JUVENILE GROWTH AND GONADAL DEVELOPMENT IN SEX REVERSED FEMALE CHINOOK SALMON (*Oncorhynchus tshawytscha*)

By

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ABSTRACT

In salmon farming, the culture of all female fish avoids the problem of precocial males and reduces the costs of production. In an all female monosex population, hormonal (17α-methyltestosterone, MT) masculinization can be used to obtain XX (chromosome) males for breeding purposes. Such treatment has been successful in chinook salmon. As adults, these XX males produced viable sperm and were fully functional. The present investigation compares the juvenile growth and gonadal development of normal XY male, female, and sex-reversed XX male chinook salmon.

Ten monosex families and 10 normal families (from two farm sources) were used for comparison. In each monosex family, half of the fish were exposed to MT treatment and were raised with equal number of untreated fish in a family tank. The body weight and fork length measurements from 940 parr (4 months of age) and 884 pre-smolt (7 months of age) fish were examined. There were no significant differences in parr weight, specific growth rate, and pre-smolt weight between MT-treated fish and non-treated fish, nor between males and females, but there were significant differences in the growth rate and fork length between the stocks from the two farm sources. Precocial males have been found at the pre-smolt stage age and have not been reported previously. There was no indication that measurements taken from MT-treated fish may bias Breeding Value estimates derived from these measurements, but continued monitoring in the next few generations may be necessary to study the relationship between selection for fast growth rate and the incidence of early precocial development in MT-treated fish.

Sex was determined by histological examination of the gonads in 159 parrs and 125 pre-smolts. Forty three percent of monosex family fish were intersex at parr stage and 19% at pre-smolt stage. The particular MT treatment protocol adopted by the fish farms may have caused

transitional intersexuality in the treated fish. It would be necessary to compare different MT treatment protocols and to examine fish of different stocks to determine the source of variation. These findings suggest that the use of MT-treatment to produce XX males may not have any adverse effect on a selective breeding program.

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ACKNOWLEDGEMENTS

DEDICATION

This thesis is dedicated to my parents: My father, Dr. Krishna Ram Amatya who always encourages me to relate science and nature, and my mother, Badrishyama Amatya who is the impetus behind my academic pursuits.

CHAPTER 1

1. GENERAL INTRODUCTION

1.1. Farmed Salmonid Production

Salmonid (Atlantic salmon, Pacific salmon, trout, and steelhead) farming is one of the world's largest growing industries providing a good source of animal protein. In 1981, total farmed salmonid production was 12,000 tons. Worldwide production of farmed salmon increased from 139,700 tons in 1988 to 551,900 tons in 1995 (Table 1.1). This represents nearly a 400% increase in the number of farmed salmon in seven years. In 1995, farmed salmon production constituted about one-third of the world total annual salmon harvest (Weber, 1997). Part of the reason for the tremendous increase in world production was due to increases in the two largest farmed salmon producers, namely Norway and Chile. Norway, Chile and the United Kingdom are the biggest producers of the farmed salmon. In 1995, the combined output of these three countries comprised 80% of world production. In 2000, world production of farmed salmonid fish increased to 1,690,752 tons (FAO on line at ftp://ftp.fao.org/fi/stat/summ_00/b-1_table.pdf). Canada maintains the fourth largest producer of farmed salmon in the world (47,800 tons in 1995 and 94,201 tons in 2000). Salmon production in British Columbia (BC) alone in 1995 was close to 28,000 tons, representing 5% of the global farmed salmon production (Table 1.1). A significant increase in BC production has occurred from 1988 to 1995. In 2000, BC farmed salmon production was 49,000 tons.

Table 1.1. World Production of Farmed Salmon (Atlantic salmon, Pacific salmon, trout, and steelhead), 1988 and 1995

Countries	1988		1995	
	'000 tons	%	'000 tons	%
Norway	80.3	56.1	251.0	44.7
Chile	3.1	2.2	126.3	22.5
United Kingdom	17.6	12.3	65.0	11.6
Canada	13.2	9.2	47.8	8.5
(British Columbia)	(6.6)	(4.6)	(27.3)	(4.9)
Ireland	4.2	2.9	16.0	2.9
United States	2.0	1.4	14.7	2.6
Japan	14.1	9.9	14.1	2.5
Faeroe Islands	3.4	2.4	12.4	2.2
Other Countries	5.1	3.6	13.9	2.5
TOTAL:	143.0	100.0	561.2	100.0

Sources: Salmon Aquaculture Review (Available on-line at http://www.intrafish.com/laws-and-regulations/report_bc/v1chp2.htm)

The data given in Table 1.1 also reinforce the growth trend in total salmon production over the later half of 1990's. More recently, Egan (2000) estimates the total salmon and sea trout production in 1999 as 1.8 million tons. Similarly, Canada's production, primarily from British Columbia and New Brunswick, increased from 57,000 tones in 1998 to 78,000 tons in 1999, a 37% increase. In 1999, Canada contributed 6.2% of the total world farmed salmon production

(1,538,529 tons) and British Columbia produced more than 65% of farmed salmon in Canada (Fisheries Statistic, 2001).

In 1999, Atlantic salmon (*Salmo salar*) made up 81 % of the total Canadian production, and Pacific salmon contributed 19 % (with chinook salmon, *Oncorhynchus tshawytscha,* 16%, and coho salmon, *O. kitsuch,* 3%) (Egan, 2000). Even though production of Atlantic salmon has dominated the industry, chinook salmon were a popular species in BC since the 1980 due to their rapid growth rate and survival under pen condition relative to other Pacific salmonids (Lamont, 1990). After 1991, however, Canada's production of Pacific salmon dropped dramatically (Egan, 2000). The decline in the production of chinook salmon occurred due to both natural and human factors. Chinook salmon reared under captive or farm conditions have high incidence of precocial males (jacks). Clarke & Blackburn (1994) reported 23% to 80% jacking for stream-type chinooks. Cheng et al. (1987) found 38% jacking of Harrison River chinook salmon reared in sea-water tanks. Precocial maturation restricts the expression of the males' growth potential (Goetz, 1979). The small size and secondary sexual characteristics of jacks lower their commercial value (Heath et al., 1991).

There has been extensive research on the precocial maturation in Pacific salmon (chinook: Rich, 1920; Taylor, 1989; coho: Iwamoto et al., 1984; Lamont, 1990; sockeye: Ricker, 1959; masu: Aida et al., 1984; Kato, 1991; steelhead: Schmidt and House, 1979). Billard (1983) reported that precocial maturation is more prevalent among farmed salmonids than among wild ones. Such maturation is more likely to develop due to improved feeding and hence increased growth. There have been many attempts to control the incidence of precocial maturation in commercial stocks. One of the popular methods followed is by farming an all-female population.

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1.2. Monosex fish populations

It is not uncommon in finfish aquaculture to produce monosex populations as one sex is more desirable than other with respect to productivity or efficiency (Donaldson, 1986; Purdom, 1986; Dunham, 1990). In tilapia, males achieve a larger market size than females (MacIntosh and Little, 1995) making it beneficial to raise males only. In Pacific salmonids, the culture of all female salmonids significantly avoids the problem of precocial males (Solar and Donaldson, 1991) and reduces the costs of captive brood stock. As a result, the monosex salmon production allows the industry to become more profitable (Donaldson and Hunter, 1982; Mires, 1995). All-female populations of salmon can be produced by direct treatment of individuals with natural or synthetic estrogens (Hunter et al., 1986; Piferrer and Donaldson, 1992). To avoid hormone treatment of market fish, however, all-female strains can be produced by gynogenesis (Chourrout, 1980; Refstie et al., 1982) or by crosses between sex-reversed XX (chromosome) males and regular females.

1.2.1. Obtaining Monosex population using gynogenesis

Gynogenesis[1] provides a useful tool for manipulation of sex in salmonids (Judith, 1993). Gynogenesis is generally induced in fish by gamma or ultraviolet irradiation of spermatozoa to destroy or inactivate a specific region of the genomic DNA without affecting their ability to swim and penetrate the egg in order to activate development. Gynogens produced by egg activation with radiation-inactivated spermatozoa are haploid, carrying a single set of maternal chromosomes. Haploid gynogens generally survive through most of embryonic development,

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[1] The term "gynogenesis" describes a process whereby embryonic development is initiated without the incorporation of a functional paternal genome.

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but show characteristic abnormalities ("haploid syndrome") and usually die before yolk absorption is complete. Viable gynogens can be obtained by making diploids of such haploids either by retaining the second polar body of the activated haploid egg (accomplished by heat shock or pressure shock treatment), which is normally extruded from the egg shortly after fertilization, or by blocking the first mitotic cell division of the zygote (Benfey et al., 2000).

All female population in rainbow trout (*Oncorhynchus mykiss*) following gynogenesis (using heat shock either at 26 or 29°C) resulted in a low rate of survival, with 1.5% and 1.6% respectively (Feist, 1995). However, all of the surviving fish were females.

1.2.2. Obtaining monosex population using hormone treatment

Unlike higher vertebrates, exogenous sex steroids can influence gonadal development in most fish. Salmonids are sensitive to steroids during the period of sex differentiation (Devlin and Nagahama, 2002). Estrogens and androgens have feminizing and masculinizing effects respectively (Yamamoto, 1969), resulting in XY chromosome fish to develop into females, and XX fish into males. Recently, a DNA probe (OtY1) specific to chinook salmon males has been isolated (Devlin et al., 1991 and 1992). With the help of the DNA probe and performing polymerase chain reaction (PCR) test, genetic females can be rapidly separated from genetic males in an androgen-treated population. By this approach, genetic sex of the fish can be determined from small amount of fin tissue or blood without sacrificing the animal. Unlike the gynogenetic process, a new monosex strain of chinook salmon can be developed in a single generation. Phenotypic masculinization (XX males) can be obtained by treating gynogenetic females with androgen. Once the stocks of male fish bearing XX sperm have been obtained, cryopreserved semen from these XX males can be used to produce all-female offspring for years

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(Donaldson and Benfey, 1987; Baker et al., 1988; Feist et al., 1995). Alternatively, once a monosex population has been established, hormonal masculinization can be done each generation to obtain XX males for breeding purposes.

There have been extensive studies towards the development of sex reversal techniques for *Pacific salmonids (Table 1.2). It has been reported that the feeding of synthetic androgen, 17α-*methyltestosterone (MT), to female rainbow trout resulted in populations of up to 100% sex reversed fish. The majority of the sex-reversed fish, however, lacks or had incomplete sperm-ducts and the semen had to be removed surgically (Bye and Lincoln, 1986). This procedure is both time-consuming and detrimental to the broodstock. The sperm, however, was viable, and was utilized by macerating the tissue in diluents. Geffin and Evans (2000) observed similar results in the same species. In European seabass (*Dicentrarchus labrax*), 100% sex reversal with MT was obtained with very low percentage of deformed testes (Chatain et al., 1999). By contrast, Fiest et al. (1995) found that feeding naturally occurring steroid 11β-hydroxyandrostenedione (OHA) had a long masculinization effect and contributed to the development of an intact sperm duct in the androgen treated fish. Dietary treatment with MT in rainbow trout (Fitzpatrick et al., 1993) and in tilapia, *Oreochromis spirulus* (Lona and Ridha, 1993) resulted in 100% masculinization.

