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INHERITANCE AND EXPRESSION IN DIPLOID AND TRIPLOID CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*): SEGREGATION DISTORTION AT THE MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) AND MICROSATELLITE LOCI

by

Sara Jamieson

A Thesis
Submitted to the Faculty of Graduate Studies and Research
through Biological Sciences
in Partial Fulfillment of the Requirements for
the Degree of Master of Science at the
University of Windsor

Windsor, Ontario, Canada

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ABSTRACT

In order to investigate the influence of triploidy on genetic inheritance and expression, DNA and mRNA were extracted from diploid and triploid offspring of a single breeding pair of Chinook salmon. Neutral (microsatellite) and a functional markers (class I major histocompability complex (MHC)) were used to analyze the DNA samples for meiotic bias. Significant segregation distortion ($P<0.02$) was found in the distribution of maternal-origin alleles in the triploid samples at two microsatellite loci and at the MHC locus. No bias was found in the diploid samples, indicating that the distortion was due to maternal dosage effects. This could be the result of a biochemical imbalance caused by dilution of the paternally-derived gene product, or to gene dosage effects of functional, deleterious, maternally-derived proteins. MRNA, converted to cDNA, was also used as a template for MHC amplification and, as expected, the MHC expression in the triploid offspring displayed a dosage effect pattern.

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STATEMENT OF ORIGINALITY

Chapter 2 of this thesis incorporates the outcome of research undertaken in collaboration with D.D. Heath relating to inheritance of microsatellite marker loci in Chinook salmon (*Oncorhynchus tshawytscha*). All three assisted with sampling, AVH gave helpful input and DDH gave feedback and guidance during the writing process. This chapter has been submitted to *Genetics* for publication. Chapter 3 of this thesis incorporates the outcome of research undertaken in collaboration with D.D. Heath, J.W. Heath and A.V. Hubberstey relating to inheritance and expression of major histocompatibility complex (MHC) loci in Chinook salmon (*O. tshawytscha*). DDH gave helpful input throughout, and, again, provided feedback and guidance during the writing process. Both collaborations were undertaken as part of a research project under the supervision of Dr. D. Heath.

I certify that, with the above qualifications, this thesis, and the research to which it refers, are the product of my own work, and that any ideas or quotations from the work of other people, published or otherwise, are fully acknowledged in accordance with the standard referencing practices of the discipline. I acknowledge the helpful guidance and support of my supervisor, Dr. D. Heath.

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TABLE OF ABBREVIATIONS

Bacterial Kidney Disease	BKD
Basepairs	bp
Diploid	2N
Infectious Haematopoietic Necrosis	IHN
Major Histocompatibility Complex	MHC
Natural Killer	NK
Natural Sciences and Engineering Research	
Council of Canada	NSERC
Peptide Binding Region	PBR
Polymerase Chain Reactions	PCR
Transporter associated with Antigen Processing	TAP
T-cell receptor	TCR
Triploid	3N
Vriable Number of Tandem Repeats	VNTR
Yellow Island Aquaculture Ltd.	YIAL

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CHAPTER 1

GENERAL INTRODUCTION

Polyploid salmon

Most vertebrate organisms are diploid, containing two complete chromosome sets: one inherited from their mother, and one from their father. Until recently, it was thought that polyploidy (the condition of containing more than two sets of chromosomes) in vertebrates was vanishingly rare compared to that seen in plants, for instance. This was thought to be due, in part, to their more complex reproductive systems, but this may be an erroneous assumption. The cell size of most vertebrates increases in response to an increase in ploidy, however, the total number of cells normally decreases, so the overall size of both internal organs and of the whole animal are maintained (BENFEY 1999; SMALL and BENFEY 1987). This means that, unless explicitly tested for, it can be difficult to tell diploid and polyploid animals apart. Detection is also complicated because, unlike plants, animals can migrate to reproduce so the existence of a polyploid line may be fleeting (OTTO and WHITTON 2000). Polyploid amphibians, reptiles, fish and even mammals have been discovered (reviewed by OTTO and WHITTON 2000), and, in fact, there is strong evidence that all vertebrates are residual polyploids (paleopolyploids; OHNO 1999). Even organisms that are strictly diploid normally harbor many cells that are in a non-diploid state, resulting from mitotic or meiotic cycles.

There are few vertebrate groups better adapted to changing ploidy states than fish. Natural, or spontaneous, polyploids have been found throughout the fish lineages (reviewed by LEGGATT and IWAMA 2003) in 37 different forms (reviewed by PANDIAN and KOTEESWARAN 1998). In salmon, triploidy can be readily induced by preventing maternal meiosis II through the application of a pressure or heat shock (Figure 1).

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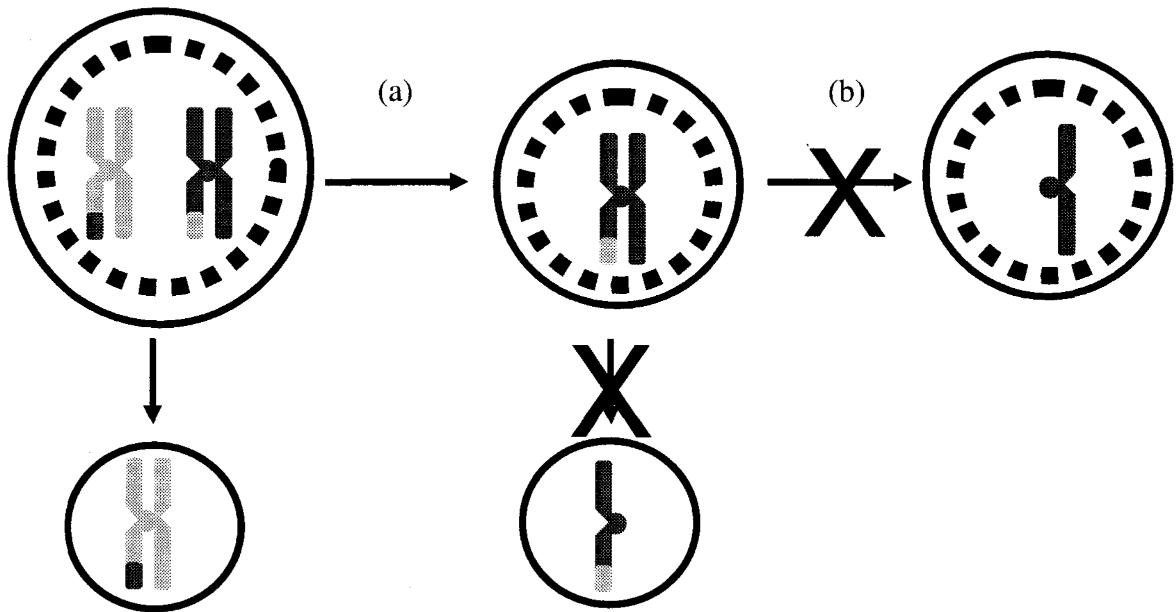
FIGURE 1

