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Inbreeding depression and segregation distortion in Chinook salmon: Conservation implications of genetic load

Kendra Komsa
University of Windsor

INBREEDING DEPRESSION AND SEGREGATION DISTORTION IN CHINOOK SALMON: CONSERVATION IMPLICATIONS OF GENETIC LOAD

By

Kendra Komsa

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By

Kendra Komsa

APPROVED BY:

_____

Dr. Doug Haffner
Great Lakes Institute for Environmental Research

_____

Dr. Oliver Love
Department of Biological Sciences

_____

Dr. Daniel Heath, Advisor
Great Lakes Institute for Environmental Research

_____

Dr. Dennis Higgs, Chair of Defense
Department of Biological Sciences

September 14, 2012

I. CO-AUTHORSHIP STATEMENT

I hereby declare that this thesis incorporates material that is result of joint research from co-authored and submitted journal articles undertaken under the supervision of my supervisor Dr. Daniel Heath (University of Windsor). The primary contributions, data collections, laboratory work, and interpretation of the data was performed by the author, with additional input on data analysis, interpretation of data, and written discussion by co-authors. I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis, and have obtained written permission from each of the co-author(s) to include the material below in my thesis.

I certify that, with the above qualification, this thesis, and the research to which it refers, is the product of my own work.

II. DECLARATION OF PREVIOUS PUBLICATION

This thesis includes 1 original paper that has been submitted for publication in a peer reviewed journal, as follows:

Komsa KR, Aykanat T, Heath JW, Heath DD. Inbreeding depression in Chinook salmon: fitness effects in offspring from hermaphrodite self-crossed and out-crossed mating. (*Manuscript submitted to Heredity: September 2012).*

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ABSTRACT

Strong evidence that shows inbreeding in many plants and animals can lead to detrimental effects at the level of the organism as well as the population. Inbreeding and its effects have been the focus of much attention in terms of conservation and captive rearing and breeding programs for many endangered species. Inbreeding depression (ID) is the fitness loss associated with individuals and populations that have experienced inbreeding. Surprisingly, ID studies within salmonids are not extensive. Here, I used inbred offspring of self-fertilized hermaphrodite parents to study the effects of ID in Chinook salmon (*Oncorhynchus tshawytscha*) at an extreme level of inbreeding. High levels of ID were found after a single generation of self-crossing, resulting in substantial fitness losses. The potential to purge genetic load from a population was shown to take place and the major genetic mechanism driving ID was due to expression of deleterious recessive alleles.

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1.0 GENERAL INTRODUCTION

Inbreeding and Inbreeding Depression

There is strong evidence to show that inbreeding in many plants and animals is destructive and can lead to detrimental effects at the level of the organism as well as the population. Darwin conducted some of the first experiments involving self-fertilization and out-crossing in many plant taxa, including over 69 families, 158 genera and 224 species – for example *Beta vulgaris L.* and *Arum maculatum* (Owen and Miller, 2009). From these experiments, Darwin's main conclusion was that inbred individuals were not as fit and did not reproduce as well as outbred individuals. Genetic diversity is therefore assumed to be a fundamental component necessary for the long-term survival of a population. Inbreeding at the individual level is simply the mating of relatives. At the level of a population, inbreeding is the mating of individuals that are more closely related than they would be if they were randomly selected from a population (Wang et al., 2002). Inbreeding can reduce genetic variation by decreasing heterozygosity and increasing genetic load. This increase in genetic load is also due to an increase in the number of homozygous individuals who are carrying recessive deleterious genes because they are carrying two copies of the deleterious allele (Wang et al. 2002; Charlesworth and Charlesworth, 1999). Therefore, the effects of inbreeding are generally considered undesirable in all populations.

Inbreeding leading to a reduction in the fitness of offspring has been recognized for centuries, and the concept of offspring that are less fit because of parents that have a high level of relatedness is referred to as inbreeding depression (ID) (Kristensen and Sorensen, 2005). Therefore, avoidance of inbreeding and maintaining genetic variation

within populations are considered primary goals in conservation biology (Brakefield and Saccheri, 1994). ID occurs as a result of inbreeding, where there is a decrease in the mean phenotypic value of traits related to fitness (Charlesworth and Charlesworth, 1999; Charlesworth and Charlesworth, 1987; Hedrick, 1994) often related to the decreased frequency of heterozygous genotypes. A decrease in heterozygosity refers to the fact that individuals who are inbred have homozygous allele combinations more often than expected. Inbreeding results in fewer alleles being passed on to the next generation, which will ultimately lead to a decrease in genetic diversity after several generations of inbreeding.

There are two main mechanisms that are accepted for how a loss of heterozygosity can lead to ID. The first explanation is that heterozygous genotypes lead to superior phenotypes compared to their homozygous alternatives. As such, when inbreeding occurs, and there are fewer heterozygous loci within a population's gene pool, the fitness of the population decreases and is thus said to be experiencing ID. This explanation is known as the Overdominance Hypothesis of ID (Charlesworth and Charlesworth, 1987, 1999). On the other hand, according to the Dominance Hypothesis, ID occurs due to the expression of deleterious recessive alleles in homozygous individuals. Such alleles, which are normally masked by dominance in heterozygous individuals, are at low frequencies in the population, so selection cannot act effectively against such alleles. Therefore, these deleterious alleles are maintained in an outbreeding population at a frequency determined by the selection-mutation balance and represent the genetic load of the population (Fox et al., 2008). Inbreeding increases the occurrence of homozygous recessive individuals, which are then at a fitness disadvantage

(Charlesworth and Charlesworth, 1987). The accumulation of these homozygous recessive genotypes will therefore decrease the populations' viability since it will be overall less fit due to the expression of its genetic load.

Purging Genetic Load

All natural populations contain some mutant genes (alleles) with mild to severe deleterious effects, which is a part of genetic variation among individuals in a population (Wallace, 1970). The genetic load refers to the sum total of all these deleterious alleles that can exist at all gene loci within the genome. In other words, genetic load decreases the fitness of the population and can therefore be measured in terms of the amount of fitness that the population has lost compared to the individual who is the most "fit" within the population and who then is carrying and expressing the least amount of genetic load (Wallace, 1970; Wang et al., 2002). Thus, the genetic load in inbred populations is usually high due to the increased number of recessive deleterious alleles occurring as homozygous genotypes, which are present within an inbred population. This all results in the decreased fitness levels observed; however, selection against deleterious recessive alleles, in a process referred to as purging, is expected to decrease genetic load within the population (Charlesworth and Charlesworth, 1999).

Purging the genetic load is a phrase that was coined by researchers to explain the rebound in fitness that populations who survive extreme inbreeding (and population decline) experience (Crnokrak and Barrett, 2002). Additionally, populations that have survived inbreeding are oftentimes more fit than the original population prior to inbreeding, and can better resist further inbreeding impacts in the future (Leberg and

Firmin, 2007; Fox et al., 2008). Populations of *Drosophila melanogaster* that have been experienced inbreeding, and have been subjected to recurring population bottlenecks, were able to regain pre-bottleneck fitness levels, suggesting the population had purged their genetic load, compared to non-inbred lines who experienced the same bottlenecks but did not regain their pre-bottleneck fitness (Miller and Hendrick, 2001). Fox et al., (2008) studied the effects of ID on the seed feeding beetle, *Stator limbatus,* by outcrossing families that were serially inbred by full-sib mating and measuring larval survival and ID. They found that the serially inbred beetles had a higher survivorship and significantly lower ID and genetic load than the outcrossed beetles, which is evidence that the genetic load had been purged. Purging relies on the expression of recessive deleterious alleles that occurs as a result of inbreeding and thus presupposes the Dominance hypothesis for ID. Individuals who express these deleterious alleles are not as fit, and are therefore less likely to survive to reproduce, reducing the number of deleterious alleles passed on to the next generation. Thus, after extreme population declines, eventually many of the individuals expressing these genotypes are lost, and the population is able to thrive and increase their survivorship until pre-inbreeding population sizes are once again reached. Therefore, purging will decrease an inbred population's genetic load by eliminating detrimental recessive alleles from the population (Hedrick, 1994; Crnokrak and Barrett, 2002).

Conservation Implications

It is because of the potentially beneficial outcome of inbreeding that conservation efforts have begun to focus on ID and whether or not allowing inbreeding to naturally occur within populations is a viable management action (Leberg and Firmin, 2007).

However, many studies identify this as a very controversial issue, and that a firm conclusion cannot be made, since ID has various effects, depending on the population and species in which it is occurring, and the environmental conditions the population is experiencing (Fox et al., 2008). Simply put, if deleterious alleles are common in a population, then ID and subsequent purging of these alleles could help to reduce the on-going cost of ID (Crnokrak and Barrett, 2002) which would be beneficial to breeding programs aimed at increasing population sizes. However, populations would first have to survive the effects of ID and extremely small population sizes without being extirpated. Additionally, it seems likely that ID is due to a combination of severely deleterious alleles that have a large effect within a population, as well as deleterious alleles that have less of an effect and which are therefore less affected by selection pressures resulting from purging (Charlesworth and Charlesworth, 1999). Accordingly, it might take many more generations to purge those alleles and the long-term benefits of purging in the population would not be realized for some time. Nonetheless, it is important to understand the mechanisms behind inbreeding and ID to determine whether or not purging is a viable conservation strategy. There are very few studies that have been able to quantify ID and genetic load within a vertebrate organism and therefore more studies are needed to explore these two concepts.

Study Species: Chinook Salmon (*Oncorhynchus tshawytscha)*

The family Salmonidae is a teleost family and includes approximately 75 extant species that are native throughout the Northern Hemisphere, but who are also present throughout the world (Allendorf and Waples, 1996). Species within this family descended from a single tetraploid ancestor that originated 50-100 million years ago

(Groot and Magolis, 1991). Salmoninae is the subfamily within Salmonidae of which salmon are a part. It is suggested that *Salmo* and *Oncorhynchus* diverged from other members of Salmoninae 30-40 million years ago and then separated into an Atlantic and Pacific group 15 million years ago (Groot and Margolis, 1991). Both of these time frames are relatively recent evolutionary events.

Most *Oncorhynchus* species, including Chinook salmon, are anadromous and semelparous. Chinook salmon have widely varying life history characteristics that depend on the population being studied, and they are often separated into "races" of Stream-type and Ocean-type salmon, depending on when they migrate to the ocean after birth and how soon they return to their natal rivers prior to spawning (Groot and Morgolis, 1991). The extraordinary diversity of environments experienced by anadromous salmonids results in extremely complex life histories, allowing them to adapt through selection to the various freshwater and marine environments they are exposed to (Allendorf and Waples, 1996). Therefore, nearly all aspects of their life history are influenced by genetic differences among individuals and populations (Allendorf and Waples, 1996), making the genetic study of these fishes extremely complex.

Inbreeding occurs naturally within many salmon populations, since their reproductive behaviour involves returning back to their natal streams to spawn at distinct times, which results in reduced population sizes and gene flow among populations and thus encourages inbreeding to take place (Allendorf and Waples, 1996). Furthermore, many salmonid species, including Chinook salmon, have been listed under the U.S. Endangered Species Act (Wang et al., 2002). The small population sizes of salmon worldwide have brought inbreeding and its potentially detrimental effects to the forefront

for salmon conservation and management programs (Fu et al., 1998; Wohlfarth, 1993; Kristensen and Sorensen, 2005; Leberg and Firmin, 2008). However, because of the residual tetraploidy in salmonid genomes, salmon are perhaps sheltered against the effects of ID at low levels of inbreeding because of the presence of a duplicated gene loci and the elevated number of gene copies may facilitate compensation (Allendorf and Waples, 1996). Although some of the duplicated genes in salmonids have diverged in function, salmonids still show a high incidence of duplicated enzymes and many duplicate genes (Allendorf and Waples, 1996). This adds to the complexity of the salmonid genome, increasing the difficulty of genetically characterizing these fishes.

Salmon Hatcheries, Aquaculture and Declining Salmon Populations

Salmon populations are drastically decreasing in size with an estimated 2.5 million anadromous salmon returning to spawn in the Columbia River Basin in the 1980s compared to over 10-16 million in the 1800s (Allendorf and Waples, 1996). An estimated 80% of the returning fish these days are from hatchery production, thus the return of wild-spawned fish is less than 5% that of historic numbers (Allendorf and Waples, 1996). As such, many salmonid populations and species are threatened with extirpation and extinction. It has become an enormous conservation concern with biological, economical and political significance, where Chinook salmon are just one of 22 species of salmonids appearing on the 1994 IUCN Red List of Threatened Animals (Allendorf and Waples, 1996).