Sex reversal treatments have been more successful in chinook salmon compared to other salmonids. Piferrer et al. (1993) reported 100% sex-reversal in chinook salmon when hormonal manipulation (immersion at 400 μg/L of MT for 2-hrs at hatch) occurred before the actual differentiation of embryonic gonads. Mature XX males had testes, which were indistinguishable both in size and structure from those of genetic males and had completed all stages of spermatogenesis. At two years of age, these males produced viable sperm capable of inducing

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normal embryonic development when used to fertilize eggs, resulting production of all-female progeny.

Table 1.2. **Effects of different androgens, routes of administration on different development stage of in salmonids**

Species	Immersion		Feed	Hormone	Resulted sex	Reference
	Egg	Alevin				
Salmo salar			X	MT	M,S	Johnstone et al., 1978
			X	MT	M	Johnstone and Youngson, 1984
O. tshawytscha		X	X	MT	M,S	Hunter et al., 1983
		X		MT	M,H	Baker et al., 1988
		X		T, MT, 11KT, DHT	M,H	Piferrer et al., 1993
O. rhodurus		X		MT	M	Nakamura, 1994
O. Kisutch	X	X	X	MT	H,S	Hunter et al., 1982
		X		MT	M,H	Piferrer and Donaldson, 1989
				MT, DHT	M,F; M	Piferrer and Donaldson, 1991
				MT, DHT	H,S; S	Piferrer et al., 1994
O. masou				MT	M	Nakamura, 1994
				MT	M	Park et al., 1993
O. mykiss			X	MT	M,H	Johnstone et al., 1978
			X	MT	M	Johnstone et al., 1979
			X	MT	M,S	Solar et al., 1984
			X	MT	M	Bye and Lincoln, 1981
			X	MT	M,H,S	van Den Hurk and Slof, 1981
			X	MT	M,F,S	Solar and Donaldson, 1985
			X	MT	M,H,S	Feist et al., 1995

M= Male, F= Female, S= Sterile, H= Hermaphrodite, MT= 17-α-methyltestosterone, DHT= 17-α-methyldihydroxytestosterone, 11KT= 11-ketotestosterone, and T= Testosterone

Most of the farmed chinook salmon production in British Columbia is based on monosex female populations (Peterson et al., 1992). In 1983, a maximum of 30 MT treated sex-reversed males from the Big Qualicum (BQ) stock was used to create all-female chinook salmon for commercial production (Hunter et al., 1983). The number of other parental fish that gave rise to the domesticated strains and their subsequent breeding history during approximately five ensuing generations of domestication was not well documented.

1.3. Chinook selective breeding program

Selective breeding based on quantitative genetics seems to be the preferred strategy to improve the performance of production fish. Most research about selective breeding was applied to rainbow trout and Atlantic salmon to increase growth rate, reproductive performance, and to decrease egg and alevin mortality (Aulstad et al., 1972; Gall, 1975; Kanis et al., 1976; Gall and Gross, 1978). Recently, the selection index has been expanded to include parameters such as sexual maturity, season and age of spawning (Wild et al., 1994; Su et al., 1999). A few coho salmon breeding studies have been carried out, e.g. survivability and alevin weight (Swift, 1991; Martinez et al., 1999), length of breeding life (van Den Berghe and Gross, 1986) and improvement of market weight in coho breeding program (Peterson and Swift, unpubl. data). There were a few genetics studies (Withler, 1986; Cheng et al., 1987; McMillan et al, 1987; Winkelman et al., 1991; Bryden, 2001), and chinook salmon selective breeding based on mass selection (R. G. Peterson, Tri-Gen Fish Improvement Ltd., Vancouver, BC, 2000, pers. comm.). The Chinook Selective Breeding Program (CSBP) is the first to utilize the Animal Model BLUP (Best Linear Unbiased Predictor) technology (Peterson and Swift, 1999).

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The goal of the CSBP was to select for growth and survival to harvest age in the monosex BQ stocks (Peterson and Swift, 1999). The challenges of monosex populations for the CSBP concern with maintaining pedigree information on sex reversed fish required for the Animal Model BLUP analyses. Pedigree information for a normal population is retained by incubating each family in a separate tray in the hatchery and at ponding the fingerlings were transferred to individual family tanks. At 5-8 g the fish were PIT tagged with the tag number associated with the original single pair mating. The application of this procedure to a monosex population requires that each family be divided into two groups and exposing one of the groups to sex-reversal treatment. Both the treated and untreated groups must then be ponded in individual family tanks to retain pedigree information. This procedure requires two family tanks per family, one for the treated group and one for the untreated group. Computer simulation studies (Winkelman and Peterson, 1992) established that a minimum of 100 families would be required to maintain adequate genetic variation for the selection program. The CSBP currently has only 100 family tanks and increasing the number of tanks would be costly (R.G. Peterson, 2002, pers. comm.). The remaining options were to 1) to use mass selection and/or pedigree selection on the XX males instead of using BLUP, or 2) to pond treated and untreated fish from the same family in a single family tank and rear them to the time when PIT tags can be applied. Option 1 would reduce selection response but option 2 had never been previously tried. Option 2 would be the logical choice since if it works, selection would be consistent with a normal XX/XY population and the costs would actually be less than Option 1. The concern was whether mixing treated and untreated fish together would affect sexual maturation, growth and survival (R.G. Peterson, 2002, pers. comm.). The CSBP also started a parallel population of normal (XY) males and females for comparison. This control population was started by a cross of

males from the Robertson Creek (RC) stocks with females from the monosex BQ stocks. Through such a cross, genetic variation brought in by the RC males can also be studied.

The present investigation focuses on the comparison of the juvenile growth and gonadal development of normal XY male and sex-reversed XX male chinook salmon. In Chapter 2, the juvenile growth (weight and length measurements) at parr (approximately 4 months after hatching) and pre-smolt (approximately 7 months after hatching) stage and survival of XX males were compared with (1) XX females to examine whether the MT treatment protocol for sex-reversal has affected these two parameters, and (2) XY males to examine the effects of RC males on growth and survival. Growth and survival of females from two BQ stocks were also compared to examine differences between these two stocks. In Chapter 3, gonadal development of XX males was examined at parr and pre-smolt stages to study the effect of the particular MT treatment protocol used by the CSBP. The XX males in the CSBP were obtained by a MT treatment protocol that is different from the ones described by Piferrer et al. (1993) (David Groves, Sea Spring Salmon Farm Ltd., Chemainus, BC, 2001, pers. comm.). While XX males obtained via this protocol showed normal male phenotype and semen production at adult stage their growth and sexual development have not been examined. The overall null hypothesis to be tested was that these XX males were going through normal male sexual and gonadal development and showed similar growth and survival compared to XY males. Information obtained from my study will be useful for the implementation of the CSBP (see Chapter 4), an integral component of sustainable fish farm production in BC (R. G. Peterson, Tri-Gen Fish Improvement Ltd., Vancouver, BC, 2000, pers. comm.)

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CHAPTER 2

2. GROWTH PERFORMANCE OF SEX REVERSED FEMALES, NORMAL MALE AND FEMALE CHINOOK SALMON (*Oncorhynchus tshawytcsha*)

Abstract

The objective of the present study was to compare juvenile growth of chinook salmon *masculinized with 17α*-methyltestosterone (MT) (XX chromosome males) with normal XX females and XY males. Fish from two fish farms raised under the same conditions were examined. Body weight and fork length of 940 parr (approximately 4 months of age) and 884 pre-smolt (approximately 7 months old) fish were measured and the specific growth rates (SGR) were calculated. Sex was determined by histological examination of the gonads in a sub-sample of fish (159 parrs and 125 pre-smolts). There was no significant difference in parr weight, SGR, and pre-smolt weight between males, females and intersex fish, and there was no evidence that juvenile growth and mortality were affected by the MT sex-reversal treatment. Consequently, measurements taken from MT treated fish, therefore, should not bias heritability estimates derived from these measurements. There were significant differences in body weights and SGR of the fish from the two fish farms. The present study also found precocial maturation in males at pre-smolt stage, which has not been previously reported in the literature for this young age. Six precocial males were encountered at pre-smolt stage and their body weight was heavier than their cohorts at both the parr and pre-smolt stages.

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2.1. Introduction

The chinook salmon (*Oncorhynchus tshawystscha*) is the preferred Pacific salmon for large scale farming in British Columbia (BC). They are selected for aquaculture for their market value, their ability to grow to a large size, and their better survival capability under pen conditions relative to other Pacific salmonids (Donaldson, 1970; Withler et al., 1987). The commercial culture of Pacific salmon began in BC in the 1980s (Winkelman et al., 1991) and since then chinook salmon has been a commonly farmed species. Its popularity is second only to Atlantic salmon (*Salmo solar*). Chinook salmon spawn in fresh water, the young migrate to salt water where they spend their adult lives and then generally return to their river of origin to spawn. This homing instinct and other aspects of the life cycle suggest that chinook salmon populations are comprised of reproductively isolated sub-populations (strains or stocks) associated with each river system (Bentzen et al. 2001). Growth parameters may differ in fish from different river systems (Withler et al., 1987) and therefore the choice of fish for broodstock development may affect their response to selective breeding. Domesticated chinook salmon strains were founded with gametes derived primarily from the Big Qualicum River hatchery (BQ) on the east coast of Vancouver Island. On the west coast of Vancouver Island, gametes were also obtained in at least one year from the Robertson Creek hatchery (RC). Moreover, gametes from different wild strains such as Nitinat and Quinsam rivers were used sporadically in the early 1980s (David Groves, Sea Spring Salmon Farm Ltd., Chemainus, BC, 2001, pers. comm.).

Realizing the benefits of all-female culture in commercial salmon production (as mentioned in Chapter 1), most of the farmed chinook salmon production in BC is based on monosex female populations (Peterson et al., 1992). In 1983, a maximum of 30 MT treated sex-

reversed BQ males were used to create all-female chinook salmon for commercial production (Kim et al., 2003; Hunter et al., 1983). The number of other parental fish that gave rise to the domesticated strains and their subsequent breeding history during approximately five ensuing generations of domestication was not well documented.

The principal objectives of most salmon selective breeding programs include increased growth rate and survival to market sizes under a commercial culture system. If selection can be carried out using juvenile body weight data, it would mean additional information for the selection index. In wild chinook salmon strains reared under commercial conditions, the heritabilities for body weight and length at 4 months of age were moderate to low (0.24 and 0.27, respectively) (Winkelman et al., 1991 and 1992). Withler et al. (1987) found heritability of smolt body weight in chinook salmon varied greatly in different stocks. Their heritability estimated from the sire component for BQ stocks was 0.00 ± 0.59, but their heritability estimate using the same method for RC stocks was 0.88 ± 0.72 (Withler et al., 1987). For comparison, heritability estimate using REML (Restricted Maximal Likelihood) for log body weight of 12-week old Grover Creek Hatchery (Pudget Sound) chinook salmon was 0.995 ± 0.058 (Hard et al., 1999) Even though some of the hereditability estimates may not seem reasonable, the above comparisons point to the importance of estimating heritability of this trait in the particular stock of concern. In commercial market, harvest weight is the most important trait. Winkelman and Peterson (1994) reported that the heritability of harvest weight was 0.27 and the genetic correlations with body weights at 9 and 12 months of ages ranged from 0.38 to 0.61, respectively. They indicated that while harvest weight heritability is not high, indirect selection for harvest weight using earlier measurements would be less than 74% as efficient as direct selection. Similar results were reported for coho salmon, *O. kisutch* (Swift et al., 1991). Springate and Bromage (1985) also reported no effect of selecting for earlier body weight on the

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adult weight in rainbow trout (*O. mykiss*). Winkelman and Peterson (1994) supported Gjerde and Schaeffer's (1989) argument that in order to improve harvest weight, selection should be on harvest weight and not on previous measurements.

