on fish individually, thus no estimate of the variance could be generated for those data. At harvest, all fish were determined to be either jacks or silvers (non-jacks). A record of mortalities was kept and the number of jacks in the low-graded group recorded. By combining the numbers of jacks in the high- and low-graded group, the total jacking rate for each cohort was determined.

**Statistical Methods**

A test for clusters within frequency distributions was used to test for bimodality (Engelman & Hartigan 1969). The null hypothesis was that the observed weight-frequency distribution was a random sample from a normally distributed fish population. The alternate hypothesis was that the observed distribution was a random sample from two normally distributed populations having equal variances but different means (i.e. bimodal). The test for clusters is based on the calculation of a series of variance (likelihood) ratios of sub-groupings of the data (for detailed descriptions see Engelman & Hartigan 1969,
McLaughlin 1989). The maximum value obtained is the $B/W_{\text{MAX}}$ statistic; the critical values are given in Engelman & Hartigan (1969). This test has two advantages over Cassie's (1954) method for the identification of bimodality: 1) the inflection point is determined and tested for significance based on a maximum likelihood ratio and 2) the test for clusters can easily be implemented on a personal computer for large data sets.

Differences in the slopes of the weight at age data were tested by analysis of homogeneity of slopes within an analysis of covariance on the means (Sokal & Rohlf 1981). We used the mean $Y$ values to maintain conservative confidence limits (see Petranka 1984, Heath & Roff 1987 for discussion of the use of means and individual values in this analysis).

1.1.3 RESULTS

Weight-Frequency Distributions

1985 Cohort (Table 1.1.2; Fig. 1.1.1). The weight-frequency data from February 22 to June 21, 1987 showed the development of a clear bimodal distribution. The distribution in February and March was not significantly bimodal, although by March the distribution was visibly skewed to the right. The isolated group of small fish (average weight = app. 100g) on the left of the main distribution for the months of February and March represented unhealthy fish which eventually died, and thus did not appear in the later samples. The distribution was statistically bimodal by May. In late May, 12.0% of the fish were high-graded as potential jacks (Table 1.1.1). Subsequently, a large size difference between the two groups was evident (June sampling).

1986 Cohort (Table 1.1.2; Fig. 1.1.2). The weight frequency data from March 10 to May 17, 1988 did not as clearly develop a bimodal distribution as did the 1985 cohort due to the smaller sample size; nevertheless, the frequency distribution in May was significantly bimodal. In early June, 15.1% of the fish were high-graded as potential jacks (Table 1.1.1); no individual sampling was done after this.
Table 1.1.2: Results of the tests for significance in bimodality for the 1987 and 1988 weight samplings in chinook salmon (see Fig. 1.1.1 and 1.1.2 for frequency distributions).

<table>
<thead>
<tr>
<th>Date</th>
<th>Sample size</th>
<th>Significance level</th>
<th>Dividing Weight(^2)(g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22 Feb. 1987</td>
<td>129</td>
<td>N.S.</td>
<td>-</td>
</tr>
<tr>
<td>26 Mar. 1987</td>
<td>154</td>
<td>N.S.</td>
<td>-</td>
</tr>
<tr>
<td>26 May 1987</td>
<td>84</td>
<td>P&lt;.01</td>
<td>658</td>
</tr>
<tr>
<td>21 June 1987</td>
<td>76</td>
<td>P&lt;.005</td>
<td>710</td>
</tr>
<tr>
<td>10 Mar 1988</td>
<td>62</td>
<td>N.S.</td>
<td>-</td>
</tr>
<tr>
<td>12 Apr 1988</td>
<td>56</td>
<td>N.S.</td>
<td>-</td>
</tr>
<tr>
<td>17 May 1988</td>
<td>89</td>
<td>P&lt;.01</td>
<td>647</td>
</tr>
</tbody>
</table>

\(^1\)Significance level generated from B/W\(_{\text{max}}\) statistic (after Engelman & Hartigan 1969).

\(^2\)Dividing weight is the weight of the individual fish that lies on the dividing point between the two distributions.
Figure 1.1.1. Wet weight frequency distribution in chinook salmon in 1987 (1985 Cohort). The shaded bars in the June sampling represent fish that had been high-graded, mostly jacks). * represents statistically significant bimodality (P < 0.01). Sample size is indicated for each distribution.
Figure 1.1.2: Wet weight frequency distribution for chinook salmon in 1988 (1986 cohort). * represents statistically significant bimodality (P < 0.01). Sample size is indicated for each distribution.
Jacking Rates

1985 Cohort (Table 1.1.1): In total, jacks made up 17.2% of the 1985 cohort; 11.4% came from the high-graded group (95.4% jacks) and 5.8% came from the low-graded group. Thus the high-graded group contained 66% of the jacks and only 0.6% of the silvers while the low-graded group contained 34% of the jacks and 99.4% of the silvers.

1986 Cohort (Table 1.1.1): Jacks made up 22.3% of the 1986 cohort; 14.8% came from the high-graded group (97.5% jacks) and 7.5% came from the low-graded group. Thus the high-graded group contained 64% of the jacks and only 0.5% of the silvers while the low-graded group contained 36% of the jacks and 99.5% of the silvers.

Growth

1985 Cohort: Growth curves of the 1985 cohort high- and low-graded fish show the large size gap between the two groups in June (Fig. 1.1.3a). This gap increased through to the early winter, however in December, 1987 the average wet weight of the low-graded fish (mostly silvers) was very close to that of the high-graded fish (mostly jacks - Fig. 1.1.3a). At this time the jacks were in extremely poor condition, with their viscera severely shrunken and their belly wall very thin. We therefore excluded the December weight measurement for jacks from the analysis of homogeneity of slopes. The slope of the best-fit line for growth of jacks was significantly greater than the slope for the low-graded fish (P = 0.0025).

1986 Cohort: The growth curves for the 1986 cohort high- and low-graded fish again show the increasingly large differential in body weight between the two groups through the summer (Fig. 1.1.3b). The slope of the best-fit line for growth of jacks (high-graded fish) was significantly greater than the slope for the low-graded fish (P = 0.0115).
Figure 1.1.3: Average wet weight of chinook salmon versus time for the 1985 (a) and 1986 (b) cohorts. The solid lines represent the low-graded fish (mostly silvers), and the dotted line represents the high-graded fish (mostly jacks). Data points enclosed in boxes were taken from mortalities, and tend to be smaller sample sizes.
1.1.4 DISCUSSION

In aquaculture, the impact of jacking on the cohort as a whole is extensive. Since the jacks are generally larger than the silvers (Fig. 1.1.3a & b) they contribute disproportionately to the total biomass. Within this study, jacks constituted 17.2% of the 1985 cohort and 22.3% of the 1986 cohort numerically, but accounted for approximately 24% and 31% of the biomass in September, respectively. Sixty-six percent of the jacks in the 1985 cohort and 64% of the jacks in the 1986 cohort were sorted out based solely on the difference in size. This sorting was done before secondary sexual characteristics were expressed, and thus at a time when the fish still retained good market value.

A bimodal growth pattern has been observed in Atlantic salmon during both the freshwater and seawater phases of their life-cycle (Simpson & Thorpe 1976, Thorpe 1977, Bailey et al. 1980, Thorpe et al. 1980, 1982, 1990). A probable mechanism for the freshwater bimodality is the reduction in the feeding activity of the fish in the lower mode group during the summer prior to the development of the bimodality due to behavioral interactions (Higgins 1985, Metcalfe et al. 1986, 1989), coupled with an increase in feeding activity in the higher mode group (Metcalfe et al. 1988). Initially bimodality in size of Atlantic salmon parr was thought to be primarily related to maturation and only incidentally to smolting (Thorpe et al. 1982); however, Villarreal & Thorpe (1985) later showed that sexual maturation was not the primary cause of the bimodality. Thorpe (1989) presented a model that proposed critical time windows for the initiation of maturation and smolting and described possible interactions between the two processes. The similarities between the bimodal frequency distributions described for the Atlantic salmon parr and those described in this chapter are striking; it would be reasonable to propose a critical window model similar to that described by Thorpe (1989) for jacking in chinook salmon.

In general for salmonids, the age of first maturity is believed to be strongly dependent on individual size and growth rate (Alm 1959, Gardner 1976, Thorpe 1986, 1991, but see Naevdal et al. 1978b, Thorpe 1989, Herbinger & Friars 1992). This is

There is strong evidence that altering food availability at critical time periods can increase or decrease the incidence of precocious maturation (Rowe & Thorpe 1990b, Thorpe et al. 1990). Rowe & Thorpe (1990b) subjected sibling groups of Atlantic salmon parr to seasonal variations in feeding opportunity and found that reduced feed availability in the spring prior to maturation suppressed early maturation whereas increased feeding during the spring enhanced early maturation, while Thorpe et al. (1990) showed a similar effect for grilsing in Atlantic salmon held in seawater netcages. One potential mechanism proposed for the effect of altered feeding levels on the incidence of sexual maturation is a spring threshold stored energy level necessary for maturation (Herbinger & Newkirk 1990, Simpson 1992, Rowe & Thorpe 1990b), although Herbinger & Friars (1992) reported that this threshold level for precocious maturation of Atlantic salmon parr was probably low, and hence not a good predictor of maturation. Rowe et al. (1991) recently published a study showing that the levels of mesenteric fat in May prior to the precocious maturation of Atlantic salmon parr are critical to precocious maturation, and proposed a model whereby the mesenteric fat stores act as sites for aromatizing circulating testosterones into estrogens, and that these estrogens then trigger the maturation process. Thus fish with larger stores of mesenteric fat would have higher rates of aromatization of testosterone into estrogen, and be more likely to sexually mature (Rowe et al. 1991).

The reported correlations between growth rate and sexual maturation do not imply that precociously maturing fish have an accelerated growth rate during the later stages of gonadal development. In fact, it is generally accepted that as fish progressively mature they
experience a reduction in growth due to allocation of energy to gonadal development (Ware 1980, Roff 1983). There are reports of reduced growth of rainbow trout and Atlantic salmon during maturation (Thorpe 1975, Moller et al. 1976, Naevdal et al. 1978b). The data presented here, however, do not support those observations; jacking males, raised in net cages, showed no decline in growth into their spawning period (i.e. September and October - see Fig. 1.1.3a & b). Possible mechanisms for the maintained growth seen in jacking chinook salmon may be; increased appetite, aggressiveness, and food conversion efficiency and/or hormonal growth stimulation, all of which may relate to the elevated androgen, thyroid, and growth hormone levels associated with sexual maturation of the male (see Chapter 3.1). Although there is little published information available on the timing and nature of the changes in behaviour, physiology, and blood chemistry associated with jacking in chinook salmon, observations suggest an increase in aggressiveness of jacks during maturation (pers. obs.).

The results presented here show that it is possible to identify groups of fish that are up to 97% jacks (up to two-thirds of the total number of jacks) before any secondary sexual characteristics are apparent. Furthermore, a bimodality in the weight-frequency distribution of chinook salmon develops in the spring prior to jacking in two separate year classes, suggesting a possible critical time for the initiation of jacking in chinook salmon.
2.1 GENETIC, ENVIRONMENTAL, AND INTERACTION EFFECTS ON THE INCIDENCE OF JACKING IN CHINOOK SALMON

2.1.1 INTRODUCTION

Jacking occurs in most natural and hatchery stocks of chinook salmon, and since jacks are generally smaller returning fish, they are considered as undesirable by both commercial and sports fishermen. Salmon farmers on the west coast of North America suffered large losses to jacking during the first years of culture (Hunter et al. 1983) since the maturing fish held little market value. Until recently, jacks were routinely excluded from Federal Salmon Enhancement Program (SEP) breeding programs, however the incidence of jacking in hatchery stocks is still higher than in wild stocks, despite the selection against jacks (Bocking & Nass 1992).

Although there is little solid evidence of a genetic component to jacking in chinook salmon, Hard et al. (1985) showed a strain effect in the incidence of jacking in transplanted stocks of chinook salmon in Alaska, and this was interpreted as a genetic effect. There is strong evidence that genetic factors play a role in determining the timing of sexual maturation in other species of salmonids (see Gardner 1976, Gjedrem 1984, Gall 1983, Naevdal 1983). By far the bulk of the detailed genetic work on sexual maturation has been with Atlantic salmon parr and grilse (Gjerde 1984, Naevdal 1983, Naevdal et al. 1978a, Myers et al. 1986, Glebe & Saunders 1986, Thorpe et al. 1983), although some work has been done on coho salmon (Iwamoto et al. 1984, Silverstein & Hershberger 1992), rainbow trout (Gall & Gross 1978b, Gall et al. 1988, Moller et al. 1976), and Arctic char, Salvelinus alpinus, (Nilsson 1992). There has been relatively little detailed research on the incidence of precocious maturation in salmonids after smolting, perhaps due to the logistic difficulties of rearing large numbers of adult fish in seawater facilities.

There is evidence that environmental effects can be highly significant contributors to the determination of the age of sexual maturation in salmonids (Alm 1959, Gardner...
1976, Randall et al. 1986, Thorpe 1991). Generally, environmental effects reported as having significant effects on the precocious maturation of salmonids are those that relate to growth acceleration (Thorpe 1991). Experience in the SEP program has shown that growth acceleration in the hatchery leads to higher jacking rates in returning adult coho and chinook salmon (Bilton 1980, 1984), however these observations were based on relatively modest returns; and therefore must be interpreted with caution. Although the effect of accelerated freshwater growth on the incidence of jacking in chinook salmon is not well known, it is likely to increase the jacking rate.

This study was designed to test for the effects of three factors on the incidence of jacking in a captive population of chinook salmon. Those were:

1) sire age, i.e. jack vs. non-jack male parent;
2) dam; and
3) temperature of early rearing water.

The mating design was unconventional in that sires were nested within dams. Although this design complicates the estimation of additive variance, this study was conceived to determine whether any significant genetic effect was present, rather than to generate heritability estimates. The use of heated water in the early rearing phase was included to test for the effect of growth acceleration on jacking rates, as has been reported by SEP and commercial hatcheries.

2.1.2 Materials and Methods

Mating Design and Incubation

The fish used for this breeding experiment were first generation domestic pure Robertson Creek stock, and originated from 15 females mated one-to-one with an equal number of males, all spawned late in the run. In September 1989, over 100 sexually maturing male and female chinook salmon were taken at random from that stock with multiple seine sets at the saltwater rearing facilities of Yellow Island
Aquaculture Ltd. (YIAL, Quadra Island, British Columbia), and transferred to freshwater facilities (aerated well water). On November 9, 1989, six 3-year-old females were spawned, and the eggs from each female were divided into two approximately equal groups. In order to increase the potential diversity of the male contribution to the offspring, half of the eggs from each female were fertilized by 2-year-old males (jacks), while the other half were fertilized by 3-year-old males (non-jacks). Each male was used only once and all fertilizations took place within 2 h of gamete collection. The resulting 12 families were further divided into two sub-groups (Table 2.1.1). The development of one of these sub-groups was accelerated by incubating the eggs and alevins in heated water (average temperature = 10.2°C; range = 9.0 - 11.1°C) while the other sub-group was incubated in unheated water (average temperature = 8.0°C; range = 7.4 - 9.0°C). All family groups were incubated in vertical stack incubation trays (Heath Technicorp., Seattle, Washington) with flows of 12-16 L·min⁻¹. The eggs were disinfected 2-3 times per week with malachite green (50-100 mg·L⁻¹, for 5-10 min. followed by flushing with fresh water) until the eyes of the developing embryo were clearly visible (eyed stage; approximately 30 days at 10°C). Once eyed, the eggs were shocked and dead eggs removed. All losses were recorded throughout the incubation period. By January 3, 1990 the accelerated (Acc) family groups were hatched, and by February 7, the alevins had reached swim-up stage (yolk-sac fully absorbed). The fish in the non-accelerated (N-Acc) family groups were hatched by January 21, 1990, and those alevins reached swim-up stage by March 13, 1990.

*Early rearing*

At swim-up, each family (500 to 1000 fry) was randomly assigned to one of 24 identical 200L outdoor tanks. All tanks received equal water flows (15 L·min⁻¹) from a common source (average temperature = 8.8°C; range = 7.8 - 10.0°C). For 14 days after swim-up, fry were fed during daylight hours with automatic feeders,
eight times per hour. Subsequently, the fry were fed to satiation by hand four times per day. The fry were fed Biomoist starter diet (Bioproducts Ltd., Warrenton, Oregon) until they reached 1.2 g when they were switched to a semi-moist diet (Moore Clarke Canada, Vancouver, B.C.). Mortalities were removed and recorded daily.

**Nose Tagging**

On May 8, 1990, 500 fry (average wt = 2.85 g) from each of the 12 Acc family groups were anesthetized (0.15 ml·L⁻¹ 2-phenoxyethanol) and a coded wire nose tag implanted (Jewell & Hager 1972). On May 23, 1990, 500 fry (average wt = 2.43 g) from each of the 12 N-Acc family groups were anesthetized and a coded wire nose tag implanted (as above). Twenty-four differently coded nose tags were used, and fish could be identified as to family of origin as well as to Acc or N-Acc groups. After the tagging, the fry were transferred to four 3000 L indoor tanks, and the Acc and N-Acc groups were kept separate.

**Rearing**

On May 22, 1990, the Acc family groups (average wt = 4.0 g) were dip-vaccinated against vibriosis following the manufacture's instructions (Microtek, Victoria, B.C.), and on June 13, 1990, the N-Acc family groups (average wt = 3.7 g) were similarly vaccinated. The Acc family groups (average weight = 8.4 g) were transferred to seawater grow-out facilities on June 30, 1990, after a two week acclimation period with 25% pumped sea water, and the N-Acc groups (average wt = 7.3 g) were similarly transferred on July 19, 1990. The Acc and N-Acc family groups were reared in separate netcages; initially 5mX10mX10m enclosures, and later 10mX10mX10m enclosures. Throughout the seawater rearing period, mortalities were removed weekly by SCUBA divers, and frozen for later nose tag recovery and decoding. The fish were fed to satiation one or two times daily using a commercially available diet (White Crest Mills, Campbell River, B.C.). The Acc and
N-Acc fish were sampled approximately monthly for weight. In the late summer / early fall of 1990, the Acc and N-Acc family groups both experienced acute losses, presumably due to vibriosis (from August 19-22 for the Acc fish, and from October 7-10 for the N-Acc fish), in each case the fish were treated for eight days with antibiotic (0.167 g·Kg$^{-1}$·day$^{-1}$; Romet, Syndel Laboratories, Vancouver, B.C.). On December 9 and 10, 1990, 415 Acc fish and 439 N-Acc fish were removed from the netcages, killed in an overdose of 2-phenoxyethanol (2.5 ml·L$^{-1}$), weighed ($\pm$ 0.1 g), and their nose tags removed and decoded to identify the fish to family of origin. The fish remaining in the netcages were individually counted to establish an inventory of surviving fish. In March, 1991, a further 425 fish were removed from the Acc family groups for the analysis of hormonal and growth changes associated with jacking (Chapter 3.2). During March and April, 1991, evidence of river otter predation in both the Acc and the N-Acc family group netcages prompted the employment of double predator nets and electrified fencing to control predation, and by May, 1991, there was no further evidence of predation.

Final Sample

By September 1991, the sexually mature males (jacks) could be distinguished by body morphology and skin colour. On September 19 and 20, 1991, all the jacks in both the Acc and N-Acc family groups were individually sorted, anesthetized in CO$_2$ saturated water and killed with a sharp blow to the head. The total wet weight and testes weight was measured, and the nose tag recovered and decoded for each jack. The remaining nonmature fish were commercially harvested during the fall of 1991; at the time of harvest they were individually weighed and their nose tags recovered and decoded. The sex ratio for each family was estimated using a Y-chromosomal sex probe for chinook salmon (Devlin et al. 1991) and DNA from blood samples for all family groups taken from randomly selected fish prior to sexual maturation of the
jacks (i.e. December 1990 sample, and March 1991 sample). The sex ratio estimate was based on 17-75 sexed fish per family (Table 2.1.1).

Analysis

Jacking rate (JR) was calculated as the total number of jacks in each family divided by the total number of surviving fish in that family (including the jacks). The male-specific jacking rate (SJR), or the percent of male fish that jacked, was calculated as follows;

\[ SJR = JR \cdot SR^{-1} \cdot 100\% \]

Where SR was the estimated sex ratio (Table 2.1.1).

A hierarchical series of log-linear models were used to test for the effect of rearing environment (i.e. Acc versus N-Acc), male parent (i.e. jack versus 3-year-old), female parent, and interactions on the observed jacking rates. The models were fit iteratively using the maximum likelihood criterion (SYSTAT, Evanston, IL, USA). The statistical significance of the various terms was determined by calculating the change in the log-likelihood statistic with the removal of the effect in question from the saturated model (Fienberg 1970).

There are five difficulties associated with estimating the heritability of jacking within this experiment;

1) Jacking is a threshold trait and is therefore scored on a binomial scale (i.e. "0" or "1"),

2) The mating design (males nested within females) does not allow an unbiased estimate of heritability based on a sib-analysis (Falconer 1981),

3) The number of surviving fish varied widely across the families,
4) The male parents were not randomly selected, but rather for 50% jacks and 50% non-jacks, and

5) Due to logistic constraints, only 12 families were used, thus any estimate of heritability would have extremely large uncertainty associated with it.

Despite these limitations two approaches were used to estimate the heritability of jacking in the population of chinook salmon at Yellow Island Aquaculture Ltd. The first was parent-offspring regressions of the jack (value = 1) and non-jack (value = 0) sires nested within a single dam on the incidence of jacking (ratio) in the full-sib offspring. Six regression coefficients were thus generated for the Acc and N-Acc family groups; these were averaged to yield a mean regression coefficient (and SE). Heritabilities for the Acc and N-Acc groups were calculated by multiplying the mean slope by two, as described by Falconer (1981) for nested parent-offspring regressions. The second method used to estimate heritability was based on an ANOVA on the incidence of jacking in the Acc and N-Acc groups separately. The model used was;

\[ Y_{ijk} = \mu + D_k + S_{jk} + e_{ijk} \]

Where \( Y_{ijk} \) was the sexual maturation status (i.e. 0-male or 1-jack) of the \( i^{th} \) progeny of the \( j^{th} \) sire nested within the \( k^{th} \) dam, \( \mu \) is the population (least square) mean, \( D_k \) is the effect of the \( k^{th} \) dam, \( S_{jk} \) is the effect of the effect of the \( j^{th} \) sire nested within the \( k^{th} \) dam, and \( e_{ijk} \) is the random error term. Since the number of surviving progeny varied widely between families, the ANOVA would be highly unbalanced, thus an approximately equal number of progeny was chosen at random for each family to include in the analysis. The data in this analysis consisted of zeros and ones, and thus the
assumption of normality for ANOVA (Sokal and Rohlf 1981) was violated. However, since the incidence of jacking in most families was high, and ANOVA is relatively robust to non-normality under these conditions (Sokal and Rohlf 1981), the results of the ANOVA are probably valid. Iwamoto et al. (1984) described a similar experimental design created to test for genetic effects in the incidence of coho salmon precocious parr. Despite low average maturation rates (app. 1%) Iwamoto et al. (1984) used the binomial ANOVA described above. Heritabilities for both the Acc and the N-Acc family groups were estimated using the calculated and expected mean squares (following Falconer 1981).

All estimated heritabilities were corrected to the continuous liability scale (Van Vleck 1972, Falconer 1981, Iwamoto et al. 1984) using;

\[ h^2 = h^2_b \frac{z^2}{[p(1-p)]} \]

Where \( h^2 \) is the continuous scale heritability, \( h^2_b \) is the binomial scale heritability (calculated from a threshold phenotype), \( z \) is the ordinate of the normal distribution at the threshold point which divides jacks from non-jacking males, and \( p \) is the frequency of jacking in the offspring (Iwamoto et al. 1984). It should be noted that these heritability estimates should be viewed with caution due to the limitations listed above.