The second meiotic division (b) is shut down in oogenesis leading to formation of a triploid salmon. After the first asymmetric cellular division (a), the resulting polar body is degraded and the secondary oocyte proceeds to the second meiotic division (b). This division produces a second polar body and a mature egg. It is this second division in maternal meiosis that is blocked during salmon triploidization.



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Normally, the egg and sperm fuse at the second oogenic division while the oocyte is dividing to form a second polar body and a mature egg. If a heat or pressure shock is applied at this point, however, the second division is prevented, leaving the mature egg with a set of maternally derived sister chromatids (2n) fusing with the haploid sperm (n) from the male parent. Specifically, the triploidization treatment causes the tubulin to depolymerize, preventing the formation of microtubules and, consequently, the development of the spindle apparatus. This, in turn, interferes with the normal movement of chromosomes during meiosis, preventing the second polar body from being extruded properly (BENFEY 2001).

There is evidence that, over time, a polyploid genome permits selection to act freely on duplicate genes, resulting in phenotypic changes that can increase the fitness of the polyploid organism (MABLE 2003). Immediate advantages to nascent polyploids are harder to identify, but may include the reallocation of energy from reproduction to somatic growth, diminished need for synthesis and maintenance of plasma membranes, and a reduction in the relative surface area across which ionic and osmotic gradients must be preserved (BENFEY 1999; BENFEY 2001). Genetically, the resulting increase in overall heterozygosity could act to reduce the effect of genetic load through masking deleterious mutations, increasing genetic diversity and improving the probability of inheriting beneficial alleles (ALLENDORF and LEARY 1984; OTTO and WHITTON 2000). At the level of the transcriptome, altered gene expression patterns could result in a favorable modification of the overall phenotype, thereby increasing performance in comparison to diploid progenitors (BONNET *et al.* 1999; JOHNSON *et al.* 2004).

In many autopolyploids (polyploidy through chromosome doubling within an individual), the majority of genes exhibit evidence of a positive dosage effect, that is, an

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increase in ploidy is usually accompanied by a proportionate increase in gene expression (BIRCHLER *et al.* 2001; GALITSKI *et al.* 1999; GUO *et al.* 1996; MARTELOTTO *et al.* 2005). Since the cell size of a polyploid vertebrate increases as well, the overall stoichiometry of gene products in a cell is maintained, and the increase in ploidy can be surprisingly undisruptive. A minority of genes in autopolyploids are often up- or down-regulated beyond the capacity of a simple positive or negative dosage effect, probably as a result of epigenetic or epistatic effects (BIRCHLER *et al.* 2001; GALITSKI *et al.* 1999; GUO *et al.* 1996; MARTELOTTO *et al.* 2005). Though, for the most part, polyploid vertebrates retain cellular stoichiometry, there may be structural changes between certain components, or irregularities caused when an excess of gene products interact with regulatory factors that were not, themselves, up-regulated (COMAI 2005). Alternatively, JOHNSON *et al.* (2006) theorized that those genes showing a straightforward dosage effect code for phenotypes under simple genetic control (e.g. lysozyme response to vaccination), while the products of genes with erratically modified expression patterns are involved in regulating highly polygenic traits and, thus, reflect a genome averaged effect. GALITSKI *et al.* (1999) found that the genes with irregular expression changes in a ploidy series of isogenic yeast had complex promoters and large upstream intergenic spaces (>1300 bp, compared to an average of 500 bp), suggesting that the gene structure could have an influence on its response to ploidy changes.

In salmon, the sole gross physical change prompted by triploidization is sterility due to incompatibilities encountered during meiosis. While triploid females are both functionally and hormonally sterile (PANDIAN and KOTEESWARAN 1998), the males are only functionally sterile, sexually maturing and spawning as normal (BENFEY 1999; PANDIAN and KOTEESWARAN 1998). A more subtle physical change in triploids is the

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increase in cell size and commensurate decrease in both cell number and in the cellular and nuclear surface area to volume ratios. Any function influenced purely by the number of cells available, such as leukocyte and lymphocyte circulation, could be affected by a change in ploidy number (JHINGAN *et al.* 2003). In addition, the alteration in surface area to volume ratios could affect processes involving cellular and nuclear membranes (exchanges of nutrients and ions, membrane-bound signaling components) or traversing intracellular distances (signal transduction from cell surface to nucleus; GALITSKI *et al.* 1999; reviewed by OTTO and WHITTON 2000). Furthermore, the stress of an induced polyploidization event can cause structural damage to polyploid cells, affecting the supply or function of embryonic or maternally supplied substances during early development (JOHNSON *et al.* 2004).

Despite this, diploid and triploid salmon generally have similar performance and development characteristics, except under situations of oxygen stress, where diploids consistently outperform triploids (reviewed by BENFEY 1999). Several studies investigating the response of diploid and triploid salmon to disease challenges have been carried out, but results have been variable: some studies found that diploids survived better (bacterial gill disease; OJOLICK 1995), others that diploids and triploid survived equally well (IHN, furunculosis and vibriosis; YAMAMOTO and IIDA 1995), and still others found that triploids outperformed diploids (bacterial kidney disease; BRUNO and JOHNSTONE 1990). These contradictory reports may have been due to variables unintentionally introduced into the experimental parameters (age, size, family, behavior; BENFEY 1999).