An interesting, but unresolved question is whether genetic merit (breeding values) of these hormone-treated fish can be estimated by taking body weight. MT is one of the potent synthetic hormones used for sex reversal. It also, however, has growth inducing capacity (Higgs et al., 1982; Schreck and Fowler, 1982). Schreck and Fowler (1982) demonstrated that all androgens (MT, testosterone and testosterone propionate) enhance growth significantly relative to controls in chinook salmon when the androgens were fed as part of the diet. Furthermore, they found that continuous exposure to high levels of MT, which caused faster sexual gonad development, might promote precocious maturation in male chinook salmon. MT may affect growth even if it is used as a sex-reversal treatment with short exposure. In their review paper, Pandian and Sheela (1995) cited Kuvampurath & Pandian (1993) that the age of maturity in MT-treated XX and XY chinook salmon was 36 months whereas untreated XY males reached maturity at 24 months. The delay in maturity helps in diverting energy to somatic growth resulting in a higher weight gain at the adult stage in the treated group. Goetz et al. (1979) revealed that growth increased by at least 15% in salmonids at the optimum doses of hormones (in sex-reversal treatments) in comparison to control fish. Baker et al. (1988) observed a positive growth response for MT sex reversed chinook salmon at around 4 months of age. In common carp (*Cyprinus carpio*), no significant difference in growth rate was observed (Komen et al., 1993).

The objective of the present study was to compare body weight of individually identified hormone treated XX males with normal XX females and XY males at the parr and pre-smolt stages using fish from the Chinook Selective Breeding Program (CSBP) (Peterson and Swift,

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1999). Specifically, the juvenile growth (weight and length measurements at parr and pre-smolt stages) and survival of XX males were compared with (1) XX females to examine whether the MT treatment protocol for sex-reversal has affected these two parameters, and (2) XY males to examine the effects of RC males on growth and survival. Growth and survival of females from two BQ stocks were also compared to examine genetic differences between these two stocks. The findings of my project will also provide information for the CSBP (see Chapter 4).

2.2. Materials and Methods

2.2.1. Experimental animals

The present study utilized the fish in families set up for the CSBP. Fish from 20 full-sib families of chinook salmon from mating fish spawned in the fall of 2000, were used for the study.

2.2.1.1. XX families

Ten families were monosex families (hereafter will be referred as XX families) originated from the BQ stocks. Six of which were BQ stocks kept by Creative Salmon Company Ltd (CS) and four were from Marine Harvest Canada (MH).

2.2.1.2. XY families

The other 10 families were normal families (hereafter will be referred as XY families) with XY males and XX females. Six were progeny of CS BQ females crossed to Robertson Creek (RC) hybrid strain (BQ X RC) males (Fig. 2.1). The other four were MH BQ females

crossed to the same RC hybrid strain males (R. G. Peterson, Tri-Gen Fish Improvement Ltd., Vancouver, BC, 2000, pers. comm.).

P_1

RC males X CS BQ females

↓

RC hybrid strain

F_1

RC hybrid strain males X   CS BQ females

↓

CS XY families

RC hybrid strain males X   MH BQ females

↓
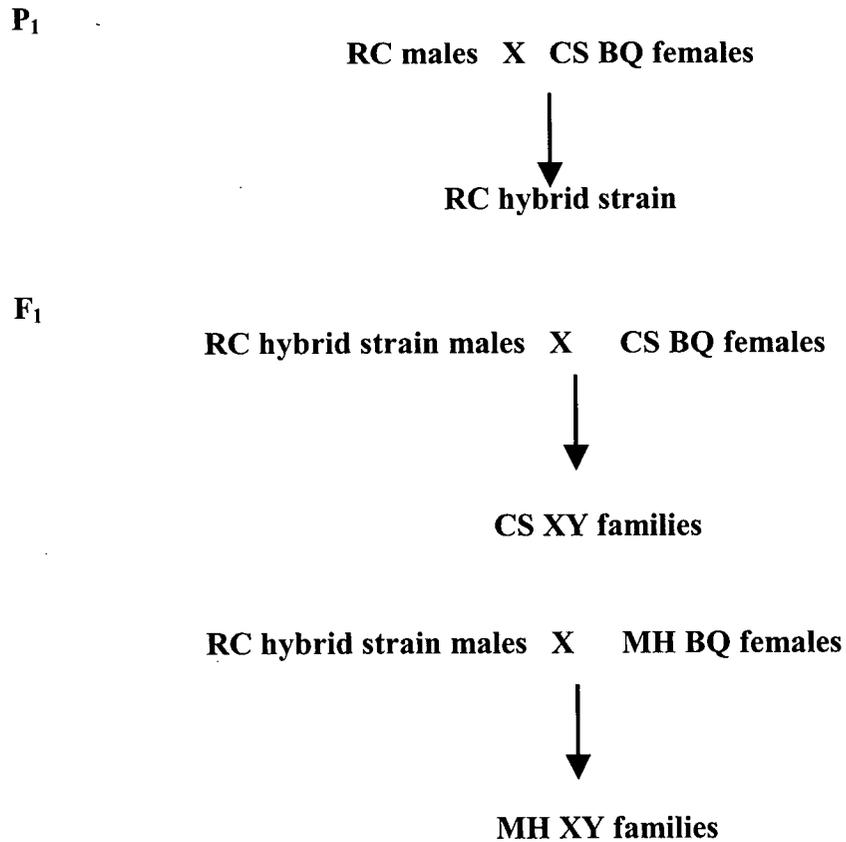
MH XY families

Figure 2.1. Flow chart showing the background of genetic make-up of the XY families of two farms (MH and CS)

2.2.2. Incubation, hatching, and rearing

Eggs were fertilized at the hatcheries of MH and CS, respectively. Eggs from MH were incubated at their own hatchery while eggs from CS were incubated at Sea Spring hatchery. The time of fertilization and the incubation environment was different, and consequently the eggs from the two companies hatched at different dates (Appendix 1). Just prior to hatching, eggs

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from MH were transported to Sea Spring Farm Ltd., Chemainus, BC, for hormone treatment. At the time of hatch, a sample of alevins from each of the XX families was treated with MT according to the protocol described below. After treatment, fish from each family were mixed with untreated fish from the same family to form the family groups. These family groups were made up to have an equal number of treated and untreated fish in each family and a sufficient number to accommodate both the selective breeding program and the fish required for this study (Appendix 1). Each family was reared separately in family tanks at Sea Spring until parr stage (June 2001, when fish were approximately 4 months after hatching and weighing approximately 8 g). At this stage approximately 100 fish per family were randomly selected for this study. Approximately 50 fish per family were sacrificed for evaluation at the parr stage and the remaining fish were tagged with passive integrated transponders (PIT). The PIT tags provide individual fish identification and a total of 966 fish were tagged for this study. Growth parameters were recorded on all fish at the parr stage and for the surviving fish at the pre-smolt stage (approximately 7 months of age). After tagging, all the families were raised in a single tank under commercial management conditions. Fish were anaesthetized with tricaine methanesulfonate (MS-222) before being handled.

2.2.3. Hormone treatment

MT treatment (500 µg/L of water in a re-circulating bath) for one hour during hatch (515 Accumulated Thermal Units in °C) was followed by two further one-hour treatments at one-week intervals (David Groves, Sea Spring Salmon Farm Ltd., Chemainus, BC, 2001, pers. comm.). Bath temperature was maintained at an average 9.8 °C (range from 9.4-10.3°C) during treatment (Piferrer et al., 1994).

2.2.4. Body weight and body length measurements

Individual body weight and fork length were measured at the parr stage. Fork length is the length measurement taken from the tip of the snout to the posterior end of the middle caudal rays (Ricker, 1979). At the pre-smolt stage (September, 2001; approximately 7 months of age), the same measurements were taken on the PIT tagged fish. A sample of parr-stage fish (non-tagged) and all pre-smolt stage fish were sacrificed (with an over-dose of MS 222) and incised laterally along the ventral surface in order to examine the gonads. Blood samples were also obtained for hormonal assays (Appendix 2). In total, 940 and 884 fish were randomly sampled at the parr stage and the pre-smolt stage, respectively. Sexes of 159 and 125 sub-samples, respectively, at the parr and pre-smolt stages were determined histologically (see Chapter 3). Three different groups were found which were male, female, or intersex. The intersex fish were those who have both testicular and ovarian tissues (Piferrer et al., 1993). Mortalities of the sampled fish in each family tank during the growing period were also recorded (Appendix 1).

2.2.5. Specific growth rate

Specific growth rate (SGR) of the individually weighed fish at parr and at pre-smolt age was calculated as follows (Taylor, 1989):

SGR = 100 x [ln (Pre-smolt weight) -ln (Parr weight)]/age difference in days

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Where ln is the natural logarithm, and the age difference in days is 97 (between June 8 and September 13, 2001).

2.2.6. Statistical Analysis

All data were analysed by Least Squares Analysis of Variance (ANOVA) or Co-varianace (ANCOVA) using JMP v4.0.4 (SAS Institute, Cary, North Carolina). Tukey's HSD test was used for mean separation for factors found significant by the ANOVA or ANCOVA. Parr body weight was analyzed using the following statistical models:

$$Y_{ijk} = \mu + T_i + F_j + (TF)_{ij} + E_{ijk}$$

or (with the smaller sample of fish with known sex)

$$Y_{ijkl} = \mu + T_i + F_j + S_k + (TF)_{ij} + (TS)_{ik} + (FS)_{jk} + E_{ijkl}$$

Where T denotes whether the fish was from an XX or XY family, F denotes whether the fish was from MH or CS, S denotes whether the fish was a male, female, or intersex. The small and unbalanced sample size prevented the examination of the TFS 3-way interaction.

Pre-smolt body weights and SGR were analysed using the same models except with parr body weight added as a covariate. SGR was arc-sine transformed before the analysis. Parr and pre-smolt fork lengths were also analysed using the same models with parr body weight or pre-smolt body weight added as covariate, respectively.

2.3. Results

2.3.1. Body measurements

2.3.1.1. Body weight at parr stage

With 940 fish sampled at the parr stage, fish from MH XX families were significantly (P< 0.0001) lighter than the other family groups (MH XY, CS XX, and CS XY; Table 2.1). With a sub-sample of 159 fish (out of 940) with known sex (by histological examination), we found no significant (P = 0.79) differences in body weight between males, females and intersex individuals, but confirmed that fish from MH XX families were significantly (P < 0.0001) lighter than fish from other family groups.

Table 2.1. Mean body weight at the parr stage (g)

	XX families	XY families
MH	6.56 ± 0.26 [b]	9.65 ± 0.25 [a]
CS	9.33 ± 0.22 [a]	9.55 ± 0.21 [a]

Means followed by different letters were significantly (P<0.0001) different from each other by Tukey's HSD test

2.3.1.2. Fork length at the parr stage

Fork length regressed significantly on body weight (P < 0.0001), with a correlation coefficient (r) of 0.94. The regression line was described as: Length = 62.8 + 3.26 x parr weight. Standardized for body weight, fish from XY families (summing over both MH and CS; 93.04 ± 0.09 mm) were significantly (P < 0.02) longer than fish from the XX families (92.71± 0.10 mm).