2.1.3 Results

Growth

The Acc and N-Acc family groups grew at similar rates throughout the duration of the experiment, although the Acc groups generally had slightly higher absolute growth rates than the N-Acc groups (Fig. 2.1.1a & b).
Figure 2.1.1: (a) Mean wet weight (± SE) of the accelerated (Acc) and non-accelerated (N-Acc) chinook salmon family groups used in the analysis of jacking rates plotted against time. (b) Absolute growth rate (i.e. linear slope of weight vs. time) of the accelerated (Acc) and non-accelerated (N-Acc) chinook salmon family groups.
Survival

There were large differences in the survival of the various family groups within the Acc and N-Acc environments from the time of marking to December 1990, as well as to the final sampling in September 1991 (Fig. 2.1.2a & b). The overall survival of the Acc and N-Acc groups were similar (17% - Acc; 25% - N-Acc), but significantly different ($X^2$ test; $P > 0.05$). Generally, families from dam 5 and jack sired families had slightly higher survival, while families from dam 3 had lower survival (Fig. 2.1.2a & b).

Sex Ratios

Although most families had roughly equal numbers of males and females, a few had skewed sex ratios (Table 2.1.1). Overall the sex ratio was not significantly different from 50:50 ($X^2$ test; $P > 0.10$).

Jacking Rates

Overall jacking rates (% jacks in population) were 19.5% for the Acc group and 17.3% for the N-Acc group. The specific jacking rates (SJR) varied between and within the Acc and N-Acc groups (Fig. 2.1.3a & b). There was a significant effect of environment (Acc vs. N-Acc) on the observed jacking rates ($G=14.85; df=1; P < .001$) with the Acc groups having a higher jacking rate. There was a significant dam effect on the observed frequencies of jacking ($G=231; df=5; P < .001$), as well as a significant sire effect (jack vs. non-jack) on the observed frequencies ($G=22.2; df=1; P < .001$). The interaction effect of dam-by-environment was found to be significant ($G=22.7; df=5; P < .001$), as was the sire-by-environment interaction ($G=9.43; df=1; P < .003$). The norms of reaction for jacking rate across the two environments (Fig. 2.1.4) show that the significant genotype-by-environment interactions were mainly due to two families (dam 6 by jack sire, and dam 3 by non-jack sire). The three-way interaction (sire-by-dam-by-environment) was found to be non-significant.
Table 2.1.1: A list of the 24 full- and half-sib family groups used for the analysis of the genetic and environmental contributions to the incidence of jacking in chinook salmon. The family groups arose from six dams, twelve sires (six jacks: J, and six third year spawners, i.e. non-jacks: N) nested within dams, and all families were reared under two early rearing regimes (accelerated - Acc, and non-accelerated N-Acc). The estimated sex ratio (% males) for each family group is given with the total number of fish used for the estimation. The totals are the overall sex ratio and the total number of fish sexed.

<table>
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<th>DAM</th>
<th>SIRE (N / J)</th>
<th>ENVIRONMENT (Acc / N-Acc)</th>
<th>SEX RATIO (% males)</th>
<th>NUMBER</th>
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<td>N</td>
<td>Acc</td>
<td>54</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>J</td>
<td>Acc</td>
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<td></td>
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<td>N-Acc</td>
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<td>29</td>
</tr>
<tr>
<td></td>
<td>J</td>
<td>N-Acc</td>
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<td>N</td>
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<td></td>
<td>J</td>
<td>N-Acc</td>
<td>43</td>
<td>23</td>
</tr>
<tr>
<td>6</td>
<td>N</td>
<td>Acc</td>
<td>36</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>J</td>
<td>Acc</td>
<td>56</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>N-Acc</td>
<td>65</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>J</td>
<td>N-Acc</td>
<td>42</td>
<td>24</td>
</tr>
<tr>
<td>TOTALS</td>
<td></td>
<td></td>
<td>48.5%</td>
<td>824</td>
</tr>
</tbody>
</table>
Table 2.1.2: Calculated heritabilities for precocious sexual maturation (jacking) in farmed Robertson Creek chinook salmon stock. The heritabilities were calculated from sire-male offspring regressions, and sib-analysis (ANOVA) with progeny scored as ones (jacks) and zeros (non-mature males). Separate heritability estimates were made for the accelerated (Acc) and non-accelerated groups (N-Acc), and are presented as binomial scale estimates ($h_b^2$), as well as transformed to continuous scale estimates ($h^2$). Standard errors are given for the heritability estimates based on sire - male offspring regressions.

<table>
<thead>
<tr>
<th>ENVIRONMENT</th>
<th>SIRE-OFFSPRING</th>
<th>SIB ANALYSIS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$h_b^2$</td>
<td>DAM</td>
</tr>
<tr>
<td>Acc</td>
<td>0.48 ($\pm$ 0.24)</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>$h^2$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.77 ($\pm$ 0.39)</td>
<td>0.53</td>
</tr>
<tr>
<td>N-Acc</td>
<td>$h_b^2$</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>0.32 ($\pm$ 0.14)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$h^2$</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>0.54 ($\pm$ 0.23)</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1.3: Results of the ANOVA on the incidence of jacking in the progeny of the 12 accelerated (Acc) and 12 non-accelerated (N-Acc) families. The sire effect was nested within the dam effect, and the Acc and N-Acc ANOVAs were run separately. Expected mean squares were taken from Falconer (1981), where $\sigma_w^2$ was the within progeny variance component, $\sigma_s^2$ was the between-sires, within dams variance component, and $\sigma_d^2$ was the between dams component.

**Acc**

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>DF</th>
<th>MS</th>
<th>EXPECTED MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAM</td>
<td>5</td>
<td>2.456</td>
<td>$\sigma_w^2 + 21\sigma_s^2 + 42\sigma_d^2$</td>
</tr>
<tr>
<td>SIRE</td>
<td>6</td>
<td>1.634</td>
<td>$\sigma_w^2 + 21\sigma_s^2$</td>
</tr>
<tr>
<td>WITHIN</td>
<td>241</td>
<td>0.152</td>
<td>$\sigma_w^2$</td>
</tr>
</tbody>
</table>

**N-Acc**

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>DF</th>
<th>MS</th>
<th>EXPECTED MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAM</td>
<td>5</td>
<td>1.865</td>
<td>$\sigma_w^2 + 29\sigma_s^2 + 54\sigma_d^2$</td>
</tr>
<tr>
<td>SIRE</td>
<td>6</td>
<td>1.217</td>
<td>$\sigma_w^2 + 29\sigma_s^2$</td>
</tr>
<tr>
<td>WITHIN</td>
<td>341</td>
<td>0.154</td>
<td>$\sigma_w^2$</td>
</tr>
</tbody>
</table>
Figure 2.1.2: Percent survival (from pre-smolt nose-tagging) for each of the 24 chinook salmon family groups in the analysis of jacking. a) family survivals for December 1990 (13 months post-fertilization), and b) for the final sampling in October 1991 (23 months post-fertilization). The December survivals are based on estimates for each family (see text). The families are the progeny of six dams (1 - 6) each mated to two males; one jack (J) and one 3-year-old, or non-jack, male (N).
Figure 2.1.3: Specific jacking rates (%) for the 24 chinook salmon family groups; a) 12 accelerated fish and b) 12 non-accelerated fish. Specific jacking rate is the ratio of jacks to the estimated total number of males (see text). The families are the progeny of six dams (1 - 6) each mated to two males; one jack (J) and one 3-year-old, or non-jack, male (N).
Figure 2.1.4: Norms of reaction for the observed specific jacking rates in the 12 full- and half-sib chinook salmon family groups reared under two environments, accelerated (Acc) and non-accelerated (N-Acc). The norms of reaction are shown for: a) the jack-sired, and b) the non-jack sired families separately. Each line represents a single family, and the dam of the family is shown on the right. The accepted effect of developmental acceleration is an increased jacking rate (see text). Genotype-by-environment interaction is identified by crossing lines.
Heritability

The heritability estimates from the Acc and N-Acc groups based on regressions of sire on male offspring (within dam) are presented in Table 2.1.2, along with standard errors. The results of the 2-way ANOVA for sire and dam effects on jacking incidence are shown in Table 2.1.3, along with expected mean squares. For the heritability estimates generated by the sib analysis (Table 2.1.2), no estimates of standard errors were calculated. Continuous liability scale heritability estimates are also shown in Table 2.1.2. Note that the sire component heritability estimates exceed 1.0 when converted to the continuous scale, indicating possible non-additive effects.

2.1.4 DISCUSSION

A genetic component to precocious sexual maturation has been demonstrated in many salmonid species. Breeding experiments designed to partition this genetic component to sexual maturation into maternal and paternal contributions have been reported for Atlantic salmon (Gjerde 1984, Naevdal 1983, Naevdal et al. 1978a, Myers et al. 1986, Glebe & Saunders 1986, Thorpe et al. 1983), rainbow trout (Gall & Gross 1978b, Gall et al. 1988, Moller et al. 1976), Arctic char (Nilsson 1992), and coho salmon (Iwamoto et al. 1984, Silverstein & Hershberger 1992). Many of the genetic analyses of precocious sexual maturation in salmonids have been done on pre-smolt parr and one of the common problems faced by these analyses has been non-genetic maternal, or "common environmental", effects (Falconer 1981 - see Iwamoto et al. 1984).

Non-genetic maternal effects have been identified as factors in precocious maturation in a number of salmonid species (Silverstein & Hershberger 1992, Bailey et al. 1980, Sutterlin & MacLean 1984, Nilsson 1992). Perhaps the most obvious non-genetic effect of a dam on her offspring is egg size, and hence early growth. The effect of egg size has been shown to influence size at age, however this effect decreases with age (Silverstein & Hershberger 1992, Sutterlin & MacLean 1984, Gall 1974, Withler et al. 1987).
maternal effect on early growth could have a profound impact on estimates of the dam component of the observed variance in maturation timing (Bradford & Peterman 1987, Silverstein & Hershberger 1992), however Gall et al. (1988) showed that maternal effects were very small for age at spawning in rainbow trout. Although it is unlikely that such an effect would be a major factor in the present study, since jacking occurred 18 months post-hatch, non-genetic maternal effects cannot be ruled out.

The effect of sire age (i.e. jack vs. non-jack) in the present study was very clear; the SJR of jack sired fish was 44.8%, while the SJR of non-jack sired fish was 26.9%, and for all but one dam the jack-sired full-sib families had higher SJR (Fig. 2.1.3). Independent of the partitioning of the genetic variance for jacking into additive and non-additive components within the population of chinook salmon examined, it is clear that jacking incidence could be profoundly affected by the choice of sires. Similar results have been reported for coho and Atlantic salmon precocious parr (Glebe & Saunders 1986, Thorpe et al. 1983, Gjerde 1984, Iwamoto et al. 1984).

In salmonids, the age of first maturity is generally believed to be strongly dependent on individual size and growth rate (Alm 1959, Thorpe 1986, Randall et al. 1986, see Chapter 1.1). Lamont (1990) showed that for individually tagged rainbow trout and coho salmon, the fastest growing individuals were precociously maturing males. The reported relationships between growth rate and precocious sexual maturation do not, however, imply causation. Iwamoto et al. (1984) conducted a controlled breeding experiment where no increase in the incidence of precocious maturation was found in coho salmon where early growth had been accelerated using heated water. Herbinger & Friars (1992) also found no consistent effect of accelerated (or decelerated) growth on the incidence of early maturation in Atlantic salmon parr. In the present study, a statistically significant increase in jacking rate was found for the accelerated family groups, however, the magnitude of the difference was modest (17.3% vs. 19.5%) despite a fairly large difference in size throughout the saltwater rearing period (Fig. 2.1.1a, b). Although the
duration of the growth or developmental acceleration (i.e. heated water) was limited in both the present study and that of Iwamoto et al. (1984), the differences in the average size of the accelerated and control fish were comparable to that reported in studies that found a correlation between size or growth rate and the incidence of precocious maturation (see Chapter 1.1). Herbinger & Friars (1992) suggested that the energy reserve threshold for precocious maturation in Atlantic salmon parr may be relatively low, and hence the effect of accelerated growth may not have a large effect. It appears that artificially accelerated growth early in development may elicit a different response than in the naturally fast growing individuals in a population.

The presence of significant sire- and dam-by-environment effects indicates that growth acceleration early in life does not simply lower the threshold for jacking within all families alike. The norms of reaction presented in Fig. 2.1.4a & b show that most of the genotype-by-environment interaction is explained by two full-sib families (dam 3, jack-sired; dam 6, non-jack-sired). Genotype-by-environment interactions have been found for temperature and growth in a number of salmonids (McKay et al. 1984, Nilsson 1992, Iwamoto et al. 1984, Heath et al. 1992 - Appendix A). Few instances of genotype-by-environment interactions for temperature and precocious maturation have been reported in salmonids (Nilsson 1992). The environmental effect in this study includes not only the early growth and size at age differences between the Acc and N-Acc family groups, but also any "netcage effects" due to the two groups being held in separate unreplicated netcages. It is thus difficult to quantify the environmental effects contributing to the interactions. However, it is fair to say that the fish in this experiment were held in a much more homogeneous environment than most wild or enhanced stocks experience.

The estimates of heritability based on intra-dam regressions of male offspring on sire (Table 2.1.2) do not include dominance effects ($V_D$) and are not confounded by non-genetic maternal effects ($V_{CE}$ - Falconer 1981), and thus are probably the best heritability estimates possible within this study. The heritability estimates based on the sib analysis
have a number of limitations (listed in Materials and Methods), however the results were presented in order to show the differences between the sire and dam estimates. The dam-component heritability is biased by any non-genetic maternal effects \((0.25V_A + V_{Ec})\), and the sire-component heritability include dominance effects \((0.25V_A + 0.25V_D)\). Since the sire-component of heritability is much larger than the dam-component, it is likely that a strong dominance effect \((V_D)\) is present, although some form of sex-linkage is also possible. The heritability estimates generated by the sire-male offspring regressions are considerably lower than those of the sire-component within the sib analysis (Table 2.1.2). This may be due to a dominance effect, or may possibly be due to a difference in the breeding values of the parental and progeny generations (i.e. a domestication effect). Table 2.1.4 shows some reported heritability estimates for early sexual maturation in salmonids. These estimates vary widely, and there is evidence that precocious maturation in saltwater is genetically controlled separately from freshwater precocious maturation in Atlantic salmon (Glebe & Saunders 1986, Gjedrem 1984).

It should be noted that the heritability estimates presented in this study are based on only twelve families and are thus should be interpreted with caution. However, since the genetic contribution to the observed variation in jacking rates was so high, the heritability estimates may be meaningful. Given the relatively consistent results of this analysis, a more detailed breeding experiment to determine the nature of the observed dominance effect in the sire contribution is warranted.

Precocious maturation in some fish may be controlled by simple genetic mechanisms. Glebe & Saunders (1986) noted that in Atlantic salmon, the frequencies of precocious male parr could be explained by a single gene model, and sexual maturation in some Xiphophorus spp. is also controlled by a single locus (Kallman & Bao 1982, Zimmerer et al. 1989). The variation in jacking rates in the present study, indicates that jacking in chinook salmon may also be determined by a few
Table 2.1.4: Estimates of heritability for mean age of first maturation in salmonids taken from the literature. Three methods of estimation are reported; sire and dam estimates refer to sib analysis, presumably on the binomial scale, and parent-offspring regressions. Standard errors were included when reported.

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>$h^2$</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chinook salmon</td>
<td>0.30</td>
<td>Ricker (1980)</td>
</tr>
<tr>
<td>Coho salmon*</td>
<td>0.05&lt;sup&gt;S&lt;/sup&gt; (± 0.05)</td>
<td>Silverstein &amp; Hershberger (1992)</td>
</tr>
<tr>
<td></td>
<td>0.13&lt;sup&gt;d&lt;/sup&gt; (± 0.11)</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>0.17</td>
<td>Iwamoto et al. (1984)</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>0.21&lt;sup&gt;S&lt;/sup&gt;</td>
<td>Gjerde &amp; Gjedrem (1984)</td>
</tr>
<tr>
<td></td>
<td>0.26&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>0.09&lt;sup&gt;s&lt;/sup&gt;</td>
<td>Moller et al. (1976)</td>
</tr>
<tr>
<td></td>
<td>0.01&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&quot;</td>
</tr>
<tr>
<td>Arctic char</td>
<td>0.45&lt;sup&gt;S&lt;/sup&gt; (± 0.17)</td>
<td>Nilsson (1992)</td>
</tr>
<tr>
<td></td>
<td>0.12&lt;sup&gt;d&lt;/sup&gt; (± 0.08)</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>0.19&lt;sup&gt;S&lt;/sup&gt; (± 0.11)</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>0.60&lt;sup&gt;d&lt;/sup&gt; (± 0.24)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Atlantic salmon</td>
<td>0.39&lt;sup&gt;s&lt;/sup&gt;</td>
<td>Gjerde &amp; Gjedrem (1984)</td>
</tr>
<tr>
<td></td>
<td>0.49&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>0.48&lt;sup&gt;r&lt;/sup&gt;</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

<sup>1</sup> as cited in Gjedrem (1984)
<sup>s</sup> sire component
<sup>d</sup> dam component
<sup>r</sup> parent-offspring regression
<sup>*</sup> precocious maturation pre-smolt
loci, although an analysis of F$_2$ progeny would be necessary to confirm this.

There is also evidence that jacking in chinook salmon may be sex linked. Two cohorts of female chinook salmon were hormonally masculinized (Hunter et al. 1983) at Yellow Island Aquaculture Ltd. These phenotypically male, genotypically female fish had very low jacking rates (1.4% in 1990, n = 2300; 2.7% in 1991, n = 1500; unpublished data) compared to jacking rates of genotypically male fish from similar stocks (30 - 40%). The incidence of third year maturation in the sex-reversed female stocks at Yellow Island Aquaculture were close to that observed for the regular males.
2.2 DNA FINGERPRINTS IN SALMONIDS PRODUCED BY HYBRIDIZATION
WITH OLIGONUCLEOTIDES.

2.2.1 INTRODUCTION

The previous chapter described a breeding experiment designed to identify genetic and environmental contributions to jacking in chinook salmon. The next two chapters describe the development of molecular-genetic tools for use in chinook salmon. The ultimate aim of developing such tools was to use them to estimate the relatedness of jacking and randomly selected chinook salmon from a random-mating population. If jacking has a family component, the average relatedness of the jacking fish should be greater than that of the randomly selected fish. A genetic component to jacking may be thus identified, prior to the large investment of time and resources necessary for extensive breeding programs.

Within fisheries science and aquaculture such molecular-genetic tools are also needed to identify stocks, families, parentage, and individual fish on a genetic basis (Hallerman & Beckmann 1988, Bentzen et al. 1991, Harris et al. 1991). Specifically, the potential uses include;

1) identification of valuable brood lines for commercial purposes,
2) identification of stocks of fish for management purposes,
3) identification of full-sib families for selection experiments without physical tags, and
4) identification of relatedness and inbreeding to determine the impact of management practices on the genetic diversity of a population.

The methods currently used to measure genetic diversity within stocks or populations of fish (such as enzyme polymorphisms, mitochondrial DNA Restriction Fragment Length Polymorphisms (RFLPs), and genomic RFLPs) are limited by either low levels of heterozygosity, high technical difficulty, or both (Hill 1987, Wetton et al. 1987, Hallerman & Beckmann 1988, Taggart & Ferguson 1990a, Bentzen et al. 1991, Wirgin et al. 1991).
An alternate method, DNA fingerprinting, allows visualization of the genetic variation present in genomic DNA tandem repeats (minisatellite DNA). DNA fingerprinting has been employed or proposed for use in the identification of populations or stocks (see Hallerman & Beckmann 1988, Gilbert et al. 1990, Rogstad et al. 1991, Wirgin et al. 1991), as well for the identification of parentage or individual animals of particular interest (Jeffreys et al. 1985, Burke & Bruford 1987, Hoelzel & Amos 1988, Harris et al. 1991). The identification of full- or half-sibs using DNA fingerprinting is problematic due to the large number of bands and the relatively large probability of band sharing due to chance alone (Anonymous 1989, Devlin et al. 1990, Taggart & Ferguson 1990b, Lynch 1991, Burke et al. 1991). However Rico et al. (1991) reported success in identifying offspring from a single parent in threespine sticklebacks, Gasterosteus aculeatus, as well as in birds (Burke & Bruford 1987, Wetton et al. 1987, Jones et al. 1991). Perhaps the most important potential application of DNA fingerprinting lies in the estimation of relatedness or genetic diversity of a population of organisms. This has been reported for example, in striped bass populations, Morone saxatilis, (Wirgin et al. 1991), isolated fox populations (Gilbert et al. 1990), inbred chicken lines (Dunnington et al. 1991), and the clonally reproducing pawpaw tree, Asimina triloba (Rogstad et al. 1991). Many of the applications of DNA fingerprinting would benefit from a wide selection of multi-locus hypervariable minisatellite probes (Harris et al. 1991, Burke et al. 1991).

Minisatellite DNA sequences (Jeffreys et al. 1985) have been shown to hybridize with DNA from a wide variety of organisms (Burke & Bruford 1987, Jeffreys & Morton 1987, Georges et al. 1988, Ryskov et al. 1988, Taggart & Ferguson 1990b, Harris et al. 1991, Rogstad et al. 1991). However, relatively few of the known minisatellite probes have been tested on salmonids. DNA fingerprints have been reported for Atlantic salmon, (Fields et al. 1989, Taggart & Ferguson 1990, Bentzen et al. 1991), rainbow trout (Fields et al. 1989, Lloyd et al. 1989, Taggart & Ferguson 1990b, Bentzen et al. 1991), brown trout, Salmo trutta L. (Taggart & Ferguson 1990b), chum salmon, Oncorhynchus keta and
coho salmon, (Fields et al. 1989, Bentzen et al. 1991), and chinook salmon, (Bentzen et al. 1991).

The use of chemically synthesized oligonucleotides as DNA fingerprinting probes has been investigated in humans (Ali et al. 1986, Schafer et al. 1988), as well as in domestic animals (Georges et al. 1988), plants (Nybom et al. 1992, Weising et al. 1992), and insects (Blanchetot 1991, 1992). Epplin et al. (1991) reported on over two hundred species ranging from fungi to humans that were successfully probed for DNA fingerprints using short repetitive-sequence oligonucleotides. A wide variety of chemically synthesized DNA fingerprint probes could be easily obtained by most laboratories and are simple and fast to label and use (Epplin et al. 1991). For this study eleven oligonucleotides were synthesized with minisatellite DNA core sequences as reported in the literature (Table 2.2.1). The synthesized oligonucleotides ranged from 11 to 18 bases and include the oligonucleotide corresponding to the core sequence of the M13 fragment, reported elsewhere as producing DNA fingerprints in salmonids (Fields et al. 1989). One oligonucleotide was examined that was very similar to the core sequence for the Jeffreys probes (1985) that have been shown to hybridize with salmonid DNA (Taggart & Ferguson 1990b). The purpose of this work was to identify potentially useful DNA fingerprinting probes for use with chinook and other species of salmon.