Triploid salmon are of great economic and ecological interest to the commercial aquaculture industry. Because the females, in particular, do not go through the

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debilitating stress of sexual maturation, they have less chance of experiencing the reduced flesh quality and mortality that accompanies this life stage. In addition, their sterility means that, in the event of a containment breach, the surrounding wild populations will remain genetically uncontaminated by a large influx of genes from farmed salmon (BENFEY 2001). Despite these advantages, the widespread adoption of triploid salmon as commercial stock has been prevented by the contradictory findings of the influence of triploidy on performance parameters (see above). Benfey (in BENFEY 2001) argues that triploid salmon are a commercially viable option, but that they should be treated as a "new species," with investigations into optimal rearing conditions and breeding for improved triploid fitness.

Studies on triploid salmon directly benefit the aquaculture industry by identifying potential sources of variation in order to improve broodstock selected for triploid stock development. In addition, triploid salmon possess unique structural, behavioral, developmental and genetic characteristics of interest in a wide variety of scientific fields. As induced triploidy blocks maternal meiosis II, triploid salmon provide a unique opportunity to study the dosage effects and resulting phenotype alterations caused by polyploidization. Since, despite changes in cell size and number, the phenotype of triploid salmon is very similar to that of diploids, they open the prospect of research on the importance of this parameter to general physiology. Behavioral differences between diploid and triploid salmon, such as an apparent decrease in aggressive behavior in triploid fish (CARTER *et al.* 1994), permit examination of the effects of ploidy changes on the neural control of behavior. Finally, polyploidization has been a significant event in the evolution of vertebrates, and triploid salmon are ideal subjects for studying the consequences of both ancient and recent polyploid events (OHNO 1999).

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The major histocompatibility complex (MHC)

The major histocompatibility complex (MHC) is a central component of the adaptive immune response found in jawed vertebrates. The key difference between the two main classes of the MHC lie in the roles they play in the immune response. The class II MHC is expressed primarily on antigen-presenting cells, such as B cells and macrophages and is involved in the humoral response to extracellular pathogens. The class I MHC is codominantly expressed on all nucleated somatic cells, and is instrumental in the cytotoxic immune response to endogenous pathogens. In mammals, at least, the class I MHC plays a role in the non-specific immune response, as well, by controlling the activity of Natural Killer (NK) cells. The MHC may also have indirect functions in kin-recognition and inbreeding avoidance (PENN and POTTS 1998).

The specific immune response has its beginnings when cellular proteins are degraded by multimeric proteasomes in the endoplasmic reticulum into peptide fragments 9 to 20 amino acids in length. The peptide is loaded onto an appropriate, empty class I MHC molecule and the complex is transported by a TAP (transporter associated with antigen processing) into the cytosol. From there, the complex is shuttled to the cell surface, where the peptide bound by the MHC is sampled by the T-cell receptors (TCRs) on passing T-cells (TOWNSEND and BODMER 1989). Once a match is made, the cytotoxic immune response is initiated, launching a cascade of immune-related activity, including the death of the infected cell.

The class I MHC is composed of two subunits (Figure 2). The heavy chain is made up of 3 extracellular immunoglobulin domains (α_1, α_2 and α_3), linked to a transmembrane domain with a cytoplasmic tail. The α_1 and α_2 domains form a groove, called the peptide binding region (PBR), where polypeptides are bound by five pockets

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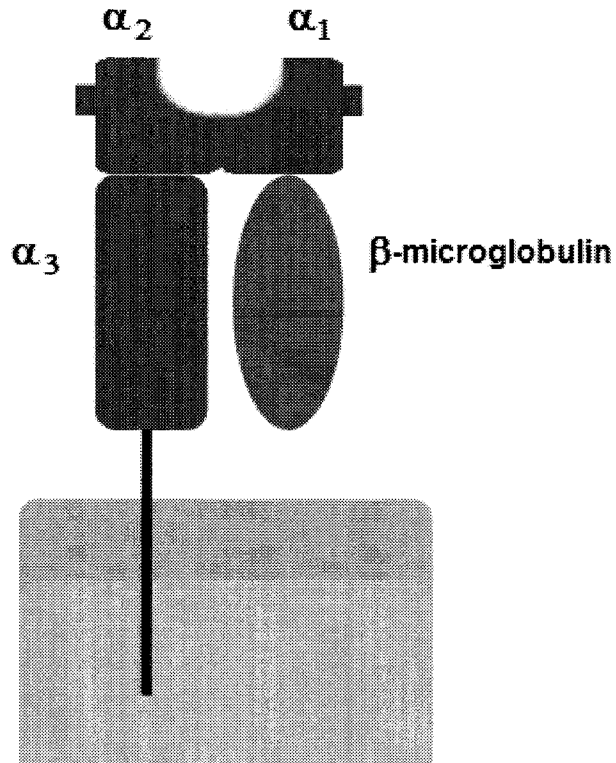
FIGURE 2

Schematic representation of MHC class I molecule, consisting of three α-domains and one β2-microglobulin molecule. The peptide-binding groove is situated between domains α1 and α2. Source: en.wikipedia.org



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(A to F) lined with residues complementary to the peptide fragments' side chains (Medzhitov et al. 1997). The α3 domain of the heavy chain is noncovalently linked to β-2microglobulin (β-2m), encoded by a gene outside the MHC. In most vertebrate organisms, the MHC is found as a large gene complex on a single chromosome (Ohta et al. 2000); in teleosts, however, the different MHC gene regions are unlinked – dispersed on three or more different chromosomes – thus, researchers often remove the word "complex" and refer to these teleost loci as the MH genes (Sato et al. 2000).