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After standardizing for body weight, fish from MH (summing over both XX and XY; 92.08 ± 0.11 mm) were significantly (P<0.0001) shorter than fish from CS (93.67 ± 0.9 mm). The two-way interaction (FT) was not significant. Examining the sub-sample of fish (n = 159) with known sex, there was no significant (P = 0.26) difference between males, females and intersex individuals in fork length given the same body weight. Fish from MH were still significantly (P<0.0001) shorter than fish from CS, but the difference between XX and XY families became non-significant (P = 0.61).

2.3.1.3. SGR between parr and pre-smolt stages

SGR regressed significantly and negatively (P<0.0001) on parr body weight (n = 884). The negative regression indicated that lighter parr fish grew faster during the period between the parr and pre-smolt stage than heavier parr fish. The regression line was described as: SGR = 0.018 – 0.0004 x parr weight. With or without standardizing for parr weight, fish from XX families (1.56 ± 0.007 % body weight/day) grew significantly (P< 0.0001) faster than fish from XY families (1.48 ± 0.007 % body weight/day). Fish from MH XX families, which had the lowest parr body weights, grew significantly faster than the other family groups (MH XY, CS XX, and CS XY; Table 2.2).

Table 2.2. Specific growth rate (SGR; % body weight/day) between parr and pre-smolt

  stage

	XX families	XY families
MH	1.60 ± 0.012 [a]	1.46 ± 0.010 [d]
CS	1.53 ± 0.008 [b]	1.49 ± 0.008 [c]

Means followed by different letters were significantly (P<0.0001) different by Tukey's HSD test.

With a sub-sample of 125 fish (out of the 884) with known sex, the difference between XX and XY families was confirmed (P< 0.05). There was no significant difference in SGR between the 3 sexes (P = 0.25) and between fish from the two fish farms (P = 0.28)

2.3.1.4. Body weight at pre-smolt stage

Even though MH XX families grew faster than the other family groups, they were still significantly (P < 0.001) lighter than the other family groups at pre-smolt age (Table 2.3). CS fish (40.1 $\pm$ 0.3 g) were significantly (P < 0.0001) heavier than MH fish (36.1 $\pm$ 0.4 g).

Table 2.3. Mean body weight at pre-smolt stage (g)

	XX families	XY families
MH	33.8 ± 0.54 [c]	38.4 ± 0.51 [b]
CS	41.2 ± 0.41 [a]	39.0 ± 0.42 [b]

Means followed by different letters were significantly (P<0.0001) different by Student's t-test.

With the sub-sample of fish with known sex (n = 125), we found that fish from CS XX families (41.56 ± 0.98 gm) were significantly (P<0.02) heavier than fish from MH XX families (34.18 ± 1.23 gm). There was no significant (P = 0.065) difference in pre-smolt body weight between the 3 sexes (males = 38.3 ± 0.7 g; intersex = 37.0 ± 3.25 g; females = 34.7 ± 1.39 g). There was no significant difference between XX males and XY males (P = 0.25). Power analysis revealed that the power of the model was 0.54 and that in order to detect 0.05 level differences between the sexes, a minimum of 137 fish would be required.

Pre-smolt body weight regressed significantly (P<0.0001) on parr body weight (n = 884). The regression line was described as pre-smolt weight= 11.1 + 3.1 x parr weight. If they started with the same parr weight, fish from XX families were predicted to be significantly (P<0.0001) heavier than fish from XY families. Fish from MH XX families would be significantly heavier at pre-smolt stage than the other family groups (Table 2.4).

Table 2.4. Predicted mean body weight (standardized for parr weight) at pre-smolt stage (g)

	XX families	XY families
MH	40.8 ± 0.37 [a]	36.8 ± 0.37 [d]
CS	39.2 ± 0.31 [b]	37.9 ± 0.31 [c]

Means followed by different letters were significantly (P<0.0001) different by Student's t-test.

2.3.1.5. Fork-length at pre-smolt stage

At pre-smolt stage, fork-length regressed significantly (P<0.0001) on pre-smolt body weight. The regression line was described as pre-smolt length= 10.6 + 0.1 x pre-smolt

weight. Given the same body weight, MH fish (144.2 ± 0.18 mm) were significantly (P<0.0001) shorter than CS fish (145.4 ± 0.24 mm). Examining a sub-sample of fish with known sex (n = 125), there were no significant differences (P = 0.062) in pre-smolt fork length between the 3 sexes. The difference between the 2 fish farms also became non-significant (P = 0.063). Power analysis revealed that the power of the model was 0.54 and that in order to detect 0.05 level differences between the sexes, a minimum of 135 fish would be required.

2.3.2. Mortality

From hatch to the parr stage (before PIT tagging), the 20 families that were examined had a mean mortality of 1.4%. There was no significant (P = 0.504) difference in mortality between XX families (1.8%) and XY families (1.3%). MH XX families had the highest mortality (3.2%) while CS XX families had the lowest (0.4%) but the difference was non-significant (P = 0.08) (Table 2.5). Power analysis revealed that the power of the model was 0.41 and that in order to detect 0.05 significant differences, a minimum of 25 families would be required. During the period between parr and pre-smolt stage, there was significantly (P<0.03) higher mortality in MH fish (0.12 ± 0.02) than CS fish (0.06 ± 0.02). There was, however, no significant (P = 0.32) difference between XX and XY families.

Table 2.5. Mortality rate for the period from hatch to parr stage

	XX families	XY families
MH	0.032 ± 0.008	0.012 ± 0.008
CS	0.004 ± 0.007	0.013 ± 0.007

2.3.3. Precocial males

Out of the six precocial males found, 5 were from XX families. All fish were from CS with 4 of the fish coming from one family (tank 205). These precocial males were longer and heavier than normal males at both parr and pre-smolt stages (Table 2.6).

Table 2.6. Mean fork length and weight of precocial males and non-precocial fish of the same age

	N	Parr stage		Pre-smolt stage	
		weight (g)	length (mm)	weight (g)	length (mm)
Precocial males	6*	12.12	104.00	63.23	161.00
Tank 205 fish**	48	10.23	98.17	45.50	151.90
CS XX fish	287	8.91	93.26	38.82	145.20

*4 out of 6 were from tank 205. ** minus the precocial males; Because of the small number of precocial males encountered, I did not conduct a statistical analysis.*

2.4. Discussion

I set out to test the null hypothesis that juvenile growth of chinook salmon was not affected by MT treatment. When I examined body weight at parr stage, I found that XX families from MH were significantly lighter than all the other family groups. XY and XX families from CS, as well as XY families from MH hatched between December 11 –18, 2000. MH XX families, however, hatched almost 3 weeks later than the oldest group (between January 3 – 8, 2001). I can, therefore, probably attribute the lighter weight of the MH XX families to their being younger than the other groups (Unwin et al., 2000). Incubation conditions that caused their

later hatching may also affect their initial growth rate (e.g. Atlantic salmon: Beer and Anderson, 2001; Johnston et al., 2000; pink salmon, *O. gorbuscha* and chum salmon, *O. keta*: Beacham, 1988). These younger fish also may not respond to the handling and PIT tagging as well as the older family groups and suffered higher mortality (Quinn and Unwin, 1993).

When I compared parr body weight of XX and XY families from CS, which were about the same age, there was no significant difference. In a smaller sample of fish with known sex from both farms, I also did not find significant (P = 0.79) differences in parr weight. I can conclude that parr weight was not affected by the MT treatment. There were no significant differences in pre-smolt body weights, and SGR between males, females, and intersex individuals. There was however, a trend that female pre-smolt weight was lighter than male and intersex pre-smolt weight, which did not seem to differ from each another. This is an indication that there may be sexual dimorphism in body weight at pre-smolt stage, and the MT-treated fish were behaving like normal XY males. Nevertheless, Peterson et al. (1992) did not find sexual dimorphism in pre-smolt weight in chinook salmon.

Withler et al. (1987) found that smolt weight was solely determined by maternal effects and non-additive genetic variance, but there is ample evidence that male and female salmon smolt weight does not differ, and divergence in growth rates takes place when maturation has begun (e.g. chinook salmon: Peterson et al. 1992; Shearer and Swanson, 1998; Atlantic salmon: Thorpe, 1977; coho salmon: Dittman et al. 1998).

During the period between parr and pre-smolt stages, fish from XX families grew significantly faster than fish from XY families. MH XX family fish grew faster than fish from MH XY families but their faster growth could be attributed to compensatory growth (Maclean and Metcalfe, 2001). The faster growth rate of the CS XX family fish compared to CS XY families cannot be attributed to compensatory growth. I regressed SGR on parr weight and the

model predicted, as expected, that if every fish started out with the same parr weight, XX family fish would be significantly heavier than XY family fish at pre-smolt stage. There was no significant difference in SGR between females, intersex fish, and males. Thus, it cannot attribute the difference between XX and XY families in SGR to MT treatment. XX family fish were mostly BQ stocks whereas XY family fish (from both MH and CS) were crossed with RC in the previous generation. The slower SGR in the XY family fish may, therefore, be attributable to the alleles brought in from the RC stock. Withler et al. (1987) have also reported that parr growth rate and smolt weight in RC chinook salmon were significantly slower and lighter, respectively, than BQ chinook salmon.

There was no significant difference in parr weight (comparison in CS fish) and mortality rate between XX and XY family fish. However, XY family fish had slower SGR than XX family fish. Heterosis that could have been generated by the BQ x RC cross was, therefore, not apparent. Cheng et al. (1987) found no heterosis for body weight in crosses between Capilano and Harrison rivers' chinook salmon. Gjerde and Refstie (1984) also did not find significant heterosis for either growth rate or survival in crosses of five strains of Atlantic salmon. My experimental design did not allow me to quantify positive or negative heterosis.

I found evidence that the BQ stocks from MH and CS were different in growth parameters. Both at the parr and pre-smolt stages, MH fish were shorter than CS fish after standardizing for body weight implying that their body shapes may be different. The SGR of MH fish was also faster than CS fish. Given that the XX families were BQ stocks from the two farms, respectively, and the XY families were the two BQ stock (either MH BQ or CS BQ) females mated to the same RC cross (RC x BQ) males, the differences can be attributed to the BQ stocks. Kim et al. (2003) using microsatellite DNA marker analysis of the same two BQ stocks also reported differences in genetic variation between them.

At the pre-smolt stage, I encountered 6 (out of 884 fish) precocial males. A search in literature found no report of precocial males at this early age. Healey (1991) and Heath et al. (1991) observed precocial males only after 1 year at sea (see also Shearer and Swanson, 1998). Schreck and Fowler (1982) also found precocial males in yearling MT-fed chinook salmon. Taylor (1989) and Foote et al. (1990) have reported precocial male chinook salmon at the pre-smolt stage (14 months), but their fish were stream-type salmon that stayed in fresh water until they were after 1 year old.

The precocial males in my study were heavier than their cohorts at parr stage. Four of them came from the same family and their sibs' body weight was also heavier than other families in the same group. The results of the present work were consistent with result that precocial males were heavier than the normal males at the early age (Rowe and Thorpe; 1990; Heath et al., 1991; Bernier et al., 1993; Heath et al., 1996), although none of these studies has measured weight differences as early as my study. More rapid growth has been associated with a higher incidence of precocious maturation in chinook salmon. In monosex all female populations, one would not have to worry about increasing the incidence of precocial males (obviously, as there are no males in the population) when selecting for faster growth rate and heavier harvest weight. Such selection, however, may alter their sensitivity to MT treatment with respect to induction of precocial development at a younger age. The results from my study provide the basis for further study to test this hypothesis.