2.2.2 MATERIALS AND METHODS

DNA Extraction

DNA was extracted from liver or testicular tissue samples from one adult male and one adult female chinook salmon (Robertson Creek stock, Vancouver Island, British Columbia), and from three of their offspring. DNA was also extracted from two presumed unrelated individuals from eleven other species of salmonids (Table 2.2.2).
Table 2.2.1: Eleven oligonucleotides screened as potential DNA fingerprint probes in chinook salmon. The sequence, reference, and origin of each oligonucleotide is given. The hybridization and wash temperatures are either the optimised conditions used to produce the autoradiograms shown in Fig. 2.2.1, or are the low stringency conditions that were used for initial screening. Only the first six (underlined) of the eleven oligonucleotides yielded multiple discreet bands.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Temperature (°C)</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hyb.</td>
<td>Wash</td>
</tr>
<tr>
<td><strong>YN24</strong></td>
<td>GGAGCAGTGGGNNNTACA¹</td>
<td>42</td>
<td>42</td>
</tr>
<tr>
<td><strong>M13</strong></td>
<td>GAGGGTGNNGGNTCT²</td>
<td>41</td>
<td>41</td>
</tr>
<tr>
<td><strong>MY01</strong></td>
<td>GGAGGTGGGCAGGGAG³</td>
<td>38</td>
<td>36</td>
</tr>
<tr>
<td><strong>GLOB</strong></td>
<td>GNGGGGNACAG⁴</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td><strong>HBV3</strong></td>
<td>GGTGAAGCANAGG³</td>
<td>38</td>
<td>36</td>
</tr>
<tr>
<td><strong>PER1</strong></td>
<td>GACNGGNACNGG⁴</td>
<td>38</td>
<td>36</td>
</tr>
<tr>
<td><strong>PER2</strong></td>
<td>TCAGGCTCAGG³</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td><strong>INS</strong></td>
<td>ACAGGGGTGGGG³</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td><strong>YN73</strong></td>
<td>CCCGTGGGGGCGCGG¹</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td><strong>YNZ22</strong></td>
<td>CTCTGGGTGGTGGC³</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td><strong>CAC⁵</strong></td>
<td>CACCAACCACCAC⁵</td>
<td>28</td>
<td>28</td>
</tr>
</tbody>
</table>

¹ Nakamura et al. (1988); ² Vassart et al. (1987); ³ Nakamura et al. (1987); ⁴ Georges et al. (1988); ⁵ Schafer et al. (1988)
Table 2.2.2: Salmonid species used for hybridizations with oligonucleotides YN24 and M13 DNA fingerprint probes. Except as noted, the mean number of bands are the averages of the number of distinct bands above 3 Kb (kilobases) counted on the original autoradiograms for two unrelated individuals of each species (digested with Hae III), and are given for both probes (YN24 and M13).

<table>
<thead>
<tr>
<th>Species</th>
<th>Common Name</th>
<th>Mean Number of Bands</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>YN24</td>
</tr>
<tr>
<td>Oncorhynchus kisutch</td>
<td>Coho salmon</td>
<td>27</td>
</tr>
<tr>
<td>O. masou</td>
<td>Masu salmon</td>
<td>33</td>
</tr>
<tr>
<td>O. keta</td>
<td>Chum salmon</td>
<td>32</td>
</tr>
<tr>
<td>O. nerka</td>
<td>Sockeye salmon</td>
<td>29</td>
</tr>
<tr>
<td>O. gorbuscha</td>
<td>Pink salmon</td>
<td>31</td>
</tr>
<tr>
<td>O. mykiss</td>
<td>Rainbow trout</td>
<td>27</td>
</tr>
<tr>
<td>Salmo salar</td>
<td>Atlantic salmon</td>
<td>31</td>
</tr>
<tr>
<td>Salvelinus alpinus</td>
<td>Arctic char</td>
<td>30</td>
</tr>
<tr>
<td>S. malma</td>
<td>Dolly Varden</td>
<td>33</td>
</tr>
<tr>
<td>S. namaycush</td>
<td>Lake trout</td>
<td>19*</td>
</tr>
<tr>
<td>Coregonus clupeaformis</td>
<td>Lake whitefish</td>
<td>28</td>
</tr>
</tbody>
</table>

* One individual counted since the other is not clear.
following the protocol described in Devlin et al. (1991). Approximately 100-200 mg of frozen or 95% ethanol preserved tissue was finely chopped and placed into 5.0 ml of proteinase K buffer (10 mM tris (pH 8.0); 10 mM ethylenediaminetetraacetic acid (EDTA); 1% sodium dodecyl sulphate (SDS)), and 200 μg·ml⁻¹ of proteinase K. The solution was incubated at 37°C, with gentle rocking overnight. NaCl was added to a final concentration of 1.5 M, and the resulting solution was mixed by inversion, and then centrifuged (15 min @ 3500 rpm). The supernatant fluid was extracted once with phenol: chloroform: isoamyl alcohol (50:50:1) and the DNA was precipitated by the addition of 0.6 volumes of isopropl alcohol. The pellet was washed with 70% ethanol, dried, and redissolved in TE (10mM tris (pH 8.0), and 1mM EDTA).

**DNA Digestion, Fractionating, and Transfer**

Five microgram aliquots of each DNA sample were digested overnight in 30 μl with Hae III restriction enzyme (BRL, Life Technologies Inc., Gaithersburg, MD., USA) according to the manufacturer’s instructions. The digested DNA was fractionated by electrophoresis on a 25 cm, 0.5% agarose gel at approximately 1.5 V·cm⁻¹ until the fragments smaller than 1 kilobase (Kb) had run off the gel. DNA was transferred to Hybond-N nylon filters (Amersham Corp. Illinois) according to Sambrook et al. (1989).

**Oligonucleotides**

The sequence for eleven oligonucleotides ranging from 11 to 18 bases long were chosen from published reports of variable number tandem repeats (VNTR) and minisatellite DNA core sequences (Table 2.1.1). The oligonucleotides were synthesized at the University of British Columbia’s Oligonucleotide Synthesis Laboratory (Vancouver, B.C.). The oligonucleotides were end-labelled with γ-[³²P]ATP using T4 kinase (Boehringer-Mannheim) as described by Sambrook et al. (1989).

Filters were pre-hybridized for 2 h in a solution comprised of 7% SDS, 1 mM EDTA (pH 8.0), 0.263 M Na₂HPO₄ and 1% bovine serum albumin (fraction V) (Westneat et al. 1988), at 42°C. End-labelled oligonucleotides were mixed with 10 ml of
pre-hybridizing solution, added to the filters, and allowed to hybridize overnight. The eleven oligonucleotides were initially screened by hybridization with chinook salmon family DNA at low temperatures (28°C). Filters were washed twice in 2 X SSC (0.3 M NaCl, 0.3 M sodium citrate, pH 7.0), and 0.1% SDS for 25 minutes at room temperature, and once at a higher temperature (28-42°C; Table 2.2.1). Filters were then wrapped in Saran wrap and exposed to X-ray film without an intensifying screen for two to seven days at room temperature. Oligonucleotides that yielded a variable banding pattern were hybridized at higher stringency to reduce the non-specific binding (30-42°C hybridization and final wash temperature: Table 2.2.1).

Other Salmonid Species

Oligonucleotides YN24 and M13 were also hybridized with DNA from two presumed unrelated individuals of 11 other species of salmonids (Table 2.2.2) under hybridization conditions determined as optimal for the chinook salmon family filters (Table 2.2.1).

2.2.3 RESULTS AND DISCUSSION

Due to the lack of clarity of even the best performing probes in this study, it was felt that an analysis of the relatedness of jacks versus non-jacks within a single population was beyond the sensitivity of the DNA fingerprint probes described here. Nevertheless the YN24 DNA fingerprint probe has been successfully applied to the analysis of gynogenesis in albino chinook salmon (B. Carswell, unpub. data), and has other potential uses in fisheries science.

Chinook Salmon Families

Eleven oligonucleotides derived from human and other sources (Table 2.2.1) were screened against Southern blots of chinook salmon family DNAs, and six yielded fingerprint-like banding patterns (Table 2.2.1, Fig. 2.2.1). Different hybridization and wash temperatures were required to reduce background noise without removing the signal,
and presumably those reflected differences in the binding strength (and hence the signal to noise ratio) of the oligonucleotides with the target DNAs (Fig. 2.2.1). Of the six successful probes, YN24 and M13 produced the strongest signal and clearest bands (Fig. 2.2.1). While probes GLOB and MYO1 also yielded multi-banded DNA fingerprints on the chinook salmon families (Fig. 2.2.1), there was more background signal, and the bands were fainter than those produced by YN24 and M13. Probes PER1 and HBV3 show multiple variable bands, however the signal strength was low and the resolution very poor (Fig. 2.2.1), although Castelli et al. (1990) reported clear banding patterns with the PER1 probe in the barbel. It is possible that PER1 and HBV3 might perform better with other hybridization and wash protocols. The five oligonucleotides that did not produce any discernible bands at even the low stringency hybridization and washing conditions (Table 2.2.1) are not likely to be useful DNA fingerprinting probes in salmonids. Rico et al. (1991) also found that probe YNZ22 (see Table 2.2.1) was not able to produce DNA fingerprints in the threespine stickleback.

Close examination of the original autoradiographs for all probes showed that most bands in the three offspring (a, b, and c in Fig. 2.2.1) were also seen in either one or both parents, with only a very few exceptions (i.e. a single band at approximately 4.5 Kb in offspring b for the M13 probe - Fig. 2.2.1). Novel bands can arise as a consequence of germ-line mutation in one of the parents, however, because only three offspring were analyzed, no meaningful estimation of the mutation rate was possible (Jeffreys et al. 1985, Hill 1987, Jeffreys et al. 1988). The limited number of offspring examined did not allow determination of the extent of alleleism or linkage for these probes, however minisatellite loci generally show little linkage (Jeffreys et al. 1985, Burke & Bruford 1987, Gyllensten et al. 1989, Castelli et al. 1990, Taggart & Ferguson 1990b).

The banding patterns produced by the six probes were quite distinct from one another (Fig. 2.2.1), this indicates that the oligonucleotides hybridized to different minisatellite loci. Since the six probes yielded information
Figure 2.2.1: DNA fingerprints of a family of chinook salmon generated by hybridization with six oligonucleotides. The family is comprised of an adult female and male, and three of their offspring (a, b, and c). Molecular size markers are shown in kilobases (Kb) on either side.
on distinct loci, the use of more than one of these probes would allow more of the genome to be sampled.

**Other Salmonid Species**

Because the oligonucleotides M13 and YN24 produced the clearest banding patterns (Fig. 2.2.1), they were further hybridized with DNA from eleven other species of salmonids (Table 2.2.2) to assess their general applicability.

Probe YN24 generated highly variable multiple banding patterns in all 11 species of salmonids considered here (Fig. 2.2.2). There is considerable variability in the number of bands observed between the species (Table 2.2.2), as well as in the clarity of those bands: lake trout has very indistinct bands, while Atlantic and sockeye salmon have very clear bands (Fig. 2.2.2). Some species, such as chum and pink salmon, appear to have greater levels of non-specific binding than others (Fig. 2.2.2). This was also noted by Burke & Bruford (1987) using Jeffreys (1985) probe 33.15 on different species of birds.

We tested probe YN24 on chinook salmon DNA digested with three other restriction endonucleases (Pst I, Hind III, and Hinf I), as well as combinations of pairs of them. We found that for Pst I and Hind III, and combinations Pst I plus Hind III, or Hind III plus Hinf I, the resulting fingerprint patterns were different, but showed essentially the same complexity and number of bands as for the Hae III digested DNA. However Hinf I, and combinations Pst I plus Hae III, Pst I plus Hinf I, Hind III plus Hinf I, or Hinf I plus Hae III, showed considerably less variation and resolution (data not shown). In general, the choice of restriction enzyme appears to be important to the performance of YN24 as a fingerprinting probe. Although the insertion-free M13 bacteriophage has been used widely for DNA fingerprinting (Vassart et al. 1987, Georges et al. 1988, Ryskov et al. 1988, Fields et al. 1989, Castelli et al. 1990, Rogstad et al. 1991, Wirgin et al. 1991), the results presented here show that the M13 oligonucleotide worked as well as the cloned M13 fragment for Atlantic, chum, and coho salmon (Fig. 2.2.3), but not as well as the fragment for rainbow trout (see Fields et al. 1989).
Figure 2.2.2: DNA fingerprints of two presumed unrelated individuals from each of eleven species of salmonids generated by hybridization with the YN24 oligonucleotide. Note that the individual fish are the same as for the M13 hybridizations (Fig. 2.2.3). Molecular size markers are shown in kilobases (Kb) on either side.
Table 2.2.3: DNA fingerprints of two presumed unrelated individuals from each of eleven species of salmonids generated by hybridization with the M13 oligonucleotide. Note that the individual fish are the same as for the YN24 hybridizations (Fig. 2.2.2). Molecular size markers are shown in kilobases (Kb) on either side.

<table>
<thead>
<tr>
<th>Species</th>
<th>Molecular Size Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coho salmon (O. kisutch)</td>
<td>12 Kb, 9 Kb, 5 Kb, 3 Kb, 2 Kb</td>
</tr>
<tr>
<td>Masu salmon (O. masou)</td>
<td>12 Kb, 9 Kb, 5 Kb, 3 Kb, 2 Kb</td>
</tr>
<tr>
<td>Chum salmon (O. keta)</td>
<td>12 Kb, 9 Kb, 5 Kb, 3 Kb, 2 Kb</td>
</tr>
<tr>
<td>Sockeye salmon (O. nerka)</td>
<td>12 Kb, 9 Kb, 5 Kb, 3 Kb, 2 Kb</td>
</tr>
<tr>
<td>Pink salmon (O. gorbuscha)</td>
<td>12 Kb, 9 Kb, 5 Kb, 3 Kb, 2 Kb</td>
</tr>
<tr>
<td>Rainbow trout (O. mykiss)</td>
<td>12 Kb, 9 Kb, 5 Kb, 3 Kb, 2 Kb</td>
</tr>
<tr>
<td>Atlantic salmon (S. salar)</td>
<td>12 Kb, 9 Kb, 5 Kb, 3 Kb, 2 Kb</td>
</tr>
<tr>
<td>Arctic char (O. aguabonita)</td>
<td>12 Kb, 9 Kb, 5 Kb, 3 Kb, 2 Kb</td>
</tr>
<tr>
<td>Dolly varden (O. kisutch)</td>
<td>12 Kb, 9 Kb, 5 Kb, 3 Kb, 2 Kb</td>
</tr>
<tr>
<td>Lake trout (O. mykiss)</td>
<td>12 Kb, 9 Kb, 5 Kb, 3 Kb, 2 Kb</td>
</tr>
<tr>
<td>Lake whitefish (Coregonus clupeaformis)</td>
<td>12 Kb, 9 Kb, 5 Kb, 3 Kb, 2 Kb</td>
</tr>
</tbody>
</table>

Figure 2.2.3: DNA fingerprints of two presumed unrelated individuals from each of eleven species of salmonids generated by hybridization with the M13 oligonucleotide. Note that the individual fish are the same as for the YN24 hybridizations (Fig. 2.2.2). Molecular size markers are shown in kilobases (Kb) on either side.
There was considerable variation in the number of discernible bands between species (Table 2.2.2), as well as in the clarity of the bands. For example, rainbow trout DNA fingerprints were not as distinct as those of the Dolly Varden (Fig. 2.2.3).

The YN24 and M13 probes produced quite different results for the same species (Fig. 2.2.2 and 2.2.3). For example, the rainbow trout fingerprint was indistinct for the M13 oligonucleotide probe, while the YN24 probe fingerprint was relatively clear. Furthermore, the number of discernible bands was different for a given species between M13 and YN24 (Table 2.2.2). Thus, the availability of a selection of different probes would allow one to choose those that yield the clearest fingerprints for the species under study.

Because of concerns regarding the negative impact of stock enhancement programs on the genetic diversity of wild populations of salmonids, there is currently much interest in estimating genetic diversity using DNA fingerprinting techniques. A number of different DNA fingerprint probes could be used to provide independent estimates of the genetic diversity of a population of salmonids, allowing the sampling of a greater proportion of the animal's genome (see Taggart and Ferguson 1990b). Overall, the use of DNA fingerprinting to assess the diversity of "neutral" (or non-coding) DNA is perhaps the method of choice for use at the population level (Hill 1987, Wetton et al. 1987, Ryskov et al. 1988, Gilbert et al. 1990, Taggart & Ferguson 1990b, Dunnington et al. 1991, Harris et al. 1991, Rogstad et al. 1991, Virgin et al. 1991).
2.3 A NOVEL APPLICATION OF PCR TO AMPLIFY HYPERVARIABLE MINISATELLITE DNA SINGLE LOCUS PROBES

2.3.1 INTRODUCTION

In the previous chapter the uses of multilocus DNA fingerprinting in fisheries science and ecology was discussed. Although DNA fingerprinting has been proposed for the analysis of relatedness between individuals (Jeffreys et al. 1985, Burke & Bruford 1987, Hoelzel & Amos 1988, see Chapter 2.2) there are serious problems with such an application (Anonymous 1989, Lynch 1991, see Chapter 2.1). In multilocus DNA fingerprints there is a relatively high probability of band sharing due to chance alone, making the identification of kinship or relatedness difficult (Anonymous 1989, Lynch 1991). However most of these limitations can be avoided by using minisatellite DNA single locus probes (Bentzen et al. 1991, Burke et al. 1991, Lynch 1991). Although minisatellite DNA single-locus probes have been isolated for a number of animals, there have been few applications of them in ecology and evolutionary biology. Although there have been some studies using single-locus probes to evaluate kinship and relatedness (Amos et al. 1991, Bruford & Burke 1991), by far the most common applications have been in human medicine and forensics (Bar & Hummel 1991, Wolff 1991). The main limitation on the use of single-locus probes in ecological and evolutionary studies has been the unavailability of suitable probes. Single locus probes have historically been technically demanding to generate (Burke et al. 1991) and often are relatively species specific.

A new Polymerase Chain Reaction (PCR) based technique, Random Amplification of Polymorphic DNA (RAPD: Williams et al. 1990, Welsh & McClelland 1990), has been used for the analysis of parentage and kinship (Arnold et al 1991, Scott et al. 1992, see Hadrys et al. 1992), stock and strain identification (Welsh & McClelland 1990, Hadrys et al. 1992), and species identification (Chapco et al. 1992, Arnold et al. 1991, Hadrys et al. 1992) in both wild and experimental populations of plants and animals. Due to the ease of
the technique, as well as the small amount of DNA required (Hadrys et al. 1992), RAPD has many potential applications in fisheries and ecology. There are, however, two limitation to RAPD; 1) relatively low levels of variation for some applications (Hadrys et al. 1992), and 2) rare PCR "artifacts", or unexplained bands (Scott et al. 1992). This study describes an extension of RAPD that generates minisatellite DNA single locus probes, without the necessity of DNA library screening, cloning or sequencing. This novel application of PCR is used to develop a minisatellite DNA single locus probe from chinook salmon DNA, as well as two other potentially useful probes from human and bird DNA. The purpose of developing chinook salmon single locus probes is to estimate relatedness in jack and randomly selected groups of fish from a randomly-mated population.

2.3.2 MATERIALS AND METHODS

Genomic DNA

DNA was extracted from liver, testes, and blood samples from three of the chinook salmon families described in Chapter 2.1 (see Chapter 2.2 for protocol). DNA from two to three unrelated individual coho salmon, chum salmon, sockeye salmon, pink salmon (Oncorhynchus gorbuscha), rainbow trout, and Atlantic salmon was also examined. Quail (Coturnix japonica) DNA from a family of birds and two unrelated individuals was extracted from blood samples kindly provided by Dr. K. Cheng (Dept. of Animal Science, UBC). Human DNA from a single family (two progeny) was provided by J. Theilman (University of BC, Vancouver, BC). Genomic DNA from all three species (human, quail, and chinook salmon) was digested with Hae III, fractionated by gel electrophoresis, and transferred to nylon membranes as described in Chapter 2.2, except that all DNA fragments smaller than 2 kilobases (Kb) in size were run off the gel.

Polymerase Chain Reaction

Approximately 0.5 μg of genomic DNA from chinook salmon, human, and quail were used as template for 50 μl polymerase chain reactions (PCR). The synthetic
oligonucleotides M13, YN73, PER2, and YNZ22 (see Chapter 2.2) were used as primers. Only one oligonucleotide was used per reaction; 0.5 µg per reaction. Note that smaller amounts of primer yielded unreliable results. The balance of the PCR was CETUS buffer (50 mM KCl, 10 mM Tris (pH=4.0 @ 20°C), 100 µg·ml⁻¹ gelatin), MgCl₂ (1.5 mM), dNTPs (2.0 mM), and Taq DNA polymerase (0.05 U·µl⁻¹: GIBCO-BRL, Gaithersburg MD, USA) as described in Innis et al. 1990. The reactions were run for 35 cycles with a 55°C annealing cycle (1.0 min), 72°C extension cycle (1.5 min), and a 95°C denaturing cycle (1.0 min). The resulting reactions were fractionated on 1.5% low melting point (LMP) agarose gels for 3-4 hr at 70V in a coldroom (app. 8°C). Under UV transillumination, clearly distinguishable bands were cut out and stored at -20°C.

**Labelling**

The frozen gel slices were heated to 68°C for 15-30 min, then 8.0 µl were immediately added to a 50 µl random priming reaction (as described in Feinberg & Vogelstein 1984) and labelled with alpha-dATP (Amersham Corp., Arlington Heights, IL, USA). The random priming reactions were incubated at 37°C to minimize the solidification of the agarose. The completed reactions were run through G50 spin columns to remove unincorporated nucleotides (Sambrook et al. 1989).

**Hybridization**

Membranes were pre-hybridized as in Chapter 2.2. The radio-labelled probes were added to approximately 15 ml of hybridization solution and added to the membranes and allowed to hybridize overnight. Hybridization temperatures were kept low (50°C) for screening but later raised (68°C) to reduce background signal with potentially useful probes. The membranes were washed as described in Chapter 2.2 for the screening process, however the concentration of SSC was lowered to 0.2 X SSC to reduce background signal. The high temperature wash was at the same temperature as the hybridization. Membranes were then wrapped in Saran wrap and exposed to X-ray film for one to two days with an intensifying screen at -70°C. Potential probes were evaluated on
the number, clarity, and inheritance pattern of the bands seen on the autoradiograph. A total of 26 gel slices (i.e. bands resulting from the PCR) were screened for useful single- (or few-) locus probes, they were comprised of; 12 chinook salmon, 8 human, and 6 quail gel slices.

**Cloning and Sequencing**

One chinook salmon DNA fragment (app. 400 base pairs) that appeared to detect a highly-variable locus was re-amplified using "touchdown" PCR protocol (Don et al. 1991). The touchdown thermal cycles started at an annealing temperature of 65°C and reduced by one degree each subsequent cycle to a minimum of 50°C, followed by an additional 20 cycles at 50°C annealing temperature. The PCR product was gel purified following the protocol of Prep-A-Gene DNA Purification Matrix (BIO-RAD, Life Science Group, Hercules CA, USA). The ends of the PCR-amplified DNA were filled in with T4 DNA polymerase (GIBCO-BRL) as described in Sambrook et al. (1989), and cloned into the pBluescript II SK+ vector (Stratagene Cloning Systems, LaJolla CA, USA). Double stranded DNA was purified from each of 18 insert-positive colonies (Sambrook et al. 1989), digested with Xho 1 and Pst 1 (GIBCO-BRL) to release the inserted DNA fragment, and the digestions were size fractionated by gel electrophoresis with LMP agarose. 13 of the 18 colonies proved to contain inserts, and 6 of these were of approximately the correct size (400 bp). These 6 inserts were screened by cutting the bands out of the gel, labelling them as described above, and hybridizing them with genomic DNA from chinook salmon (as above) to identify clones that produced the single locus pattern.

Double- and single-stranded DNA was prepared from the positively screened clone (Sambrook et al. 1989). Sequencing was performed in both orientations using a Sequenase kit (United States Biochemical Corp.) and was analyzed with PCGene software (Intelligenetics).
Applications

The cloned chinook salmon probe was labelled and hybridized to three chinook salmon families; two of the families consisted of two parents and four offspring, while the third consisted of two parents and 23 offspring. The probe was also hybridized with 17 presumed unrelated chinook salmon and two to three presumed unrelated individuals from each of 6 species of salmonids (see above). One of the human-derived DNA fragments was hybridized with DNA from a human family (parents and two progeny), while one of the quail-derived DNA fragments was hybridized with quail genomic DNA (two parents, four progeny, and two unrelated individuals).