The MHC is noted for its high level of polymorphism, both in the alleles in a population, and in the extent of sequence variation among alleles. Only non-coding VNTR loci (variable number of tandem repeats), such as mini- and microsatellites, show more variation (AMMER *et al.* 1992; GIBBS *et al.* 1990). The number of loci vary among species, as MHC genes are repeatedly lost and gained through expansion and contraction of the MHC region (MILLER *et al.* 2002). For instance, salmon are thought to have only one classical class I MHC locus (AOYAGI *et al.* 2002), while Atlantic cod may have as many as 42 (MILLER *et al.* 2002). Whether this is the result of random duplications and deletions or has functional significance is still unknown.

The PBR is the primary source of allelic diversity, and the genetic sequence in this region can vary by as much as 11.2% – or 50 amino acids – among species (BJORKMAN and PARHAM 1990; KLEIN and FIGUEROA 1986). The class I loci are normally more polymorphic than the class II, probably due to the stronger selection pressures acting upon it (FLAJNIK *et al.* 1991). A study of a Chinook salmon population, for example, found 12 class I alleles, but only six class II (GARRIGAN and HEDRICK 2001; MILLER *et al.* 1997). Species that are more social will show higher levels of allelic variation due to the ease with which disease can be transmitted through the population. For example,

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compare the class I polymorphism of mice (55 alleles per locus) to that of the solitary, geographically isolated Syrian hamster (1 allele; J.W. Streilen in LAWLOR *et al.* 1990). With more alleles comes immunity to more disease, but there appears to be an optimal number that balances diversity with the potential depletion of the T-cell repertoire (LAWLOR *et al.* 1990). Consequently, in many polyploid lineages, the MHC complement has been "diploidized" over time. For instance, tetraploid (4n) *Xenopus ruwenzoriensis* could potentially express 12 MHC molecules but, instead, expresses only four; the others are silenced, perhaps through deletion (KRUISWIJK *et al.* 2004).

The extraordinarily high polymorphism of the MHC is thought to be maintained through both disease-based and reproductive mechanisms (reviewed by BERNATCHEZ and LANDRY 2003). The disease-based mechanisms are in response to the constantly evolving "arms race" between pathogens and hosts. They include heterozygote advantage (overdominance hypothesis) and rare allele advantage (frequency based selection; HUGHES and NEI 1988; TAKAHATA and NEI 1990). The hypothesis is that individuals with heterozygotic MHC alleles will have higher fitness than a homozygote because they can resist a broader array of pathogens. Also, since parasites and pathogens will evolve to elude the most common host genotype, there will be a selective advantage to those individuals carrying new, rare MHC alleles. Reproductive mechanisms (probably olfactory-based) include disassortative mating preferences and inbreeding avoidance, operating so that individuals can maximize MHC diversity in their offspring and, thus, improve their immune response (PENN and POTTS 1998). Diversity in the MHC is maintained through duplication, deletion, point mutation, recombination, conversion, insertion and translocation of retroelements operating at mutational "hot spots" throughout the MHC. The mutational mechanisms at work may differ between MHC

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classes and even among exons of the same gene (BERNATCHEZ and LANDRY 2003; CULLEN *et al.* 2002; KULSKI *et al.* 1997; MARTINSOHN *et al.* 1999).

The first fish MHC was discovered in carp in 1990 (HASHIMOTO *et al.* 1990). Since then, hundreds of MHC sequences have been found in over 30 fish species and interest in studying fish MHC continues to grow (DIXON *et al.* 1995). The teleost group encompasses about half of all living vertebrate species occupying a wide range of habitats (SHAND and DIXON 2001); the study of teleost MHC, therefore, can provide crucial information regarding adaptive radiation and species origins. The teleost and mammalian MHC differ in genetic structure and organization, providing an avenue to explore the evolutionary origin of the adaptive immune system. The MHC has been linked to resistance to numerous pathogenic diseases (reviewed by APANIUS *et al.* 1997), and this link may be even stronger in salmon, as they have relatively few classical MHC genes (MILLER *et al.* 2004). Thus, MHC studies in salmon populations could be of use to commercial aquaculture, increasing our knowledge of infectious diseases relevant to aquaculture and potentially aiding in vaccine development. Finally, its polymorphic character and central function in the immune system make the MHC a good choice as a marker for stock identification and investigating adaptive variation and for in salmon populations.

Study objectives

This study uses both neutral (microsatellite) and functional (MHC) markesr to investigate inheritance of loci in diploid and triploid Chinook salmon (*Oncorhynchus tshawytscha).* Because induced triploidy in salmon blocks maternal meiosis II, if a bias is present, we will be able to determine where in the meiotic cycle it is occurring: by deduction, the diploid samples will give information about meiosis II, and the triploid

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about meiosis I. We will also be able to determine if meiotic drive is random or directed (i.e. do certain alleles get transmitted preferentially). Finally, because the MHC produces a functional gene product, we will then investigate if an increase in ploidy causes an alteration in the gene expression of the MHC.

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CHAPTER 2[1]

Segregation distortion in diploid and triploid Chinook salmon (*Oncorhynchus tshawytscha*): deleterious dosage effects

[1] This chapter is the outcome of joint research undertaken in collaboration with D.D. Heath and has been submitted to *Genetics*

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INTRODUCTION

New mutations appear constantly in every part of the genome and the vast majority of active new mutations are either deleterious or lethal (BLOT 1994; CROW and SIMMONS 1983; DRAKE *et al.* 1998). Despite this, most genomes harbor significant numbers of such mutations, which constitute the "genetic load" of a species (MULLER 1950). The majority of new mutations are believed to be non-functional and slow to be eliminated in a diploid population because selection against them is not efficient (BOURGUET 1999; DRAKE *et al.* 1998; SZAFRANIEC *et al.* 2003). The inefficient selection is probably due to: 1) the masking effects of dominant wildtype alleles, 2) the low levels of expression associated with a mutant allele, and/or 3) the safety margin built into most cellular pathways (BOURGUET 1999; HALDANE 1937; KEIGHTLEY 1996; SZAFRANIEC *et al.* 2003; WRIGHT 1929). It is widely agreed that lethal mutations appear far less frequently than deleterious mutations, although the precise rates are disputed (MUKAI *et al.* 1972; WLOCH *et al.* 2001). In addition, studies of yeast (SZAFRANIEC *et al.* 2003) and *Drosophila* (reviewed by CROW and SIMMONS 1983) found that the heterozygotic effects of lethal and mildly deleterious alleles are remarkably similar, indicating either that both lethal and deleterious mutations can be masked completely, or that the wildtype allele displays complete dominance. SZAFRANIEC *et al.* (2003) found that lethal mutations in diploid yeast were masked eight times more efficiently than non-lethal mutations, indicating that complete dominance was not the prime factor in determining the heterozygotic effects of lethal and deleterious alleles.