2.5. Conclusions

There were no significant differences in parr weight, SGR, and pre-smolt weight between MT-treated fish and non-treated fish or between males and females. Consequently, my results

indicate no bias estimates of genetic merit derived from the measurements taken from MT-treated fish. There were significant differences in the growth rate and fork length between the MH and CS BQ stocks. Juvenile growth rate has been found to affect the overall productivity (Jonasson and Gjedrem, 1997). Body length may also relate to adult survival (Taylor, 1986; Ewing and Ewing, 2002) in wild fish. The relationship between body length and adult survival in farmed population has not been studied Further studies on older fish are therefore necessary. Precocial males have been found at a very early age in these populations. Continued monitoring in the next few generations will be necessary to study the relationship between selection for fast growth rate and the incidence of early precocial development in MT-treated fish.

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CHAPTER 3

3. GONADAL DEVELOPMENT IN SEX REVERSED FEMALE, NORMAL MALE AND FEMALE CHINOOK SALMON (*Oncorhynchus tshawytcsha*)

Abstract

In order to compare gonadal development of sex-reversed female (fish carrying XX chromosomes masculinized with 17-α-methyltestosterone (MT)) with normal male (XY) chinook salmon, 10 monosex (all XX) families, with half of the fish in each family exposed to MT treatment, were compared with 10 normal families (with both XX and XY fish). At parr stage (approximately 4 months of age), 43% of the fish from XX families were histologically identified as intersex, based on gonads of predominantly testicular tissue interspersed with some ovarian tissue. In the normal families, the few intersex fish (7%) found had predominantly ovarian tissue interspersed with little testicular tissue. At pre-smolt stage (approximately 7 months of age), the percent of intersex fish in XX families were reduced to less than 19%, indicating that some MT treated XX fish may have transitional intersexuality due to the prolongation of the indifferent stage or extension of the differentiation process. Intersex fish were not common even in other hormone treated chinook salmon studies and the transitional intersexuality found in this study may be related to the particular MT treatment protocol employed. Five of the 6 precocious males encountered at pre-smolt stage in this study were from XX families and histological examination showed complete spermatogenesis in their gonads.

3.1. Introduction

The chromosomal or genetic sex of an embryo is determined at fertilization. Sex-linked morphological characteristics, however, do not begin to develop until a certain stage of development depending on species. In most fish, the early genital systems in the two sexes are similar. Therefore, the initial period of genital development is referred to as the indifferent stage of sexual development. In the indifferent teleosts, both male and female germ cells are grouped into areas containing germ cells of similar sex or, even more often, are scattered throughout the gonadal tissues (Gilbert, 1991). For testicular differentiation, the number of female germ cells is reduced, and vice versa for ovarian differentiation. Sex-determining mechanisms in these lower vertebrates are diverse, labile and easily influenced by environmental factors (Adkins-Regan, 1987). Due to the plasticity of gonadal development, sex can be mediated by exogenous sex steroids, with the estrogens and androgens having, respectively, feminizing and masculinizing effects. If applied properly, XY chromosome fish can develop into females, and XX fish into males. Salmonids are sensitive to exogenous steroids immediately after hatching, prior to sex differentiation and first feeding (Donaldson and Hunter, 1982; Hunter et al., 1986; Baker et al., 1988; Piferrer et al., 1993). Histological evidence of sex differentiation occurs only after hatching (Foyle, 1993).

In chinook salmon, Piferrer et al. (1994) obtained sex-reversed females (XX) by exposing alevins to 17-α-Methyltestosterone (MT) at 400 µg/ L for two hours. The XX males at 2 years of age were indistinguishable from regular XY males in appearance, growth, gonadosomatic index, and rate of maturity. Both fertilization rate and progeny embryonic development rates were normal, and 100 % of the progeny were females. Similar results were reproduced in rainbow trout (*O. mykiss*) when treated with MT (Feist, 1995). However, this sex-reversal technique

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failed to achieve success with Atlantic (*Salmo salar*) (Johnson & MacLachlan, 1994) and coho salmon (*O. kisutch*) (Piferrer and Donaldson, 1989). The gonads of fish are generally believed to become refractory to steroids after the sexes are determined (Ashby, 1957; Yamamoto, 1969; Piferrer et al., 1993). Piferrer et al. (1993) have found no intersex in 6 and 11 month old MT treated (400 – 2000 µg/L) XX chinook salmon. To the best of my knowledge, no intersex has been detected in normal male gonadal development during the parr and pre-smolt stages.

The XX males in the CSBP were obtained by a MT treatment protocol that is different from the ones described by Piferrer et al. (1993) (David Groves, Sea Spring Salmon Farm Ltd., Chemainus, BC, 2001, pers. comm.). While XX males obtained via this protocol showed normal male phenotype and semen production at adult stage, their growth and sexual development have not been examined. The main objective of the present study was to compare histological gonadal development in XX males and XY males in chinook salmon at both parr and pre-smolt age.

3.2.   Material and Methods
3.2.1. Experimental animals

The present study utilized fish in families set up for the breeding program. Fish from 20 families of chinook salmon, which were spawned in the fall of 2000, were used for the study. Ten families were monosex families (XX families) originated from the BQ stocks. The other 10 families were normal families (XY families). Six were progeny of CS BQ females crossed to a Robertson Creek (RC) hybrid strain (BQ X RC) males. The other 4 were MH BQ females crossed to the same RC hybrid strain males (Ray G. Peterson, 2001, pers. comm.).

3.2.2. Incubation, hatching, rearing, and hormone treatment

See Chapter 2 for details.

3.2.4. Visual and histological sex determination

Total of 940 fish were randomly sampled at the parr stage and 884 fish were sampled at the pre-smolt stage. The fish were killed with a lethal dose of MS-222. Gonads were exposed by laparotomy, examined visually for sex determination. They were then dissected and collected in individually marked histocassettes. The gonads were fixed and preserved in 10% phosphate buffered formalin for histological examination. A sub-sample of the gonad specimen (n = 215 at parr stage and 125 at pre-smolt stage) were sectioned at 5 μm following paraffin embedding and then stained with Hematoxylin-Eosin. The remaining ones were archived. In order to confirm the sex of fish, single and deeper sections (for pre-smolt stage fish) and serial sectioning (for parr stage fish) histological slides were made. At parr stage, the male gonads were very small. A few samples (n = 38) were lost during processing and could not be examined histologically for sex identification. Fish were categorized as female, male, or intersex. The intersex category consisted of fish having gonads which contained both spermatogonia and oocytes, either segregated in different parts of the gonad or intermingled together (Piferrer et al, 1993). At parr stage, about equal number of visually identified males and females were examined histologically. Since more intersex were found in visually identified males at parr stage, many more visually identified males than females were examined histologically at pre-smolt stage to obtain more intersex fish for study.

3.2.4. Mortality

Mortalities of the sampled fish in each family tank during the growing period were recorded daily up to the date of PIT tagging. Mortality between hatch (after initial MT treatment for the XX families) and parr stage and between parr and pre-smolt stage was calculated separately (See Chapter 2).

3.3. Results

3.3.1. Sex determination by visual examination

The sex of fish was visually identified by the morphology of its gonads. At parr stage, while females could be distinguished from males by a pair of thin yellow colored strands (ovaries) along the ventral side of the air bladder, testes appeared to be thin white strands that were difficult to be seen by the naked eyes. On the other hand, at pre-smolt stage, both ovaries and testes were clearly distinguishable from one another. Ovaries were orange colored granular enlargements at the anterior portion of the gonads and testes were pale colored and smooth textured (Figure 3.1). At both stages, less easily distinguishable gonads were examined under a dissecting microscope for confirmation.

Figure 3.1. Visual examination of male gonads at the pre-smolt stage

Visual sex determination showed that the male-female sex ratio at parr stage was about 47:53, whereas at pre-smolt stage, it was 52:48 (Table 3.1). All sex ratios were not significantly different from 1:1 male:female ratio.

Table 3.1. Sex ratio by visual sex determination (%)

		N	Male : Female	χ^2 value
Parr stage	XX family	465	42 : 59	2.56
	XY family	423	52 : 48	0.12
	Total		47 : 53	
Pre-smolt stage	XX family	435	52 : 48	0.06
	XY family	448	53 : 47	0.28
	Total		52 : 48	

3.3.2. Sex determination by histological examination

3.3.2.1. Gonad histology of parr stage fish

At the parr stage, histological analysis of, XY and XX males had unrestricted lobular testes with smaller and darker staining germ cells (Figure 3.2A), whereas females showed the presence of oocytes and a gonad with a lamellar structure (Figure 3.2B and 3.2C). By definition, XX males and XY males had testicular tissues only and XX females (from both XX and XY families) had ovarian tissues only (Figure 2.2A). From XX families, we also observed intersex fish that had large amount of testicular tissue interspersed with some ovarian tissue (Figure 3.2D).

(A)

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(B)