Salmon aquaculture has been occurring for close to 2000 years (Bentsen and Olesen, 2002), and is rapidly expanding with salmon farming exceeding wild caught

salmon by 70% (FOA, 2003). Interestingly, over the years, the need to improve the efficacy, output and productivity of fish farming in general has not been met (Bentsen and Olesen, 2002) and therefore, further research in this area should be pursued. Studies have focused on the effects that farmed salmon have when released from captivity (e.g. Ford et al., 2012), estimating the ability of captive-bred salmon to survive in the wild (e.g. Blanchet et al., 2008) and the differences between wild and farmed salmon in terms of reproductive success (e.g. Lehnert et al., 2012). However, it is also important to explore the genetic impacts of aquaculture practices on captive salmon in terms of inbreeding and possible resulting ID. Both commercial aquaculture and government hatcheries concerned with stocking programs and conservation need to address the effects of ID among their salmon, since artificial rearing and breeding can and does increase inbreeding levels. Under captive conditions, inbreeding is likely to occur because over generations, the degree of relatedness between fish increases and genetic diversity decreases, simply because the gene pools are small. Many stocking programs over the last two decades have been focused on increasing the numbers of salmon in wild populations. However, even though stocking programs have increased the number of fish they are introducing, studies have shown that there is not always an increase in the number of fish caught that result from the stocking program (Peck et al., 1999 studied Chinook salmon stocking programs and showed these results for Lake Superior, Ontario). Furthermore, captive offspring bred from wild parents and released into the wild at an early date, still show significantly reduced levels of genetic variability and allele frequency variation relative to the wild born offspring (Blanchet et al., 2008). As a result, any advances in understanding the genetic makeup of salmon will inform hatchery

stocking programs and the aquaculture industry to ensure continued successful culture and rearing of these economically and ecologically important fishes.

1.1 THESIS OBJECTIVES

The overall aim of this thesis was to increase our knowledge surrounding the genetic aspects of inbreeding in vertebrates, and salmonids in particular, and to explore the phenotypic effects and genetic mechanisms of inbreeding and the associated inbreeding depression.

Chapter 2 Objectives

The effects of inbreeding and its relation to decreased fitness, or ID, was quantified phenotypically using specific life history traits that have known fitness effects within Chinook salmon (Wang et al., 2002). Specifically, wet weight, specific growth rate and survivorship over an 18 month period was monitored in self-crossed (inbred) versus outbred fish. Significant differences were found between the inbred (self-crossed) and outbred control fish, providing evidence that ID does exist within salmon populations, despite mixed results reported in the past (Wang et al., 2002). I can therefore recommend that conservation programs remain cognoscente of the effects of ID among captive bred salmon, as well as the fact that wild populations continuing to decline in population size could be experiencing these adverse effects as well. Overall, these phenotypic results helped characterize the effect of inbreeding on individuals, highlighting the intriguing genetic aspects of inbreeding and ID.

Chapter 3 Objectives

Microsatellite markers were used to genotype inbred (self-crossed) fish and control (out-crossed) fish. Genotype inheritance analyses allowed us to study the effects of inbreeding on genetic load by determining deviations from Mendelian inheritance. Deviations from Mendelian inheritance were discovered in the inbred offspring and more thorough analyses were completed to determine the possible causes for the deviations. I was able to show evidence of the potential for populations to purge genetic load within a single generation of extreme inbreeding, as well as define the mechanisms by which highly inbred populations suffer fitness effects of ID.

This thesis provides a comprehensive examination of phenotype and genotype variation with and between inbred and outbred Chinook salmon families, which helps increase our understanding of the mechanisms of ID in vertebrates in general, and specifically within Chinook salmon. I also explore the implications of my findings for conservation and commercial aquaculture practices, surrounding the possible effects of ID and population genetic load, which should lead to protocol changes for rearing salmon effectively for both enhancement and commercial purposes.

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2.0 INBREEDING DEPRESSION IN CHINOOK SALMON: FITNESS EFFECTS IN OFFSPRING FROM HERMAPHRODITE SELF-CROSSED AND OUT-CROSSED MATING

2.1 INTRODUCTION

Inbreeding is the mating of relatives, and the resulting offspring may experience a loss of fitness, which is defined as inbreeding depression (ID) (Charlesworth and Charlesworth, 1987; Hedrick, 1994). The negative fitness effects of inbreeding first became apparent through experiments performed by Charles Darwin involving self-fertilization and out-crossing of many plant taxa, where offspring from "selfed" or inbred parents did not reproduce as well as the individuals produced by outcrossing (Darwin, 1876). There are two current hypotheses, first described by Wright (1977), that address the genetic basis of ID: 1) The Dominance Hypothesis, which explains ID as resulting from the increased expression of deleterious recessive alleles in their homozygous state when inbreeding occurs, and 2) The Overdominance Hypothesis, which explains ID as the result of the loss of beneficial heterozygote allele combinations across the genome (Charlesworth and Charlesworth, 1987; 1999). Charlesworth and Charlesworth (1999), conclude that the fitness loss observed in inbreeding populations is likely due to the expression of partially recessive deleterious alleles (Dominance Hypothesis) which are often not expressed when inbreeding within the population is low. However, studies have shown that the Overdominance Hypothesis does seem to hold as the best explanation for the fitness loss associated with inbreeding for several traits, specifically shown in *Drosophila melanogaster* (Charlesworth and Charlesworth, 1999; Leberg and Firmin,

2008). In natural situations, ID is likely due to a combination of the two mechanisms (Charlesworth and Charlesworth, 1999; Wang et al., 2002).

A variety of phenotypic traits have been shown to be affected by ID (DeRose and Roff, 1999). ID is expected to affect traits that are closely related to an individual's fitness, such as reproductive traits and survival, as well as traits that are indirectly related to fitness, such as body size and condition, and others associated with reproductive capacity or physiological efficiency. Those traits are therefore often used to study the effects of ID (Wang et al., 2002). Falconer (1989) first proposed the idea that life history traits should exhibit higher ID than morphological traits, and DeRose and Roff (1999) empirically showed that to hold over a broad taxonomic range. Additionally, it has been shown that the severity of ID in the wild is much higher than under captive conditions, which is likely due to the fact that ID is more intense under harsher conditions and when the individuals are experiencing stress (Crnokrak and Roff, 1999).

The effects of inbreeding have been shown in many different species. For example, the plant *Collinsia* was shown to have higher ID when less pollen was available and thus when more inbreeding was occurring (Lankinen and Armbruster, 2007). Additionally, invertebrate species such as *C. remanei* were noted to display dramatic reductions in brood size and relative fitness in highly inbred lines (Dolgin et al., 2007). Finally, vertebrate species have also been shown to exhibit ID, where significant ID was demonstrated in the number of offspring per litter of an Australian rodent, *Rattus villosissimus*; however there were no signs of ID in the survival or growth rate of the young (Lacy and Horner, 1997). ID has also been shown to occur in several fish species, such as the channel catfish, where Bondari and Dunham (1986) showed increased

inbreeding coefficients after two generations of full-sib matings, which was associated with an increased number of days required for inbred offspring to hatch. Surprisingly, there have not been many quantitative studies characterizing ID in salmonid fishes, and the few studies that have been performed do not agree, since some studies have shown significant effects of inbreeding and others none (Reviewed by Wang et al., 2002).

Inbreeding is expected to occur naturally within salmon populations since their life history includes isolation due to strong natal homing (phylopatry) and small population sizes (Allendorf and Waples, 1996; Quinn, 2005) both of which increase the probability of inbreeding (Wang et al., 2002). Furthermore, Yeates et al. (2009) showed using sperm competition studies in Atlantic salmon, *Salmo salar,* that males gained significantly greater relative fertilization success when competing for eggs of females who were genetically similar to themselves (at MHC II), which means the offspring in this population are at higher risk of inbreeding. Populations of wild Alaskan steelhead, *Oncorhynchus mykiss,* were shown to have significant ID after a single generation of full-sib mating in terms of adult body size after two to three years in the ocean, coupled with decreased survival of the offspring they produced (Thrower and Hard, 2009). However, there are several salmonid studies that have tested for ID, but found little or no evidence of ID. For example, Su et al. (1996) reported no significant ID for body size in rainbow trout, *Onchorynchus mykiss,* at early life stages (prior to 256 days post-fertilization) when the levels of inbreeding were low (Inbreeding coefficient, F=0.064). However, ID did appear to have more of an effect on the individuals that survived to spawning. This was postulated to be due to a cumulative effect of ID in these individuals as the fish grew larger. This is consistent with Houde et al. (2011) who found no evidence of ID in body

size traits throughout the early life stages (from egg to alevin stage) in Atlantic salmon (F=0.125 and F=0.25). It can be postulated that the limited evidence for inbreeding effects in salmon, despite the expectation for high levels of inbreeding, may relate to the tetraploid ancestry of salmonids, providing this group of fishes with the potential for duplicated loci and perhaps genetic buffering for both proposed mechanisms of ID (Allendorf and Waples, 1996; Wang et al., 2002). Another possible factor in the apparent low levels of ID in salmon may be the occurrence of "purging" deleterious alleles, a process whereby several consecutive generations of inbreeding leads to a reduction in the number of deleterious alleles (or genetic load) present within the population (Crnokrak and Barrett, 2002). Thus, populations with a history of inbreeding are expected to be less susceptible to the effects of ID. As salmon populations are expected to naturally inbreed (due to isolation and small effective population size), they may be purging deleterious alleles and therefore are less affected by ID under the Dominance Hypothesis. Evidence of purging has been shown in *Drosophila melanogaster,* where the magnitude of ID in an inbred population after 19 generations of full-sib mating was approximately one third that observed in the original base population (Swindell and Bouzat, 2005). Additionally, evidence of purging has been shown in the seed-feeding beetle, *Stator limbatus,* where the outcrossing of serially inbred beetles resulted in higher survivorship and significantly lower ID and genetic load than in the non-inbred beetles (Fox et al., 2008).

The decline of many wild stocks of anadromous salmonids is well documented (e.g. Allendorf et al., 1997; Brown et al., 1994; Parrish et al., 2011; Bradford and Irvine, 2000) and inbreeding effects may be contributing to this decline in the smaller populations (Wray and Thompson, 1990). Chinook salmon (*Oncorhynchus tshawytscha)*

populations in British Columbia studied over a 20-year period have shown population declines and small effective population sizes (Shrimpton and Heath, 2003). Declining population sizes and the presumed effects of ID in wild salmonids has resulted in a perceived management crisis, bringing ID and its potentially detrimental effects to the forefront for salmon conservation and management programs (Fu et al., 1998; Kristensen and Sorensen, 2005; Leberg and Firmin, 2008). Thus, the quantification of the effects of ID on life history traits in salmon is a high priority from a conservation, as well as an evolutionary perspective.

Here we test for ID in captive Chinook salmon. We explore the effects of self-crossing (or "selfing") using artificially-produced functional hermaphrodites, a novel approach that allows us to study inbreeding in a vertebrate organism at an extreme level. We predict that ID in the self-crossed hermaphrodite offspring would express itself as reduced growth and survival throughout their life cycle. We address variation in fitness (survival and growth) as genetic ID and gamete quality effects (i.e. the quality of the sperm and eggs used in the crosses) by comparing performance among self-crossed offspring, out-crossed offspring and mixed parentage offspring (one hermaphrodite parent and one control parent – reciprocal crosses) from early freshwater stages through saltwater netcage rearing to 18 months post-fertilization. We show that ID effects on both growth and survival occurs at high levels in the self-crossed offspring, providing evidence that inbreeding can have potentially detrimental effects within salmon populations, given sufficient inbreeding intensity. Furthermore, the effects of ID were life stage specific, intensifying later in life. Thus, we conclude that conservation efforts should continue to focus on the potential impacts of inbreeding in small and isolated

populations of salmon, despite past work showing limited evidence for ID that commonly focused on low levels of inbreeding and early life stage performance.

2.2 MATERIALS AND METHODS

Study Fish

The Chinook salmon used in this study are farmed salmon from Yellow Island Aquaculture Ltd. (YIAL), an organic fish farm located on Quadra Island, British Columbia. Three types of parental fish were used in controlled crosses for this experiment – 1) hermaphrodite, 2) control female and 3) control male. The hermaphrodite fish resulted from incomplete sex-reversal. Sex-reversal is used to create fish that are genetically female (XX) but phenotypically male (Hunter et al, 1983) and involves exposing known female (XX) larvae to two treatments of alpha-methyltestosterone (aMT). The treatment consists of immersion of the larvae in 0.4mg/L aMT at 50 percent hatch (i.e. 50 percent of the eggs have hatched) and again at 100 percent hatch for two hours. We modified this protocol to increase the incidence of incomplete masculinization (which can lead to intersex individuals and functional hermaphrodites) by reducing the concentration of aMT (to 0.2mg/L) and by reducing the exposure time to 1 hour for each immersion. We identified sexually mature hermaphrodite fish as those with intersex secondary sexual characteristics (<1% of the treated fish). We systematically searched for hermaphrodite mature fish in the treated fish, and in November 2009 we identified and bred two hermaphrodite fish. Below we describe the breeding experiments.

Breeding and Rearing

Two hermaphrodite fish were collected on November 3, 2009 among the treated fish that were created in the fall of 2004. The control male used in this study was a sex-reversed female fish (i.e. XX male) from the same year class to avoid phenotypic effects of XY offspring among the crosses that were performed (described below), while the control female was a regular female of the same year class. Therefore, all the parental fish were genetically XX and all of the offspring created were also genetically and phenotypically female.