Thus, we generally expect that the strength of masking should be proportional to the ratio of wildtype to mutant alleles, and, hence, an increase in an organism's ploidy number should result in an accompanying increase in fitness. Alternatively, if the

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wildtype allele displays complete dominance, there would be no fitness variation in organisms with higher ploidy. In practice, MABLE AND OTTO (2001) found that extra masking abilities may give tetraploid yeast only an initial, if fleeting, advantage over diploid strains. Similarly, experiments investigating the impact of ploidy level on salmonid fitness have indicated that triploid fish display similar or only slightly reduced performance levels relative to diploid fish, except when under oxygen stress, when diploid fish outperform triploid (reviewed by BENFEY 1999; BENFEY 2001). Such findings indicate that both the masking ability of the dominant allele and the expression levels of the recessive allele are not dosage dependent, thus, the wildtype alleles show complete penetrance.

While an increase in ploidy appears to have little or no effect on fish phenotype, it is expected to have genetic and epigenetic consequences at all levels of the genome (see MABLE 2004). In plants, for instance, there is evidence of widespread genetic and epigenetic events immediately following an increase in ploidy level, including rapid gene loss, gene silencing and genome reorganizing (reviewed by CHEN and NI 2006). Spontaneous polyploidy in fish has been widely reported (reviewed by LEGGATT and IWAMA 2003) and polyploidy can be induced in many fish species (reviewed by PANDIAN and KOTEESWARAN 1998). The plasticity of the salmonid genome, in particular, makes it amenable to various forms of artificial ploidy manipulation, including induced triploidization (PANDIAN and KOTEESWARAN 1998). An efficient method of producing triploid salmon is to subject the egg to a temperature or pressure shock within an hour of fertilization (BENFEY and SUTTERLIN 1984). Unusually, female salmon delay meiosis II until after fertilization, and this shock causes the egg to retain the second polar body during oogenesis, producing a fish with three chromosome sets: two from the female

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parent and one from the male. Thus, since triploid salmon eggs undergo only meiosis I, while their diploid counterparts proceed normally through the meiotic cycle, induced triploidy in salmon provides a novel opportunity to examine segregation and recombination in meisosis I and II independently among triploid and diploid siblings.

Polyploidy in salmon is of general interest as a model for the evaluation of the role of chromosome duplication in genome evolution. Increasing evidence of the importance of ploidy changes in the evolution of the vertebrate genome has caused an intensification of interest in studying vertebrate organisms displaying evidence of recent ploidy changes and tolerance to ploidy manipulation (OHNO 1999; VENKATESH 2003). The salmon genome is of particular interest in studies examining the fates of duplicated genes resulting from past polyploidization events, since salmonid species are the product of an ancient tetraploidization event (ALLENDORF and THORGAARD 1984). The plasticity of the salmon genome facilitates the investigation of factors involved in and affected by genome duplication, thereby shedding light on ancestral polyploidization events. Polyploidy in salmon can also be used to investigate the impact of cell size and number on physiological processes and gene expression, and to test theories about the mechanisms, effects and origins of polyploidy derived from work on plants (BENFEY 1999; MABLE 2004).

Triploid salmon are normally morphologically indistinguishable from their diploid counterparts (SMALL and BENFEY 1987) but are rendered sterile, with little or no gonad development in the female. Thus, they represent an important opportunity for commercial aquaculture. Sterile stocks are valuable for salmon aquaculture, as they do not lose flesh quality nor develop the secondary sexual characteristics of normally maturing salmon (reviewed by BENFEY 1999). Ecologically, sterile farmed salmon have been of critical

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interest as a method to minimize the impact of escaped farmed salmon via genetic "swamping" of local wild populations (BENFEY 2001).

Here we describe an experiment involving ploidy manipulation of the genome of the Chinook salmon, *Oncorhynchus tshawytscha*, in order to investigate the occurrence of segregation distortion in allelic inheritance at meiosis I (triploid) and meiosis II (diploid). We track the inheritance of microsatellite DNA alleles from a single mated pair into diploid and triploid offspring. By genotyping diploid and triploid siblings we ascertain whether segregation distortion is taking place and, if so, infer where in the meiotic cycle it is occurring. Segregation distortion found at marker loci only in the diploid offspring suggests that biased allele transmission is occurring during meiosis II; found in all offspring, regardless of ploidy status, it infers that the distortion is happening during meiosis I. These theoretical expectations arise as a consequence of blocking maternal meiosis II during induced triploidization but could be confounded by unexpected effects due to disruptions in the genome and transcriptome as a result of polyploidization.

METHODS AND MATERIALS

Breeding and sampling

In November 2002, half the fertilized eggs from a breeding pair of Chinook salmon were triploidized. Salmon eggs proceed only through meiosis I before fertilization and a thermal or hydrostatic shock applied within an hour of fertilization will prevent completion of the meiotic cycle, resulting in a triploid embryo (BENFEY and SUTTERLIN 1984). The eggs to be triploidized were pressure-shocked 20 min after fertilization (69 MPa for 8 to 10 min) inducing triploidization by retention of the polar body. The remaining half of the fertilized eggs were permitted to complete meiosis and were retained, untreated, as a diploid control group. Both groups of eggs were incubated

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at Yellow Island Aquaculture Ltd. (YIAL) on Quadra Island, British Columbia, Canada following standard hatchery practice in vertical stack incubation trays at 7.8°C (average) and approximately 12 $L \cdot min^{-1}$. Parental adipose fin tissue was sampled and stored in 95% ethanol for later DNA extraction. In order to minimize possible selection effects, the eggs were sampled as early in development as possible; the earliest stage from which DNA could be successfully extracted was one month after fertilization at the "eyed egg stage" when the embryonic eye is clearly visible through the shell of the egg (260 accumulated thermal units). Once embryonic development had reached the eyed egg stage, sub-samples of eggs from both triploid (N=20) and diploid (N=20) groups were removed, the egg cases were slit and the samples were stored in 95% ethanol for later DNA extraction. We sampled a limited number of eggs because of the need to conserve stock for future projects, coupled with the possibility of unexpected mortality events later in life.