(C)
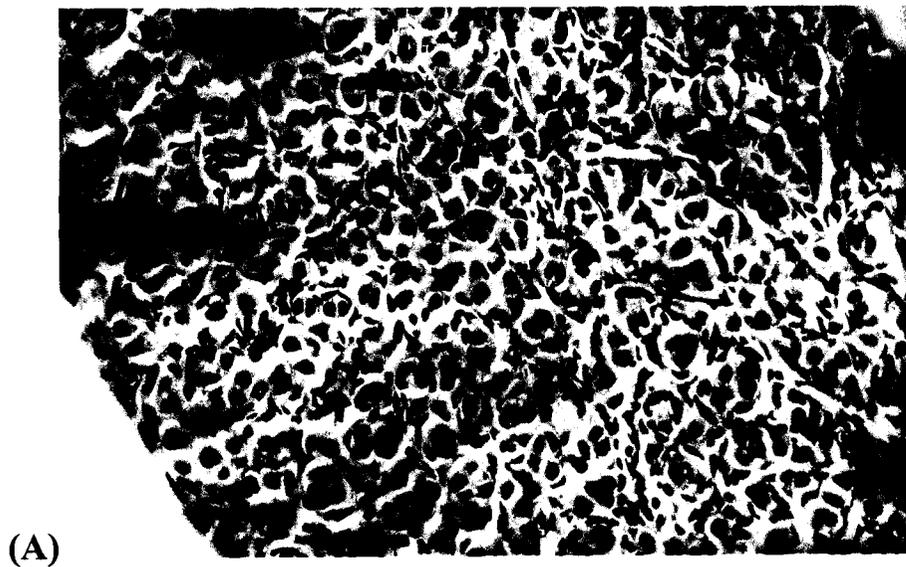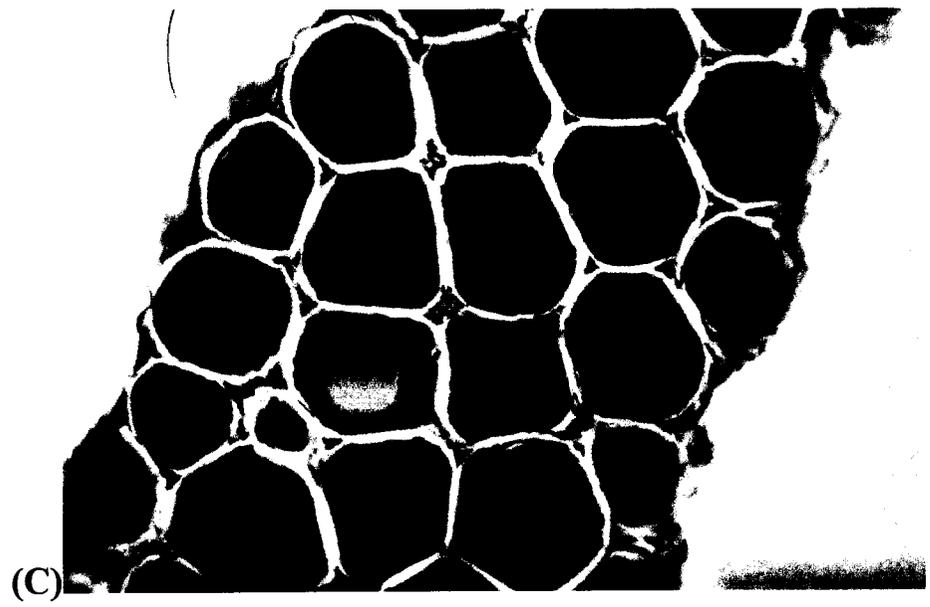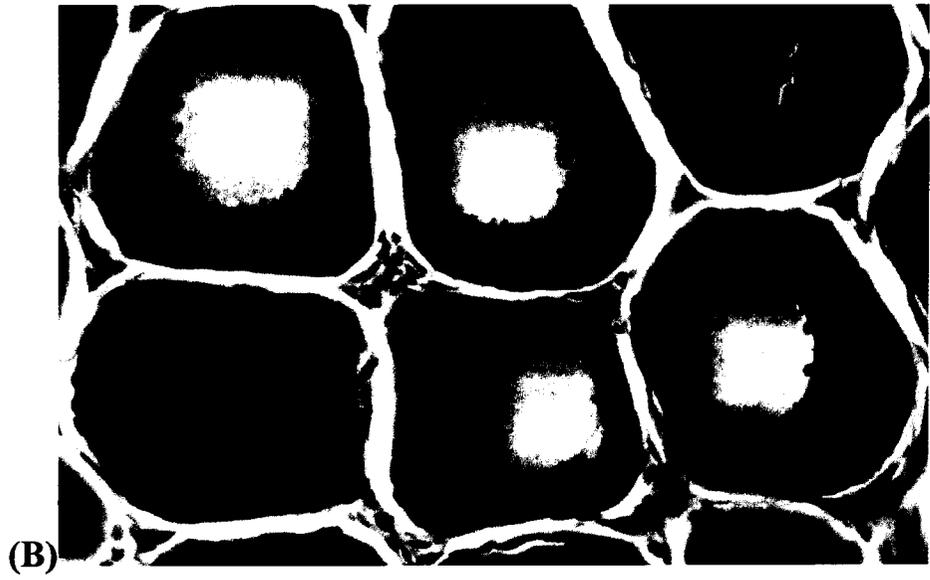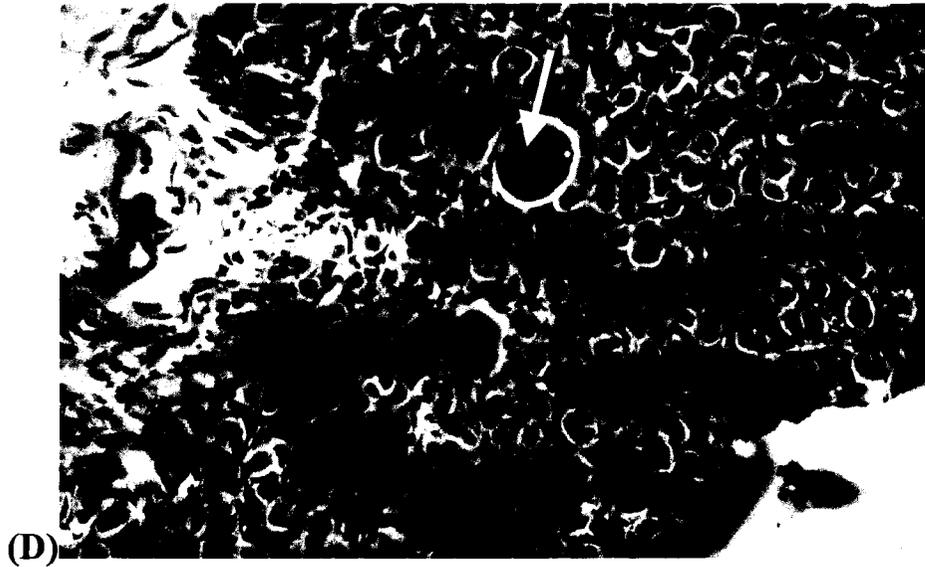
Figure 3.2. Histological slides of gonads of chinook salmon at the parr stage. (A). Testis. Circular cells are spermatogonial cells (350X). (B) and (C). Ovary. In chinook, development of ova is synchronous. Small cells surrounding ova are granulosa cells (350X and 175X). (D). Intersex gonad from an MT-treated fish with developing ova (arrow) amidst testicular tissue (350X).

Seven individuals from XY family (6 from tank 222), visually sexed as females, turned out to be intersex fish with a large amount of ovarian tissue interspersed with testicular tissue during the parr stage, whereas no intersex individuals were observed in this family at the pre-smolt age (Table 3.3).

3.3.2.2. Gonad histology of pre-smolt stage fish

At the pre-smolt stage, only testicular tissues were observed in visibly identified males in both XX and XY families (Figure 3.3A). Similarly, only ovarian tissues were observed in females (Figure 3.3B). Intersex fish had both testicular and ovarian tissues. Intersex fish from XX families had testicular tissue with interspersed ovarian tissue, whereas intersex fish from XY families had predominantly ovarian tissue with a small amount of testicular tissue at the tip of the gonads (Figure 3.3C).

(A)
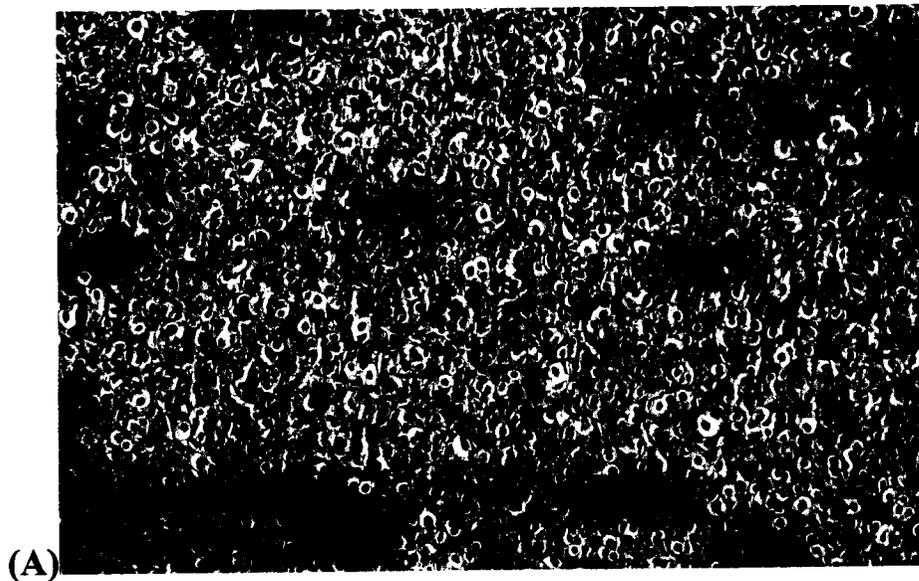
Figure 3.3. Histological slides of chinook salmon gonads at pre-smolt stage. (A). Testis. Circular cells are spermatogonial cells (175X). (B). Ovary (175X). (C). Intersex gonad from an MT-treated fish. Testicular tissue interspersed with developing ova (arrow) (175X)

3.3.3. Occurrence of intersex

At parr stage, 43 % of the fish from XX families were identified by histology as intersex. At pre-smolt stage, the occurrence of intersex was reduced to 19% (Table 2.2). It should be noted that at parr stage, about equal number of visually identified males and females were examined histologically. But at pre-smolt stage, many more visually identified males than females were examined (Table 3.2). The percentage of intersex would have been lower than 19% if the same number of visually sexed males and females were examined.

Table 3.2. Sex determination by histological examination

	Males	Females	Intersex	Unidentifiable	Sample size	% Intersex
Parr stage						
XX families	13	30	47	19	109	43
XY families	35	45	7*	19	106	7
Pre-smolt stage						
XX families	35	23	14	0	72	19
XY families	46	7	1	0	54	2

* 6 out of 7 from one family (tank 222)

Among XX families, 58 % of CS fish were intersex individuals at the parr stage while 43% of MH fish were intersex individuals. The difference was not significant (χ^2 = 2.04, P< 0.05)

3.3.4. Accuracy of visual sexing

Accuracy of visually sexing at parr stage was 52% and 69% for males and females respectively and at pre-smolt stage, 85% and 90% respectively for males and females (Table 3.3). The accuracy of visual sexing at parr stage would be significantly higher if intersex fish were not encountered (see XY families).

Table 3.3. A comparison of visual sexing and histological sexing

Stage		Visual M	Histology of visual-sexed M			Visual F	Histology of visual-sexed F		
			M	F	F/M		M	F	F/M
Parr	XX families	56	13	11	32	53	2	37	14
	XY families	53	44	6	3	53	3	46	4
	Total	109	57	17	35	106	5	73	28
Pre-smolt	XX families	49	35	2	12	22	0	21	1
	XY families	45	45	0	0	9	1	7	1
	Total	94	80	2	12	31	1	28	2

M = males; F = females; F/M = intersex

3.3.5. Precocial males

During the course of gonad sampling at pre-smolt stage, 6 "bloated" males were found. When their gonads were examined, they were found to be precocial males with well-developed testes that filled up most of the abdominal cavity (Figure 3.4A). Of the 6 precocial males, 5 were from XX families and only one from XY family. Four of the 5 XX males were from one family (tank 205). Histological examination shows complete spermatogenesis in their gonads (Figure 3.4B).

(A)
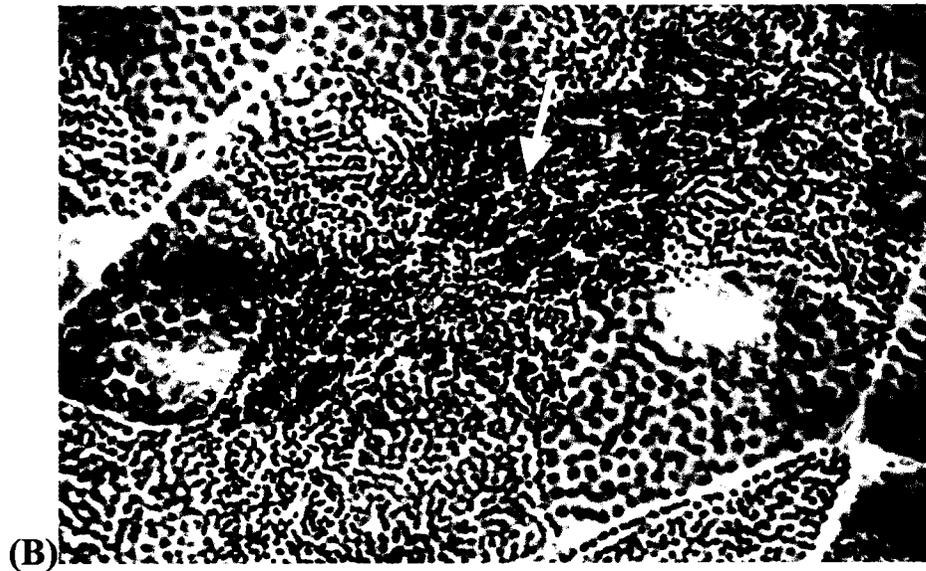
Figure 3.4. (A) Precocious male and well-developed testis covering most of the abdomen. (B) Testis filled with seminiferous tubules containing mature spermatozoa (arrow) (175X)

3.3.6. Mortality

Mortality rate from hatch to parr stage was 1.4% and between parr and pre-smolt stages was 8.4%. There was no significant difference in mortality between XX and XY families during both stages. (see Chapter 2).