Hermaphrodite and control fish gametes were collected from the parents at the netcage site and transferred to the hatchery where the crosses were performed following standard hatchery fertilization protocols. Approximately 3000 eggs were collected each from Hermaphrodite 1 and Hermaphrodite 3 (Hermaphrodite 2 had unviable eggs). The eggs from each of the hermaphrodites were divided into two roughly equal groups and the control female eggs were divided into roughly three equal groups. Each grouping of eggs was fertilized using 300-500μL of milt from selected males following a 2x2 factorial cross (see Figure 2.1). In total, seven different families were created; although two different hermaphrodite parents were used, we used the same control male and female fish for both 2x2 crosses involving the hermaphrodite gametes (Figure 2.1). As per convention when describing crosses, the female is listed first, followed by the male when referring to these crosses.

The outcrosses between hermaphrodite and control gametes were performed to evaluate the viability of the hermaphrodite gametes, while the CxC cross was performed

as a control to provide a standard for relative performance. We considered the possibility of reduced hermaphrodite gamete viability and quality resulting from the intersex endocrinological environment experienced during development. Such gametic quality effects may contribute to reduced fitness in offspring that were created using one or both of the hermaphrodite gamete types; however such effects are expected to be primarily expressed in early development (Heath et al., 1999; Aykanat et al., 2012).

Once fertilized, the eggs from each cross were held in replicated separate compartments in vertical incubation trays with a freshwater flow of 12L/min (half of the CxC offspring were incubated with the H1xH1 offspring and were therefore deemed the C1xC1 offspring and the other half were incubated with the H3xH3 offspring and are therefore the C3xC3 offspring). The offspring were subsequently transferred into freshwater tanks (200L) on March 2, 2010. Coded passive integrated transponder (PIT) tags, which allow the identification of individual fish, were injected (Figure 2.1) at seven months of age (approximately 4g wet weight) on June 9-10, 2010. After being PIT tagged, the fish were weighed and transferred into a single large freshwater holding tank (3000L) in the hatchery where they were vaccinated with the *Vibrio anguillarum* vaccine by immersion on June 17, 2010, as per normal practice at the fish farm. All the fish were moved into a single saltwater netcage on July 1, 2012 where they were held for the duration of the project.

Sampling

The offspring were sampled on three separate occasions, on June 9-10, 2010 (during PIT tagging, 218 days post-fertilization), October 15-16, 2010 (339 days post-

fertilization) and April 8-9, 2011 (514 days post-fertilization). The fish were anesthetized using 50mg/L (Kennedy et al., 2007) of clove oil before being individually handled to determine their wet weight (g), which was recorded with their PIT tag code. The surviving fish were also identified at each sampling period to calculate survivorship. Specific growth rate (House et al., 2011; Fraser et al., 2010) for individual surviving fish was calculated between each sampling period and overall from June 2010 to April 2011.

Statistical Analyses

We predicted that ID would be detected as decreased performance in the HxH crossed offspring, which would be determined by comparing the HxH crosses to the CxC cross for both hermaphrodite parents. However, differences between the HxH and CxC families could be confounded by possible hermaphrodite gamete quality effects and therefore, the HxC and CxH families were also compared to the CxC family. It is important to note that egg quality effects are expected to be most prevalent early in the life of the offspring (Heath et al., 1999). Differences between the reciprocal crossed offspring and the control offspring performance is likely to be due to poor hermaphrodite gamete quality and not ID (since the mixed-cross offspring are not inbred). This would indicate that any observed reduced performance in the HxH crosses (relative to the CxC cross offspring) could be, at least in part, attributed to gamete quality factors. Therefore, all of the statistical analyses performed are relative to the CxC families.

Survival: The survivorship for each family was determined for the periods between June and October 2010 and October 2010 and April 2011. Overall survivorship was also determined as the total number of offspring who survived in each family from June 2010

to April 2011. Survivorship was calculated as the number of surviving offspring divided by the number at the start of the time period. Statistical analyses of the differences in survivorship between crosses were performed as Cross-tab analyses on the numbers of fish that lived or died. In all cases our analyses compared the treatment crosses (HxH, HxC and CxH) to the CxC control cross.

Size and Growth: To assess the effect of ID on the size of the offspring, we tested for differences between the treatment crosses (HxH, HxC and CxH) and the CxC control cross for wet weight and specific growth rate. Specific growth rate was calculated using the following formula:

$$SGR = 100 \times [\ln(\text{wet weight}_{time2}) - \ln(\text{wet weight}_{time1})]/\text{time}_{days}$$

A Shapiro-Wilk test was performed to test the normality of the continuous variables at all three sample times; most were not normally distributed ($p<<0.05$) within families. Therefore, Mann-Whitney U tests were performed to test for differences between the treatment (HxH, HxC and CxH) and the CxC control cross offspring. Since Mann-Whitney U tests are conservative, we replicated all of our analyses using randomization tests (Manly, 1997) performed on the wet weight and growth rate data, which provided qualitatively similar results (results not shown).

2.3 RESULTS

Survival

Overall, the hermaphrodite offspring showed significantly lower survivorship (H1 $p=0.009$ and H3 $p=0.004$) than the control offspring from June 2010 to April 2011 (Figure 2.2). The general trend in terms of the reciprocal cross offspring is that those

families do not differ significantly from the control family in terms of overall survivorship (CxH1 p=0.6, H1xC p=0.9, CxH3 p=0.9, H3xC p=0.8; Figure 2.2). Across the whole study period, there were no significant differences in the wet weight of the offspring that died relative to those that survived within each family (results not shown), indicating no evidence for size-biased mortality.

Size and Growth

Interestingly, when the fish were sampled for the first time in June 2010 (at PIT tagging), the HxH offspring were significantly larger in terms of wet weight compared to the control CxC families (Table 2.1). However, the HxH offspring were found to be smaller in terms of wet weight (mg) (Table 2.1), and exhibited lower growth rates (Figure 2.3) relative to the control cross offspring by October 2010. The most apparent differences in terms of size were seen when the offspring were 514 days post-fertilization in April 2011 (Table 2.1). In terms of growth rate, the HxH offspring grew at a significantly slower rate than the CxC offspring over all three sampling periods (Figure 2.3).

The reciprocal crossed offspring were also significantly larger than the CxC offspring in June 2010 and there were no clear signs of the mixed offspring being smaller than the control CxC offspring in October 2010 (especially in the CxH1 offspring who were still significantly larger than the CxC offspring; Table 2.1). By April 2011 the hermaphrodite female gametes appeared to be having some effect on the offspring, since the HxC offspring were not as large as the CxC offspring, but there were no differences seen

between the CxH offspring and CxC offspring (Figure 2.4). The mixed offspring grew at a significantly slower rate than the CxC offspring over all time periods (Figure 2.3).

2.4 DISCUSSION

This study is the first to show evidence for substantial inbreeding depression in salmon, specifically in Chinook salmon, perhaps due to our use of self-crossed hermaphrodite parents. We found ID effects on survivorship and body-size traits that are directly related to fitness in salmonids (Wang et al., 2002) after only a single generation of intense inbreeding. Specifically, our data shows ID effects on growth and body size of hermaphrodite self-crossed offspring. Body size is an important component of reproductive fitness in salmon. Several studies have shown that salmonid female weight is closely related to fecundity, a direct measure of female fitness (e.g. Barnes et al., 2011; Estay and Diaz, 1999; Quinn and Bloomberg, 1992). Additionally, Williamson et al. (2010) used genetic parentage assignment to show that body size was positively correlated with reproductive success in male Chinook salmon, with larger and older fish producing more offspring than smaller individuals, presumably due to sexual selection. Body size is also indirectly related to fitness in salmon, as salmon fry survival rate is generally correlated with body size (Bilton, 1984; Heath and Blouw, 1998; Malick et al., 2011).

Although the inbred offspring clearly exhibited lower performance, we can quantitatively estimate the magnitude of the fitness loss experienced by the HxH offspring relative to the control CxC offspring using body-size fecundity relationships and our survival data. Based on the mean wet weights of the fish in April 2011 and assuming the relative body size relationship between the hermaphrodite self-cross versus

the control out-cross fish remains the same to maturity, the relative fecundity of the H1xH1 and H3xH3 self-crossed offspring at maturity will be 0.89 and 0.80, respectively, that of the CxC offspring. In the same way, we can estimate relative survivorship, where the survivorship of the H1xH1 and H3xH3 offspring compared to the control offspring is 0.82 and 0.85 respectively. Thus, the fitness relative to the control offspring of the H1xH1 offspring is 0.73 (=0.89 · 0.82) and 0.68 (=0.80 · 0.85) for the H3xH3 offspring (based on fitness=fecundity x survivorship). Therefore, our ID selection pressure estimates are 0.27 and 0.32 for the H1xH1 and H3xH3 offspring respectively. These are the first such estimates for salmonids, with very few experimental studies able to quantify ID selection pressure in any other vertebrates, indeed such estimates are also rare among invertebrates as well (i.e. Mallet et al., 1990; Crnokrak and Roff, 1995). This is a very large fitness cost associated with inbreeding, especially given other studies in salmonids report less than a 15% decrease in fitness with every 10% increase in inbreeding (Wang et al., 2002). Furthermore, our estimate of fitness loss and selection pressure resulting from ID is likely conservative, since we observed an increasing trend for ID effects on body size and survival over time, perhaps leading to even greater size and survival differentials by the time of maturation of our study fish.

ID has been reported to exhibit developmental variation where levels of ID are higher later in an individual's life. Wolfe (1993) was one of the first to explore this and showed that in a normally outcrossing plant, *Hydrophyllum appendiculatum,* the magnitude of ID detected resulting from self-crossing was small during the first year of life, but increased with age and had significant effects on adult body size and reproductive traits (Wolfe, 1993). Husband and Schemske (1996) showed this to be a

common trend among many plant species, where ID was expressed later in the life cycle of 14 out of 18 naturally selfing plant species and in 19 out of 40 outcrossing species. It has been postulated that the effects of ID may be initially masked by maternal effects that reduce the effects of inbreeding early in life (Wolfe, 1993). Data on the timing of the effects of ID in species other than plants is limited. However, indirect evidence that ID may be more prevalent later in life for salmonids can be inferred from the lack of detection of ID in early life history traits (e.g., Su et al., 1996; Houde et al., 2011). Our results support the general pattern of ID effects in plants (increasing later in life), since we do not detect signs of ID until the offspring are one year old. Curiously, the HxH offspring were the largest fish followed by the mixed-cross offspring, with the control CxC offspring being the smallest at approximately seven months of age (at the time of tagging). This unexpected finding may be explained by rearing environment (tank) effects or maternal effects. We estimated tank effects using the replicated CxC crosses and found highly significant tank effects for body size at the time of tagging; however, the tank effects were non-significant at all subsequent sampling times. Additionally, the variance explained by cross-type effects was over 10 times higher than that explained by tank effects (results not shown).

The reciprocal (mixed) crosses generally displayed intermediate performance between the hermaphrodite self-crossed and control out-crossed families in terms of growth, but the same performance as the control in terms of survival. This pattern is indicative of factors other than ID affecting growth performance in our crosses, since the mixed cross offspring are not inbred and thus should not exhibit ID. A reduction in the growth performance of the mixed cross offspring due to reduced hermaphrodite gamete

quality effects is possible, although not expected since the reduction in performance occurred well after the expected early life time frame for gamete quality effects, which, in terms of female gamete quality effects (maternal effects), normally manifest themselves shortly after fertilization (Heath and Blouw, 1998; Heath et al., 1999). Generally we found that the CxH offspring are, on average, more fit than the HxC offspring (indeed in some instances they exhibit better growth than the control CxC offspring as well) indicating that the hermaphrodite sperm quality is likely not contributing to the reduced performance of the reciprocal or inbred crosses. Although both the reciprocal crosses show signs of being less fit than the CxC offspring, our data indicate a greater long-term fitness cost associated with the hermaphrodite eggs than with the hermaphrodite sperm, since the CxH offspring are 99% as fit as the control offspring, whereas the HxC offspring are almost 8% less fit than the control offspring, with a relative fitness of 92% using wet weights measured in April 2011 (calculated as above). Such long-term egg quality effects have not been previously reported, and the mechanism behind such effects are not clear. It is possible that there were epigenetic effects during the development of the hermaphrodite eggs due to their intersex hormonal environment, or that hermaphrodite maternal effects may have affected developing offspring past the normal maternal effect time span. Although such epigenetic effects have yet to be demonstrated in salmonids (Aykanat et al., 2010), maternal effects on growth and survival in fish well past early developmental stages are well documented (i.e. Heath and Blouw, 1998; Tyndale et al., 2008). Despite the apparent hermaphrodite gamete quality effects on performance, the hermaphrodite self-crossed fish consistently performed worse than the

mixed-crossed fish, indicating that ID is the major contributor to the reduced fitness of the hermaphrodite self-crossed offspring.