DNA extraction and microsatellite analysis

DNA was extracted from embryos and parental fin clips using the Wizard® Genomic Purification Kit (Promega, Madison, WI) following manufacturer's protocol for DNA extraction from animal tissue. The parents were screened using 98 primers suitable for use on Chinook salmon DNA, and we identified 11 microsatellite loci that amplified reliably and were both maternally heterozygous and amplified a combined minimum of three alleles for both parents (Table 1). Polymerase chain reactions (PCR) were carried out with both parental and offspring samples in reactions comprised of: 10X PCR Buffer (10 mM Tris-HCl (pH-8.4) 50mM KCl), 2.5 mM $MgCl_2$, 200 μM dNTP's, 0.05 μg of each primer, 0.5 units DNA Taq polymerase (Invitrogen, Burlington, ON), and 50-100 ng of genomic template DNA (25 μL total reaction volume). PCRs were performed with the

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TABLE 1

Primer sequences, annealing temperatures (T_a), and allele sizes at the 11 Chinook salmon (*Oncorhynchus tshawytscha*) microsatellite loci used in this study. Allele sizes correspond to the four alleles that offspring can inherit from the maternal (M) and paternal (P) parents. All primers from Williamson *et al* (2002), except *One114* (OLSEN *et al.* 2000), *Ots104* and *Ots107* (NELSON and BEACHAM 1999) and *Ssa85* (O'REILLY *et al.* 1995)

Locus Name	Primer sequence 5´-3´	T_a (°C)	Allele sizes (bp): Maternal parent M_1	M_2	Paternal parent P_1	P_2
One114	TCATTAATCTAGGCTTGTCAGC	59	283	311	259	287
	TGCAGGTAAGACAAGGTATCC					
Ots104	GCACTGTATCCACCAGTA	55	224	228	220	228
	GTAGGAGTTTCATTTGAATC					
Ots107	ACAGACCAGACCTCAACA	55	246	242	238	250
	ATAGAGACCTGAATCGGTA					
OtsG249	TTCTCAGAGGGTAAAATCTCAGTAAG	55	237	245	213	233
	GTACAACCCCTCTCACCTACCC					

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OtsG311	TGCGGTGCTCAAAGTGATCTCAGTCA	55	299	310	306	326
	TCCATCCCTCCCCCATCCATTGT					
OtsG432	TGAAAAGTAGGGGAAACACATACG	55	111	133	107	137
	TAAAGCCCATTGAATTGAATAGAA					
OtsG68	TATGAACTGCAGCTTGTTATGTTAGT	59	185	234	246	262
	CATGTCGGCTGCTCAATGTA					
OtsG78b	GTCCCTTGAATTGAATTGATTAGA	55	249	265	249	325
	CAGCCTACTGCAGTTCAATAGACT					
OtsG83b	TAGCCCTGCACTAAAATACAGTTC	55	187	216	167	191
	CATTAATCTAGGCTTGTCAGCAGT					
RT36	GCAAGTTATACACTCGTCCAGGTACGC	59	100	122	114	122
	ACACTCCTTTACACCCCTTCACCAAC					
Ssa85	AGGTGGGTCCTCCAAGCTAC	55	128	168	150	168
	ACCCGCTCCTCACTTAATC					

following reaction profile: 2 min initial denaturation (94°); followed by 35 cycles of 1 min denaturation (94°), 1 min annealing (see Table 1), 1 min extension (72°); 3 min concluding extension cycle (72°). Amplifications were analyzed for fragment size using a CEQ 8000 automated DNA sequencer with appropriate size standard (Beckman-Coulter, Fullerton, CA).

Data analysis

Confirmation of Ploidy Status: Diploid and triploid ploidy status was confirmed using the genotypic microsatellite data; one of the pressure-shocked offspring failed to triploidize and was diploid. This sample was removed from further analyses, as the stress of the triploidization process may have affected the embryo in ways that could compromise the allelic data. Five samples were discarded due to poor DNA quality. The final sample sizes were 16 diploid and 18 triploid embryos.

Segregation Distortion in Meiosis: Triploid salmon eggs only undergo meiosis I (see above), while their diploid counterparts proceed normally through the entire meiotic cycle. By inference, then, we can examine triploid data in light of meiosis I only and diploid in light of meiosis I and II. Thus, to determine if segregation distortion had occurred over the course of the complete meiotic cycle, the frequencies of the maternal- and paternal-origin alleles in the diploid offspring, and of the paternal-origin allele in the triploid offspring were determined. Based on the expectation of random segregation, both maternal and paternal alleles should occur at 50% frequency in the diploid offspring as should the paternal alleles in the triploid offspring. Significant divergence from the expected frequencies was tested using the chi-square test. To account for multiple, simultaneous tests across loci, these results were adjusted for significance using the sequential Bonferroni correction procedure (RICE 1988).

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Similarly, the genotype frequencies for the diploid offspring were calculated and departures from the expected Mendelian 1:2:1 ratios were tested for significance using the chi-square test. Triploid genotype frequencies could not be tested in the same fashion due to complications peculiar to salmon recombination. Because triploidization blocks maternal meiosis II, maternal alleles in the triploid fish are inherited as a homozygotic pair unless recombination occurs between the centromere and the marker locus in maternal meiosis I. Since salmon experience 100% interference during meiosis (THORGAARD *et al.* 1983), the occurrence of recombination depends directly on the location of the marker on the chromosome arm: the more distal the locus, the greater the likelihood of recombination. Salmonid recombination rates are also influenced by differences specific to family and parental sex (e.g. SAKAMOTO *et al.* 2000); however, our experiment used a single family and, thus, should not be affected by such considerations. Since we do not know, *a priori*, the location of the microsatellite loci relative to the centromere, we cannot predict expected triploid genotype frequencies based on Mendelian inheritance.