3.4. Discussion

The present study was designed to test the hypothesis that XX fish masculinized with MT (using the treatment protocol adopted by the CSBP) have similar gonadal development as XY males. Visual observation of the gonads of MT treated XX fish detected no difference from XY males, except at pre-smolt stage 5 precocious males were found in the XX families and only one in the XY families. The sex ratio in the XX families as determined by visual observation was not

different than 1:1, indicating, by this parameter, that the MT treatment was close to 100 %

effective in masculinization. Histological examinations, however, revealed that, at parr stage,

there were 43 % intersex fish in the XX families (among all fish in the families, only half of

which were MT treated) but only 7 % intersex fish from the XY families. Pifferrer et al. (1993)

found no intersex fish with MT (400 to 2000 µg/L) treated XX chinook salmon at 6 and 11

months of age. Even with lower dosages, less than 10 % intersex fish were detected (among MT-

treated fish). In the present study, all the intersex fish in the XX families may not be the result of

hormone treatment. Hormone treated XX fish (unmarked at this stage) were not raised separately

but were mixed in equal numbers of untreated fish. Therefore, the possibility that some of the

intersex fish may develop from untreated fish cannot be eliminated. Nevertheless, Piferrer et al.

(1993) found no intersex fish at 6 and 11 month of age in untreated XX chinook salmon. In the

present study, only 7% of the fish from the XY families were intersex at the parr stage. These

intersex fish mostly came from one family and their intersex condition (mostly ovarian tissue

interspersed with little testicular tissue) seems to be different from those encountered in the XX

families. I have not encountered any intersex fish in XY families that have similar gonadal

histology as the intersex fish (with gonad histology similar to that described by Piferrer et al,

1993) in our XX families. I can, therefore, assume that most if not all of the intersex fish from

the XX families were the result of MT treatment, and my results were different from those

reported by Piferrer et al (1993). Contrary to the present study, Pandian and Sheela (1995) also

observed no intersex fish at 6, 14 and 18 months of age of MT treated chinook. Similarly, Feist

et al. (1995) has reported no intersex in rainbow trout with similar hormone treatment. On the

other hand, Baker et al. (1988) described intersex also at an early age (122 days post-hatch) of

androgen treated chinook salmon. Piferrer and Donaldson (1989) exposed XX coho salmon to

400 µg/L MT at hatch, one week, two weeks, and 3 weeks after hatch, respectively. They found

5% intersex fish in the group that was treated at hatch and 2% intersex fish in the group that was treated 3 weeks after hatching. These fish were sampled at 4 months of age.

The genetic background of the fish stocks (XX families) in the present study was similar to Piferrer et al. (1993) i.e. mostly fish from the Big Qualicum River (Kim et al., 2003). There was also no difference in the percentage of intersex fish between MH BQ and CS BQ families in my study. The incubation conditions were similar to that reported by Piferrer et al (1993). The MT treatment protocol used in this study, which is different from that of Piferrer et al (1993), may cause the high frequency of intersex fish at the parr stage.

At the parr stage, there were three times as many intersex fish as males in the XX families. By the pre-smolt stage, the number of intersex fish decreased drastically (less than half the number of males). The reduction in intersex fish was not because of differential mortality. There was no difference in the mortality rate between XX and XY families (see Chapter 2), and the mortality rate was too low to account for the disappearance of intersex fish at the pre-smolt stage. Piferrer et al. (1993) reported no significant difference in mortality between their treated and untreated fish. Frederick et al. (1979) mentioned that treatment of fish with steroids during early life stages might result in transitory gonadal conditions that could be partially or completely reversed by the time of sexual maturity. It can be hypothesized that some of these MT treated XX fish in my study may have extended the sexual differentiation process. Although the dosage of MT used in the treatment protocol was within the optimum range recommended by Piferrer et al (1993), a multiple exposure scheme was adopted with the initial exposure for one hour. The total water volume used and the number of fish to be treated in the same volume of water also differed significantly. Piferrer et al. (1993) and Piferrer and Donaldson (1989) commented that timing is crucial in the effectiveness of the MT treatment. The response to exogenous androgen changes dramatically during the ontogenetic process. Piferrer and

Donaldson (1989), after reviewing studies by Yamamoto (1969) and Hackman and Reinboth (1974), concluded that whereas the administration of exogenous female hormone to XY fish would reinforce an already occurring process, the administration of exogenous male hormone to XX fish would have to counteract the effect of endogenous female hormone. A much higher dosage and a critical timing would therefore be required. Without the co-administration of an aromatase inhibitor, some MT may also be aromatized into estrogen (Piferrer et al., 1994). There may also be genetic variation in the sensitivity to MT treatment (see Chapter 2 discussion).

In the present study, histological examination of immature females from both XX and XY families found synchronously developed oocytes in the ovaries. These oocytes were organized in lamella (Figure 3.2 and 3.3). Testicular development with very small spermatogonial cells was observed in immature XX and XY males, and the XX male gonads were indistinguishable from those of XY male controls. The histology of these immature gonads was similar to those described (Patt and Patt, 1969; Robertson, 1953; Nakamura et al., 1974; van den Huwk and Slof, 1981; Piferrer and Donaldson, 1989; Fitzpatrick et al., 1993; Piferrer et al., 1994; and Lombardi, 1998). XX males have been used extensively to produce monosex chinook salmon and there has been no report of abnormality in their semen production and fertility. No intersex fish has been encountered after sexual maturation (David Groves, Sea Spring Salmon Farm Ltd., Chemainus, BC, 2001, pers. comm.). Whether the transitional intersexuality of the treated fish has long-term effects, however, remains to be determined. The intersex fish encountered in the XY families were predominantly female with only very little testicular tissue remaining at the tip of their ovaries. It is likely that either a genetic effect or a tank effect has caused a slight delay in completing the differentiation process. No intersex fish was seen from this family at the pre-smolt stage.

In a sampling of 884 pre-smolt fish, 6 precocial males were encountered, 5 of which came from XX families (4 from one family and 1 from another). The occurrence of precocious maturation of males is common in cultured and wild Pacific salmon (Billard, 1983; Bernier et al., 1993), but precocious males have not been reported at pre-smolt age. Foote et al. (1990) observed precocious males in yearling chinook salmon, whereas Healey (1991) and Heath et al. (1991) observed them after 1 year at sea. Schreck and Fowler (1982) administered MT in fish feed to chinook salmon to examine its growth promoting effect and reported encountering precocious males (older than 7 months of age). They speculated that high exposure to the hormone may have induced precocious development in the treated fish. In the present study, very few precocious males were encountered at the pre-smolt stage and no conclusion can be drawn from such a small sample. The onset of precocious development seems to be quick, as we have not encountered any fish in the intermediate stages of testicular maturation in our histological examinations. Encountering "bloated" fish at this stage, however, was not uncommon in these stocks (Bruce Swift, pers comm.). Before histological identification of these as precocious males, bloated fish were treated as sick fish and were eliminated from the population. Whether there is genetic variation in the sensitivity to MT treatment, which may facilitate precocious sexual development in the more sensitive fish remains to be determined.

3.5. Conclusion

In the present study, the gonadal development of XX and XY males were different at the two ages that they were examined. It was hypothesized that the particular MT treatment protocol adopted by the fish farms may have caused transitional intersexuality in the treated fish. The duration of this transitional intersexuality did not seem to last much beyond the pre-smolt stage.

It would be necessary to compare different MT treatment protocols and also to examine older fish to answer some of the questions raised in the present study.

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CHAPTER 4

4.   GENERAL SUMMARY AND CONCLUSION

The tendency for precocial maturation of cultured chinook salmon results in males with small size, poor flesh pigmentation and deteriorating flesh quality prior to harvest (Hunter et al., 1982, Gobantes et al., 1998). Precocial males represent a significant loss of income to fish farmers. Female chinook salmon can be grown consistently to optimal market size prior to sexual maturation, making the development of all-female monosex stocks for aquaculture desirable. Monosex chinook salmon strains have been developed in the BC aquaculture industry through use of MT treated sex-reversed males (Hunter et al., 1983). All sperm produced by these phenotypic (and functional) males carry the X chromosome, and the mating of such males with normal females results in all-female progeny production. Each generation some of the all-female eggs are sex-reversed with MT during hatching in order to provide the monosex male parents for the next generation.

It has been demonstrated that all-female culture has advantages over a regular culture with males and females in commercial salmon production, both in terms of management and in economics. Selective breeding programs to improve production performance with monosex populations, however, have seldom been carried out prior to the development of monosex chinook stocks, and represent some challenges relative to dealing with normal populations with both XX and XY fish (R. G. Peterson, 2002, pers. comm.). Following are some concerns either general to monosex populations or specific to the CSBP.

4.1. MT treatment for sex-reversal

Androgen treatment has in most cases been very effective in inducing masculinization of fish (Hunter and Donaldson, 1983). The most common androgen employed in sex-reversal has been MT, being effective in over 25 species examined (Devlin and Nagahama, 2002). Effective immersion dosages are approximately 50 – *1000 µg/L of water, but the requirement varies* widely with different species and treatment regimes (Devlin and Nagahama, 2002). The treatment protocol adopted by the fish farms under the CSBP involved exposing alevins to 500 *µg of MT pe*r litre of water for an hour during hatching, and two weekly hour-long exposures after that. This protocol has been used since the 1980's when the monosex populations were first developed. Despite more recent findings that 100% masculinization can be achieved in chinook *salmon with only a single immersion in MT at 400 µg/L of water for two hours at hatching* (Piferrer et al., 1993), the protocol continued to be used. This is probably because the protocol has been effective and XX males obtained via this protocol showed normal male phenotype and semen production at adult stage, thus changes were not warranted. There have been serious concerns, however, with this protocol (Bruce Swift, 2002, pers. comm.). While the concentration *of MT (500 µg/litre of water),* bath temperature (9.4-10.3°C), and the regime of exposure (three weekly hour-long exposures starting at hatch) have been standardized, the volume of solution used and the number of fish placed in a given volume of solution varied greatly from farm to farm, from hatch to hatch, and from one exposure to another. These are factors not specified in the treatment protocol. In my study, 43% of the fish sampled at parr stage were intersex fish. Assuming equal numbers of treated and untreated fish were sampled, 86% of the treated fish would be intersex individuals at this stage. All other MT sex-reversal studies reported less than

12% intersex fish at various stages. While the intersexuality appeared to be a transitional one, as many fewer intersex individuals were found three months later at pre-smolt stage, it nevertheless indicated that the treatment protocol might be sub-optimum. Without further studies, I would not know whether the large amount of intersexuality observed was particular to this hatch (because of the variables mentioned above) or particular to these BQ stocks. I also raised the question whether selective breeding for improving growth rate, and repeated exposure to MT generation after generation, would alter the fish's sensitivity in response to MT treatment.