In conclusion, we have shown that although the fitness cost associated with inbreeding in salmon may not be apparent at low inbreeding levels, populations experiencing high or sustained levels of inbreeding may suffer substantial fitness losses. It appears as though the salmonid ID may be a threshold effect, where there is little or no effect at low or moderate levels of inbreeding; however, above a certain level of inbreeding, ID increases greatly. This may be due to the ancestral tetraploidy of salmon, although past purging effects are not likely to contribute to such a threshold type of response. Although it is apparent that ID clearly can affect salmonids, it is important to explore whether it is ecologically or conservation relevant. The inbreeding coefficient, F, is a measure of the probability that genes within a population are identical by descent, and reflects mean inbreeding levels (Wang et al., 2002). Offspring resulting from self-crossing (of a non-inbred parent) have an average F value of 0.50. Although self-crossed salmon are not likely to occur in natural populations, small isolated salmon populations may have effective populations sizes (Ne) on the order of 10 (e.g. Hedrick et al., 2000; Heath et al., 2002; Shrimpton and Heath 2003; Wang et al., 2002). Inbreeding coefficients have been shown to increase by (1/Ne) per generation (specifically as $F_t = 1-[1-(1/2Ne)]^t$) in salmon populations of constant size (Wang et al., 2002; Falconer and Mackay, 1996). Thus, after 10 generations, a population with Ne = 10 reaches an F value of 0.40 and after 20 generations F=0.60. Consequently, the effects of ID found in this study, although extreme for a single generation, are relevant for the conservation of small and endangered salmonid populations in the wild. Also, the offspring in this study were

held in a relatively benign environment, and thus it is expected that inbreeding in the wild will likely have an even greater fitness cost due to increased exposure to stress (Crnokrok and Roff, 1999; Wang et al., 2002). Conservation programs should thus monitor levels of inbreeding (and ID) in small populations of wild salmon, and inbreeding avoidance measures should continue to be a high priority for government supplementation hatcheries. This study is the first to show an apparent threshold effect on the expression of ID, perhaps driven by a high genetic load in Chinook salmon buffered by their residual tetraploidy. However, an alternative explanation may be the presence of high levels of overdominance among salmon, which, when mediated by the duplication of functional genes, may mask the effects of ID until levels of homozygosity at many loci across the genome reach critical levels. The present study is not able to distinguish between Dominance and Overdominance models of ID; however, in Chapter 3 we explore the genetic mechanisms contributing to ID in Chinook salmon.

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Table 2.1. Inbred and Outbred Offspring Size Data with Significant Differences

Mean wet weight (WW) of the Chinook salmon offspring (with SE in brackets) from all eight families at three sampling times: June 2010, October 2010 and April 2011. The asterisks represent significant differences between the mean compared to the mean of the control family via Mann Whitney U tests (*p<0.05, **p<0.01, ***p<0.001).

		June 2010	October 2010	April 2011
Offspring		WW(SE)	WW(SE)	WW(SE)
Hermaphrodite 1 Families	H1xH1	6.69 (0.09) ***	42.6 (0.67) *	117 (3.37) **
	CxH1	6.00 (0.06) ***	45.0 (0.51) ***	138 (2.36)
	H1xC	5.78 (0.15) ***	39.4 (0.96)	116 (3.92) **
	C1xC1	4.59 (0.08)	40.6 (0.69)	133 (3.11)
Hermaphrodite 3 Families	H3xH3	5.09 (0.05) **	35.0 (0.39) ***	109 (1.91) ***
	CxH3	4.91 (0.10)	39.8 (0.73) *	130 (3.65)
	H3xC	5.08 (0.05) **	40.8 (0.41)	128 (1.93) **
	C3xC3	4.86 (0.05)	41.8 (0.53)	137 (2.17)

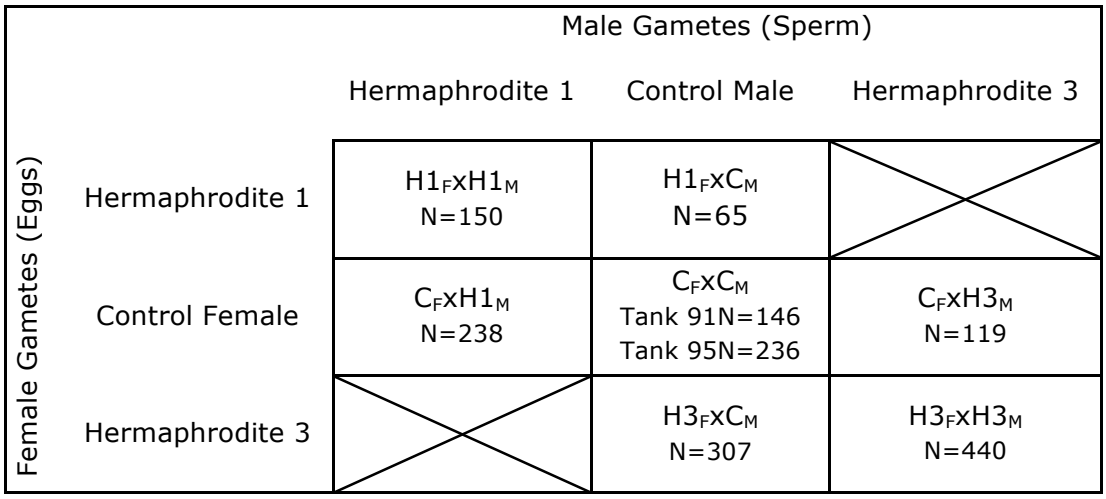

Figure 2.1. Family Breeding Design Summary of the crosses performed using gametes from 4 parental fish: Hermaphrodite 1 ($H1_F$ & $H1_M$), Hermaphrodite 3 ($H3_F$ & $H3_M$), Control (Sex-reversed) Male (C_M) and Control Female (C_F) to create a total of 7 offspring families. The number of offspring in each family PIT-tagged and measured in June 2010 is shown (N). The Control Family was split into two groups at fertilization – Tank 91 contained offspring that were housed in compartments of the incubation trays where the H3 offspring were held and Tank 95 contained offspring that were housed in compartments of the incubation trays where the H1 offspring were held.

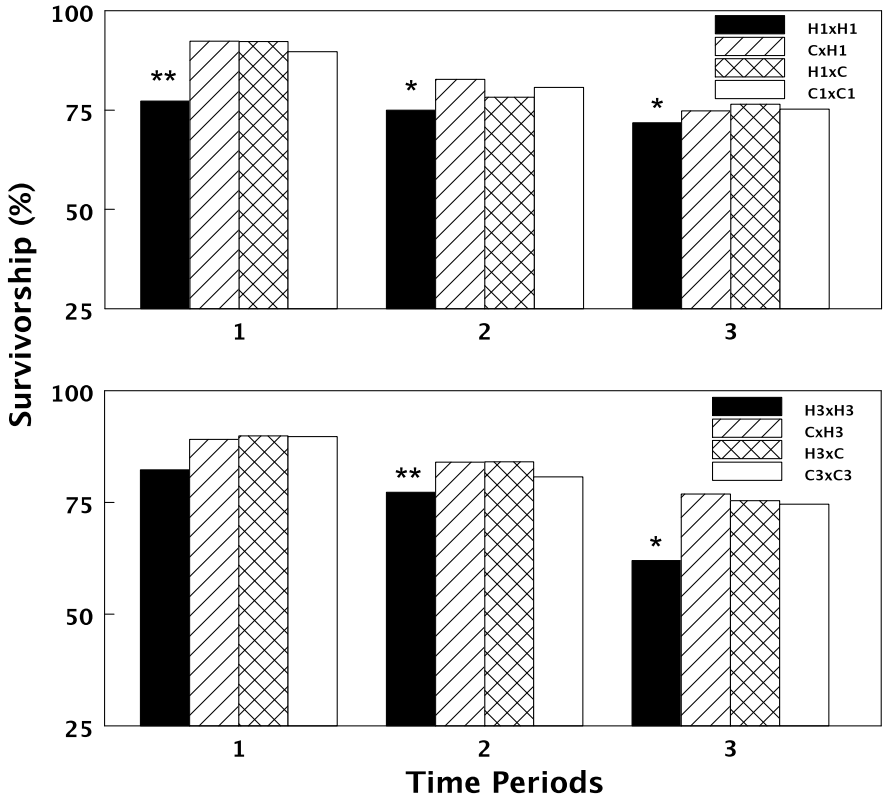

Figure 2.2. Survivorship Cross survivorship (%) from tagging (June 2010) until April 2011, displayed over three different time periods (1 - June 2010-October 2010, 2 - October 2010-April 2011 and 3 - overall from June 2010 – April 2011) – the top panel shows results for the H1 crosses, the bottom panel shows results for the H3 crosses. Significant differences were determined between all the crosses compared to the CxC cross (*$p<0.05$, **$p<0.01$, ***$p<0.005$).

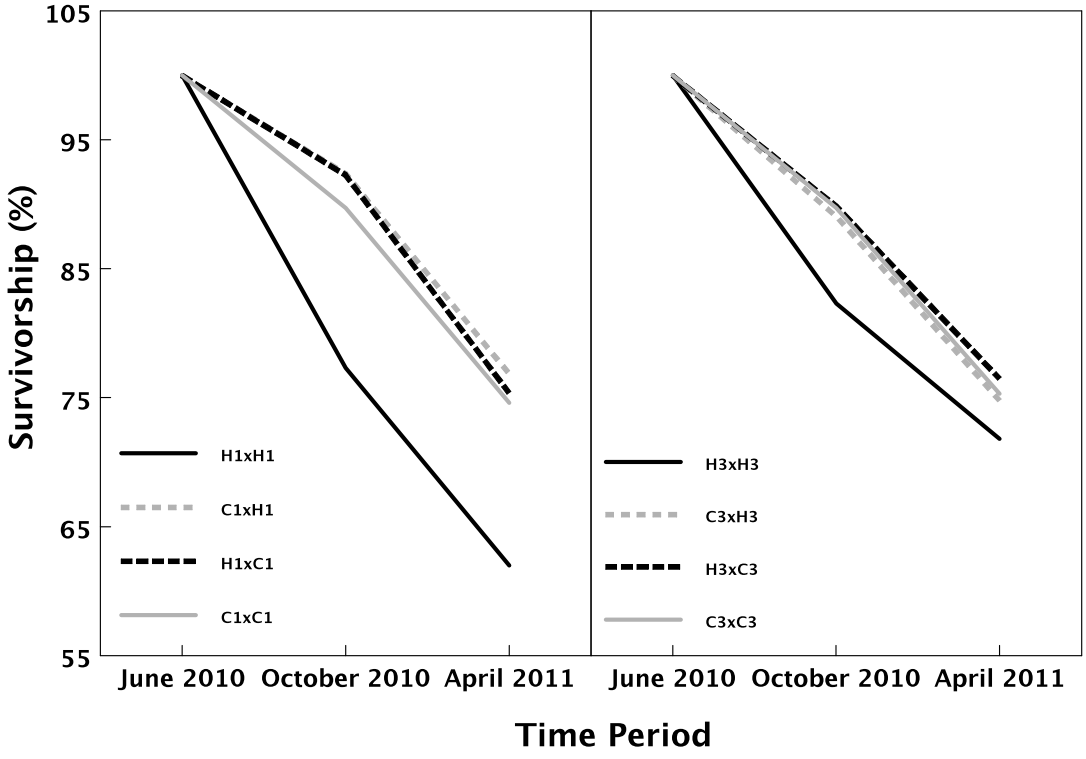

Figure 2.3 Overall Survivorship Cross survivorship (%) overall at each time period (June 2010, October 2010 and April 2011). The left graph depicts survivorship for the H1 and the right panel depicts survivorship for the H3 crosses. Survivorship was calculated as a percentage of offspring that survived compared to number of offspring in June 2010.

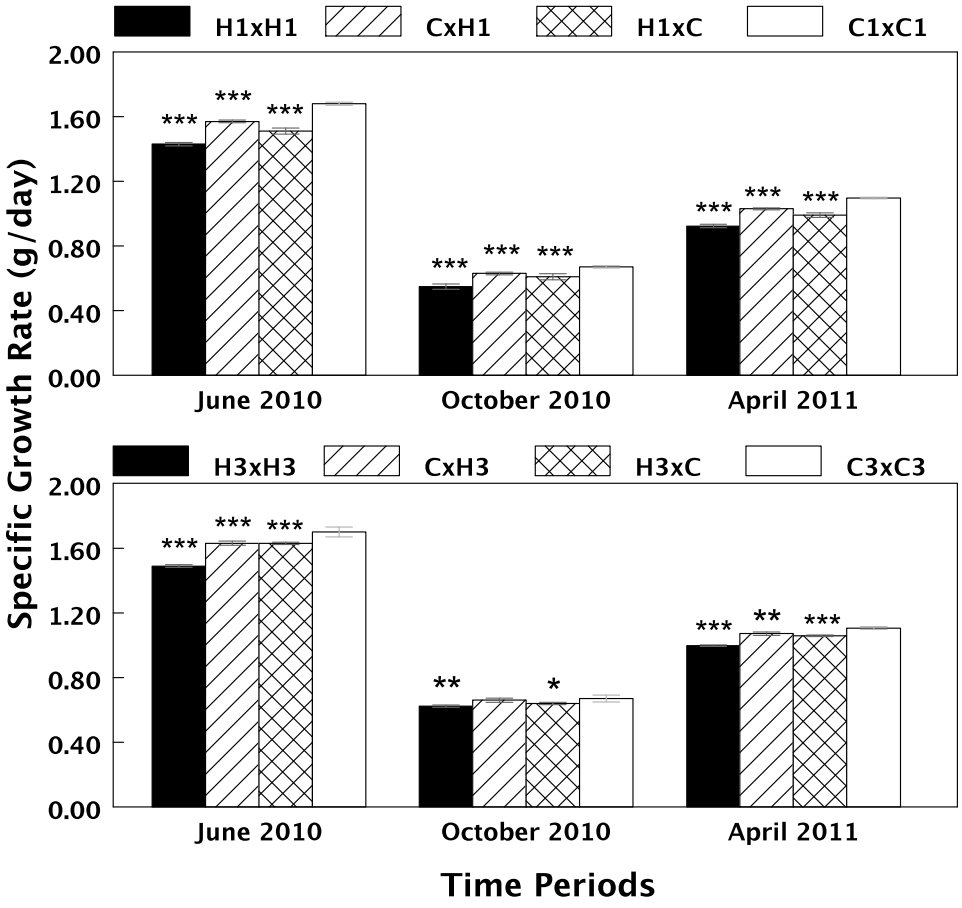

Figure 2.4. Specific Growth Rate Mean specific growth rate for all crosses (H1 crosses in the top panel and H3 crosses in the bottom panel) over three time periods (1 – June 2010-October 2010, 2 – October 2010-April 2011, 3 – overall from June 2010-April 2011). The number of asterisks indicate significant differences of the mean compared to the control cross using Mann-Whitney U tests (*p<0.05, **p<0.01, ***p<0.001).