2.3.3 RESULTS AND DISCUSSION

The PCR using the single oligonucleotides yielded complex banding patterns with 1 to 10 clearly distinguishable bands, depending on the primer and the species (Fig. 2.3.1). These patterns were evident for the chinook salmon, human and quail template reactions, although the number and positions of the bands differed between species (Fig. 2.3.1a). The PCR amplification of a number of different individual chinook salmon shows that although there is some variation between individuals, most of the bands are not variable (Fig. 2.3.1b). One hypothesis that explains these bands is presented in Fig. 2.3.2. It is possible that a DNA inversion could transfer non-repetitive DNA into the VNTR region. This would account for the PCR amplification of DNA with only one primer since the inverted repeats would be in the reverse orientation relative to the undisturbed VNTRs (Fig. 2.3.2). The occurrence of such an inversion would be infrequent, thus accounting for the low level of variation seen in the banding patterns.

The PCR protocol described here is an extension of the RAPD protocol. The essential difference lies in the use of a single known VNTR core sequence as the primer, as opposed to random primers in RAPD. The VNTR core sequence primer directs the amplification to minisatellite regions, thus the process is a Directed Amplification of
Minisatellite (region) DNA (DAMD). It appears that DAMD yields species-specific banding patterns (Fig. 2.3.1a, b), and may also be useful for stock or population identification (this has not been tested - all of the chinook salmon shown in Fig. 2.3.1b are from a single stock). A PCR based method of identifying closely related species or stocks would have many applications in stock management (Hadrys et al. 1992).

If the hypothesized model shown in Fig. 2.3.2 is correct, then the non-VNTR DNA amplified by DAMD could be specific to a single VNTR locus. When 26 of these putative near-VNTR DNA fragments from chinook salmon, human, and quail were screened as probes on Southern blots of genomic DNA, the results were one highly variable single locus probe for chinook salmon (OTSL1) amplified with the M13 oligonucleotide, as well as one human and one quail probe that gave relatively simple banding patterns.

The quail probe (amplified with the YNZ22 oligonucleotide) yielded two or three loci; it is difficult to determine the segregation of the bands in the quail since the family showed very little variation (Fig. 2.3.3a), however the two unrelated quail show that there is variation at these loci.

The human probe (amplified with the YN73 oligonucleotide) yielded a high molecular weight single-locus banding pattern, with a multilocus pattern at lower molecular weights (Fig. 2.3.3b). The segregation of the male parent band at approximately 10 Kb appears to show incomplete inheritance (i.e. no male parent band in offspring "b"), although a second male parent band may exist at a lower molecular weight, and may thus be obscured by the multi-locus signal, or run off the gel.

The chinook salmon probe, OTSL1, clearly hybridized to a single highly variable locus (Fig. 2.3.4), and appeared to be conservatively inherited (Fig. 2.3.5), except for one obvious mutation within the progeny of the first of three families shown in Fig. 2.3.5. To investigate the mutation further, 23 offspring from that family were hybridized with OTSL1, and an analysis of the inheritance of the alleles is given in Table 2.3.1. Although the inheritance of the alleles appears to be
Figure 2.3.1: Results of PRC using a single oligonucleotide as primer; 1 Kb ladder marker lanes are identified on each gel. **Gel a:** the first lane (lane a) is chinook salmon DNA as template with YN73 oligonucleotide as primer, the next three lanes (lanes b, c, & d) are human DNA as template with YN73, M13, and PER1 respectively as primer, the last three lanes (lanes e, f, & g) are quail DNA as template and INS, YN73, and YNZ22. **Gel b:** the first nine lanes (lanes 1 - 9) are chinook salmon DNA from nine unrelated individuals as template with M13 oligonucleotide as primer, after the marker lane the next two lanes (lanes a & b) are Atlantic salmon DNA from two unrelated individuals as template with M13 as primer, the last two lanes (lanes c & d) are rainbow trout DNA from two unrelated individuals as template with M13 as primer.
Figure 2.3.2: A schematic diagram of the hypothesized inversion event that allows PCR amplification of multiple-sized DNA fragments (see Fig. 2.3.1) using a single primer with the core sequence from known VNTRs. The ancestral organization shows the edge of a VNTR region with non-repetitive DNA represented by the straight lines and the tandem repeats represented by the arrows. The diagram shows the boxed section of DNA inverted in the present day organization. In the present day organization a PCR with the core sequence of the VNTR as the primer would amplify the non-repetitive section of DNA enclosed in the VNTR (indicated by the shaded bar). If this hypothesized inversion event model is correct, then the PCR amplified fragment should; 1) show little variation between individuals since the inversion event would be rare, and 2) be homologous to only the minisatellite DNA locus where the event took place.
Figure 2.3.3: Autoradiographs of membranes probed with radio-labelled potential single-locus minisatellite DNA probes. The quail film ("QUAIL") shows two unrelated individuals (lanes 1 & 2), and a family; two parents and four offspring (lanes a, b, c, & d). Note the very low level of variation within the family, compared to the two unrelated individuals. The Human film ("HUMAN") shows a family; two parents and two offspring (lanes a & b). The small number of progeny precludes an interpretation of the inheritance of the bands, however offspring "a" (female) clearly inherited the paternal band, while offspring "b" (male) did not. The missing allele in offspring "b" may be of low molecular weight and thus obscured by the multi-locus signal.
Figure 2.3.4: Autoradiographs of membranes with 17 unrelated chinook salmon probed with the chinook salmon single-locus minisatellite DNA probe OTSL1. Lanes a - f represent adult female salmon, lanes g - l represent adult male salmon, and lanes m - q represent sexually precocious male salmon (i.e. jacks).
Figure 2.3.5: Autoradiographs of membranes with three families of chinook salmon probed with the chinook salmon single-locus minisatellite DNA probe OTSL1. All alleles were conservatively inherited except for the paternal allele in offspring "c" in family 1 (marked by an arrow). This mutation represents an increase in molecular weight of the allele of between 1 and 2 Kb, depending on which paternal allele mutated.
Table 2.3.1: An analysis of allele inheritance in a chinook salmon family for the minisatellite DNA locus OTSL1. Twenty-three progeny were scored for allele frequency; expected frequencies were based on Mendelian inheritance. A total of five alleles were observed, one of which was not observed in either parent. Chi-square analysis showed that the observed frequencies were not significantly different from the expected frequencies ($P > 0.10$).

<table>
<thead>
<tr>
<th>ALLELE #</th>
<th>PARENT</th>
<th>FREQUENCY IN OFFSPRING</th>
<th>EXPECTED FREQUENCY</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FEMALE</td>
<td>8 (17%)</td>
<td>11.5 (25%)</td>
</tr>
<tr>
<td>2</td>
<td>FEMALE</td>
<td>15 (33%)</td>
<td>11.5 (25%)</td>
</tr>
<tr>
<td>3</td>
<td>MALE</td>
<td>13 (28%)</td>
<td>11.5 (25%)</td>
</tr>
<tr>
<td>4</td>
<td>MALE</td>
<td>9 (20%)</td>
<td>11.5 (25%)</td>
</tr>
<tr>
<td>5</td>
<td>-*</td>
<td>1 ( 2%)</td>
<td>0 ( 0%)</td>
</tr>
</tbody>
</table>

* Mutant allele from male parent
unequal, the segregation of the alleles was not significantly different from Mendelian inheritance (Table 2.3.1). The relatively high mutation rate seen at the OTSL1 locus (Table 2.3.1) is consistent with estimates from other VNTR loci (Jeffreys et al. 1988, Bentzen et al. 1991, see Jeffreys et al. 1991a). OTSL1 was found to hybridize with other species of salmonids at low stringencies (Fig. 2.3.6), however the banding pattern does not reflect clear single locus variation.

OTSL1 was cloned and sequenced in order to investigate the origin of OTSL1 with respect to the hypothesized inversion event model (Fig. 2.3.2). The nucleotide sequence of OTSL1 is 382 bases long and the sequence is shown in Fig. 2.3.7. The M13 oligonucleotide is present at either end of the sequence in opposite orientation (Fig. 2.3.7), consistent with the inversion event model. The OTSL1 fragment has a total of four Hae III restriction sites (Fig. 2.3.7). There are two repeated sequences within OTSL1 (Fig. 2.3.7), one of these has a Hae III restriction site and thus it is probably not involved in the multilocus fingerprint-like banding patterns observed in the other salmonid species (Fig. 2.3.6) as well as in chinook salmon at low stringencies (data not shown). The second repeat may hybridize with the salmonid DNA to give the multi-locus banding pattern.

The results of the sequencing do not clearly support the hypothesized inversion event model (Fig. 2.3.2). The large number of Hae III sites within OTSL1 make it unlikely that the cloned fragment exists as genomic DNA at the OTSL1 locus, although the observed single-locus banding pattern may be due to hybridization with the sequence from 291bp - 382bp. The balance of the sequence could hybridize with the Hae III digested genomic DNA, however the bands would be off the gel (i.e. < 2.0 Kb). Independent of the origin of the OTSL1 cloned fragment, some portion of the sequence does hybridize with salmonid genomic DNA, in chinook salmon the hybridization occurs at a single locus, furthermore, it is likely that the variation in allele size detected at the OTSL1 locus in
Figure 2.3.6: Autoradiograph of a membrane with six species of salmonids probed with the chinook salmon single-locus minisatellite DNA probe OTSL1 at low stringencies. The species shown are: rainbow trout (*O. mykiss*) - 3 lanes; Atlantic salmon (*S. salar*) - 3 lanes; chum salmon (*O. keta*) - 3 lanes; coho salmon (*O. kisutch*) - 3 lanes; pink salmon (*O. gorb.*) - 3 lanes; sockeye salmon (*O. nerka*) - 2 lanes.
Figure 2.3.7: The base pair sequence of OTSL1. Arrows identify the M13 oligonucleotide (and orientation) PCR primers, HaeIII restriction sites are labelled, and the single and double underlined sequences are the two repeated sections.
chinook salmon is due to VNTR variation (i.e. OTSL1 hybridizes with a VNTR region).

The unrelated individuals used for Fig. 2.3.4 were the parents used for the breeding experiment in Chapter 2.1. Inspection of the banding patterns revealed that all the possible progeny were uniquely identifiable using the probe OTSL1. Consequently the logistical difficulties with tagging and/or rearing of large numbers of separate families could be greatly reduced by the use of single locus probes such as OTSL1. Furthermore, the fish would not have to undergo the stress of tagging and their kinship could be determined from a (less stressful) tissue sample without sacrificing the fish.

Although the use of multi-locus DNA fingerprinting probes for the analysis of relatedness between individuals is currently being re-examined (see Chapter 2.2), many of the objections do not apply to single-locus probes (Lynch 1991, Jeffreys et al. 1991b). Single-locus probes are, however, technically demanding to generate (Burke et al. 1991) and are often relatively species specific. DAMD may provide a quick and simple method for the generation of single-locus probes for a species under study. Although in this study the probe (OTSL1) was cloned and sequenced, this generally would not be necessary to apply DAMD-generated probes to biological or ecological problems.
2.4 APPLICATION OF SINGLE-LOCUS MINISATELLITE DNA PROBES TO IDENTIFY GENETIC COMPONENT IN JACKING AND PREOCIOUS CHINOOK PARR

2.4.1 INTRODUCTION

In Chapter 2.1, a controlled breeding experiment was described that was designed to test for a genetic component to jacking in chinook salmon. Chapters 2.2 and 2.3 described two molecular biological techniques that could be used to test for genetic contribution to the incidence of specific phenotypes within a random mating population. If jacking has a genetic basis, then the jack group should be the progeny of a sub-group of the potential parents, and hence have different allele frequencies than randomly selected individuals. Minisatellite DNA probes would allow measurement of the genetic diversity (or "relatedness") of jacking fish and of randomly selected individuals from the same random-mated population at non-coding or neutral loci (Castelli & Philippart 1990, see Chapter 2.2).

Historically, a number of molecular biological techniques have been used to estimate genetic diversity in animals. Protein electrophoresis has been used to demonstrate differences in genetic diversity between populations of salmonids (Ryman 1983, Stahl 1983; Cross & King 1983; Utter et al. 1989, Vespoor et al. 1991), however the relatively low levels of variation makes protein electrophoresis unsuitable for stock, or within population, comparisons of genetic diversity (Hallerman & Beckmann 1988, Davidson et al. 1989, Triggs et al. 1992). Mitochondrial Restriction Fragment Length Polymorphisms (RFLP) have been extensively used to show genetic distance between populations and between phenotypes within fish populations (Wilson et al. 1987, Bentzen et al. 1989, Bernatchez & Dodson 1990, Chapman 1990, Birt et al. 1991, Knox & Verspoor 1991, Shields et al. 1992). Although mitochondrial RFLP analysis may allow stock identification within a species, the level of variation is usually insufficient to measure relatedness
between groups, within a population. RAPD has also been shown to distinguish populations or closely related species of plants and animals (see Hadrys et al. 1992, Chapter 2.3). The potential applications of RAPD to the measurement of within-population genetic diversity has not been extensively examined, however RAPD does have promise (Hadrys et al. 1992). Minisatellite DNA fingerprinting has been used to estimate genetic diversity in a number of populations of plants and animals (Wirgin et al. 1991; Rogstad 1991; Dunnington et al. 1991; Gilbert et al. 1990, Triggs et al. 1992, see Chapter 2.2). Single-locus minisatellite DNA probes have been developed for a number of species (Taggart & Ferguson 1990a, Bentzen et al. 1991, Hanotte et al. 1991, see Burke et al. 1991), however very few applications of single-locus probes to animal populations have been published.

Amos et al. (1991) reported on the use of multi-locus and single-locus probes to measure relatedness in pods of pilot whales, and a single-locus probe has been used to compare the genetic diversity of runs of Atlantic salmon on the east coast of North America (P. Bentzen - pers. comm.; Marine Gene Probe Lab, Dalhousie University, Halifax N.S.). There have been extensive applications of single-locus minisatellite DNA probes in humans for medical and forensic purposes (Jeffreys et al. 1991a, Wolff et al. 1991). Although the genetic diversity of distinct populations (or stocks) has been estimated and compared using a variety of molecular biological techniques, there have been no reports of comparisons of the genetic diversity of phenotypes within a population.

This study describes the application of two hypervariable single locus probes (OTSL1 - see Chapter 2.3, and Ssa1, Bentzen et al. 1991) to examine the genetic component of jacking in chinook salmon, and of precocious sexual maturation in chinook salmon under-yearling parr. The jacks were from the same breeding population as the parental fish used in the breeding experiment described in Chapter 2.1, where a substantial genetic component to jacking was demonstrated (see Chapter 2.1). Therefore, if the analysis of allele distribution of the jacks and randomly selected individuals shows no
differences, then we can conclude that the approach is not sensitive enough for this type of application. The analysis of precocious chinook salmon parr is an example of this approach applied to an uncontrolled situation.

2.4.2 MATERIALS AND METHODS

Fish Stocks

Two groups of fish were used for this experiment; underyearling chinook salmon parr from the Spius Creek Hatchery, Nicola River stock (see Appendix B), and two-year-old chinook salmon (Robertson Creek (RC) stock) from the commercial grow-out site of Yellow Island Aquaculture Ltd (YIAL). The Nicola River (NR) fish were of uncertain parentage, however hatchery personnel estimate that approximately 70 females contributed to the yearclass. The adult RC chinook salmon population was the progeny of one to one fertilization of fifteen females and an equal number of males. The NR parr were incubated in varying temperature water (1 - 14°C), while the RC stock was incubated in relatively constant temperature water (average T = 8.2°C). The NR parr were reared (post-hatch) in a concrete raceway. The RC fish were reared in 3000 L rectangular tanks, then transferred to seawater netcages at 5-10 g average weight. The RC stock was reared to sexual maturity under typical commercial rearing conditions at YIAL (see Chapter 1.1).

Sampling

Nicola River parr: Approximately 50-70 precociously sexually mature male parr were selected at random from the population (approx. 80,000 individuals) of NR parr held at Spius Creek hatchery in the fall of 1989. The precocious parr were identified as described in Bernier et al. (1992, see Appendix B). At the same time, an equal number of non-mature parr were also taken at random. The fish were killed by a blow to the head, individually wrapped in plastic, and frozen (-20°C) until DNA was extracted.

Robertson Creek adults: In the fall of 1989 approximately 150 jacks and 200 non-mature fish were taken from a single netcage (approx. 12,000 individuals) at YIAL using a hook
and line as well as by recovering recent mortalities and moribund fish by SCUBA diving.
All live fish were killed by a blow to the head. Liver or testes tissue samples were taken
from all fish and were individually bagged and frozen (-20°C) until DNA was extracted.

**DNA Extraction and Southern Transfer**

The whole fish samples from the Nicola River parr were dissected while still frozen
and liver tissue was taken for the DNA extraction. The extraction protocol for all samples
was that described in Chapter 2.2. Sex was determined for all the non-mature fish using
the Y-chromosomal probe developed by Devlin *et al.* (1991). Approximately 3 µg of DNA
from 74 jacks, 95 females, and 22 non-mature males from the RC stock, and from 45
precociously mature male parr, 29 females, and 22 non-mature males from the NR stock
were digested overnight with *Hae* III, size fractionated by gel electrophoresis (0.6%
agarose), and transferred to nylon membranes (as described in Chapter 2.2). DNA from
one individual was run on all gels to act as a control for the band position determination.
Molecular weight standards (1 Kb ladder DNA - Gibco-BRL) were run on both end lanes
and in the middle lane of all gels. Polaroid photographs were taken of the UV
transilluminated gels (0.5 µg•m-1 ethidium bromide added to the gels) with a ruler
included for reference to allow band size determination based on the positions of the
molecular weight standards.

**Probes and Hybridization**

The filters were hybridized with two probes; OTSL1, the chinook hypervariable
single locus probe (SLP) described in Chapter 2.3, and Ssa1, a highly variable SLP
developed for Atlantic salmon (Bentzen *et al.* 1991). The hybridization protocol used for
both probes is described in Chapter 2.3. Each filter was stripped of probe (following
manufactures instructions - Amersham Corp. IL, USA) after being hybridized with
OTSL1, then re-hybridized with Ssa1. The hybridized filters were exposed to X-ray film
with intensifying screens at -70°C for one to three days.

**Allele Scoring**
To determine the molecular size of each allele, a linear relation was generated between the natural log of the migration distance (with respect to the loading wells) and the natural log of the size (Kb) of the marker bands for each gel, based on measurements made on the Polaroid photos. Distance measurements (to the nearest mm) were made from the loading wells to the top (closest to loading wells) of all bands (alleles) for the OTSL1- and Ssa1-probed autoradiographs. These distance measurements were converted to approximate fragment sizes (in Kb) using the log-log relation described above. The individual that was run on all gels was used to confirm the allele size conversion for each gel. All patterns which consisted of a single band were identified as homozygous for that probe, although, formally, hemizygosity cannot be ruled out. The Ssa1 probe yielded higher background signal than OTSL1, however the bands were still distinguishable.

**Allele Binning**

Once all individuals had both OTSL1 and Ssa1 allele sizes scored, an estimation of the error associated with the calculated allele sizes was made. Error in allele size originates from two sources; within gel errors (measurement errors, non-uniform migration speed, etc.), and between gel errors. The repeated individual lane allowed an estimation of the range of between gel error, while the frequently occurring "common alleles" allowed an estimate of the within gel error. Using this information, allele "bins" were defined consisting of ranges of allele sizes that were slightly larger than the total observed error range. Since the error range increased with allele size, the bin width was set using a simple linear function based on $\pm 2.5\%$ of the median allele size for OTSL1 and $\pm 3.5\%$ of the median allele size for Ssa1. Thus at small allele sizes (i.e. 3.0 Kb) the bin width was small (i.e. 2.93 Kb - 3.07 Kb), while larger allele sizes (i.e. 15 Kb) had correspondingly larger bin widths (i.e. 14.62 Kb - 15.38 Kb). Once a series of bins had been defined that covered the range of measured allele sizes (for both probes and stocks) the bins were shifted by 0.1 Kb increments until the alleles that were known to be of the same fragment size fell into a
single bin. The range of bin shifts that allowed the known alleles to fall into a single bin was determined and the bin shift was set to the center of this range.

**Data Analysis**

To test for differences in the distribution of the allele sizes between; 1) the jacks and the non-mature adult chinook salmon, and 2) the precocious parr and the non-mature controls, a log-linear model was fitted to the frequency distribution;

\[
\log M_{ij} = \mu + S_i + B_j + SB_{ij}
\]

Where \(M_{ij}\) is the expected number of alleles for the \(i^{th}\) sexual maturation category (mature or non-mature), and \(j^{th}\) bin number, \(\mu\) is the grand mean of the expected counts, \(S_i\) is the main effect of the \(i^{th}\) sexual maturation category, \(B_j\) is the main effect of the \(j^{th}\) bin number, and \(SB_{ij}\) is the interaction of the \(i^{th}\) sexual maturation category and the \(j^{th}\) bin number. This model is saturated and gives expected cell counts equal to the observed cell counts (SYSTAT, Evanston, IL, USA). To test the interaction between sexual maturation status and bin number for significance the above model was modified by dropping the interaction term, then rerun. If the modified model gives expected counts that are significantly different (based on the G statistic described in Sokal & Rohlf 1981) from the observed then the interaction is significant. A significant interaction indicates the frequency distribution of the two sexual maturation categories (mature versus non-mature) are statistically different. Two factors combine to make this analysis highly conservative; 1) as a non-parametric analysis, the log-linear model is inherently conservative (Sokal & Rohlf 1981), and 2) the use of wide band widths for the allele binning (see above) probably suppresses some allelic variation (i.e. true allele differences are binned together). The overall effect of this analysis is therefore a higher probability of a Type II error (Sokal & Rohlf 1981), that is, accepting an incorrect null hypothesis.
Differences in the frequency of homozygous loci between 1) jacks and females, and 2) precocious parr and controls, were tested using the 2-way contingency table analysis (Sokal & Rohlf 1981).

Mean cumulative frequencies of novel alleles were calculated by counting the number of "novel" alleles per fish within a single gel. An allele was defined as novel when it had not been observed in any of the previously scored fish. Counts were made within gels to allow visual scoring of alleles, rather than the using the conversion and binning routine described above. Since the sexually mature and non-mature controls from both stocks occurred on more than one gel, the numbers of novel alleles for each fish were averaged to give a mean cumulative frequency distribution. The use of the average reduces the variation in the first few fish scored (i.e. the impact of a homozygous fish would be lessened). The mean cumulative frequency of novel alleles for OTSL1 and Ssa1 was plotted against fish number for the mature and non-mature groups from the RC and NR stocks. The cumulative frequency of novel alleles for OTSL1 was also plotted against fish number for a full-sib family and for 17 unrelated individuals (these were not repeated thus the cumulative frequencies could not be averaged). High relative relatedness appears as a lower sloped curve on such a plot. The cumulative frequency distributions were tested for significant differences using the two-sample Kolmogorov-Smirnov test (Sokal & Rohlf 1981).