4.2. Rearing treated and untreated fish in the same tank

The challenges of monosex populations for the CSBP concern with maintaining pedigree information on sex reversed fish required for the Animal Model BLUP (Best Linear Unbiased Predictor) analyses. Pedigree information for a normal population has been retained by the following procedure: Single pair matings (one male x one female) were carried out to create full-sib families. Each family was incubated in a separate tray in the hatchery and at ponding the fingerlings were transferred to individual family tanks. At 5-8 g the fish were PIT tagged with the tag number associated with the original single pair mating. This would provide the appropriate pedigree information. The application of this procedure to a monosex population requires that each family be divided into two groups and exposing one of the groups to sex-reversal treatment. Both the treated and untreated groups must then be ponded in individual family tanks to retain pedigree information. This procedure requires two family tanks per family, one for the treated group and one for the untreated group. Computer simulation studies (Winkelman and Peterson, 1992) established that a minimum of 100 families would be required

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to maintain adequate genetic variation for the selection program. The CSBP currently has only 100 family tanks and increasing the number of tanks would be costly (R.G. Peterson, 2002, pers. comm.). The remaining options were to 1) to use mass selection and/or pedigree selection on the XX males instead of using BLUP, or 2) to pond treated and untreated fish from the same family in a single family tank and rear them to the time when PIT tags can be applied. Option 1 would reduce selection response but option 2 had never been previously tried. Option 2 would be the logical choice since if it works, selection would be consistent with a normal XX/XY population and the costs would actually be less than Option 1. The concern was whether mixing treated and untreated fish together would affect sexual maturation, growth and survival (R.G. Peterson, 2002, pers. comm.).

In my study, I attempted to compare all three of these traits between XX males and females and XY males. There was no significant difference in the mortality between XX and XY families before PIT tagging when the treated and untreated fish of each family were in the same tank. Fish from some XX families had significantly lighter body weight than fish from XY families at PIT tagging time, but the difference could be attributed to the younger age of these XX families. When XX and XY families of similar age were compared, there was no significance difference in body weight. There was also no difference in body weight among males (both XX and XY), females, and intersex fish at this stage. I encountered a large number of intersex fish in the XX families but that could be attributed to the MT treatment and not because of the mixing of the treated and untreated fish. Gonadal development of XX males was not different than that of the XY males. Gonadal development of the females in the XX and XY families was similar. In summary, I detected no adverse effect of mixing the treated and untreated fish in the same family tank.

4.3. MT treatment affecting juvenile growth parameters

One of the concerns in the selection program utilizing XX males is whether taking body weight measurements in these hormone-treated fish will affect the estimation of genetic merit (breeding values) of treated fish relative to normal males and females. MT is one of the potent synthetic hormones used for sex reversal. It also, however, has growth inducing capacity (Higgs et al., 1982; Schreck and Fowler, 1982). MT may affect growth even if it is used as a sex-reversal treatment with short exposure (Kuvampurath and Pandian, 1993; Goetz et al., 1979; Baker et al., 1988; Piferrer and Donaldson, 1993). In my study, however, I found no evidence that juvenile growth parameters of the treated fish were different than those of the untreated fish. Taking body weight measurements in these treated fish may not bias the estimation of breeding values of these individuals.

4.4. Precocial males

The occurrence of precocial maturation of males is common in cultured and wild Pacific salmon (Billard, 1983; Bernier et al., 1993), but precocial males are more prevalent in cultured population. More rapid growth has been associated with a higher incidence of precocial males in chinook salmon. In my sampling of 884 pre-smolt fish I encountered 6 precocial males, 5 of which came from XX families. All 6 precocial males were found with well-developed testes that filled up most of the abdominal cavity and histological examination shows complete spermatogenesis in their gonads. This is the first report of precocial development at such an early age. In a normal XX/XY fish culture, precocial males would not be used for breeding. There is a

consistent selection against precocial development. In a monosex population, genes affecting precocial development may not be exposed to the same type of selection pressure. The use of exogenous androgens to stimulate sex reversal may also alter the expression of these genes. Whether there is genetic variation in the sensitivity to MT treatment, which may facilitate precocious sexual development in the more sensitive fish remains to be determined.

4.5. Sexual dimorphism in meiosis

Salmonids are believed to be descended from a single tetraploid event estimated to have occurred 25-100 mya (Allendorf and Thorgaard, 1984, See also Phillips and Ráb, 2001 for the evolution of salmonid karyotypes). In present day stocks, many homologous chromosome arms still exchange chromatid segments as a result of quadravalent formations during meiosis (Lee and Wright, 1981). However, this phenomenon appears to be almost exclusive to males (Lee and Wright, 1981; Allendorf and Thorgaard, 1984), resulting in differences in recombination rate and linkage relationship between males and females (Sakamoto et al., 2000). Whether this sexual dimorphism in recombination rate will be an important factor in the use of quantitative selective breeding methods to improve performance of the monosex population is of concern (Kim Cheng, Department of Animal Science, University of British Columbia, Vancouver, BC, 2001, pers. Comm.). My study will not be able to answer this question but my data can be included in the data-base for further studies. Alternatively, one can also use FISH (flourecent in-situ hybridization) to compare XX male chromosome segregation pattern to see if they act like males or females (K.M. Cheng, 2002, pers. Comm.).

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4.6. Genetic differences in the fish stocks

Growth parameters may differ in fish from different river systems (Withler, et al., 1987) and therefore the choice of fish for broodstock development may affect their response to selective breeding.

In my study, there were significant differences in body weights and SGR of the fish from the two farms. The slower specific growth rate (SGR) in XY families may be attributable to genes brought in by these males as XY families were sired by RC hybrid strain males and XX families were lacking this genetic input from the RC stock. Furthermore, differences in body weight and growth rate were evident between two BQ stocks (MH stock and CS stock). My findings supported previous reports (Withler et al., 1987; Kim et al., 2003) of genetic differences between the fish stocks used by the CSBP.

4.6. Summary and conclusions

4.6.1. The MT treatment protocol used by the CSBP, or the sensitivity of response to MT treatment in the particular BQ stocks used by the CSBP, resulted in transitional intersexuality in a large proportion of treated fish.

4.6.2. There were no differences in the juvenile growth parameters measured in treated and untreated fish.

4.6.3. There were significant differences in juvenile growth parameters measured in the two BQ stocks. Genes brought in by the RC stock hybrid strain also affected these growth parameters.

4.6.4. A small number of precocial males were encountered at the pre-smolt stage. Precocial development at this young age has not been reported before.

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Appendix 1

Hatch date and mortality data of the 20 chinook salmon families under study

Family ID	Tank Number	Family Type	Stock Cross	Date of hatch	Mortality		Total fish in tank at ponding	Total fish sampled at parr stage*	Total PIT tagged fish at parr stage
					Ponding to parr stage	Parr to pre-smolt stage (tagged fish)			
100084	407	MH XX	MH BQ x MH BQ	Jan 3	6	4	350	48	43
100086	409	MH XX	MH BQ x MH BQ	Jan 3	24	4	350	48	43
100091	414	MH XX	MH BQ x MH BQ	Jan 8	2	7	350	43	35
100093	416	MH XX	MH BQ x MH BQ	Jan 8	13	15	350	51	35
100017	117	CS XX	CS BQ x CS BQ	Dec 15	5	6	350	48	41
100023	123	CS XX	CS BQ x CS BQ	Dec 15	2	1	350	49	47
100028	202	CS XX	CS BQ x CS BQ	Dec 15	1	2	350	48	45
100031	205	CS XX	CS BQ x CS BQ	Dec 15	0	1	350	48	46
100034	208	CS XX	CS BQ x CS BQ	Dec 15	1	4	350	48	43
100038	212	CS XX	CS BQ x CS BQ	Dec 15	0	0	350	48	47
100043	217	CS XY	CS BQ x RC hybrid	Dec 14	2	5	200	49	42
100046	220	CS XY	CS BQ x RC hybrid	Dec 14	0	1	200	49	47
100047	221	CS XY	CS BQ x RC hybrid	Dec 14	4	6	200	48	41
100048	222	CS XY	CS BQ x RC hybrid	Dec 14	9	3	200	49	45
100001	101	CS XY	CS BQ x RC hybrid	Dec 11	0	2	200	47	45
100003	103	CS XY	CS BQ x RC hybrid	Dec 11	1	4	200	48	44
100066	315	MH XY	MH BQ x RC hybrid	Dec 18	1	5	200	49	43
100074	323	MH XY	MH BQ x RC hybrid	Dec 18	2	4	200	46	51
100077	326	MH XY	MH BQ x RC hybrid	Dec 18	7	4	200	48	43
100079	402	MH XY	MH BQ x RC hybrid	Dec 18	0	3	200	49	45

* Fish collected for at parr stage for sex determination and growth measurement

Appendix 2

PLASMA TESTOSTERONE, EXTRADIOL AND THYROXINE LEVELS OF JUVENILE SALMON

At the pre-smolt age, 385 fish out of 20 families (10 families were treated families and 10, untreated; 19 fish per tank in an average; for detail refer chapter 2) were taken randomly and anesthetized in water containing MS-222. Blood was collected from tail vein in heparinized hematocrit tubes after severing the tail (caudal peduncle) (Shearer and Shawnson, 2000). Samples were kept on ice. Plasma (50 – *150 µL per sample) was obtained after centrifugation* (1500 * 15 mins), and was stored at –80°C until they could be analyzed for respective hormones: Testosterone (T), 17-*β*-Estradiol (E2) and Thyroxine (T4) hormones. Fish were euthanized immediately after blood sampling.

Because of the small quantity of plasma available from the chinook salmon sampled, I was advised to use similar age coho salmon plasma for calibrating the hormone assay kits. The period for maximum steroid sensitivity in coho and chinook salmon is similar (coho: Goetz et al., 1979; chinook: Baker et al., 1988), and both have similar sexual development at pre-smolt stage (L. Afonso, 2002, pers comm.). Plasma samples from pre-smolt coho salmon (n=40; Males, 20 and Females, 20) were collected from fish maintained at the Department of Fisheries and Oceans, West Vancouver, Canada, using similar protocol as the Chinook salmon.

Hormonal analyses

Total plasma E_2 and T of pre-smolt coho salmon were assayed using enzyme-linked immunosorbent assay (ELISA) (17-β-Estradiol (E_2) Enzyme Immunoassay Kit and Testosterone (T) Enzyme Immunoassay Kit; Cayman Chemical) and Thyroxine (T4) were assayed using a radioimmunoassay (RIA) (Amerlex-M Thyroxin (T_4) RIA Kit; Trinity Biotech). Assay was performed according to the prescribed protocol that came with the kits, except for a slight modification for Thyroxine assay (extending the incubation period to allow better binding). Samples were run in duplicates and a range of dilutions (straight, diluted 2x and diluted 10x) wherever possible. While testing for T_4, several samples of plasma from the same sex fish were pooled to obtain enough plasma to run the assay. In the outcome, if the percent bound was greater than 80% (i.e. off the linear part of the standard curve), the samples were taken to have less than detectable levels for the assay. The detection limits of the kits were 6 pg/ml, 9 pg/ml and 3.108 ng/l, respectively for T, E_2, and T_4.

Results and Discussion

Plasma concentrations of T, E_2 and T_4 of the pre-smolt coho salmon sampled were under or borderline on the lower detection limits of the kits. Nagahama et al. (1982), found in yearling amago salmon (*Oncorhynchus rhodurus*) during smoltification, that plasma testosterone levels were low in males and that plasma estradiol was undetectable (less than 30 pg/ml) in females. In most of the literature that I reviewed, reliable results of plasma T, E_2 and T_4 assay were from tissues sampled from yearling salmonids which would be a few months older than the fish I sampled.

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Based on the results obtained during calibration of the hormone assay kits, it was decided that these kits were not sensitive enough to detect the low hormone concentration in the pre-smolt chinook salmon plasma (L. Afonso, 2002, pers. Comm.)

References

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