3.0 SEGREGATION DISTORTION AT MICROSATELLITE LOCI IN SELF-CROSSED HERMAPHRODITE CHINOOK SALMON: GENETIC LOAD AND PURGING

3.1 INTRODUCTION

Inbreeding results from the mating of individuals that are related, and inbreeding depression is the resulting loss of fitness associated with inbreeding (Charlesworth and Charlesworth, 1999). Inbreeding depression (ID) can affect individuals and populations and the two common mechanistic hypotheses that explain how inbreeding depression occurs are the Overdominance and Dominance Hypotheses. Under the Overdominance Hypothesis, inbreeding depression is explained as a loss of advantageous heterozygote loci, where the heterozygote allele combinations increase fitness of the individuals expressing them (heterosis) (Charlesworth and Charlesworth 1987; 1999) and should result in a relative decrease in the number of homozygote genotypes at affected loci (either directly or by linkage disequilibrium). According to the Dominance Hypothesis, inbreeding depression is due to an increase in the frequency of recessive deleterious alleles being expressed within an inbred population, and selection against individuals homozygous for the deleterious alleles will lead to decreased population size and individual fitness (Charlesworth and Charlesworth, 1987; 1999). We thus would expect to see a reduction in the frequency of inbred offspring that are homozygous for the deleterious alleles (directly or by linkage) at the affected loci. In theory, it is possible to classify ID as resulting from the Overdominance or Dominance effects by the pattern of relative genotype frequencies at affected loci, or at marker loci in linkage disequilibrium with the functional loci.

The Dominance Hypothesis presupposes recessive deleterious alleles to be present in populations, and within individuals' genomes. Genetic load, first proposed by Muller (1950), is the total of all the deleterious alleles in a population at all gene loci (Wallace, 1970). In terms of fitness, genetic load defined by Crow (1958) is the proportional amount by which the average fitness of a population is reduced relative to the optimal genotype by the presence of deleterious alleles (Wang et al., 2002). Genetic load can also be measured at an individual level, where it is the total number of deleterious (often lethal or "lethal equivalent") alleles in a single individual. The Overdominance Hypothesis is predicted on heterosis, the genetic process whereby individuals who are "outcrossed", where gametes with dissimilar genetic makeups are combined, produce offspring that have increased vigour and other traits that make them more suited to survive and reproduce (Shull, 1948). The concepts of heterosis and genetic load combined should therefore drive the mating of individuals who are genetically unrelated, the exact opposite of inbreeding, where individuals who are genetically similar mate, producing offspring with higher levels of homozygosity, which should lead to decreased fitness expressed (under both hypothesized mechanisms) as increased ID. For example, in an African species of butterfly, *Bicyclus anynana,* a study designed to measure the effects of inbreeding on genetic load showed that seven generations of inbreeding resulted in intermediate to high levels of ID and high expression of genetic load of lethal equivalents (van Oosterhout et al., 2000). This led to sterile egg clutches, reduced zygote and juvenile survival and reduced adult male and female longevity (van Oosterhout et al., 2000). Additionally, Launey and Hedgecock (2001) used inbred lines of Pacific oysters, *Crassostrea gigas,* and segregation patterns at microsatellite DNA loci in

offspring to show that inbred lines carried a genetic load of 8-14 highly deleterious recessive mutations. This was interpreted as strong support for the Dominance Hypothesis in that species (Launey and Hedgecock, 2001).

Inbred individuals carrying high levels of genetic load will exhibit decreased fitness (due to ID) resulting from higher levels of homozygosity in general, and thus more recessive deleterious alleles being expressed (Kirkpatrick and Jarne, 2000). This sets up the potential for genetic "purging" to occur. Genetic purging occurs when the deleterious alleles are expressed, selected against and lost to the population leading to a reduction in the genetic load of the population overall, and potentially an increase in the fitness of the population (Leberg and Firmin, 2008). Moreover, populations that experience purging are less susceptible to future negative effects of inbreeding and are subsequently able to recover faster from population bottlenecks (Leberg and Firmin, 2008). The potential for beneficial effects resulting from inbreeding has caught the attention of conservation biologists and managers (Hendrick, 1994; Kristenson and Sorenson, 2005; Leberg and Firmin, 2008).

As genetic diversity is thought to be necessary for the long-term viability of a population, one of the main goals of conservation programs for endangered or exploited species is the avoidance of inbreeding (eg. Hendrick, 1994; Leberg and Firmin, 2008) and the associated loss of genetic diversity (Charlesworth and Charlesworth, 1987; Kristensen and Sorensen, 2005). One of the main tenants of programs aimed at the conservation and management of salmon is the avoidance of inbreeding and the maintenance of genetic diversity (Hendrick, 1994; Kristensen and Sorensen, 2005). Salmon populations are thought to naturally experience inbreeding, due to fluctuations in population sizes (i.e.

bottlenecks) and population isolation resulting from their strong natal site homing tendencies (philopatry). Interestingly, brief bottlenecks of only a few generations, followed by population expansion provide opportunity for purging of highly deleterious alleles through drift, without the necessity of inbreeding (Leberg and Firmin, 2008). However, salmon have diverse life histories and unusual genomic makeup making genetic conservation complex (Allendorf and Waples, 2002). Salmon have a tetraploid ancestry, which complicates their system of inheritance (Allendorf and Waples, 2002), and although their polyploidy ancestry likely buffers them from the effects of deleterious mutations or alleles (Comai, 2005; Chapter 2), they may also not experience positive effects of purging for the same reason.

Here we analyze the mechanisms of inbreeding and ID at the genotypic level by characterizing segregation at microsatellite loci in inbred (selfed) versus outbred Chinook salmon. To accomplish this, functional hermaphrodite Chinook salmon were used to create self-fertilized offspring that were compared to outbred families of Chinook salmon. Segregation distortion detected in the inbred hermaphrodite family may reflect selection acting on the developing fish resulting in departures from Mendelian genotype inheritance expectations. Under the Dominance Hypothesis for ID, we expect to observe the loss of one homozygote class in the inbred offspring, and that would be evidence for the presence of genetic load and purging occurring in a single generation. Alternatively, the Overdominance Hypothesis would predict that we would observe reduced frequencies of both homozygote classes (relative to the heterozygotes). We genotyped parents and inbred and outbred offspring at 20 microsatellite loci to detect segregation distortion resulting from linkage with functional loci (Chinook salmon exhibit very low

recombination rates making the linkage groups large; Young et al., 1997). Some loci exhibited abnormal genotype ratios in the inbred offspring suggestive of ID effects, and the pattern of deviation from Mendelian expectations provide insight into the mechanism of ID in salmon. Our results inform both applied conservation and management scientists interested in how ID can affect population viability in salmon, and also contribute to our basic understanding of the genetic principles that underlie ID, genetic load and purging in vertebrates.

3.2 MATERIALS AND METHODS

Hermaphrodite Production

As described in Chapter 2, two functional hermaphrodite Chinook salmon were created using a hormonal embryo incubation treatment, where known female embryos were treated with alpha-methyltestosterone (AMT) at two separate developmental stages (50% hatch and 100% hatch). The eggs were fertilized in the fall of 2004 and the mature inter-sex (hermaphrodite) fish were collected in November 2009.

Experimental Crosses

On November 3, 2009, three hermaphrodite salmon were identified by inter-sex secondary characteristics within over 800 treated fish, as detailed in Chapter 2. Two of the three salmon carried viable male and female gametes (sperm and eggs), which were collected and used, along with control female and control male gametes, to create seven offspring families (see Chapter 2, Figure 2.1). The control male fish used in the crosses was a sex-reversed XX male fish, which eliminated the potential complications associated with having XY offspring (such as differential performance, no recombination,

etc.; Lehnert et al., 2012; Heath et al., 2002). All of the crosses took place at Yellow Island Aquaculture Limited (YIAL), located on Quadra Island British Columbia. Fin clips were taken from the parents at the time of fertilization for later DNA extraction. Offspring from each family were reared in replicated, separate incubation trays (freshwater flow of 12L/min), and later in separate freshwater tanks (200L). On June 9-10, 2010, offspring were injected with individual Personal Integrated Transponder (PIT) tags. Only a subset of each of the families were used in this experiment – adipose fin clips were taken from 200 of the hermaphrodite (HxH) offspring, 100 HxC offspring, 100 CxH offspring and 150 CxC offspring. The fin clips were held in individual 200μL microcentrifuge tubes containing 95% ethanol. Subsequently, the PIT-tagged fish were held in a common saltwater netcage. These fish were monitored for overall saltwater growth and survival from June 2010, when they were moved into netcages, until April 2011.

Genotyping

DNA from the parental fin clips (two 2009 hermaphrodites, one male XX and one female fish) was extracted using Phenol-Chloroform Isoamyl alcohol DNA extraction. In this type of extraction, the tissue is digested overnight in Proteinase K and buffer, and the digest solution is isolated with a phenol/chloroform/isoamyl alcohol mixture to remove protein contaminants and then precipitated with 100% ethanol. The DNA is pelleted after the precipitation step, washed with 70% ethanol and resuspended in TE buffer. The offspring DNA was extracted from offspring fin clip tissue samples using an automated plate based extraction protocol (Elphinstone et al., 2003).

The parental DNA was screened at 42 microsatellite loci known to yield polymorphic alleles in Chinook salmon. Dye-labelled (IR-700 and IR-800) forward and reverse primers were used for each of the 42 loci. Amplification at these loci were performed in 50μL polymerase chain reactions (PCR) using 1μL of DNA template, 1μL of each primer (0.1 μg μL^{-1}, 1X PCR buffer (Promega), 1μL deoxy-nucleoside triphosphate (dNTP) (200μM) and 0.5U Taq polymerase. Amplification was performed according to conditions optimized for each primer set using parental DNA and modified PCR solution and amplification conditions using a gradient of $MgCL_2$ concentrations and annealing temperatures. Parental PCR product sizes were determined on a LiCor 4300 DNA Analyzer with GeneImagir 4.05 software (Scanlytics), which allowed the determination of homozygosity or heterozygosity at each locus for the two hermaphrodite parents. Only microsatellite loci that were heterozygous in at least one hermaphrodite parent were genotyped in the hermaphrodite offspring, so that the inheritance and segregation patterns of the parental alleles into the HxH offspring could be determined.

Out of the 42 loci screened in the hermaphrodite parents, 20 loci were found to be suitable for this study because they were reliable and heterozygous in both the hermaphrodite parents. To determine if there was segregation distortion occurring, we tested observed genotype frequencies against the expected 1:2:1 homozygous small: heterozygous: homozygous large expected Mendelian ratio. Any locus that was found to deviate from the normal Mendelian ratio was then genotyped in the control (CxC) and reciprocal cross (CxH and HxC) offspring to characterize the nature of the segregation distortion. As with the hermaphrodite parental DNA, DNA from the each of the offspring was amplified at each of the microsatellite loci being studied using PCR reactions

described above, and then individually genotyped using the LiCor 4300 DNA Analyzer with GeneImagir 4.05 software. There were 100 H1xH1 offspring and 100 H3xH3 offspring genotyped at each of the 20 microsatellite loci. The loci that were segregating abnormally in either the H1xH1 or H3xH3 offspring were then genotyped in the 150 control (CxC) offspring to determine whether or not there was an inherent problem with the microsatellite loci. Next, 50 H1xC, 50 H3xC, 50 CxH1 and 50 CxH3 offspring were genotyped at those loci.