2.4.3 RESULTS

OTSL1: Frequency histograms for the range of allele bins generated by OTSL1 are shown in Fig. 2.4.1 for the adult RC fish, and in Fig. 2.4.2 for the NR parr. Few alleles fell on the borders of the allele bins, indicating that the bin positions were probably adequate. The RC jack and female OTSL1 allele frequency distributions were significantly different (G=57.9, df=9, P<.001), while the NR precocious parr and control parr frequency distributions were not (G=11.1, df=7, P>.13).
Figure 2.4.1: OTSL1 locus allele frequencies (within allele bins) plotted against median allele size for the RC adult chinook salmon; a) adult female salmon (N=95), b) jacking salmon (N=74), and c) the difference of (a) and (b) allele frequencies. The allele frequency distribution of the jacks is significantly different from that of the females.
Figure 2.4.2: OTSL1 locus allele frequencies (within allele bins) plotted against median allele size for the NR chinook salmon parr; a) non-mature control parr (N=51), b) precociously mature male parr (N=45), and c) the difference of (a) and (b) allele frequencies. The allele frequency distributions of the mature parr and the non-mature control fish are not significantly different.
Figure 2.4.3: Ssa1 locus allele frequencies (within allele bins) plotted against median allele size for the RC adult chinook salmon; a) adult female salmon (N=87), b) jack salmon (N=68), and c) the difference of (a) and (b) allele frequencies. The allele frequency distribution of the jacks is significantly different from the females.
Figure 2.4.4: Ssa1 locus allele frequencies (within allele bins) plotted against median allele size for the NR chinook salmon parr; a) non-mature control parr (N=47), b) precociously mature male parr (N=41), and c) the difference of (a) and (b) allele frequencies. The allele frequency distributions of the mature parr and the non-mature control fish are not significantly different.
Ssal: Frequency histograms for the range of allele bins generated by Ssal are shown in Fig.s 2.4.3 & 2.4.4 for the adult RC fish the NR parr, respectively. Few alleles fell on the borders of the allele bins, indicating that the bin positions were probably adequate. The RC jack and female Ssal allele frequency distributions were significantly different ($G=45.1$, $df=12$, $P < .001$), while the NR precocious parr and control parr frequency distributions were not ($G=15.2$, $df=9$, $P > .08$).

The observed frequencies of heterozygotes are given in Table 2.4.1 for both loci and both stocks. There were no significant differences in heterozygosity found between sexually mature and non-mature fish at either locus, or within either stock (Table 2.4.1). However the RC stock (females) had a significantly higher frequency of heterozygotes than the NR stock (controls) at the OTSL1 locus ($P < 0.05$). The overall heterozygosity for the OTSL1 locus in the RC and NR stocks combined was 82%. The heterozygosity for the Ssal locus in the RC and NR stocks combined was 91%.

The mean cumulative frequency of novel OTSL1 alleles for the RC stock (jacks vs. females), the NR stock (precocious parr vs control parr), and 17 unrelated and 17 full sib individuals are shown in Fig. 2.4.5. There were no significant differences between the mean cumulative frequencies of the sexually mature and non-mature fish for either the RC or NR stocks. The mean cumulative frequency of novel Ssal alleles for the RC stock (jacks vs. females), and the NR stock (precocious parr vs control parr) are shown in Fig. 2.4.6. There were no significant differences between the mean cumulative frequencies of the sexually mature and non-mature fish for either the RC or NR stocks.
Table 2.4.1: The frequency of heterozygote chinook salmon at the minisatellite DNA loci OTSL1 and Ssa1. Two stocks and two phenotypes in each stock of chinook salmon are presented; jacks and females from a population of Robertson Creek (RC) adults, and sexually precocious male parr and non-mature controls from a population of Nicola River (NR) parr. There were no significant differences between the phenotypes for frequency of heterozygotes at either locus (G test, P > 0.05).

<table>
<thead>
<tr>
<th>LOCUS</th>
<th>STOCK</th>
<th>PHENOTYPE</th>
<th>HETEROZYGOSITY (%)</th>
<th>SAMPLE NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTSL1</td>
<td>RC</td>
<td>JACK</td>
<td>85.1</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FEMALES</td>
<td>87.2</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>NR</td>
<td>MATURE MALE</td>
<td>57.6</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CONTROLS</td>
<td>73.2</td>
<td>56</td>
</tr>
<tr>
<td>SSA1</td>
<td>RC</td>
<td>JACK</td>
<td>79.1</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FEMALES</td>
<td>90.3</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>NR</td>
<td>PRECOCIOUS PARR</td>
<td>93.1</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CONTROLS</td>
<td>92.9</td>
<td>56</td>
</tr>
</tbody>
</table>
Figure 2.4.5: Mean cumulative number of novel alleles at the OTSL1 locus plotted against cumulative fish numbers for: a) precocious mature male and non-mature control NR chinook salmon parr, b) jack and female (non-mature) adult RC chinook salmon, and c) a full-sib chinook salmon family and 17 unrelated chinook salmon.

Differences in allele diversity are identified by differences in the elevation of the plots. There were no significant differences between the mean cumulative novel allele frequencies for the mature and non-mature fish, however the RC adult stock did have a significantly higher allele diversity than the NR parr stock.
Figure 2.4.6: Mean cumulative number of novel alleles at the Ssa1 locus plotted against cumulative fish numbers for: a) precocious mature male and non-mature control NR chinook salmon parr, and b) jack and female (non-mature) adult RC chinook salmon. Differences in allele diversity are identified by differences in the elevation of the plots. There were no significant differences between the mean cumulative novel allele frequencies for the mature and non-mature fish.
2.4.4 DISCUSSION

Before the results of the allele frequency analysis can be discussed in detail, it is important to make two points:

1) Our analysis is based on only two loci, and thus "relatedness" and diversity based on these two loci do not necessarily reflect the whole genome; and
2) The two loci examined in this analysis have not been mapped, or extensively checked for linkage with phenotypes, thus it is possible that one or both loci may be under some form of selection pressure.

Nevertheless, since both OTSL1 and Ssa1 are minisatellite DNA-associated probes, they probably behave similarly to Variable Number Tandem Repeat (VNTR) loci in humans in that they hybridize with non-coding or "neutral" areas of the genome (Castelli & Philippart 1990), and can be used as unbiased measures of kinship or relatedness.

The analysis of the allele frequency distributions for the OTSL1 and Ssa1 loci showed that jacks represented a genetic subset of the RC population, i.e. there were significant differences in the allele frequency distributions at both loci; some alleles were over-represented in the jacks, while others were under-represented (Fig. 2.4.1 & 2.4.3). Thus the jacks did not represent a random sample from the population, assuming the randomly selected females adequately represented the population (this assumption would be violated if the populations had experienced significant sex-specific selection or mortality).

The differences in the allele frequency distributions may have been due to either: 1) genetic effects (i.e. certain families being predisposed to jacking), or 2) non-genetic maternal and "tank" effects leading to non-uniform mortality (selection): our approach cannot distinguish between these possibilities. However, since the fish were held in a common environment from hatch, and non-genetic maternal effects are generally not significant for fish at these ages (see Chapter 2.1), it is likely that the differences in allele frequencies reflects genetic contributions to the incidence of jacking. The analysis described here also shows that single-locus minisatellite DNA probes are sensitive enough
to identify differences between phenotypes in random-mated populations. In the case of chinook salmon jacks, it is not surprising that the mature males formed a (genetic) subset of the population, since the breeding experiment (based on the same population of fish) showed strong family effects for jacking (see Chapter 2.1).

There was no difference between the precocious and non-mature control NR parr in allele frequencies for the OTSL1 and Ssa1 loci, however, one of the limitations of our analysis is that non-significant results are ambiguous. There are three possible explanations for the lack of differences:

1) there was no family component to precocious maturation;

2) there was too little allelic variation in the parental generation; and

3) the sample size was not large enough, given the conservative nature of the analysis.

Although it is possible that there was no family component to the incidence of precocious parr in the NR stock, it is unlikely since there is evidence for family or stock effects in other chinook populations (Chapter 1.1). If the parental generation had low levels of variation at the OTSL1 and Ssa1 loci, then the resolving power of our analysis would be greatly reduced. For example, consider the extreme case where all parents have the same alleles at both loci; in such a case, no differences would be detectable between the allele frequency distributions of any sub-groups of the progeny. There is evidence that the parents of the sampled NR parr were, in fact, fairly related. Inspection of the allele frequency histograms of their progeny (Fig. 2.4.2 & 2.4.4) shows that the NR parr had considerably fewer alleles than the RC adult chinook salmon, and furthermore, the NR parr had one or two extremely common alleles at both the OTSL1 and Ssa1 loci (Figs 2.4.2 & 2.4.4). In general, the NR parr appear to be inbred, relative to the RC adults. It is difficult to determine how much of an increased sample size would be necessary to yield significant results, or even if any sample size would be large enough, given the probable limited allele diversity in the parental generation. The results do indicate, however, that the NR chinook
salmon population at the Spius Creek hatchery is probably not a good candidate for an extensive breeding experiment.

Although the mean heterozygosity at the OTSL1 and Ssa1 loci were comparable to other single-locus minisatellite DNA probes (Henke et al. 1991, Jeffreys et al. 1991a), there were no differences between the maturing and non-maturing groups in either stock. This reflects the difference between relatedness and inbreeding; it is possible for a group of fish to be related, but not be inbred. Thus, although the jacks were found to be a genetic subset of the adult chinook salmon population, they did not have a lower level of heterozygosity. It is obvious that a comparison of heterozygosity levels cannot generally be used to infer genetic differences between phenotypes in random mating populations. It is interesting to note that the heterozygosity of the adult female RC chinook was significantly higher than the control NR chinook parr. This suggests that the NR stock has lower genetic diversity than the RC stock.

The mean cumulative novel allele frequencies for both loci showed no differences between the maturing and randomly selected fish (Figs. 2.4.5 & 2.4.6). This type of presentation is designed to reflect differences in allele diversity, not allele distribution. Since the analysis of the allele frequency distribution showed a difference between the jacks and the females, and the mean cumulative novel allele plots showed no difference, the genetic differences detected between jacks and females were due to differences in allele distribution, not allele diversity. The mean cumulative allele plots also show that, in general, the NR parr were less genetically diverse at the OTSL1 and Ssa1 loci than the RC adults, at least within the hatchery-reared population. That is, the curves for the NR parr are generally lower than the RC adults (Figs. 2.4.5 & 2.4.6). The extreme examples of unrelated individuals and a full-sib family (Fig. 2.4.5) show the potential range of the mean cumulative allele frequencies for the OTSL1 locus; the NR parr and the RC adults are both of intermediate allele diversity, between unrelated and full-sib individuals.
The analysis of the allele distribution frequencies for phenotypes of interest (life history strategies) in random mating populations has two important potential applications. One of these is screening wild populations for family effects in traits of interest. In theory, any measurable phenotypic trait could be used, however quantitative traits would have to be arbitrarily divided into two or more groups (such as "large" and "small" for size at age differences). This study describes the first attempt to determine family effects in a specific trait within a random mating population, using DNA-based technology. Although the use of single-locus minisatellite DNA probes simplifies the analysis, multi-locus fingerprinting probes could also be used, provided the signals were clear. The use of minisatellite DNA variation to estimate relatedness in groups of animals should prove to be a valuable tool for studying natural populations, especially those that cannot be sampled or selectively bred. The second potential application is the comparison of genetic diversity between populations. Although the absolute magnitude of the allele diversity would have little practical value (it would depend on the specific loci sampled), a comparison of diversities would be useful to determine the effects of various management practices on the genetic diversity of threatened populations.
3.1 HORMONAL AND GROWTH CHANGES ASSOCIATED WITH JACKING IN CHINOOK SALMON

3.1.1 INTRODUCTION

Five months prior to maturation, cultured chinook salmon develop a bimodal weight frequency distribution (Chapter 1.1). The upper mode, or the largest fish, were shown to consist almost exclusively of jacks, while the lower mode was mostly composed of non-jacks, or silvers (Chapter 1.1). Although the work described in Chapter 1.1 implied that jacking chinook salmon experienced an elevated growth rate during the late spring prior to maturation, it did not identify individual growth curves until June, nor could the relative position of the jacks within the normally distributed weight frequency distribution prior to the bimodality be determined.

A growth pattern similar to that described in Chapter 1.1 for chinook salmon jacks has been observed in a number of salmonid species (see Chapter 1.1), the bulk of the work having been done on precocious male Atlantic salmon parr (Chapter 1.1). One difference, however, between maturing parr and the results of Chapter 1.1 is that precocious parr experience a growth reduction during the summer prior to maturation (Leyzerovich 1973, Rowe & Thorpe 1990a, Foote et al. 1991, Randall et al. 1986, see Chapter 1.1). Berglund et al. (1992) showed that testosterone implants in non-mature Atlantic parr produced a pattern of growth very similar to that of the precocious maturing parr, as well as increased incidence of sexual maturation. Hunt et al. (1982) measured growth, 11-oxotestosterone, and testosterone in a group of adult male Atlantic salmon over the course of their sexual maturation, and showed that growth increased in the spring prior to maturation (correlated with serum testosterone concentration), then decreased through the summer. A number of experiments have shown that testosterone enhances growth in salmonids, often with an accompanying increased rate of sexual maturation (Higgs et al. 1982, Schreck & Fowler 1982, Borghetti et al. 1989).
Thyroid hormones, triiodo-L-thyronine (T3) and thyroxine (T4), are involved in the mediation of growth and metabolism in fish (Higgs et al. 1982). There has also been extensive work showing maturation-related changes in circulating thyroid hormone concentrations in salmonids; Atlantic salmon (Dickhoff et al. 1989, Mayer et al. 1990a,b, Rydevik et al. 1989), sockeye salmon (Biddiscombe & Idler 1983), rainbow trout (Pavlidis et al. 1991), amago salmon (Nagahama et al. 1982), coho salmon (Leatherland & Sonstegard 1981a,b, Sower & Schreck 1982); brook trout (Audet & Claireaux 1992); and pink salmon (Leatherland et al. 1989).

Elevated circulating levels of cortisol have been shown to be associated with final maturation in salmonids (Morrison et al. 1985, Audet & Claireaux 1992, see Barton & Iwama 1991). High levels of cortisol, often associated with stress, have also been shown to down-regulate testosterone in maturing salmonids (Pickering et al. 1987, Pickering et al. 1991, Donaldson 1990, Barton & Iwama 1991). Finally, in coho salmon, injected or implanted cortisol resulted in reduced plasma T3, but not T4, concentrations (Redding et al. 1984, Vijayan & Leatherland 1989), although there are reports of inconsistent response of T3 to cortisol during smoltification (Redding et al. 1991). It is likely that circulating testosterone, thyroid hormone, and cortisol concentrations may be involved, additively or synergistically, to influence the growth pattern characteristic of jacking chinook salmon.

The study described here was designed to test three main hypotheses:
1) jacking chinook salmon experience elevated growth in the spring prior to maturation, relative to the non-mature fish;
2) jacking chinook salmon have different circulating T3, testosterone, and cortisol concentration profiles during the seven months prior to sexual maturation; and
3) the circulating hormonal concentrations of jacking chinook salmon correlate with their (elevated) growth rate.
This study described the growth and changes in circulating levels of cortisol, total T3 thyroid hormone, and total testosterone of individually tagged non-mature males, females, and jacking chinook salmon from identical familial backgrounds.

### 3.1.2 MATERIALS AND METHODS

#### Sampled Fish

On December 9 & 10, 1990 and March 17 & 18, 1991, 415 and 425 fish, respectively, were randomly selected from multiple seine sets made in the 10mX10mX10m netcage at Yellow Island Aquaculture Ltd. (YIAL - Quadra Island, B.C.) that contained the accelerated family groups described in Chapter 2.1. The sample population consisted of 12 full- and half-sib chinook salmon families, all identified by coded wire nose tag implants to their family of origin (see Chapter 2.1).

**December Sample:** The selected fish were exposed to a lethal dose of 2-phenoxyethanol (2.5 ml·L$^{-1}$) immediately after capture. The fish were weighed (± 0.1 g) and blood was collected in heparinized syringes from the caudal vessel. The blood samples were centrifuged and the plasma and packed cells were separated and frozen at -20°C for approximately seven days until transferred to a -70°C freezer where they were held until analysis.

**March Sample:** The selected fish were anesthetized with 2-phenoxyethanol (0.15 ml·L$^{-1}$) immediately after capture, weighed (± 0.1 g), and blood was collected in heparinized syringes from the caudal vessel. Each fish was also injected intraperitoneally with a Passive Integrated Transponder (PIT) tag (Canadian Biosonics Ltd., Vancouver, B.C. - see Moore 1992). The PIT tag was read at the time of injection and the 10 digit alpha-numeric code recorded, this allowed the identification of individual fish through-out the experiment. The blood samples were centrifuged and the plasma and packed cells were
separated and frozen at -20°C for approximately three days until transferred to a -70°C freezer where they were held until analysis.

The PIT tagged fish were held for the duration of the experiment in a 4mX4mX4m netcage at YIAL; the characteristics of the grow-out site are described in Chapter 1.1. The fish were fed to satiation one or two times daily with a commercially available feed (White Crest Mills, Campbell River, B.C.). The fish were orally treated with oxytetracycline, as a prophylactic measure, for approximately 10 days following handling. Mortalities were recovered on a weekly basis and frozen pending PIT and nose tag recovery.

Further Sampling

On April 25, May 26, and June 15, 1991, all surviving PIT tagged fish were anesthetized, weighed, and blood samples taken (for plasma extraction) following the protocol of the first sampling. On August 13, 1991, the PIT tagged fish were killed, weighed, their PIT tag read and recorded, and blood samples taken (for plasma extraction - as above). The fish were then dissected to recover nose tags for later decoding. Jacks were clearly identifiable by their developed testes. Testes were removed and weighed (± 0.1 g) for all jacking fish.

All fish were sexed using DNA from the packed blood cells taken during the sampling in December and March with the Y-chromosomal DNA probe for chinook salmon developed by Devlin et al. (1991). Fish were selected for analysis from the total sample size based on their sex (male - female), maturation state (jack - non-jack), and familial background (family of origin - determined by nose tag recovery). To reduce the possibility of family effects on the measured parameters, the jack, male (non-mature), and female groups of chinook salmon were selected to have identical familial representation (Table 3.1.1). Ideally, a single full-sib family would have been used for the analysis, however families with large numbers of jacks tended to have few non-mature males, and vice-versa, thus no single family had sufficient numbers of jacks and non-mature males.
Table 3.1.1: Family composition of the fish used for the analysis of hormonal and growth differences between jacks and non-maturing chinook salmon.

<table>
<thead>
<tr>
<th>FEMALE</th>
<th>MALE (J / N)</th>
<th>JACKS (#'s)</th>
<th>FEMALES (#'s)</th>
<th>MALES (#'s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>J</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>N</td>
<td>2</td>
<td>2</td>
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<tr>
<td>2</td>
<td>J</td>
<td>3</td>
<td>3</td>
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<tr>
<td>3</td>
<td>J</td>
<td>1</td>
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<td>4</td>
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<td>J</td>
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<td>J</td>
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<tr>
<td>6</td>
<td>J</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>
A total of 20 jacks, 20 non-mature males, and 20 females were selected for the experimental period, and 16 males and 16 females were chosen at random from the December sampling for growth and hormonal analysis.

**Assays**

Plasma concentrations of cortisol, total testosterone, and total T3 were determined by $^{125}$I radiolmmunoassay clinical kits (COAT-A-COUNT, Diagnostic Products Corp. Los Angeles, CA, USA: cat. #s; TKC01, TKTT1, and TKT31). Cross reactivity was reported low for all of the kits. The clinical assay kits were validated for use with chinook salmon by evaluation of serially diluted chinook salmon plasma (using zero standard), as well as chinook salmon plasma serially spiked with known concentrations of the pure hormone. The measured values were in close agreement with the known concentrations, with the exception of very high values of cortisol ([cortisol] > 300 ng·ml$^{-1}$), where the measured values were approximately 10-15% lower than the known concentrations, probably due to saturation effects. The samples with measured values of plasma cortisol greater than 300 ng·ml$^{-1}$ were corrected for this effect.

**Statistical Analysis**

Gonadosomatic index (GSI) was calculated by;

\[ \text{GSI} = \frac{W_{\text{testes}}}{W_{\text{total}}} \times 100\% \]

Where $W_{\text{testes}}$ was the weight of the testes and $W_{\text{total}}$ was the weight of the whole fish (including the testes).

Relative growth rate (RGR) was calculated for the time periods between sampling dates for each individual fish;

\[ \text{RGR} = \frac{(W_{\text{final}} - W_{\text{initial}})}{(W_{\text{initial}} \times \text{Days})} \times 100\% \]
Where $W_{t_{\text{initial}}}$ was the weight of an individual fish at one sampling date, $W_{t_{\text{final}}}$ was the weight of the same fish one sampling date later, and Days was the number of days between the two consecutive sampling dates.

Weight-frequency distributions were generated for the sampled fish (jacks, non-mature males, and females combined) for the five sampling dates and tested for bimodality using the test for clusters described in Chapter 1.1. A one way ANOVA was used to test for differences in plasma cortisol, T3, and testosterone concentrations between jacks, non-mature males, and females within sampling dates (hormone concentrations were natural logarithm transformed). One way ANOVA was also used to test for differences in RGR between jacks, non-mature males, and females within sampling dates (RGR was ARCSIN $\sqrt{\ }$ transformed - Sokal & Rohlf 1981). Correlations between RGR, wet weight, plasma hormone concentrations, and GSI were tested using regression analysis on the transformed data. All data is presented before transformation.

3.1.3 RESULTS

The weight-frequency distribution for the jacks, males and females combined is shown in Fig. 3.1.1. The frequency distribution became significantly bimodal in May, and the spread increased through the summer (Fig. 3.1.1). Mean wet weights for the non-mature male, female, and jack groups are plotted against time, and mean relative growth rates (RGR) are shown for the three intervals between the weight samples in Figure 3.1.2a and b. The jacks had a significantly higher wet weight from March to August, however the jacks had higher mean RGR during the April - May and the May - June time periods only (Fig. 3.1.2a & b). Generally, RGR decreased into June, then increased through summer. The significantly higher RGR of the jack group is due mostly to a reduction in growth of the non-mature fish, rather than an increased jack growth rate,
Figure 3.1.1: Weight-frequency distributions for the combined male, female and jack chinook salmon in salt water used for the hormonal analyses. The total number of fish at all sampling periods was 60. The shaded bars represent fish that eventually jacked. The distribution became significantly bimodal in May: * = P < 0.05; ** = P < 0.01; *** = P < 0.001.
Figure 3.1.2: a) Mean wet weight (± SE) of the female, male and jack chinook salmon groups in salt water used for the hormonal analysis (N=20). The jack group was significantly heavier from March to August. b) Mean relative growth (± SE) of the three groups of fish used for the hormonal analyses (N=20). *** = P < 0.001.
nevertheless the development of the bimodal distribution in May - June is explained by the growth differences.

Cortisol: Mean plasma cortisol concentration is presented for the male, female, and jack groups in Fig. 3.1.3a. Although there was a general rise in the mean values throughout the sample period, no differences were found between the three groups within sampling dates. The mean plasma cortisol concentrations for all groups were extremely high, ranging from approximately 85 to 220 ng·ml\(^{-1}\).

Testosterone: Mean plasma testosterone is presented for the male, female and jack groups in Fig. 3.1.3b. The non-mature male and female groups mean values were not significantly different from zero, however the jack group mean values were significantly greater than either of the non-mature group means throughout the duration of the experiment (Fig. 3.1.3b). The jack group mean plasma testosterone concentration remained relatively low from March to May, then increased dramatically in June and August (Fig. 3.1.3b).

T3: Mean plasma T3 concentration is presented for the male, female, and jack groups in Fig. 3.1.3c. The male and female group mean values were not significantly different from each other throughout the sampling experiment, however jack group mean was significantly higher than the non-mature group means in March only (Fig. 3.1.3c). The jack group mean plasma T3 was not different from the non-mature groups means from April to the end of the experiment (Fig. 3.1.3c).