Segregation Distortion in Meiosis I: In order to determine if segregation distortion had occurred in meiosis I, the maternal-origin allele segregation in the triploid offspring was analyzed. According to the principles of random segregation, the maternal alleles were expected to occur in the offspring at 50% frequency, although there would be twice the number of maternal alleles in the triploid offspring relative to the paternal alleles. The maternal-origin allele frequencies were determined, and a chi-square test was performed to establish if the values significantly departed from the predicted Mendelian ratio (1:1). The significance of the results was adjusted using the sequential Bonferroni correction procedure to account for multiple, simultaneous tests (RICE 1988). Although

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we could not test for specific genotypic ratios, we do expect the ratio of maternal-origin allele homozygotes in the triploid offspring to be equal. Finally, to confirm that segregation at different microsatellite loci was independent, linkage analysis was performed among the 11 microsatellite loci by estimating pair-wise maternal recombination among the marker loci; 50% recombination is indicative of no linkage.

Mapping: The 100% interference found in salmon permits a simple estimate of map distances of the 11 loci relative to the centromere in triploid salmon. This was calculated as the total number of recombinants divided by the total number of genotypes at that locus, multiplied by 100%.

RESULTS

Of the 11 loci selected for use, four had three alleles in the two parents, while the remaining eight loci had four parental alleles (Table 1).

None of the diploid maternal- or paternal-origin allele frequencies were significantly different from the expected Mendelian frequencies (Table 2). As well, all genotype frequencies in diploid offspring conformed to Mendelian expectations (1:2:1; Appendix A), as did distribution of the paternal-origin component of the triploid genotype (Appendix B).

Maternal-origin alleles at two microsatellite loci in the triploid offspring (*Ots107* and *OtsG68*) showed significant departure from Mendelian 1:1 ratios after sequential Bonferroni correction (Table 2, Appendix B). These departures were reflected in the maternal genotype frequencies with one of the homozygous maternal genotypes never or seldom appearing (Table 3). No significant linkage was found between any of the 11 loci. Finally, recombination rates of the different loci were distributed from 0% (centromeric) to 50% (distal; Table 2).

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TABLE 2

Maternal- (M) and paternal-origin (P) microsatellite allele frequencies found in diploid (2N) and triploid (3N) Chinook salmon offspring from a single cross. Recombination rates reflect the maternal genotype from 0% (all homozygotes) to 50% recombination (all heterozygotes).

	2N				3N				
	Maternal		Paternal		Maternal		Paternal		Recombination Rates (%)
Locus Name	M_1	M_2	P_1	P_2	M_1	M_2	P_1	P_2	
One114	0.438	0.563	0.438	0.563	0.471	0.529	0.647	0.353	47.1
Ots104	0.438	0.563	0.563	0.438	0.500	0.500	0.611	0.389	50.0
Ots107	0.563	0.438	0.438	0.563	0.794	0.206*	0.647	0.353	20.6
OtsG249	0.467	0.533	0.333	0.667	0.472	0.528	0.722	0.278	8.3
OtsG311	0.563	0.438	0.438	0.563	0.500	0.500	0.471	0.529	50.0
OtsG432	0.313	0.688	0.625	0.375	0.556	0.444	0.611	0.389	0.0
OtsG68	0.438	0.563	0.563	0.438	0.750	0.250*	0.667	0.333	13.9

OtsG78b	0.538	0.462	0.538	0.462	0.563	0.438	0.500	0.500	43.8
OtsG83b	0.467	0.533	0.467	0.533	0.485	0.515	0.647	0.353	50.0
RT36	0.688	0.313	0.438	0.563	0.528	0.472	0.667	0.333	2.8
Ssa85	0.563	0.438	0.750	0.250	0.472	0.528	0.500	0.500	2.8

Values with asterisks (*) indicate significant departures from expected 1:1 segregation ($P < 0.05$) after sequential Bonferroni correction.

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TABLE 3

Maternal genotype frequencies in triploid Chinook salmon offspring at marker loci identified as departing from expected Mendelian segregation frequencies. Expected values are calculated as the value resulting from equal numbers of homozygote maternal allele. Due to the single obligate crossover that occurs in salmon meiosis, nonconformity to Mendelian expectations could only be assessed in homozygotes.

Locus	Actual		
Name	M_1M_1	M_2M_2	Expected
Ots107	0.588	0.000*	0.294
OtsG68	0.611	0.111*	0.361

Values with asterisks (*) indicate significant departures from expected values (P < 0.05).

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DISCUSSION

Among vertebrates, fish – especially salmon – genomes are remarkably permissive of ploidy manipulation. Furthermore, the fish appear to suffer few physiological or phenotypic perturbations as a consequence (BENFEY 1999). This is despite the expectation for widespread and fundamental genetic disruptions in polyploid vertebrates (MABLE 2004). Our project was designed to test for distortions in marker allele transmission from parent to triploid offspring. These distortions would arise as a consequence of epistatic effects and dominance modification resulting from the presence of the extra maternal chromosome in the polyploid offspring. Due to the relatively minor phenotypic consequences of triploidy in salmon, we expected, at most, modest evidence for segregation distortion; instead, we found dramatic departure from Mendelian expectation at two unlinked loci in triploid offspring, but none in diploid offspring.

To conserve the stock for future experimentation, the sample size for this analysis was necessarily small. The segregation distortion effects demonstrated in the results are so dramatic, however, that in this case the small sample size is overcome by a very large treatment effect. The number of fish examined does leave open the possibility that other markers, less strongly linked to deleterious alleles, were also affected, but that their weaker effects were undetectable due to the small sample size.