Statistical Analysis

The analysis of segregation patterns consisted of hierarchal steps: 1) we tested for segregation distortion in the offspring of the HxH crosses; 2) if we detected significant departure from Mendelian expectations, we next tested for segregation in the CxC control cross (microsatellite loci exhibiting segregation distortion in the control cross resulting from technical issues); and finally, 3) if the locus exhibited normal segregation in the control crosses, we tested genotype frequencies in the reciprocal hybrid crosses (CxH and HxC). All tests for departure from Mendelian genotype frequency expectations were analyzed using Pearson Chi-square analysis. A modified Bonferonni correction (Hochberg variation) was applied, where Chi-square values were ranked from largest to smallest before alpha values were determined, in order to accurately establish whether a significant deviation from Mendelian ratios was present. In this variation, the largest value of p is compared to alpha – if it is significant, then so are all the smaller values of p. If it is not significant, we moved on to the next value of p, which is compared to the critical value alpha/(T-1), where T is the number of tests.

The hierarchical approach to our analysis provides a series of possible explanations as to why the hermaphrodite (HxH) offspring deviated from normal Mendelian ratios. If Mendelian deviations are also present in the CxC offspring at the locus, it is likely due to a problem associated with the microsatellite marker itself (such as the presence of a null allele) or it could have resulted from a technical error. If a locus exhibits departure from Mendelian expectations in the HxH offspring but segregated normally in the CxC offspring, the segregation pattern in the reciprocal mixed offspring allows us to discriminate between selection acting on the inbred HxH offspring and true meiotic segregation distortion that could affect hermaphrodite gametes independent of the cross type. For example, if deviations are detected in the CxH offspring (hermaphrodite sperm), it would signal a problem with segregation in the hermaphrodite's sperm, while deviations detected in the HxC offspring (hermaphrodite eggs) would indicate a problem with segregation in the hermaphrodite's eggs. Finally, if there are only deviations detected in the HxH offspring and there is normal segregation in the reciprocal crosses as well as the CxC cross offspring, we have evidence of segregation distortion resulting from selection arising due to inbreeding and further analysis of the genotype frequencies in the HxH crosses will allow the characterization of the nature of the inbreeding effects.

We expanded our investigation of the microsatellite loci that showed abnormal Mendelian segregation in the HxH offspring (but normal segregation in the CxC, HxC and CxH offspring) by examining the performance of the offspring after tissue sampling (June 2010). We predicted that the genotypes observed at lower than expected frequency (presumably due to selection against them during development) would also exhibit lower growth rates and reduced size later in life. Wet weight measurements of the HxH

offspring were taken at three different sampling times (June 2010, October 2011, April 2011). The mean wet weights were determined for offspring of each genotype at the candidate microsatellite loci and compared using a one-way ANOVA. If there was a significant difference detected, a Tukey post-hoc test was applied to determine which specific genotype classes were different. The specific growth rate was also calculated for the offspring of each genotype at the same microsatellite loci (SGR = 100 x [ln(wet $\text{weight}_{\text{time2}}$) – ln(wet $\text{weight}_{\text{time1}}$)/$\text{time}_{\text{days}}$) over the time period June 2010 to April 2011. SGR of the various genotypes were then compared using a one-way ANOVA. Finally, survivorship of the offspring was determined and differences in the frequency of offspring that survived versus those that did not survive from each genotype category were analyzed using cross-tab analyses.

3.3 RESULTS

Twenty of the forty-two screened microsatellite loci were reliable and heterozygous in at least one of the hermaphrodite parents, making them suitable for segregation analyses in the offspring fish. Of the 20 loci that were genotyped in the 2009 hermaphrodite offspring, 5 microsatellite loci were found to significantly deviate from normal Mendelian genotypic ratios after sequential Bonferonni correction in the HxH families. The H1xH1 offspring significantly departed from Mendelian genotype frequency expectations at three microsatellite loci (Omy325, $p<0.01$; Ssa85, $p<0.001$; Ots107, $p<0.01$; Figure 3.1). Additionally, the H3xH3 offspring were found to deviate significantly from Mendelian ratios at two loci (OtsG311, $p<0.01$; Ots212 $p<0.04$; Figure 3.2). Next we analyzed the segregation patterns of these 5 microsatellite loci in the control offspring (CxC) and found that there were no significant deviations from

Mendelian inheritance patterns at any of the loci (Table 3.1). Segregation patterns of the microsatellite loci in the reciprocal hybrid crosses (CxH and HxC) showed mixed results. At Omy325, the CxH1 offspring segregated normally according to Mendelian inheritance, however there was significant deviation in the H1xC offspring ($p<0.017$; Table 3.1). The microsatellite locus Ssa85 was found to segregate normally in the CxH1 crossed offspring, however could not be analyzed further because the H1xC crossed genotypes had an allele that was not present in either parent, indicating either a technical error, or a null allele. At the remaining three microsatellite loci (Ots107, OtsG311 and Ots212), the HxC and CxH crossed offspring were found to segregate according to Mendelian inheritance (Table 3.1). Thus 3 loci fulfilled the requirements for differential survival among offspring genotypes in the hermaphrodite crosses that is consistent with the effects of ID in these highly inbred families.

For the three microsatellite loci (Ots107, OtsG311 and Ots212) that segregated normally in the reciprocal hybrid offspring, but departed from Mendelian expectations in the HxH offspring, we tested for microsatellite genotype differences in wet weight, survivorship and specific growth rate of each genotype at each locus. For the Ots107 microsatellite locus, the fish with the homozygous allele combination 228:228 were found to be significantly smaller than the heterozygous allele combination 228:236 genotype and the 236:236 homozygous genotype in terms of wet weight ($p=0.0004$) at the initial sampling period in June 2010 (Figure 3.3). The fish with the homozygous 228:228 genotype were also significantly smaller in terms of wet weight ($p=0.025$) than the 236:236 homozygous genotype in April 2011 (Figure 3.3). As shown in Figure 3.3, there were no significant differences noted between the HxH fish of different genotypes

at the loci OtsG311 and Ots212. Survivorship was calculated as a percentage of the number of offspring that were present for each genotype in June 2010. There were no significant differences found between the genotypes at any of the three microsatellite loci (Figure 3.4). In terms of specific growth rate, overall there were no significant differences noted between any of the genotypes at all of the microsatellite loci; however at OtsG311, the genotype 325:325 grew at a rate that approached significance ($p=0.064$; Figure 3.5).

3.4 DISCUSSION

It is clear from our findings that abnormal segregation is occurring in the hermaphrodite offspring, resulting from extreme inbreeding (i.e. selfing). Of the 20 microsatellite loci screened, 5 microsatellites deviated from Mendelian inheritance patterns in the HxH loci. However, after analyzing these five loci in the HxC and CxH crosses, two were found to be segregating abnormally in these reciprocal crosses and therefore segregation distortion in the inbred HxH lines was detected at 3 loci (Ots107, OtsG311 and Ots212). The distortion in at least two of these loci was likely due to low survival of one homozygous state. Thus, those loci show a pattern consistent with the Dominance hypothesis where there is an increase in the expression of deleterious alleles in homozygote genotypes. The locus Ots107 is deviating from Mendelian inheritance due to a loss of 228:228 homozygotes (dominance) and OtsG311 is also due to a loss of 329:329 homozygotes (dominance); however, OtsG311 also exhibits a surplus of heterozygote genotypes, which is consistent with the Overdominance hypothesis. On the other hand, Ots212 appears to be deviating due to a reduced frequency of heterozygotes, which is not an outcome we expect to occur. Therefore, there is a potential that Ots212 is in fact not deviating from expected inheritance patterns due to selection resulting from

ID, but may be due to either a technical artifact or an undescribed form of segregation distortion. The next step is to explore segregation at this microsatellite in greater detail. Therefore, there is evidence that both Overdominance and Dominance are contributing to ID in Chinook salmon, although Dominance effects appear to be larger.

Omy325 was a locus that was ruled out as segregating abnormally due to ID because the hybrid cross H1xC was also found to be segregating abnormally. Thus, the deviations seen at this locus could be due to true meiotic distortion in the salmon gametes, and not differential survival. Since the segregation distortion (from the hermaphrodite) is via the egg, these data provide evidence that during meiosis II of oogenesis in the eggs, the extra set of chromosomes ejected may not be a random process, thereby resulting in segregation distortion (Guraya, 1986). This is evidence that meiotic drive may be taking place, where meiotic drive is any alteration of normal meiosis which results in an effective gametic pool with an excess of one genotype (Zimmering et al., 1970). Therefore, in our case, one microsatellite allele is present in more than 50% of the female's eggs (Zimmering et al., 1970; Guraya, 1986) and thus the offspring display an excess of genotypes with this allele. The Ssa85 genotype could not be analyzed past the point of deciphering the abnormal segregation in the HxH offspring due to some technical difficulties with the H1xC crossed offspring alleles. However, it is important to note that both of these two loci (Omy325 and Ssa85) segregated normally in the CxC cross offspring, indicating that their unusual inheritance pattern is unlikely, although possibly, due to a simple technical error or marker failure.

Surprisingly, 3 out of 20 loci (15%) segregated abnormally, probably due to the effects of ID, which is indicative of a very high genetic load. The "C value" is the total

genomic DNA content of the gametes of a species, which has been determined for rainbow trout (*Oncorhynchus mykiss*) to be 5.17pg (Vinogradov, 1998) and therefore we can assume it is likely similar for Chinook salmon since they are within the same genus. Of the 3 microsatellite loci that were segregating abnormally in the HxH crosses, 2 were shown to be in linkage disequilibrium with highly deleterious recessive alleles, indicative of genetic load. Those microsatellite markers reflect selection acting on all functional gene alleles within their linkage group. Since female salmon have been found to only recombine once per chromosome arm (Young et al., 1997), the size of the linkage groups should be, on average, half that of a chromosome arm. This means the total number of linkage groups should be twice the number of chromosomes. Phillips et al. (1985) have found Chinook salmon to have 68 chromosomes, therefore 132 linkage groups. Thus, since we have found 2/20 loci to be high in genetic load (replicated in 2 fish), assuming all the microsatellites were unlinked, there are 13 deleterious alleles per fish. This estimate of genetic load is comparable to the levels of genetic load that have been shown in invertebrate species as well, such as the Pacific Oyster, *Crassostra gigas,* who have been found to carry a minimum of 8-14 highly deleterious recessive mutations within inbred lines, which was described as being very high (Launey and Hedgecock, 2001). These findings of high genetic load among inbred populations support the Dominance theory of ID. Other estimates of the number of lethal alleles per gamete have resulted in mean values per individual of 2.8 for mammals, 4.3 for birds, 2.8 for *Drosophila* and 8.1 for conifers (Lynch and Walsh, 1998). Those estimates would be much lower if the authors had used marker based genetic analyses of genetic load, as this study and Launey

and Hedgecock (2001) did since such approaches should provide more accurate estimates of genetic load in highly fecund species (Launey and Hedgecock, 2001).

Our results have conservation as well as evolutionary implications. In terms of conservation, homozygote genotypes tend to exhibit reduced fitness, especially later in life, which was shown at the Ots107 microsatellite locus as smaller individuals carrying the 228:228 homozygous genotype (Figure 3.3). Our results also highlight evidence for the potential for inbreeding in salmon to lead to purging of the genetic load. Theoretically, purging takes place after a series of bottlenecks occurs which drives increased inbreeding levels and eventually ID. The resulting ID, if due to the expression of recessive deleterious alleles, will lead to selection against the deleterious alleles, reducing their likelihood of being passed on to the next generation (Hedrick, 1994). Thus, for genetic purging via selection to act, the dominance hypothesis of ID must be in effect. Our results support the Dominance hypothesis for ID, and there is evidence that purging is in fact able to occur as a result. However, because of the high genetic load that our salmon exhibit coupled with the threshold type of effect shown for ID (Chapter 2) purging is not likely a common factor in salmon population genetics, especially at low levels of inbreeding (Wang et al., 2001). However, it is important to note that the magnitude of selection acting at the genetic load loci we detected is not very high. For instance, at Ots107, the observed genotype frequencies are 26:51:8 for 236:236, 228:236, 226:226. Therefore, 18 of the homozygous genotypes that are lacking (226:226) were "lost" according to Mendel's 1:2:1 ratio of inheritance. Therefore, the selection rate against these fish is only 18/26 or 70%. Nonetheless, to the best of our knowledge, this is the first time that evidence for effective purging has been reported in a vertebrate

organism. Since the hermaphrodite salmon self-crossing used in this study represents very extreme levels of inbreeding, it was expected that a more pronounced level of ID, and hence purging, would result. However, salmonids may only experience the potentially beneficial effects of purging at extremely high levels of inbreeding due to their ancestral genome duplication (Allendorf and Waples 1996) and genetic buffering that take place. From a conservation perspective this is somewhat unfortunate, since the potential beneficial effects of purging could act to increase population viability in salmon, which naturally experience population bottlenecks and isolation making them prone to inbreeding (Miller and Hendrick, 2001; Legerg and Firmin, 2007).