Individual plasma cortisol and testosterone concentrations were not correlated with relative growth rates within the jack group at any time during the experiment. The significant correlation of jack plasma T3 concentration in March with the May-June relative growth rates is shown in Fig. 3.1.4a. No other combination of jack hormone concentration and relative growth yielded a significant correlation. The August plasma testosterone concentrations were found to be highly correlated with the GSI of the jacks (Fig. 3.1.4b), however,
Figure 3.1.3: Mean plasma concentrations (± SE) of; a) cortisol, b) T3, and c) testosterone for the male, female, and jack chinook salmon groups in salt water. The December data are from 16 randomly selected fish, while the other data are from 20 fish sampled over the whole period. ** = P < 0.01; *** = P < 0.001.
Figure 3.1.4: Correlations between transformed hormonal values, growth, and GSI for the individual jacking chinook salmon: a) correlation between the March plasma T3 concentrations for individual jack chinook salmon in salt water and relative growth (RGR); b) correlation between the August plasma testosterone concentrations for individual jack chinook salmon and gonadosomatic index (GSI); c) correlation between the June plasma testosterone (TEST) and cortisol (CORT) concentrations in the jacks; d) correlation between the August plasma testosterone concentrations in the jacks and the cortisol. All regression equations are given, and all slopes were significantly different from zero.
no correlation was found between jack plasma testosterone concentration and GSI for any other sample date. A significant negative correlation between jack plasma cortisol and testosterone concentrations was found for June and August sampling dates (Fig. 3.1.4c,d).

All of the hormonal observations presented here are single data points within a dynamic system. Seasonal and diel fluctuations for all three hormones have been documented (Barton & Iwama 1991, Audet & Claireaux 1992, Baynes & Scott 1985). Furthermore, plasma concentrations do not reflect differences in clearance rates or production levels, and may thus be misleading.

3.1.4 DISCUSSION

The PIT tagged fish in this experiment generated a bimodal weight-frequency distribution very similar to those reported in Chapter 1.1. However, since the fish were individually identified (PIT tags), the jacks, males (non-maturing) and females were individually identifiable from March through August. As is the case for precociously maturing male fish from other species of salmonids (Randall et al. 1986, Foote et al. 1991, see Chapter 1.1), the jacks were the largest fish before any bimodality could be identified (Fig. 3.1.2a). Although the growth of the jacks was higher than either the males or females from March to June, this was not due to an increase in the growth of the jacks, but rather a decrease in growth of the non-mature fish (Fig. 3.1.2a,b). Unlike the growth described for precociously maturing parr, the growth of the jacks did not decrease during the summer, although the growth of the non-mature fish increased dramatically. The results of this work support the findings described in Chapter 1.1; the general pattern of the growth of chinook jacks during the nine months prior to sexual maturation is similar to that reported for other precociously maturing salmonids except for the maintained fast growth through the summer (see Chapter 1.1).

The rapid increase in circulating testosterone observed in the jacks during the summer months prior to maturation (Fig. 3.1.3b) is also typical of maturing salmonids
(Hunt et al. 1982, Baynes & Scott 1985, Scott & Sumpter 1989, Mayer et al. 1990b, Mayer et al. 1992). The slightly elevated levels of testosterone in the jacks from March to May, forming a small peak, is similar to those reported by Hunt et al. (1982), Schultz (1984), and Mayer et al. (1990b) in maturing male salmonids, and has been tentatively identified as a possible trigger for the initiation of testicular development (Hunt et al. 1982). Although the testosterone levels of the jacks were already elevated relative to the non-mature fish in March, there was no evidence of higher testosterone levels in any of the male fish assayed in December. Based on the known jacking rate (Chapter 1.1), six or seven of the male fish sampled in December would have been expected to be jacks, yet there was no dichotomy of testosterone levels in the male fish (most were below the level of detection). Thus the circulating levels of testosterone in the jacks probably began to increase between December and March.

The drop in jack circulating T3 level in April (Fig. 3.1.3c) coincided with the development of the bimodality in the weight-frequency distribution. T3 has been shown to stimulate growth when fed to salmonids (see Higgs et al. 1982). It is thus surprising that circulating T3 levels dropped in the jacking fish just prior to the development of the bimodality. A considerable body of work has shown that T3 drops during final maturation, however the drop is usually closely associated with spawning (Biddiscombe & Idler 1983; Pavlidis et al. 1991; Audet & Claireaux 1992; Leatherland et al. 1989), although Rydevik et al. (1989) reported elevated circulating T3 levels through the breeding season in precocious male Atlantic salmon parr. Audet & Claireaux (1992) found a T3 peak in the spring prior to the maturation of brook trout, and Dickhoff et al. (1989) also reported a T3 peak in the spring prior to maturation in adult Atlantic salmon. The elevated T3 levels in the jacks in March could be interpreted as a peak if the T3 of the jacks in December was low relative to the March value. Although it is not possible to positively identify the potential jacking fish in the December sample, Figure 3.1.5 shows log (weight) plotted against log (T3) for the 16 male fish measured in December. Approximately 6 - 8 of
Figure 3.1.5: Transformed December wet weight plotted against December plasma T3 concentrations for 15 male chinook salmon. The points within the circle may be male fish that eventually become jacks, while the points outside the circle may be non-maturing males (see text). The purpose of this figure is to identify any possible difference in T3 levels between the jacks and non-mature males in December.
these 16 randomly selected fish are expected to be jacks based on observed jacking rates (app. 40%; see Chapter 2.1). Two groups of fish can be identified in Fig. 3.1.5; high T3 - high weight, and low T3 - low weight, these two groups fit the expected numbers of fish in the jack and non-jack categories. It is possible that the high T3 - high weight group may be jacks, if this were the case these "jacks" would have a mean T3 value in December of 9.0 ng·ml\(^{-1}\). Thus the March circulating T3 levels in the jacks represent a peak, independent of whether the two groups seen in Fig. 3.1.5 correspond to jacking and non-maturing fish.

Although the elevated T3 levels of the maturing males in March may constitute the spring "peak" reported elsewhere, the subsequent drop in circulating T3 has not, to our knowledge, been reported elsewhere for maturing male salmonids.

There was no difference between the jack and non-maturing fish circulating levels of cortisol throughout the course of the experiment. Although a rise in cortisol has been reported in maturing salmonids (Morrison et al. 1985, Audet & Claireaux 1992, see Barton & Iwama 1991), circulating cortisol increased in all fish in this study. It is thus unlikely that the rise in cortisol through the spring and summer is related to sexual maturation. The actual plasma cortisol concentrations reported here are extremely high (10-20 X reported resting values) compared to published values for salmonids (see Barton & Iwama 1991); however, most of the reported cortisol concentrations are from juvenile freshwater fish, and may not be comparable to the values reported here. Morrison et al. (1985) measured circulating cortisol levels in sexually mature adult coho salmon and reported values comparable to those presented here. It is unlikely that the circulating cortisol levels in our study represent a stress response, since the values were so high as to normally represent a severe acute stress response, at least in rainbow trout and juvenile salmon (Barton & Iwama 1991). It is unlikely that such a stress response could be maintained for eight months. It is unclear what the function is of these high levels of circulating cortisol, although it may be that resting values are naturally high in adult Pacific salmon.
Although a number of the characteristics of the hormone profiles shown in Fig. 3.1.3 coincide with the development of the bimodal weight-frequency distribution, the critical test was to correlate individual hormonal measurements with growth rates within the jacking group, as well as within the data set as a whole. The only variable that correlated with growth was T3 concentration in the jacks in March, showing a negative relationship with growth (Fig. 3.1.4a), that is, the jacks with the highest T3 concentration in March were generally the slowest growing from May to June. This agrees with the observation that circulating T3 levels in jacks decreased during the development of the bimodal weight-frequency distribution. It would seem that, in this experiment, elevated circulating T3 concentrations did not stimulate somatic growth in a dose dependent fashion, as has been reported elsewhere for juvenile fish (see Higgs et al. 1982). The significant negative correlations between circulating levels of cortisol and testosterone in June and August, agrees with other work in non-mature salmonids (Pickering et al. 1987, see Barton & Iwama 1991). Cortisol has also been shown to reduce levels of circulating T3 in immature and smolting salmonids (Redding et al. 1984; Vijayan & Leatherland 1989), possibly through a reduction of the T4 - T3 conversion or an increase in T3 clearance rates (Redding et al. 1984). In our study there was no correlation between measured levels of cortisol and T3 within the jack group, or within the data set as a whole. Finally the significant correlation between the circulating levels of testosterone and GSI seen in August indicates that; 1) the rate of gonad development was dependent on the level of testosterone, or 2) the rate of testosterone production was dependent on the size of the gonad. In either case, since the jacks were not expressing milt at the time of the August sample, their testes were not fully developed.

Three hypotheses were tested in this study; 1) jacking chinook salmon experience elevated growth, relative to the non-maturing fish, during the spring prior to maturation and this leads to the observed bimodality in the weight-frequency distribution, 2) jacking chinook salmon have characteristic circulating levels of T3, testosterone, and cortisol
during the 7 months prior to maturation, and 3) the circulating T3, testosterone, and/or cortisol levels correlate with the (elevated) growth of the jacks. The first two hypotheses were consistent with the data (although there was no difference in the cortisol levels between the jacks and non-jacks); however, no clear correlate with the elevated growth of the jacks was identified. The observed differences in circulating T3 is probably not stimulating the growth of the jacks, since it decreased during the period of elevated growth, and was found to be negatively correlated with individual growth rates. Although circulating testosterone was slightly elevated at the time of divergent growth, and indeed this spring "peak" has been proposed as a potential factor in the growth of precocious Atlantic salmon parr (Hunt et al. 1982, Schultz 1984, Mayer et al. 1990b), testosterone did not correlate with individual growth rates within the jacking group or the whole data set. It is possible, but unlikely, that the elevated testosterone levels in the jacks was stimulating growth but was not acting in a dose dependent manner (see Higgs et al. 1982). It is also possible that another circulating hormone may be more directly stimulating the growth of the jacks, relative to the non-maturing fish, and one potential candidate is somatotropin. Somatotropin has been shown to be positively correlated with cortisol (Pickering et al. 1991), thus the increased levels of circulating cortisol in the fish through the spring and summer would not be unexpected. Somatotropin has been shown to stimulate the release of testosterone (see Planas et al. 1992), and may also explain the slightly elevated circulating testosterone levels in the spring observed in this study and others. Somatotropin can increase circulating levels of T3 and T4 (see Leatherland & Farbridge 1992), thus we would expect circulating T3 levels to increase with growth, however this was not the case. Most of the work that has been done with somatotropin in salmonids has been done on immature juvenile salmonids, and hence our discussion of its possible role in the precocious maturation of adult chinook salmon is highly speculative, and represents only one possibility.
3.2 PHYSIOLOGICAL CORRELATES WITH THE INCIDENCE OF JACKING IN CHINOOK SALMON

3.2.1 INTRODUCTION

There are a variety of physiological variables that have been shown to correlate with age of maturity in salmonids. Although there has been some work published on physiological correlates with precocious sexual maturation in Pacific salmon, Atlantic salmon parr have been particularly well studied. This may be due to the relative ease of rearing these fish to maturity (less than 2 years in freshwater). Precocious sexually mature male rainbow trout, coho, Atlantic, and chinook salmon parr and Atlantic salmon grilse have been shown to be the largest and/or fastest growing individuals in the population (Foote et al. 1991, Lamont 1990, Hagar & Noble 1976, Saunders et al. 1982, Berglund 1992, Randall et al. 1986, Thorpe et al. 1990, Herbinger & Newkirk 1990, Thorpe 1991, see Appendix B). Although Bilton’s (1980, 1984) work with coho and chinook salmon indicated that pre-smolt growth (or size at age) correlated with jacking rates, much of the work on Atlantic salmon indicates that the critical time period for growth and/or size, is during the winter or spring preceding maturation (see Thorpe 1991).

Recently there has been work published indicating that fat stores may be more critical to sexual maturation of Atlantic salmon parr than is total weight or growth during the spring prior to maturation (Herbinger & Friars 1992, Rowe et al. 1991, Simpson 1992). A correlation between condition factor and incidence of precocious maturation in chinook and Atlantic salmon parr has been interpreted as potentially a minimum energy "threshold" necessary for maturation (Rowe & Thorpe 1990a, Leyzerovich 1973, Dalley et al. 1983, Herbinger & Newkirk 1990, Foote et al. 1991). A correlation between fat stores and condition factor for individual Atlantic salmon parr has been demonstrated, explaining a possible mechanism for the correlation between condition factor and sexual maturation (Herbinger & Friars 1992, Simpson 1992).
There has been work done that showed that non-genetic maternal effects could have an effect on the incidence of precocious maturation salmon parr (Silverstein & Hershberger 1992, see Chapter 2.1). Bradford & Peterman (1987) pointed out that reported correlations between age of maturation in dam and offspring could be due to non-genetic maternal effects, specifically differences in early growth due to egg size.

The environmental stress response in salmonids has been shown to interfere with sexual maturation and reproduction (Donaldson 1990; Pickering 1989; Wedemeyer et al. 1984).

The analyses described here was designed to test some of the hypothesized correlates with precocious sexual maturation in chinook salmon using the 24 chinook salmon family groups in the breeding experiment described in Chapter 2.1. A total of 25 variables were measured for the 24 family groups (accelerated and non-accelerated - see Chapter 2.1). The variables included measurements of parental characteristics as well as measurements of physiological parameters of the family groups themselves from the fry stage to sexually mature adults (two years post fertilization). The variables were classified as;

1) parental characteristics (4 variables),
2) growth and size at age parameters (10 variables),
3) stress response (7 variables), and
4) survival/mortality (4 variables).

Table 3.2.1 gives a list of the specific variables measured in each category.

### 3.2.2 MATERIALS AND METHODS

The parental characteristics were measured at spawning and included; total weights (± 10 g), individual egg weights (average of 100 eggs), and total egg weights (from which female GSI was calculated as described in Chapter 3.1).
Table 3.2.1: A list of the 25 variables within the four categories that were used in the correlation analysis. Each of the variables are means or ratios (percent) for the 24 full- and half-sib families used for the analysis of the genetic component to jacking.

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<th>VARIABLE DESCRIPTION</th>
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<tr>
<td>STRESS RESPONSE</td>
<td>CONTROL HEMATOCRIT (HEMCON)</td>
<td></td>
<td>%</td>
</tr>
<tr>
<td></td>
<td>STRESSED HEMATOCRIT (HEMSTR)</td>
<td></td>
<td>%</td>
</tr>
<tr>
<td></td>
<td>CONTROL CORTISOL CONCENTRATION (CORTCON)</td>
<td></td>
<td>ng·ml⁻¹</td>
</tr>
<tr>
<td></td>
<td>STRESSED CORTISOL CONC. (CORTSTR)</td>
<td></td>
<td>ng·ml⁻¹</td>
</tr>
<tr>
<td></td>
<td>CONTROL GLUCOSE CONC. (GLUCON)</td>
<td></td>
<td>mg·dl⁻¹</td>
</tr>
<tr>
<td></td>
<td>STRESSED GLUCOSE CONC. (GLUSTR)</td>
<td></td>
<td>mg·dl⁻¹</td>
</tr>
<tr>
<td></td>
<td>DECEMBER CONTROL CORTISOL CONC. (DECCORT)</td>
<td></td>
<td>ng·ml⁻¹</td>
</tr>
<tr>
<td>SURVIVAL/ MORTALITY</td>
<td>EYED EGG SURVIVAL (EGGSURV)</td>
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<td>%</td>
</tr>
<tr>
<td></td>
<td>DECEMBER SURVIVAL (DECSURV)</td>
<td></td>
<td>%</td>
</tr>
<tr>
<td></td>
<td>FINAL SURVIVAL (FINSURV)</td>
<td></td>
<td>%</td>
</tr>
<tr>
<td></td>
<td>VIBRIO MORTALITY (VIBMORT)</td>
<td></td>
<td>%</td>
</tr>
</tbody>
</table>
The fry and alevin growth and wet weight measurements were made before the family groups were pooled in May 1990, and included the measurements made during the stress response experiments (see Appendix A). The fry condition factor was based on measurements of weight and length taken during the freshwater growth period (Heath et al. 1992 - Appendix A), and was calculated as the wet weight divided by the total length cubed (X 100%). Since the identification of family of origin required the recovery and decoding of nose tags after the family groups had been pooled, the December, 1990 and final sample (September, 1991) jack and non-jack mean family weights were made on fish that had been killed (see Chapter 2.1). The mean weight estimates of the jacks and non-jacks at the final sample was based on a random sample of the non-jacks, and all of the recovered jacks. All relative growth rates (RGR) were calculated as described in Chapter 3.1.

The data on the stress response of the fry family groups were taken from the stress response study (Heath et al. 1992 - see Appendix A), while the December plasma cortisol concentrations were determined from the plasma samples taken during the December sampling (see Chapter 2.1). The stress related parameters were taken following standard practices for the measurement of hematocrit and plasma concentrations of glucose and cortisol (Heath et al. 1992 - Appendix A).

The eyed egg to hatch survivals were based on twice-weekly dead egg removal and counting (see Chapter 2.1). The vibrio mortality (vibriosis was identified as the pathogen based on gross clinical signs) estimates were based on the numbers of mortalities recovered by SCUBA diving during the August, 1990, vibrio outbreak (accelerated) and the October, 1990, vibrio outbreak (non-accelerated) as described in Chapter 2.1. The inventory of surviving fish in each family group for December, 1990, was calculated by multiplying the proportion of each family group taken in a random sample (app. 420 fish from the accelerated and non-accelerated groups - see Chapter 2.1) by the total number of fish in the netcages. The estimate of fish alive in December was divided by the original number of
fish tagged in each family (500 fish) to give percent survival. The final survival rate was calculated as above except that the number of surviving fish in each family group was counted, rather than calculated.

Tables 3.2.2a & b give the mean values for all variables as well as jacking rate (i.e. specific jacking rate (SJR) as defined in Chapter 2.1) for all families, the abbreviations are as defined in Table 3.2.1.

Analysis

The analysis was designed to test the 25 variables for correlation with the observed jacking rates for the 24 family groups reared in the breeding experiment. All variables expressed as percents were ARCSIN $\sqrt{}$ transformed, while all other variables were natural logarithm transformed for analysis.

The four categories of variables were initially screened for correlation with SJR rate using a stepwise multiple regression routine (SYSTAT, Evanston, IL, USA). The general form of the model was;

$$SJR = \{CONSTANT + D + S + E\} + VAR_1 + VAR_2 + ... + VAR_n$$

Where SJR was the (transformed) specific jacking rate for each family; D, S, and E were categorical (non-transformed) variables for dam, sire (jack or non-jack) and environment (accelerated or non-accelerated) respectively, and VAR_1 to VAR_n were the (transformed) variables in the category being examined. The categorical variables (D, S, & E) were included to account for genetic and environmental differences between the family groups.

The stepwise multiple regression routine tested the inclusion of each variable for a significant increase in the explained variance (over that explained by the sire, dam and environment variables); only variables that contributed significantly to the final model were retained. It is important to note that the stepwise multiple regression
Table 3.2.2a: A list of the family values for the 25 variables used for the correlation analysis. Data shown are for the accelerated groups only. See Table 3.2.1 for meaning of abbreviations.

<table>
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<tr>
<th>VARIABLE</th>
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<th>1N</th>
<th>2J</th>
<th>2N</th>
<th>3J</th>
<th>3N</th>
<th>4J</th>
<th>4N</th>
<th>5J</th>
<th>5N</th>
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<th>6N</th>
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<td>7</td>
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<td>-</td>
<td>1.16</td>
<td>-</td>
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<td>1.091</td>
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<td>1.102</td>
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<td>-</td>
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<td>-</td>
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<td>0.56</td>
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<td>0.77</td>
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Table 3.2.2b: A list of the family values for the 25 variables used in the correlation analysis. Data shown are for non-accelerated groups only. See Table 3.2.1 for the meaning of the abbreviations.

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</tr>
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<td>ALVGRO</td>
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</table>
routine is extremely prone to Type I errors (rejecting a true null hypothesis), especially with a large set of independent variables, and thus the routine is useful only to identify potential correlates, not as a method of generating predictive relations.

Linear regression analysis (SYSTAT Evanston, IL, USA) was performed on the variables identified by the stepwise multiple regression analysis as significant, to test for individual correlations with SJR.

3.2.3 RESULTS
Stepwise analysis

Parental characteristics: The final multivariate linear model for the parental characteristic category was;

\[ SJR = \{\text{CONSTANT} + (S) + (E)\} - 4.28(\text{DAMWT}) + 7.82(\text{GSI}) \]

Thus variables that were retained in the model by the stepwise routine were dam weight and dam GSI. Note that the CONSTANT, S, AND E terms are as described above.

Growth and size at age parameters: The final model found for the growth and weight variables was;

\[ SJR = \{\text{CONSTANT} + (S) + (D)\} + 0.467(\text{DIFF}) \]

The only variable retained in the stepwise routine was the differential jack-nonjack final weight. No significant model resulted from only growth rate variables included in the model.

Stress response parameters: The final model found for the stress related variables was;
\[ \text{SJR} = \{\text{CONSTANT} + (D)\} + 0.087(\text{CORTCON}) - 1.55(\text{GLUCON}) - 3.99(\text{HEMCON}) \]

Three variables were retained by the stepwise routine; control fry cortisol concentration, control fry glucose concentration, and fry control hematocrit. None of the post-stress response variables were retained in the model.

\textit{Survival/mortality parameters:} The final model found using the survival/mortality variables was;

\[ \text{SJR} = \{\text{CONSTANT} + (D) + (S)\} -1.07(\text{FINSURV}) \]

The only variable retained by the stepwise routine was final survival.

\textbf{Linear regression}

Linear regressions were performed on all the variables identified as potential predictors in the stepwise routine; only DIFF, dam weight, and control fry glucose concentration were found to be significantly correlated with SJR. These regressions are shown in Fig. 3.2.1a & b, and Fig. 3.2.2a. Figure 3.2.2b shows the significant linear regression between control fry glucose concentration and fry relative growth (FRYGRO).

\subsection*{3.2.4 DISCUSSION}

Non-genetic maternal effects on growth and size at age have been reported in chinook salmon up to the smolt stage (Withler \textit{et al.} 1987). Silverstein & Hershberger (1992) showed that wet weight at three (but not five) months post-hatch in coho salmon fry was correlated with egg size, and they also found a significant, but weak, correlation between egg size and incidence of precocious maturation in parr. Similarly, Bradford & Peterman (1987) hypothesized that the observed correlation between age of maturation of female parents and offspring in Pacific salmon may be due to non-genetic maternal effects (egg size differences). However, no significant
Figure 3.2.1: Results of the correlation analyses for family means of physiological variables versus family specific jacking rates (SJR): a) transformed difference in wet weight between the jacks and non-mature fish at the final sample (DIFF) plotted against the transformed family jacking rates (SJR); and b) transformed wet weight of the dam for each family (DAMWT) plotted against transformed family jacking rates (SJR). Regression equations are given for both relations, all slopes were significantly different from zero.
Figure 3.2.2: Results of the correlation analyses for mean family plasma glucose concentrations against growth and jacking rates: a) transformed control (non-stressed) plasma glucose concentration in chinook salmon fry (GLUCON) plotted against transformed family jacking rates (SJR); and b) transformed control (non-stressed) plasma glucose concentration (GLUCON) plotted against transformed relative growth (FRYGRO) for chinook salmon fry. Regression equations are given for both relations, all slopes were significantly different from zero.
correlation between egg size and jacking rates was found in this study, although a significant negative correlation between dam wet weight and jacking rates was observed (thus the larger females produced, on the average, fewer jacks). Since there was no correlation between egg size and jacking rates, it is not clear how dam wet weight could affect the incidence of jacking. One possibility is that the size of the 3-year-old female parents was negatively correlated with the presence of "jacking genes". That is, the smaller females have genetic jacking tendencies that could not be expressed phenotypically since only males jack (see Chapter 1.1). Alternately, since the full-sib families used for this analysis were the progeny of only six females, the sample size of the regression analysis may be inflated and thus the significance of the regression is potentially suspect. However, the four full-sib families from each dam had different sires and different environmental conditions thus they are, at worst, pseudo-replicates.