Here we showed evidence for ID operating under both the Dominance and Overdominance mechanistic hypotheses of ID. Dominance effects (genetic load) on ID in the self-crossed Chinook salmon offspring in this study are perhaps not surprising, but this is the first study to show direct evidence for genetic load, plus a quantitative estimate of genetic load in a captive population of Chinook salmon. However, our estimate of the genetic load is not consistent with substantial purging having acted on our study population. The contribution of overdominance effects to Chinook salmon ID was also expected, although past studies in salmon have shown only weak heterosis effects in controlled breeding experiments (e.g. Bryden et al., 2004). Therefore commercial breeding programs and salmon enhancement conservation programs should address the potential harmful effects of ID rather than risk the potentially beneficial effects of purging or heterosis when breeding to maximize production on fish farms or long term viability in the wild. We have shown that purging is possible in highly inbred populations; however, the potential for the populations to suffer fitness losses, and

possible extirpation prior to experiencing post-purging increases in fitness may outweigh the benefits. Further study into the techniques of purging genetic load in salmonids should be pursued; however, there is now concrete evidence that substantial genetic load does exist in vertebrate organisms.

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Table 3.1. Offspring Genotypes Summary of the offspring genotypes at microsatellites where significant deviation from Mendelian inheritance were found in the HxH crossed offspring. Shown are the number of offspring of each of the four possible genotypes in the CxC offspring, CxH offspring and HxC offspring. p values indicate Pearson's Chi-square values and were ranked using a modified Bonferonni correction to be analyzed. * indicates a significant p-value.

Microsatellite	Cross	Genotype	Observed	Expected	p-value
Ots235	CxC	86x86	22	24	0.3
		86x100	29	24	
		86x105	28	24	
		100x105	17	24	
	CxH1	86x86	9	12.25	0.3
		86x96	17	12.25	
		86x105	9	12.25	
		93x105	14	12.25	
	H1xC	86x86	6	11	0.01*
		86x93	21	11	
		86x100	10	11	
		93x100	7	11	
Ssa85	CxC	120x132	25	24.25	0.3
		120x151	18	24.25	
		132x166	32	24.25	
		151x166	22	24.25	
	CxH1	120x136	10	12.25	0.09

		120x166	9	12.25	
		136x166	10	12.25	
		166x166	20	12.25	
	H1xC	Not available	/	/	/
Ots107	CxC	223x236	22	21.5	0.05
		223x248	16	21.5	
		228x235	17	21.5	
		228x248	31	21.5	
	CxH1	228x236	14	12	0.6
		236x236	15	12	
		228x247	10	12	
		236x247	9	12	
	H1xC	223x228	6	12	0.02
		223x235	12	12	
		228x228	9	12	
		228x235	21	12	
OtsG311	CxC	265x325	22	24	0.6
		313x325	27	24	
		265x333	19	24	
		313x333	28	24	
	CxH3	325x325	8	11.25	0.07
		325x329	17	11.25	
		333x325	10	11.25	
		329x333	10	11.25	

	H3xC	325x265	10	11.75	0.3
		265x329	9	11.75	
		325x313	18	11.75	
		329x313	10	11.75	
Ots212	CxC	132x161	27	24.75	0.9
		132x175	24	24.75	
		161x255	25	24.75	
		175x255	23	24.75	
	CxH3	132x175	13	11.5	0.7
		132x255	13	11.5	
		175x255	9	11.5	
		255x255	11	11.5	
	H3xC	161x175	14	12.5	0.3
		175x175	8	12.5	
		161x255	12	12.5	
		175x255	16	12.5	

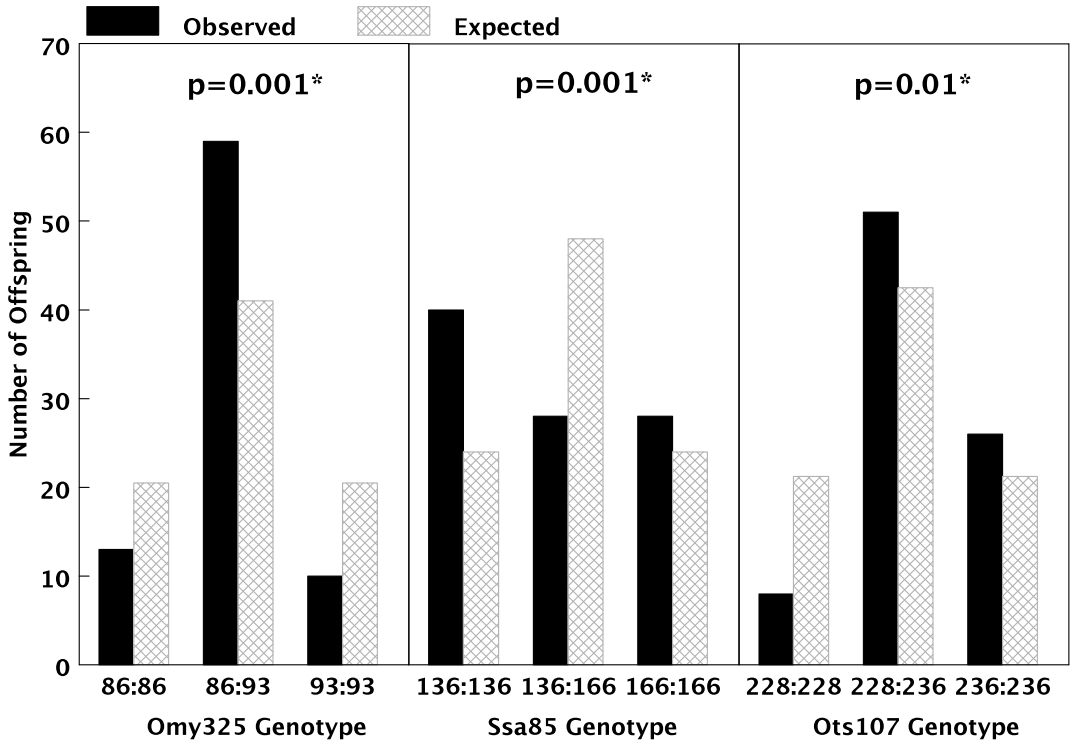

Figure 3.1. H1xH1 Offspring Microsatellites Genotype Frequencies These microsatellite loci showed departures from expected Mendelian genotype frequencies in the hermaphrodite self-crossed (H1xH1) offspring. * indicates the Pearson Chi-Square value was significant after applying modified Bonferonni correction.

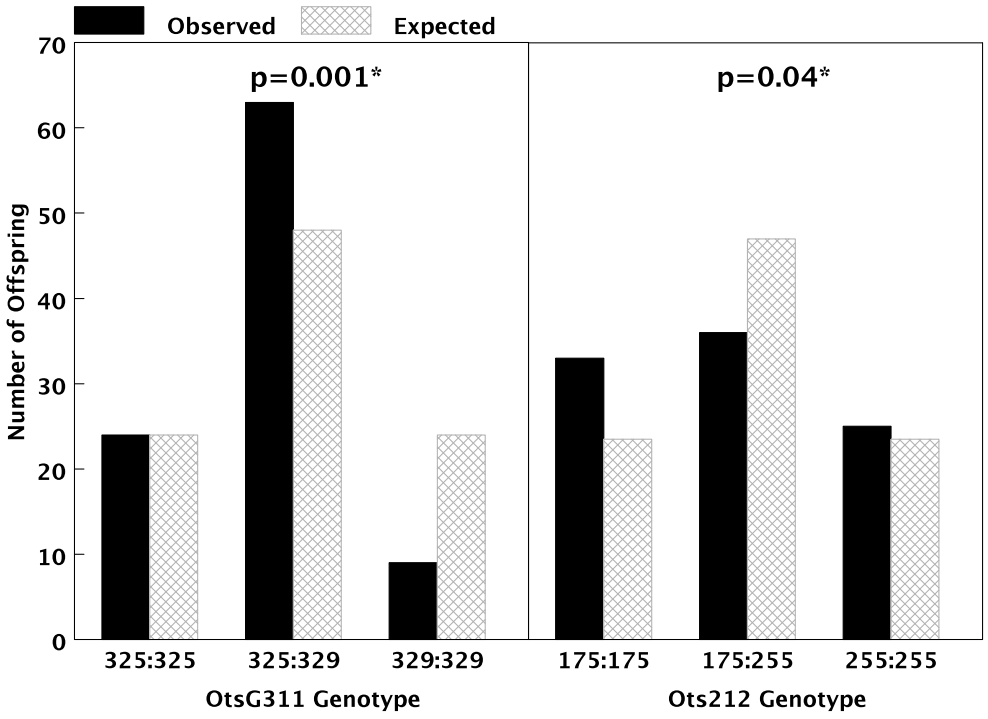

Figure 3.2. H3xH3 Offspring Microsatellites Genotype Frequencies These microsatellite loci showed departures from expected Mendelian genotype frequencies in the hermaphrodite self-crossed (H3xH3) offspring. * indicates the Pearson Chi-square value was significant after applying modified Bonferonni correction.

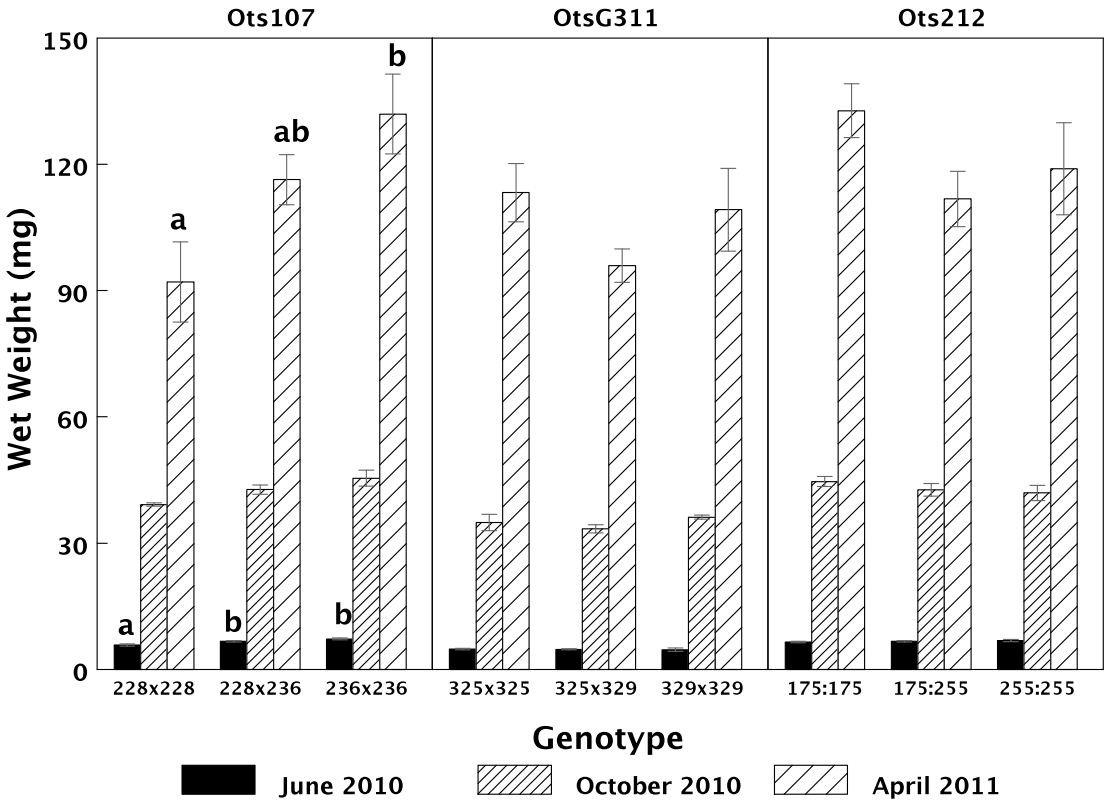

Figure 3.3. Inbred Offspring Wet Weight Shown by Microsatellite Genotype Mean wet weight (± SE) of inbred (hermaphrodite self-crossed) fish by microsatellite genotypes (at the three microsatellites that deviated from Mendelian inheritance). The three deviant loci are Ots107, OtsG311 and Ots212. Differences among the wet weights were found at the Ots107 loci using ANOVA and Tukey Post-hoc analyses and are depicted with letters in the above graph.