It is widely believed that freshwater growth rate and/or size at release has a profound effect on jacking rate, and this is supported by evidence that shows that the return rate of jacks increases with growth acceleration in coho and chinook salmon (Bilton 1980, 1984). Recently published escapement statistics for the Stamp River system (Robertson Creek Hatchery, Vancouver Island, British Columbia) show that the incidence of 2-year-old returning male chinook salmon (jacks) in the hatchery-reared (i.e. accelerated growth) fish was more than twice that of the river-reared fish (9.1% vs. 3.4% - Bocking & Nass 1992). Lamont (1990) also showed that size at age was critical in determining sexual maturation in male rainbow trout and coho salmon parr. There is extensive evidence that growth rate or size at age plays an important role in the precocious sexual maturation of Atlantic salmon parr (see Chapter 1.1, & 2.1). Thorpe (1986) described a growth rate threshold model and hypothesized that early sexual maturation was determined, in part, by energy reserves during a critical time "window". Support for such a model came from work that showed ration limitation during the spring prior to maturation could significantly reduce the incidence of precocious parr and grilsing in Atlantic salmon (Rowe & Thorpe
1990b; Thorpe et al. 1990). Some correlation of fry growth and/or size at age with jacking rates was expected, however, none of the freshwater growth or size at age variables were significantly correlated with jacking rate, despite a large range in observed jacking rates and the growth/size related variables (see Table 3.2.2a & b). This does not agree with results published by Bilton (1980, 1984), or with conventional belief within the SEP and commercial hatcheries in British Columbia. There was also no correlation between jacking rates and fry condition factor, however it is not surprising that condition factor would not be correlated with jacking 15 months before maturation (Berglund 1992). The work that has associated condition factor with sexual maturation has been almost exclusively with Atlantic salmon precocious parr (Simpson 1992, Herbinger & Newkirk 1990, Dalley et al. 1983, Leyzerovich 1973, Rowe & Thorpe 1990a). Unfortunately, length measurements were not taken during the December or final sampling, thus condition factor differences closer to maturation could not be tested for correlation with jacking in this experiment.

The single size-related variable that did correlate significantly with jacking rates was the difference in mean weights between the jacks and non-jacks at the final sampling (DIFF). That is, the families that had the greatest separation in weight between jacks and the non-maturing fish also had the highest jacking rates. This correlation may have little to do with the energy reserve thresholds discussed above, since final jack weight or final non-jack weight should have reflected such an effect better. It is possible that families with higher jacking rates have, in general, higher androgen levels in the maturing fish, and hence, enhanced jack growth rates prior to maturation (see Chapter 3.1). Further work is necessary to investigate this correlation.

Since freshwater size at age or growth rate is often reported as being involved in precocious maturation in salmonids (see above), it was theorized that the stress response in fry may also be involved, since changes in growth is a tertiary stress response (Heath et al. 1992 - see Appendix A). The December plasma cortisol concentrations were included in the analysis because elevated plasma cortisol may have negative effects on reproductive
processes (Donaldson 1990, Barton & Iwama 1991, see Chapter 3.1). The only stress-related factor that was significantly correlated with jacking rates was control fry glucose concentration (GLUCON). Our results indicate that families with low GLUCON tended to have high jacking rates. Since we know that metabolic rate, growth and circulating glucose levels are inter-related (Higgs et al. 1992, Umminger 1977, Jobling 1983, Barton 1988), we tested freshwater growth variables for correlation with glucose levels and found a significant positive correlation of fry relative growth rate with GLUCON. Although the positive correlation of GLUCON with growth is expected (Heath et al. 1992 - Appendix A), these results imply that full-sib families with high GLUCON have high growth, but also low jacking rates, in contradiction of previous work and accepted beliefs. The anomalous correlation of GLUCON with jacking rate represents the only freshwater correlate with jacking rate found in this study and since there is clearly some freshwater effect on jacking rates in Pacific salmon (Bilton 1980, 1984, Bocking & Nass 1992), the correlation of GLUCON with jacking rate should be investigated more thoroughly.

The results of the correlation analysis presented here should be interpreted with caution. It should be noted that the correlations described in this report are mostly based on family means, and are thus somewhat insensitive to effects on the individual level. For example, although our results showed that families with higher freshwater growth or larger size at age do not have a higher incidence of jacking, the individual fast growing or larger fish within the families may indeed have had a higher probability of jacking. The latter hypothesis cannot be tested using our approach (but see Chapter 3.1).

Although the relations presented here are statistically significant, even allowing for the relatively large number of correlations tested (Bonferroni-adjusted probabilities - SYSTAT Evanston, IL, USA), they are correlations only and cannot be interpreted as implying causation. The results of a correlational analysis such as this has two main purposes; 1) to generate predictive models, and 2) to identify potentially productive areas of research. Since the relations presented here are probably not quantitatively applicable to
other stocks under other rearing conditions, they are not useful predictors of jacking rates in general. On the other hand, our results have identified three previously unreported correlates with jacking, indicating areas of potentially useful future research.
GENERAL DISCUSSION

The results of the breeding experiment showed that jacking in chinook salmon is controlled, to a great extent, by maternal and paternal genetic effects. Although the possibility of non-genetic maternal or early-rearing tank effects cannot be formally excluded, their contributions are probably small (see Chapter 2.1). The magnitude of the genetic component found for jacking in chinook salmon is greater than most other estimates for precocious maturation in salmonids (see Chapter 2.1). Glebe & Saunders (1986) speculated that a simple (single locus) genetic model might explain their observed maturation rates in an Atlantic salmon breeding program. It is possible that a simple genetic model might also apply to jacking in chinook salmon. The existence of a strong genetic effect in jacking supports previous work showing that jacking in Pacific salmon may be an evolutionary stable state (Gross 1984, 1985). Independent of the theoretical implications of a major genetic contribution to jacking, it also indicates that jacking rates should respond to selection. Although the use of mono-sex (all female) stocks of chinook salmon has eliminated the economic impact of jacking in the culture of that species (Hunter et al. 1983), genetically high- and low-jacking chinook strains could still be useful in breeding programs.

The analysis of allele frequencies at two minisatellite DNA loci (OTSL1 and Ssa1) agreed with the results of the breeding program; the incidence of jacking was found to be family dependent. Allele frequency analysis does not allow partitioning of the observed family (genetic) variation into maternal and paternal effects. Therefore, minisatellite allele analysis cannot replace breeding experiments for quantitative traits, but rather may serve as a method to determine whether an extensive breeding program is worthwhile.

The manipulation of water temperature to control early development was limited in duration, yet had a significant effect on jacking rates in the chinook salmon family groups. It is likely that the much greater difference in early rearing environments experienced by wild versus hatchery-reared salmonids would have a correspondingly larger effect on
jacking rates. Indeed it is possible that the genetic effects could be masked by environmental effects at very low rates of development in the early life stages (i.e. cold water and limited food). One most unexpected result of the correlational analysis was the absence of significant growth- and size-related correlations with jacking rates. Although there was considerable variation in jacking rates, growth, and size at age among the full-sib families, the fast-growing, (or high mean weight) families experienced no higher jacking rates than the slow-growing (or low mean weight) families. This is at odds with much of the published information for other salmonids (see Chapters 1.1, 2.1, 3.2), as well as with the results of the breeding experiment described here. At least two possible explanations present themselves; 1) the between-family growth variation was too small to significantly affect jacking rates, and/or 2) the artificially accelerated development was fundamentally different from naturally occurring fast-growth families. Further experiments to test these two possibilities would be conceptually simple, however the resources required to rear large numbers of salmon to sexual maturation in sea water are daunting.

The presence of a significant genotype-by-environment interaction effect on the incidence of jacking indicates that the accelerated early development did not affect all families in the same manner. The significant interaction was mainly due to two families that responded to the acceleration treatment with a drastic increase in jacking rate (Fig. 2.1.6). Inspection of the reaction norms presented in Fig. 2.1.6 indicates that jacking rates in chinook salmon are clearly not a linear function of early developmental rates.

The results of the weight-frequency distribution analyses indicated that chinook salmon exhibit an unusual growth pattern during the spring and summer prior to sexual maturation. The jacks experienced high growth relative to the non-maturing fish in April and May, with little or no reduction in growth during gonadal development through the summer (see Chapters 1.1 and 3.1). There is no obvious explanation for this result as it contradicts published reports on the growth of precociously maturing chinook, coho, and Atlantic salmon parr reared in captivity (see Chapters 1.1 and 3.1). One possible
explanation may be behavioral differences between jacking chinook salmon and precociously maturing parr. Observations of marked increases in aggressiveness in jacking chinook salmon during gonadal development have been noted (pers. obs.). Metcalfe et al. (1986, 1988, 1989) showed that the development of a bimodal size distribution in smolting and non-smolting Atlantic salmon parr could be explained by differences in feeding behavior; similar experiments with chinook salmon jacks might prove valuable.

The analysis of hormonal changes associated with the elevated growth of jacks relative to non-maturing fish showed a number of clear differences that coincided with the development of a bimodal weight-frequency distribution. However, the more direct analysis of hormonal correlates with growth and size at age on an individual fish basis failed to identify any potential causative relations. Thus, some other unidentified hormone, or combinations of hormones acting synergistically, stimulated the growth of the jacking fish 5-6 months prior to final maturation. One potential candidate identified for such a growth stimulation was somatotropin, or growth hormone. The somatotropin hypothesis would account for some of the changes in hormonal profiles described in Chapter 3.1. Unfortunately the reliable measurement of salmon growth hormone is still possible in only a few laboratories, and since the assay is in high demand, the measurement of growth hormone was not feasible within this thesis.

The minisatellite DNA probes developed for use in salmonids (Chapters 2.2 & 2.3) are potentially important tools for the study of individual, groups, and populations of salmon. Although the DNA fingerprint probes could not be used effectively for the estimating relatedness in the analysis of the genetic component of jacking (Chapter 2.2), they are effective in identifying parentage and individual fish. The chinook salmon single-locus minisatellite DNA probe (OTSL1) represents a valuable tool for the study of chinook salmon at the individual or population level (Burke et al. 1991). However, the method used to isolate OTSL1 (i.e. "DAMD") is perhaps more significant, since it means that
other probes, specific to a species of interest, can be developed with relatively low investment in time and equipment.

The application of OTSL1 and Ssa1 to the question of genetic involvement in jacking of chinook salmon, described in this thesis, is one of very few such studies in animal populations (Chapter 2.3), although extensive applications of single-locus probes have been documented in human medicine and forensic science. The comparison of relatedness estimates generated by single-locus probes for specific phenotypes within a random-mated population (Chapter 2.4) is a novel use of DNA fingerprinting technology. The success of this technique in identifying allele distribution differences between jacks and females indicates that it is sensitive enough to be a potentially valuable approach, especially for wild populations where breeding experiments are not feasible. Another possible application of relatedness estimation using single-locus probes is the measurement of inbreeding or genetic diversity. The Nicola River chinook salmon parr were not previously suspected of having low genetic diversity, however the analysis in Chapter 2.4 shows that this population exhibits low variability at the OTSL1 locus, relative to another chinook population. An extensive survey of chinook salmon stocks using single-locus minisatellite DNA probes to estimate relatedness could provide valuable information on the level of genetic diversity in chinook salmon stocks. Such information would be of great benefit to stock enhancement and preservation efforts.

CONCLUSIONS

The key findings of this research on the incidence of jacking in chinook salmon were;

Primary Findings

1) There is a strong genetic contribution to jacking.
2) The genetic contribution has both sire and dam components.
3) There is a positive effect of increased early-rearing development rate on jacking rates.
4) There are genotype-by-environment interactions for the incidence of jacking.
Secondary Findings

5) Mean family growth and/or size at age do not predict jacking rate.

6) Jacking chinook salmon experience an elevated growth rate relative to non-maturing fish in the spring prior to maturation.

7) Jacks do not experience reduced growth during gonadal development under culture conditions.

8) Plasma concentrations of testosterone and T3 are elevated in jacks up to eight months prior to final maturation.

9) Neither testosterone, T3, nor cortisol is positively correlated with the elevated growth of jacks in the spring.

Figure D.1 shows the diagram presented in the General Introduction, with the areas that have been addressed in this research marked. The physiological triggers of jacking in chinook salmon have received the least attention in this thesis; however, these triggers have been extensively investigated, particularly in the Atlantic salmon parr (see Chapters 1.1, 3.1, 3.2).

Although questions have been addressed and answered by the research constituting this thesis, an even greater number of additional questions have been suggested by the results of this research. A number of possible avenues of research have been proposed through-out the text, and other approaches will occur to researchers interested in the phenomenon of jacking in Pacific salmon. Research into the phenomenon of jacking in Pacific salmon is valuable, not only for the physiological aspects of precocious maturation, but also as a relatively straightforward example of an alternate male life history strategy in those species.
Figure D.1: A schematic diagram of the reported factors hypothesized to affect the incidence of precocious sexual maturation in male salmonids, as described in the General Introduction. The factors investigated in this thesis are identified with arrows. The two factors not dealt with were: energy (or fat) stores 6-9 months prior to maturation, and the effect of photoperiod.
Appendix A


Abstract

Eight full- and half-sib families of chinook salmon were held during egg development at two temperatures (8.0°C and 10.2°C). As fry, these families were measured for: relative growth rate, initial and final wet weight, hematocrit values before and 2 h after a 30 s handling stress, and plasma cortisol and glucose concentrations before and after stress. Significant sire effects were found for all measured traits, and significant dam effects were found for all traits except for the post-stress increases in cortisol concentrations. There were significant genotype-by-environment interactions for all traits except unstressed (control) plasma glucose concentrations. Incubation temperature had a significant effect on relative growth rate and final wet weight only. We found a significant correlation between post-stress plasma glucose concentration and relative growth rate for all fish, independent of family; while resting plasma cortisol concentration and post-stress hematocrit correlated with wet weight only when analyzed within the eight individual families. Genetic contributions to stress-related parameters and genotype-by-environment interactions should be considered as a factor in stress-related research with fish.

Introduction

Chinook salmon are reared extensively in government hatcheries on the west coast of North America as part of stock enhancement programs. In the last decade, chinook salmon have also been reared as food fish by private hatcheries and salmon farms. Many culture techniques result in stress to the fish (Flos et al. 1988, Barton & Iwama 1991). The
effects of chronic and acute stressors on these artificially reared fish have been shown to impact their performance and survival (see Wedemeyer et al. 1984, Maule et al. 1989, Pickering 1989, Barton & Iwama 1991).

A genetic component to stress response has been proposed for salmonids (Schreck 1981, Barton 1988, Wedemeyer et al. 1990, Barton and Iwama 1991); however, there are few experimental data to support this hypothesis. Differences in the endocrine stress response between species of fish have been shown (Davis & Parker 1983, 1986, Sumpter et al. 1986, Fevolden et al. 1991), as well as differences between stocks within a species (coho salmon, McGeer et al. 1991; wild and hatchery-reared rainbow trout, Woodward & Strange 1987). Fall and spring emigrating stocks of chinook salmon fry in the Columbia River system have different sensitivities to handling and transportation stress as measured by plasma cortisol concentration, \([\text{cortisol}]_\text{pl}\), and plasma glucose concentration, \([\text{glucose}]_\text{pl}\), (Maule et al. 1988). These differences were attributed, in part, to genetic effects. Refstie's (1982, 1986) work on the genetic contribution to stress response within and between species using controlled breeding experiments for rainbow trout and Atlantic salmon indicated a heritable component to both \([\text{glucose}]_\text{pl}\) and \([\text{cortisol}]_\text{pl}\) responses. Fevolden et al. (1991) also found a heritable stress response in rainbow trout and Atlantic salmon in a selection experiment for high and low \([\text{cortisol}]_\text{pl}\) response to stress. It is possible that physiological responses such as the primary and secondary stress responses (see Wedemeyer et al. 1990, Barton & Iwama 1991) may be under relatively simple genetic control.

Genotype-by-environment interactions within stress responses have not been investigated in fish. If these interactions exist, such phenotypic plasticity of the endocrine stress response would indicate that a fish's response to stress would, at least in part, depend on its environment and thus the environmental history of the study fish would be important to the interpretation of investigations into the genetic basis of the stress response.
Although the genetic component of the stress responses of salmonids is not well understood, the genetics of growth and size at age has been examined extensively (Reisenbichler & McIntyre 1977, Gall & Gross 1978a,b, Thorpe & Morgan 1978, Ayles & Baker 1983, Bailey & Saunders 1984, Gjerde & Gjedrem 1984, Robison & Luempert 1984, deMarch 1991). Such investigations with chinook salmon fry have shown that growth and growth-related characteristics generally have significant genetic components (Cheng et al. 1987, Withler et al. 1987). The effect of rearing temperature on growth and size at age of salmonid fry has been extensively described (Clarke et al. 1981, Iwama & Tautz 1981, Baker 1983, Clarke & Shelbourn 1985, Pereira & Adelman 1985), and genotype-by-environment interactions involving growth rate and rearing temperature have been reported for rainbow trout (McKay et al. 1984).

The main purpose of this study was to investigate the effects of genetic and environmental (incubation temperature) factors, as well as the genotype-by-environment interaction, on growth-related and stress-related traits. Specifically, this paper reports experiments using a nested mating design with two incubation temperatures to test for sire, dam, environmental and interaction contributions to: freshwater growth, wet weight at eight months of age, stress responses, as measured by hematocrit (Hct), [cortisol]p] and [glucose]p]. We also investigated correlations between the growth and stress related variables within the full-sib families as well as across all families.

Materials and Methods

Mating Design and Incubation

In September, 1989, over 100 sexually maturing male and female chinook salmon (first generation domestic Robertson Creek stock) were taken at random from multiple seine sets at the saltwater rearing facilities of Yellow Island Aquaculture Ltd. (YIAL, Quadra Island, British Columbia), and transferred to freshwater
facilities (aerated well water). On November 9, 1989, four 3-year-old females were spawned, and the eggs from each female were divided into two approximately equal groups. In order to increase the potential diversity of the male contribution to the offspring, half of the eggs from each female were fertilized by 2-year-old males, while the other half were fertilized by 3-year-old males. Each male was used only once and all fertilizations took place within 2 h of gamete collection. The resulting eight families were further divided into two sub-groups (Table A.1). The development of one of these sub-groups was accelerated by incubating the eggs and alevins in heated water (average temperature = 10.2°C; range = 9.0 - 11.1°C) while the other sub-group was incubated in unheated water (average temperature = 8.0°C; range = 7.4 - 9.0°C). All family groups were incubated in vertical stack incubation trays (Heath Technicorp., Seattle, Washington) with flows of 12-16 L·min⁻¹. The eggs were disinfected 2-3 times per week with malachite green (50-100 mg·L⁻¹ flush treatment) until the eyes of the developing embryo were clearly visible (eyed stage; approximately 30 days at 10°C). Once eyed, the eggs were shocked and dead eggs removed. All losses were recorded throughout the incubation period. By January 3, 1990 all the accelerated family groups were hatched and the alevins had reached swim-up stage (yolk sac fully absorbed) by February 7, 1990. All of the fish in the non-accelerated family groups were hatched by January 21, 1990, and those alevins reached swim-up stage by March 13, 1990.

**Family Groups**

At swim-up, each family (500 to 1000 fry) was randomly assigned to one of 16 identical 200L outdoor tanks. All tanks received equal water flows (15 L·min⁻¹) from a common source (average temperature = 8.8°C; range = 7.8 - 10.0°C). For 14 days after swim-up, fry were fed during daylight hours with automatic feeders, eight times per hour. Subsequently, the fry were fed to satiation by hand four times per day. The fry were fed Biomoist starter
Table A.1; Schematic diagram of the nested mating design used, across two incubation temperatures. The dams were all 3-year-old chinook salmon and the sires were aged as noted, family numbers are those referenced in Fig. A.1. The survival data over the first two periods are based on 520-977 fish. The survival data given for the trial period are presented as percent values, with initial number in parentheses.

<table>
<thead>
<tr>
<th>Dam</th>
<th>Sire</th>
<th>Family Number</th>
<th>Incubation Strategy</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fertilization To Swim-up</td>
<td>Swim-up To Trial</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female 1</td>
<td>Male 1</td>
<td>1</td>
<td>Accel.</td>
<td>82.0 97.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Non-accel.</td>
<td>92.5 95.6</td>
</tr>
<tr>
<td></td>
<td>Male 2</td>
<td>2</td>
<td>Accel.</td>
<td>94.4 97.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Non-accel.</td>
<td>89.6 96.7</td>
</tr>
<tr>
<td>Female 2</td>
<td>Male 3</td>
<td>3</td>
<td>Accel.</td>
<td>97.1 97.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Non-accel.</td>
<td>94.4 97.9</td>
</tr>
<tr>
<td></td>
<td>Male 4</td>
<td>4</td>
<td>Accel.</td>
<td>98.5 98.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Non-accel.</td>
<td>93.5 99.0</td>
</tr>
<tr>
<td>Female 3</td>
<td>Male 5</td>
<td>5</td>
<td>Accel.</td>
<td>97.2 99.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Non-accel.</td>
<td>97.6 99.2</td>
</tr>
<tr>
<td></td>
<td>Male 6</td>
<td>6</td>
<td>Accel.</td>
<td>93.3 98.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Non-accel.</td>
<td>97.9 98.4</td>
</tr>
<tr>
<td>Female 4</td>
<td>Male 7</td>
<td>7</td>
<td>Accel.</td>
<td>96.4 98.0</td>
</tr>
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<td>Non-accel.</td>
<td>91.9 98.3</td>
</tr>
<tr>
<td></td>
<td>Male 8</td>
<td>8</td>
<td>Accel.</td>
<td>96.2 98.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Non-accel.</td>
<td>94.1 97.3</td>
</tr>
</tbody>
</table>

1 No significant differences (chi square=21.2; df=15); 2 No significant differences (chi square=0.99; df=15); 3 No significant differences (chi square=3.83; df=15)
diet (Bioproducts Ltd., Warrenton, Oregon) until they reached 1.2 g when they were
switched to a semi-moist diet (Moore Clarke Canada, Vancouver, B.C.). Mortalities
were removed and recorded daily.

On May 24, 1990, about 40 fish from each of the 16 family groups were
anaesthetized in 20 mg·L⁻¹ MS-222 (Syndel Laboratories, Vancouver, B.C.), and
each was injected intraperitoneally with a passive integrated transponder (PIT) tag.
The PIT tag allowed the identification of each individual fish throughout the duration
of the experiment. At the same time wet weight (±0.1 g) was measured; this was the
initial weight measurement for subsequent growth calculations. Tagged fish were
transferred into a single 3 000 L indoor tank receiving a flow of 40 L·min⁻¹ under
simulated natural photoperiod and fed with automatic feeders every 5 min during
daylight hours. To minimize the possibility of tank effects during the experiment, the
fish were held in this common environment from May 25 to July 01, 1990, (37
days). All mortalities were removed and frozen for later identification. The fry were
starved from June 29 to July 1, at which time the pooled fish were anaesthetized
(0.12 mg·L⁻¹ 2-phenoxyethanol), weighed (±0.1 g), and identified by their PIT
tag. This weight was the final weight that was used in the growth calculations. The
fish were then returned to their 16 original family tanks and left undisturbed for
approximately three days (until July 3-4).

To investigate the role of genetics in the stress response, six to 16 fish from
each of the 16 family groups were subjected to an acute handling stress by placing
them on a flat screen suspended in the air for 30 s, and then returning them to a
holding tank for recovery. At the same time, a similar number of fish from the same
family groups were immediately transferred to a lethal dose of 2-phenoxyethanol (5
ml·L⁻¹); they lost equilibrium and were dead within 20 s. These fish were the non-
stressed controls, while the stressed fish were sampled 2 h after the handling stress
(see Barton & Iwama 1991). At the time of sampling, all fish were identified by
their PIT tag code and, within 15 min of capture, bled from the caudal vasculature into an ammonium-heparinized capillary tube after the caudal peduncle was severed. The capillary tubes were centrifuged, Hct measured (as percent packed cell volume), and the plasma was frozen (-20°C) and stored for later cortisol and glucose analyses. All sampling was carried out between 9:00 and 17:00 to reduce the possibility of diurnal effects (Thorpe et al. 1987).

Plasma cortisol concentration was determined by 125I cortisol radioimmunoassay (Clinical Assay No. 529; Baxter Healthcare Corp., Cambridge, Mass.). This procedure is based on the competitive binding principles of radioimmunoassay as described by Sumpter & Donaldson (1986) and has been used to measure [cortisol]pl in salmonids (Wedemeyer & Yasutake 1977, Iwama et al. 1989, Wedemeyer et al. 1990). Plasma glucose concentration was measured using a modification of Trinder's (1969) colorimetric procedure with premixed 4-aminoantipyrine (Glucose [TRINDER] procedure No. 315; Sigma Chemical Company, St.Louis, Missouri).

**Statistical Analysis**

Survival of the 16 family groups during three rearing periods: (1) incubation (fertilization to swim-up), (2) rearing from swim-up to the beginning of the experiment, and (3) the experimental period was compared using the chi-square goodness of fit procedure (Zar 1974). Survival was not significantly different among the 16 family groups for any of the time periods analyzed (Table A.1).

Since the growth of the fish during the experiment was approximately linear, we used relative growth rate (RG) in our analyses. Because the PIT tags allowed us to identify individual fish we calculated RG for each fish using:

\[
\text{RG} = \frac{(W_{t_{\text{final}}} - W_{t_{\text{initial}}}) \times 100}{(W_{t_{\text{initial}}} \times \text{Time})}
\]
Where \( W_{t_{\text{initial}}} \) and \( W_{t_{\text{final}}} \) are the individual weights measured at the time of tagging, and the final sampling, respectively, and Time is the number of days between the initial and final weight measurements.

The changes in \([\text{glucose}]_{\text{pl}}\) and \([\text{cortisol}]_{\text{pl}}\) in response to the handling stress were standardized by subtracting the family mean value for the control fish from the individual post-stress measurements (referred to as \( \triangle[\text{glucose}]_{\text{pl}} \), and \( \triangle[\text{cortisol}]_{\text{pl}} \)). The use of \( \triangle[\text{glucose}]_{\text{pl}} \), and \( \triangle[\text{cortisol}]_{\text{pl}} \) allowed the analysis of the change in \([\text{glucose}]_{\text{pl}}\) and \([\text{cortisol}]_{\text{pl}}\) due to the stressor, without the confounding unstressed component.

Since RG was a percentage value, it was transformed using the arcsine square root transformation, however the Hct measurements were within the 30-70% range, thus the arcsine square root transformation was not used (Sokal & Rohlf 1981). All variables (except RG) were found to be heteroscedastic and were therefore transformed using the natural logarithm (Sokal & Rohlf 1981). Analyses were performed on the transformed data and reported means and 95% confidence limits are back-transformed values (Sokal & Rohlf 1981).

To test for sire, dam, and environmental (incubation temperature) effects, as well as incubation temperature-by-sire and -by-dam interactions (genotype-by-environment interactions) a three-way nested ANOVA (males nested within females) was used. The model was:

\[
Y_{p_{mn0}} = \mu + E_{o} + D_{n} + S_{mn} + E_{D_{on}} + E_{S_{omn}} + e_{mnop} \quad \text{(Model A)}
\]

Where \( Y_{p_{mn0}} \) is the observation on the \( p \)th progeny of the \( m \)th sire nested within the \( n \)th dam, within the \( o \)th incubation strategy; and \( \mu \) is the population (least square) mean. \( E_{o} \) is the fixed effect of the \( o \)th incubation strategy (accelerated or non-
accelerated); $D_n$ is the random effect of the $n$th dam; $S_{mn}$ is the random effect of the $m$th sire nested within the $n$th dam; $E_{D_on}$ is the interaction of the $o$th incubation strategy and the $n$th dam (genotype by environment interaction); $E_{S_{omn}}$ is the interaction of the $o$th incubation strategy and the $m$th sire nested within the $n$th dam (genotype by environment interaction); and $e_{mnop}$ is the random error term.

Since the sire component estimated in Model A would also include any sire age effects (i.e. differences between the progeny of the 2- and 3-year-old sires), we tested for the effect of sire age using a saturated four-way ANOVA. The main effects within the model were; incubation strategy (fixed effect), treatment (fixed effect), dam (random effect), and sire age (two and three years of age - fixed effect), and all interaction terms were also included. The purpose of this analysis was to test for strong sire age effects which, if present, would contribute to the random sire effect described in Model A.

A two-way ANOVA was used to test for family by incubation temperature interactions (genotype by environment interactions). The model used was:

$$Y_{ijk} = \mu + C_j + E_i + CE_{ij} + e_{ijk} \quad \text{(Model B)}$$

Where $Y_{ijk}$ is the observation of the $k$th progeny of the $j$th cross from the $i$th incubation strategy, $\mu$ is the population (least square) mean, $C_j$ is random effect of the $j$th full-sib family, $E_i$ is the fixed effect of the $i$th incubation strategy, $CE_{ij}$ is the interaction between the $j$th cross and the $i$th incubation strategy (the genotype-by-environment interaction), and $e_{ijk}$ is the random error term. It should be noted that Model B does not include the half-sib relationships of some of the families making the analysis conservatively suboptimal. Model B is thus prone to Type II errors (Sokal & Rohlf 1981), and any nonsignificant results within Model B must be viewed with caution.
A four-way nested ANOVA (males nested within females) was used to test for the effect of the applied stress (treatment). The model was:

\[ Y_{qpmno} = \mu + T_p + E_0 + D_n + S_{nm} + TD_{pn} + TS_{pmn} + e_{mnopq} \] (Model C)

Where \( Y_{qpmno} \) is the observation on the qth progeny of the mth sire nested within the nth dam, within the oth incubation strategy, within the pth treatment; and \( \mu \) is the population (least square) mean. \( T_p \) is the fixed effect of the pth treatment (stressed or non-stressed); \( E_0, D_n, \) and \( S_{mn} \) are as in Model A; \( TD_{pn} \) is the interaction of the pth treatment and the nth dam; \( TS_{pmn} \) is the interaction of the pth treatment and the mth sire nested within the nth dam; and \( e_{mnopq} \) is the random error term.

Since all of the ANOVA models described above are mixed models, the error terms and degrees of freedom used for the tests of significance of the interaction terms, as well as the main effects, were estimated as described in Zar (1974).

Regression analysis was used to test for correlations between final weight and control and post-stress: (1) \([\text{glucose}]_p\); (2) \([\text{cortisol}]_p\); and (3) Hct. Regression analysis was also used to test for correlations between RG and control and post-stress: (1) \([\text{glucose}]_p\); (2) \([\text{cortisol}]_p\); and (3) Hct. The regression analysis was done within each family separately, as well as across all families. To test for the possible effect of the incubation temperature a dummy variable coding for accelerated versus non-accelerated groups was included in a multiple regression analysis.

Results

Sire and Dam Effects

The nested ANOVA (Model A) allowed the estimation of the sire and dam components of the observed variance. Sire effects were significant for all traits measured (Table A.2), while the dam effect was significant for all traits except for
Table A.2: ANOVA table (model A; see text) for growth and stress-related parameters measured on 8 full-sib families of chinook salmon fry. Mean squares (MS) are given for sire (S), dam (D), incubation temperature (IT), and interaction effects. Sires were nested within dams and both are random effects, while IT was a fixed effect.

<table>
<thead>
<tr>
<th>Source -&gt;</th>
<th>Dam (D) (df=3)</th>
<th>Sire (S) (df=4)</th>
<th>Incubation Temperature (IT) (df=1)</th>
<th>D X IT Interaction (df=3)</th>
<th>S X IT Interaction (df=4)</th>
<th>MS Error (df)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trait</td>
<td>MS (g)</td>
<td>MS (pg'dl-1)</td>
<td>MS (mg'dl-1)</td>
<td>MS (%)</td>
<td>MS (%)</td>
<td></td>
</tr>
<tr>
<td>Wet weight (g)</td>
<td>1.3***</td>
<td>0.12**</td>
<td>11.3***</td>
<td>0.022NS</td>
<td>0.069*</td>
<td>0.025 (354)</td>
</tr>
<tr>
<td>RG ((\mu g'\text{dl}^{-1}))</td>
<td>0.004***</td>
<td>0.002***</td>
<td>0.013**</td>
<td>0.001NS</td>
<td>0.001**</td>
<td>0.0002 (354)</td>
</tr>
<tr>
<td>[Cortisol]pl ((\mu g'\text{dl}^{-1}))</td>
<td>3.0**</td>
<td>4.5***</td>
<td>7.5NS</td>
<td>8.6***</td>
<td>1.7***</td>
<td>0.24 (177)</td>
</tr>
<tr>
<td>[Glucose]pl ((\text{mg'dl}^{-1}))</td>
<td>0.21***</td>
<td>0.26***</td>
<td>0.068NS</td>
<td>0.043NS</td>
<td>0.048NS</td>
<td>0.032 (166)</td>
</tr>
<tr>
<td>Control Hct (%)</td>
<td>0.098***</td>
<td>0.028**</td>
<td>0.002NS</td>
<td>0.026*</td>
<td>0.025*</td>
<td>0.008 (177)</td>
</tr>
<tr>
<td>(\circ)[Cortisol]pl ((\mu g'\text{dl}^{-1}))</td>
<td>0.20NS</td>
<td>0.54***</td>
<td>0.017NS</td>
<td>0.96***</td>
<td>0.092NS</td>
<td>0.10 (168)</td>
</tr>
<tr>
<td>(\circ)[Glucose]pl ((\text{mg'dl}^{-1}))</td>
<td>0.91**</td>
<td>0.43*</td>
<td>0.74NS</td>
<td>1.18***</td>
<td>0.18NS</td>
<td>0.042 (167)</td>
</tr>
<tr>
<td>Post-stress Hct (%)</td>
<td>0.27***</td>
<td>0.049***</td>
<td>0.24NS</td>
<td>0.053**</td>
<td>0.066***</td>
<td>0.01 (168)</td>
</tr>
</tbody>
</table>

NS Not significant at P < 0.05; * Significant at P < 0.05; ** Significant at P < 0.01; *** Significant at P < 0.001.
The effect of sire age (2-year-old versus 3-year-old sires) was found to be not significant (P > 0.05) for all of the traits measured when analyzed with the four-way saturated ANOVA. The effect of sire age was therefore not a major contributor to the observed individual sire effects described above.

Genotype-by-Environment Interactions

The family-by-incubation temperature interaction was found to be significant for all traits measured except for final wet weight and [glucose]pl (Fig. A.1). The norms of reaction (Fig. A.1) indicated possible incubation temperature interactions with the sire and dam effects. The nested ANOVA (Model A) allowed analysis of the sire-by- and dam-by-incubation temperature interactions separately. The dam-by-incubation interaction was significant for all traits except for final wet weight, RG, and [glucose]pl (Table A.2), while the sire-by-incubation temperature interaction was significant for all traits except for [glucose]pl, △[glucose]pl, and △[cortisol]pl (Table A.2).

Treatment Responses

The nested ANOVA (Model C) showed that the effect of the applied stress was significant for [cortisol]pl (P < 0.005) and [glucose]pl (P < .01), however there was no significant treatment (stress) effect for the Hct values (P > .05). The [cortisol]pl and [glucose]pl mean values were higher 2 h post-stress than the control values (i.e. △[cortisol]pl and △[glucose]pl are not negative in Table A.3)

Incubation Temperature Effects

Within the nested ANOVA (Model A), there was a significant incubation temperature effect for both final wet weight, and RG over the duration of the experiment (Table A.2). Mean wet weight of the fish in the accelerated groups was greater than that of the fish in the non-accelerated groups (Table A.3), and RG was higher in the fish in the non-accelerated groups than in the accelerated groups (Table A.3). None of the stress related traits showed significant incubation temperature effects (Table A.2), and the mean
Figure A.1: Back-transformed means for the measured traits for the eight chinook salmon families (numbered as in Table A.1). Means are given for the two incubation temperatures (accelerated and non-accelerated), and the lines joining the full-sib group means are norms of reaction. Genotype-by-incubation temperature interactions are identified by crossing of these lines. RG is relative growth, Hct is hematocrit, $[\text{cortisol}]_{pl}$ and $[\text{glucose}]_{pl}$ are the plasma concentrations of cortisol and glucose respectively, $\triangle[\text{cortisol}]_{pl}$ and $\triangle[\text{glucose}]_{pl}$ are the changes in plasma concentrations of cortisol and glucose 2 h post stress. $P$ is the statistical significance of the full-sib family-by-incubation temperature interaction from the Model B ANOVA (see text).
Table A.3: Back-transformed means and confidence intervals of the traits measured on chinook salmon fry reared at two incubation temperatures (accelerated and non-accelerated). RG is relative growth rate, \([\text{cortisol}]_{pl}\) and \([\text{glucose}]_{pl}\) are the plasma concentrations of the control fish, and \(\Delta[\text{cortisol}]_{pl}\) and \(\Delta[\text{glucose}]_{pl}\) are the changes in plasma concentration due to handling stress.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Accelerated (Heated Water) Group Mean</th>
<th>Non-Accelerated (Un-heated Water) Group Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wet weight (g)</td>
<td>7.27 (7.06-7.49)(^1)</td>
<td>5.06 (4.92-5.21)(^1)</td>
</tr>
<tr>
<td>RG (% day(^{-1}))</td>
<td>3.00 (2.92-3.06)</td>
<td>3.49 (3.42-3.57)</td>
</tr>
<tr>
<td>([\text{Cortisol}]_{pl}) ((\mu g\cdot dl^{-1}))</td>
<td>5.42 (4.80-6.11)</td>
<td>3.57 (2.82-4.41)</td>
</tr>
<tr>
<td>([\text{Glucose}]_{pl}) (mg\cdot dl(^{-1}))</td>
<td>77.5 (74.4-80.6)</td>
<td>83.1 (79.8-86.5)</td>
</tr>
<tr>
<td>Control Hct (%)</td>
<td>45.2 (44.3-46.1)</td>
<td>45.2 (44.3-46.1)</td>
</tr>
<tr>
<td>(\Delta[\text{Cortisol}]_{pl}) ((\mu g\cdot dl^{-1}))</td>
<td>9.26 (7.81-10.8)</td>
<td>9.14 (7.85-10.5)</td>
</tr>
<tr>
<td>(\Delta[\text{Glucose}]_{pl}) (mg\cdot dl(^{-1}))</td>
<td>10.5 (5.32-15.9)</td>
<td>20.9 (15.2-26.8)</td>
</tr>
<tr>
<td>Post-stress Hct (%)</td>
<td>47.7 (46.5-48.6)</td>
<td>45.6 (43.6-46.8)</td>
</tr>
</tbody>
</table>

\(^1\) 95% confidence interval.
values were not very different between the treatments (Table A.3).

**Growth Rate and Body Size Effects**

To test for the overall effect of final weight and growth during the study period on the stress related traits, regression analysis was performed on the data across all family groups. Few of the stress-related parameters were correlated with either RG or final weight. However, $\Delta [\text{glucose}]_p$ was positively correlated with RG ($\text{slope} = 6.69; P < 0.01; r^2 = 0.18; n=180$). To test for family-specific relationships between final weight or RG and the stress related parameters, regression analysis was performed separately on the data from each full-sib family. Significant relationships between $[\text{cortisol}]_p$ and final weight were found in six of the eight families ($P < 0.05$). Five of the eight families had significant relationships between the Hct and wet weight values in the post-stress fish ($P < 0.01$). Multiple regression analysis showed that the incubation temperature effect was not significant ($P > 0.05$) within the regressions.

**Discussion**

**Genetic Effects**

We found significant dam and sire effects for most of the traits that we measured. Previous researchers have shown that wet weight and growth are heritable in chinook salmon fry (Cheng *et al.* 1987, Withler *et al.* 1987), and our results for the Robertson Creek stock are consistent with this work. A genetic component to stress-related traits in chinook salmon has not been previously reported, although significant sire and dam effects for $[\text{glucose}]_p$ and $[\text{cortisol}]_p$ have been found for rainbow trout and Atlantic salmon (Refstie 1982, 1986, Fevolden *et al.* 1991). Furthermore, stock and strain differences in $[\text{cortisol}]_p$, $[\text{glucose}]_p$ and Hct, have been reported for chinook salmon (Barton *et al.* 1986, Maule *et al.* 1988), coho salmon (McGeer *et al.* 1991), rainbow trout (Casillas & Smith 1977, Refstie 1982, Woodward & Strange 1987), and largemouth bass, *Micropterus salmoides*, (Williamson & Carmichael 1986); these differences have been interpreted as
evidence for a genetic component to these parameters. Our results support those interpretations.

Our results indicate that the pedigree of the fish may have a major impact on the stress-response of salmonids, and thus some knowledge of the genetic history of the fish is necessary for meaningful comparisons between groups of control and challenged fish. Furthermore, the variability among family members was considerably less than that usually reported for randomly chosen groups of individuals (Barton & Schreck 1988, Maule et al. 1989), thus full-sib or, ideally, clonal fish should be used when high sensitivity is desired for stress response experiments. The genetic effects observed in this study for [cortisol]_{pl} and [glucose]_{pl} may have been due to genetic differences in endocrine system response time, peak plasma hormone concentration, clearance rate, or any combination thereof. We were not able to determine which mechanism(s) led to the observed differences from this study.

Our mating design allowed the partitioning of the observed variation in the measured traits into sire and dam components but since the sires were nested within the dams, and only four females were used there was little power to resolve dam effects. Furthermore, the dam effects included "maternal" or "common environmental" effects (i.e. \( \sigma_D^2 = 0.25V_A + V_{CE} \), where \( V_{CE} \) is the maternal common environment effect - Falconer 1981). Maternal common environment effects are non-genetic influences, such as egg size, egg quality, etc., of the dam on her offspring. These effects have been shown to be relatively small for growth and size related measurements in salmonids of the size of those in this study (Iwamoto et al. 1984, Withler et al. 1987). However, no studies have reported the magnitude of maternal effects for stress response traits. Furthermore, since the sire component of the observed variance (i.e. \( \sigma_S^2 = 0.25V_A + 0.25V_D - Falconer 1981 \)) includes dominance effects (\( V_D \)), no unbiased estimate of \( V_A \) is possible. No estimate of heritability (\( h^2 \)) was attempted due to the uncertainty of the magnitude of the contribution of the non-additive effects (i.e. \( V_{CE} \) and \( V_D \)). Furthermore, even if the non-additive
effects were assumed to be negligible, the standard error of heritability estimates based on only 8 families would be unacceptably large.

**Genotype-by-Environment Interactions**

The family by environment interactions found for RG, [cortisol]p1, △[cortisol]p1, △[glucose]p1, and Hct of control and post-stress fish indicate that the relative ranking of the eight families is different between the two incubation temperature regimes (Fig. A.1), and thus the genetic component of the stress responses of fish varies widely depending on their environmental history. The genotype by environment interaction is even more evident when the full-sib families are broken down into sire and dam components (Table A.2).

Social interactions can affect chronic stress responses (Schreck 1981, Schreck 1990), and since the fish in this study were held in a single tank for the duration of the trial, complex behavioral interactions may have contributed to the observed genotype-by-environment interaction terms. Since the family groups were held in separate tanks prior to the trial period, tank effects would also contribute to the incubation temperature-by-genotype interaction, thus the environmental effects are not just due to incubation temperature differences but represent a combination of environmental effects. The genotype-by-environment interaction is quite apparent upon inspection of the norms of reaction presented in Fig. A.1.

Independent of the source of the interaction, our results indicate that environmental history and developmental stage of the fish is critical to the comparison and interpretation of most of the stress-related traits examined in this study. Furthermore, these results indicate that selection on these traits would be most effective on family groups with identical rearing histories. Gains realized by this selection could be lost by changes in rearing practices in subsequent generations.

**Environmental Effects**

One of the most obvious differences between the fish from the two incubation temperatures was size (Table A.3). Since smolting in chinook salmon is strongly dependent
on fish size (Clarke 1982, Clarke & Shelbourn 1985), it is possible that the accelerated and non-accelerated family groups were at different physiological stages within the smolting process. Although there was no significant differences between the accelerated and non-accelerated fish for the stress-related traits (Table A.2), it is possible that the differences in the final weight or RG of the two groups may have indirectly influenced the stress related traits. If this were the case, we would expect to find correlations between weight or RG, and the stress-related traits. It has been previously shown that resting [cortisol]p1 is not correlated with body size in salmonids (Mazur 1991, Fevolden et al. 1991), and our results support this conclusion based on the analysis across all families. We found, however, significant correlations between wet weight and [cortisol]p1 within six of the eight full-sib families. Correlations between [cortisol]p1 and weight have probably not been previously observed because of the family dependent nature of the relationship (i.e. the slope of the regression varies between families). The positive correlation between post-stress [glucose]p1 and RG probably reflects the fact that both are positively related to metabolic rate (Umminger 1977, Jobling 1983, Barton 1988).

The control values of [cortisol]p1 were relatively high (Table A.3) compared to those reported elsewhere for chinook salmon (see Barton & Iwama 1991), and may be associated with smoltification in these fish, similar to that which occurs in coho salmon (Barton et al. 1985; Young et al. 1989). Despite the high resting values, mean [cortisol]p1 increased almost three-fold two hours after the handling stress (Table A.3), indicating that the interrenal tissue was still capable of releasing cortisol in response to acute stress. However, the family groups with the highest average resting [cortisol]p1 tended to be the groups with the lowest mean [cortisol]p1 increase due to stress ($r^2=0.29; P<0.05$; slope = -0.30; N = 16). This supports the hypothesis of a negative feedback mechanism that limits the release of cortisol when the existing plasma concentrations are already elevated (Fryer & Peter 1977).
The response of fish to chronic and acute stress is very important to fish husbandry, and these results indicate that selection for tolerance to stress is probably possible. However, until the nature of the genotype-by-environmental interaction is better understood, the implementation of an effective selection program would be problematic. The genetic contribution to the variance observed in the growth-related and the stress-related traits considered in our study is very important to research which involves making comparisons between groups of fish. Precise comparisons would only be valid if the fish were of similar genetic background, or from a randomized genetic mixture. Genetically randomized groups of fish are logistically difficult to obtain, and would greatly increase within-group variance, thus reducing the precision of the experiment. Due to the limitations of our mating design no unbiased estimate of the additive genetic variance for the traits studied was possible. Our results do indicate, however that further research using a more appropriate mating design (i.e. dams nested within sires and more families) is warranted.
Appendix B


Abstract

Precocious sexual maturation in salmonid parr occurs under wild and culture conditions. We investigated the possibility of repeat maturation in chinook salmon precocious parr from the Nicola River, British Columbia. Precocious and immature (control) yearling parr were reared in fresh water from March, 1990 to mid-June, and then transferred to salt water (29-30 ppt) until September, 1990. The precocious parr were significantly larger than the control from March to July and there were no differences in relative growth rate between the groups throughout the study. Total mortalities were 45.7% and 5.9% for precocious and control fish, respectively. All the precocious, but none of the control fish expressed milt in March in fresh water. None of the fish expressed milt soon after the transfer to salt water in June, but all precocious fish and 18.8% of the control expressed milt in September. There were no significant differences in the average plasma concentrations of Na\(^+\), Cl\(^-\), and cortisol between groups in September, suggesting that both precocious parr and control groups were saltwater competent. These data demonstrate that male precocious chinook salmon parr have the physiological capability to mature more than once in sea water.
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