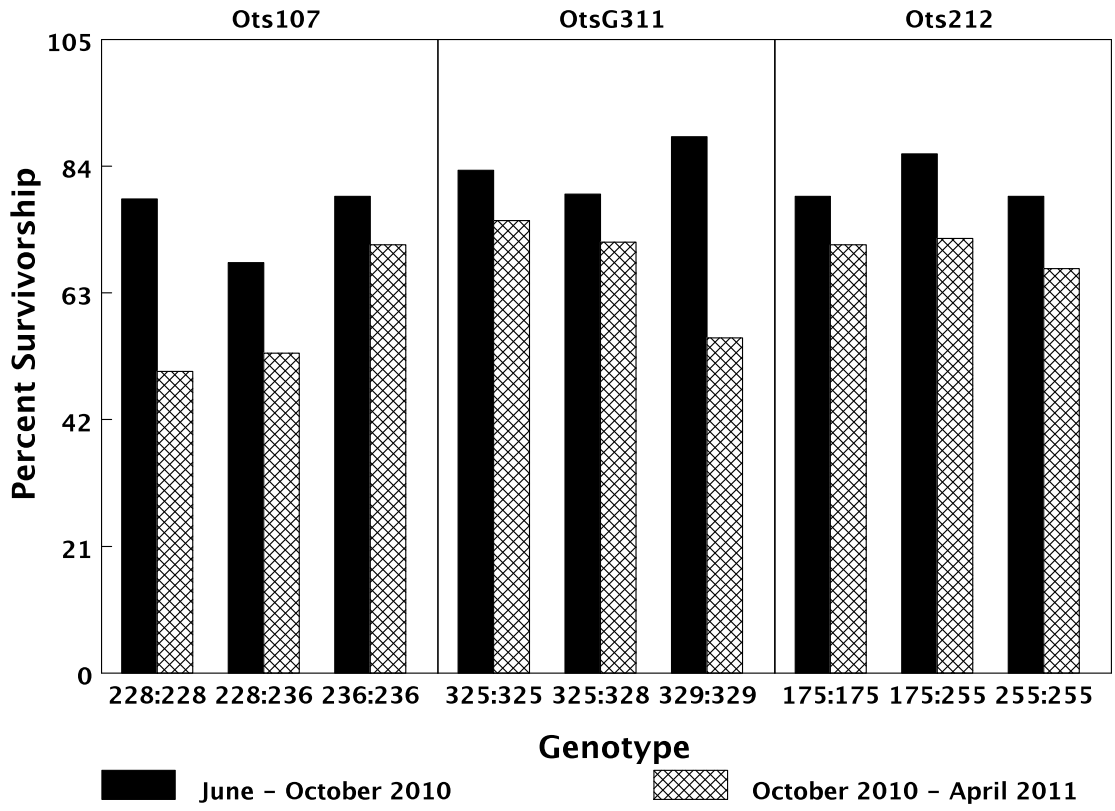

Figure 3.4. Inbred Offspring Survivorship Survivorship of inbred (hermaphrodite self-crossed) fish by microsatellite genotype (at the three microsatellites that deviated from Mendelian inheritance). The three deviant loci are Ots107, OtsG311 and Ots212. Crosstab analyses were performed to detect any significant differences between the genotypes, and no differences were found.

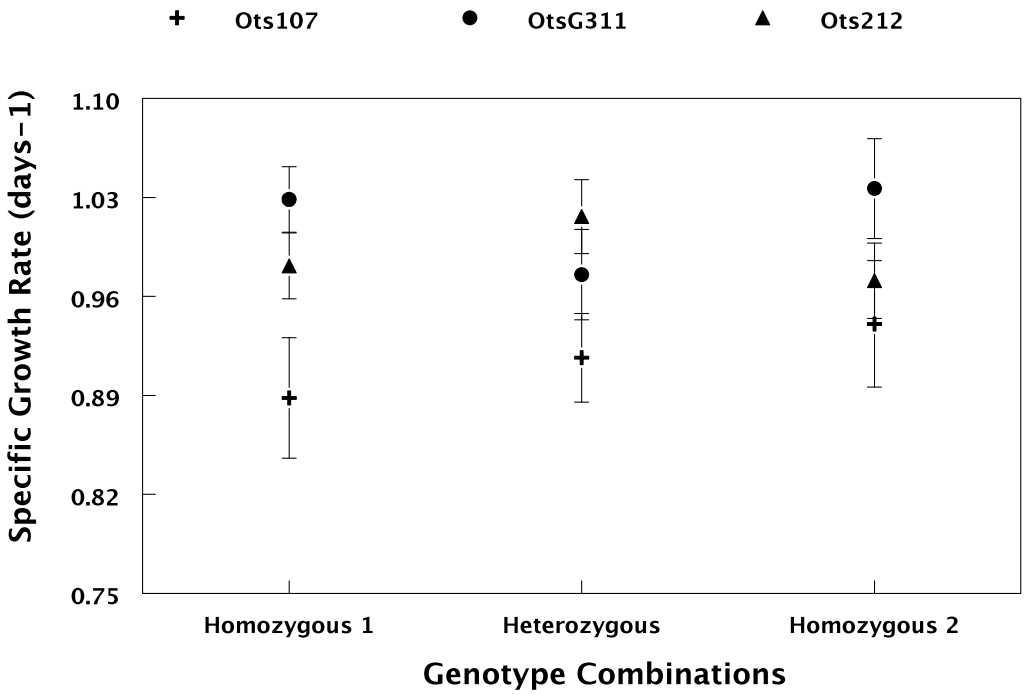

Figure 3.5. Inbred Offspring Specific Growth Rate Mean specific growth rate (± SE) of inbred (hermaphrodite self-crossed) Chinook salmon by microsatellite genotype (at the three microsatellites that deviated from Mendelian inheritance). The three deviant loci are Ots107 (223:223, 223:236, 236:236), OtsG311 (325:325, 325:329, 329:329) and Ots212 (175:175, 175:255, 255:255). The specific growth rate was calculated over the time period of June 2010 to April 2011 and no significant differences in SGR were found among the genotypes at any of the three loci.

4.0 GENERAL DISCUSSION

Inbreeding has the potential to depress an individual's or a population's fitness which is inbreeding depression (Charlesworth and Charlesworth 1987; Hedrick, 1994). Inbreeding occurs naturally within many salmonid populations and the potential effects of ID on these populations have many possible repercussions. Salmon are fecund organisms, producing many eggs and therefore, potentially many offspring. In both a natural and captive environment, this can lead to a large census population size, with a small effective population size, since although there is a large number of fish, there is a high degree of relatedness among them and few are genetically distinct. Therefore, even though the fish (or human breeders) have the potential to choose from many mates, they are likely to choose a mate that is genetically similar and to experience inbreeding. Population sizes in many salmon populations are decreasing and this will add to the level of inbreeding, simply due to the fact that there are fewer potential mates to choose from. Additionally, the fact that many salmon are philopatric, returning to their natal rivers to spawn, also increases the likelihood that they will mate with a relative naturally in the wild. It is therefore crucial to understand as much as possible about the effects of inbreeding and ID in salmon to aid the efforts in place to save these fish from extinction, as stocks are drastically falling (Allendorf and Waples, 1996).

This study showed that inbreeding depression (ID) negatively affects captive populations of highly inbred Chinook salmon. I can also infer that, in the wild, populations of salmon are able to reach effective population sizes where inbreeding coefficients can approach the high level of inbreeding I artificially created in this study (Hedrick, et al., 2000; Heath et al., 2002). Given that in the wild salmon are exposed to

higher levels of environmental stress (relative to in a salmon farm) they are therefore more likely to be heavily affected by the impacts of ID than farmed fish (Crnokrak and Roff, 1999). Indeed, Thrower and Hard (2008) evaluated the effects of inbreeding in a captive population while simultaneously measuring its effects in a wild, but inbred, population and they found ID levels to be much higher in the wild population. Thus, I conclude that the results of my studies are conservative relative to the fitness effects expected under wild conditions, and hence my graduate research is highly relevant to the effects of inbreeding and ID, under culture and wild settings.

To the best of my knowledge, this is the first study to examine the effects of purging using marker genotype analyses in a vertebrate organism and it was clear from my findings that purging does have the potential to occur within a highly inbred vertebrate population. However, I did expect the levels of purging to be much more pronounced, since other studies have shown high levels of ID and purging after only a few generations of serial inbreeding of full-sibs (i.e. Fox et al., 2008; Crnokrak and Barrett, 2002; Kirkpatrick and Jarne, 1999). Therefore, I believe this brings up two important points: 1) purging can occur within salmonid populations, although the tetraploid ancestry of these fish does make it more difficult to predict the level and effectiveness of purging, and 2) the potential beneficial and detrimental components of purging for salmon populations should continue to be studied. For the time being, however, my work indicates that the potential cost of ID outweighs the possible benefits of purging in salmonids. Thus, fish farms and conservation/enhancement programs should continue to manage to minimize inbreeding that takes place in the small populations being cultured.

There are many reasons why understanding the genetic basis of ID in salmon is relevant. Because of the conservation and commercial fishing industry's interest and investment in these fish, it is important that they understand the potential risks they are facing when captively rearing these fish. For instance, I show that inbreeding can negatively impact captive populations, which is an important finding in and of itself, since studies prior to this have debated this point (Wang et al., 2002). However, the added knowledge of the genetic basis for ID in Chinook salmon could specifically help with reintroduction programs of Chinook into the wild, as well as potentially other members of the *Oncorhynchus* genus. For instance, Blanchet et al. (2008) explore the potential benefits of supportive breeding (Wang and Rymen, 2001) where wild fish are caught and kept in captivity, bred and then their offspring are released at an early developmental stage to provide the offspring with the best possible chance to adapt to wild conditions. Under such a program, it would be critical to be able to screen the parental fish prior to breeding, to ensure that unplanned inbreeding is not taking place.

Curiously, I found evidence for substantial hermaphrodite gamete effects that extended over the course of my study. This is not expected, since egg (maternal) effects are expected to reduce rapidly after hatch (Heath and Blouw, 1998; Heath et al., 1999) and sperm (paternal) effects have not been reported in salmon. Although my work does not provide an explanation for these effects, they represent a novel finding and should be followed up in future studies.

4.1 FUTURE DIRECTIONS

My graduate work has contributed to our understanding of the relationship between inbreeding, genetic load and purging in vertebrates. ID has now been

conclusively shown to occur in salmon for the first time, and the potential for purging of the genetic load to take place was realized. This also led to a better understanding of the hypotheses surrounding the genetic basis of ID and the fact that both the Dominance and Overdominance hypotheses play a role in ID effects in salmon populations. With this base information as a starting point, however, there are exciting and important future projects that suggest themselves:

Purging: The premise of purging along with its potentially beneficial effects need to be explored further. I show evidence that there is the potential for purging to eventually be incorporated into conservation programs, once we fully understand the variation in fitness costs so that it is ensured the population does not die off before the purging is able to take place. Additionally, now that the effects of potential purging have been shown within the salmon genome experimentally using a captive population of fish, I think it would be valuable to quantify purging in a wild population of salmon. The biggest obstacle would be to find a small, isolated inbred population of salmon in the wild. Assuming that the population was small enough to allow inbreeding to take place, genotyping a sample of the fish in this population over several generations and comparing their genotypes to the previous year's genotyped fish would allow differences in the allele frequency distribution to be quantified and significant departures from Mendelian expectations would be an indicator that purging is taking place.

Vertebrate Inbreeding Depression: I provided evidence that ID takes place in vertebrates using phenotypic traits directly and indirectly associated with fitness, as well as microsatellite markers showing that increased homozygosity (Dominance) and decreased heterozygosity (Overdominance) contribute to ID in salmon. An important next step

could be targeted DNA sequencing or whole genome (Next Generation) DNA sequencing (Allendorf et al., 2010). Using this new technology, one would be able to sequence the entire genome of individuals, and compare genome sequences between fish, which is much more specific and would provide much more information than comparing individual microsatellite locus markers. Through comparing sequence differences with the fitness traits of the individuals, one would be able to determine the genes that are being affected by ID and to further classify the number of deleterious alleles present within that fish's genome. Conservation programs would then be able to screen potential parents prior to breeding for these deleterious alleles, ensuring those fish who have the potential for passing on the deleterious allele to the next generation are not mated. The idea behind this is to create an "artificial purging" set up, where the genes that caused decreased fitness levels would not be passed to the next generation.

Hermaphrodite Reproductive Performance: While this study showed the effects of inbreeding within a timeframe of approximately 7 months post fertilization to 22 months post fertilization, it would be valuable to evaluate the gametes produces by the inbred, self-crossed offspring of the hermaphrodites. I could do this by using hermaphrodite offspring to study the eggs and milt they produce, as well as early life history traits such as fertilized egg survivorship, early disease resistance, etc. Since the hermaphrodite offspring used in this study are all genetically female, it would be necessary to treat a subset of the hermaphrodite offspring with alpha-methyltestosterone, using a sex-reversal protocol, to generate phenotypically male fish to test whether: a) the hermaphrodite offspring produce viable sperm and test for sperm quality (number, longevity, swimming speed), b) the eggs produced by the female hermaphrodite offspring are viable, and

finally, c) the milt and eggs are able to produce viable embryos via hatchery fertilization protocols. This would allow us to determine the reproductive fitness of the hermaphrodite offspring, plus to explore the effects of ID at an even higher level of inbreeding (self-fertilized full sib-mating).

In conclusion, my research has proven to be effective in capturing a fundamental and conceptual understanding of the effects of inbreeding and genetic load in an economically valuable and ecologically important fish species. It has also opened a new avenue for the study of inbreeding in an experimental setting using artificially created hermaphrodite salmon as a study system. It will be interesting to see the future directions and the type of research that will be pursued using this model study system. The global decline in salmonids has been ongoing for many years and it has spawned extreme controversy and political confusion for generations (Allendorf and Waples, 1996). However, I believe that my research will contribute to a better understanding of the risks and benefits of natural inbreeding in small populations, and artificial inbreeding under culture condition. This will, in turn allow better management decisions for a dwindling resource.

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VITA AUCTORIS

NAME:	Kendra Rose Komsa
YEAR OF BIRTH:	1988
EDUCATION:	St. Basil Secondary School, Sault Ste. Marie, ON 2006 – High School
	Wilfrid Laurier University, Waterloo, ON 2006-2010 – H. Bsc. Biology
	Great Lakes Institute for Environmental Research University of Windsor, Windsor, ON 2010-2012, MSc. Environmental Science