Due to the effect of induced triploidy on the meiotic cycle, we had anticipated two possible patterns of segregation distortion: 1) only loci in the diploid samples would have unexpected Mendelian ratios, in which case the distortion would be occurring at meiosis II or 2) marker loci in both diploid and triploid samples would show evidence of segregation distortion, in which case the distortion would be occurring at meiosis I. Instead, we found strong evidence of biased allele transmissions at loci only in the

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triploid samples. While this indicates little about meiosis I or II, it does posit linkage of a deleterious gene to the marker loci used in this study. Selection at the early zygotic stage is occurring, and its strength is dependent on the dosage of the deleterious maternally-derived allele. The diploid allele frequency data (Table 2) showed nearly equal frequencies of each of the alleles at the loci implicated in the triploid data, indicating that a single dose of the putatively deleterious maternal has no discernible effects on embryo survival. Under our deleterious allele dosage model, once the dose of the deleterious maternal allele was doubled in the triploid offspring, the single paternal allele could no longer mask the effects, and the zygote died before reaching even the eyed egg stage of development. Thus, the deleterious maternally inherited allele approaches 100% lethality when its dosage increases from 50% to 67%.

Since gene dosage is driving the observed segregation distortion, one possibility is that the product of the maternal allele is actively deleterious, not merely nonfunctional. This would be a surprising outcome, as most characterized deleterious and lethal alleles are recessive and form no functional protein (BLOT 1994; DRAKE *et al.* 1998). The fitness of organisms heterozygotic for such mutations is substantially unchanged because cellular pathways are thought to have a large safety margin (HALDANE 1937; KEIGHTLEY 1996); only one wildtype allele need be expressed for the pathway to operate at full capacity. In other words, the number of maternal deleterious alleles (i^m) present in the genome should not have an impact on fitness, as long as there is a single paternal wildtype allele (i^+) whose functional gene product will mask the effects of the allele producing a nonfunctional protein. We would further expect the fitness of a diploid homozygotic for the maternal allele ($i^m i^m$) to be very low, but the fitness of a diploid heterozygote ($i^m i^+$) and triploid maternal homozygote ($i^m i^m i^+$) to be equal. Instead, we found that the paternal

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allele was able to mask expression of a single dose of the maternal deleterious allele, but could not mask the double dose present in the triploid offspring. This indicates that the maternal allele is likely linked to a gene whose product is actively deleterious. A breeding experiment to confirm the low fitness of homozygote diploid deleterious offspring ($i^m i^m$) is presently underway. Such an experiment may also be able to account for the low survival characteristic of some specific salmon families observed at the YIAL hatchery.

Due to both likelihood and selection, the occurrence of a mutation resulting in a functional protein – deleterious or not -- is expected to be rare, so it is surprising that we would find two loci in the same family linked to two actively deleterious alleles. However, it must be remembered that linkage groups in salmonids can be very large due to the 100% chiasma interference found in these species (YOUNG *et al.* 1998). In addition, preliminary results from a Chinook salmon study using self-crossed hermaphrodites also identified several loci showing segregation distortion of genetic markers that could only be attributed to a high frequency of deleterious alleles (data not shown). Finally, salmon are residual tetraploids and, thus, have many functional, duplicated gene loci (ALLENDORF and THORGAARD 1984). Perhaps the prevalence of these duplicated loci equip the salmon genome with cellular mechanisms able to cope with these excess functional gene copies. The fact that salmon can regulate cell number and organ size in response to polyploidization implies they could also have sophisticated mechanisms to deal with changes in gene dosage and developmental pathways.

Although it is widely believed that cellular pathways can operate efficiently with variable component concentrations (HALDANE 1937; KEIGHTLEY 1996), it is also well-established that regulatory hierarchies are dosage-dependent (reviewed by BIRCHLER *et*

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al. 2005). Perhaps, then, it is not the increased dosage of the maternal allele that is problematic, but, instead, the proportional decrease in dosage of the paternal allele. If the maternal allele produced a nonfunctional protein, this could cause a dose-dependent dilution of the paternal-origin protein, possibly resulting in a debilitating alteration of cellular pathways. In our diploid salmon, for instance, the paternally-derived, active protein is present at 50% and a theoretical regulatory signal with which it interacts is present in a 100% dose. If expression levels were dose dependent, the triploid salmon would then have 100% regulatory signal but only 33% paternally-derived proteins, causing a potentially deleterious distortion in the concentration of the functional protein.

Alternatively, the observed segregation distortions could be caused by an environmental effect, such that the triploidization process preferentially eliminates one type of nuclear allele arrangement, perhaps one associated with pressure sensitivity. Such a process would act immediately after fertilization at the one cell stage, at the time of polar body retention and before significant gene expression. Recent studies examining heritability in triploid salmon theorized that there is, in fact, a genetic component to triploidization success in salmon, however, no gene-by-environment effects were observed (JOHNSON *et al.* 2006; JOHNSON *et al.* 2004), providing little evidence for environmental effects on allele segregation. Although direct effects of early pressure shock on the survival of specific genotypes is theoretically possible, we are aware of no published evidence for such a mechanism in any organism.

In summary, we provide strong evidence that dosage dependent lethality is occurring in triploid Chinook salmon embryos resulting in segregation distortion at two unlinked marker loci. We are unsure if the distortion operates through a dilution mechanism with an inactive protein or through a dose-dependent effect of an actively

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deleterious protein. A breeding experiment to confirm the low fitness of deleterious allele homozygotes (i^mi^m) is presently underway and will cast further light on the mechanism employed. It might be expected that, in most cases, a polyploid organism would have an extra layer of protection from deleterious alleles, instead we found that polyploidy exposed them to unexpected risks due to dose dependence. However, as the damage occurs prezygotically, this study finds no ecological or economic reasons preventing the use of triploid stock in commercial ventures.

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CHAPTER 3[2]

Segregation distortion in diploid and triploid Chinook salmon (*Oncorhynchus tshawytscha*) at the MHC: evidence of dose-dependent lethality and dosage effects

[2] This chapter is the outcome of joint research undertaken in collaboration with J.W. Heath , A.V. Hubberstey and D.D. Heath.

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INTRODUCTION

Salmon have an extremely plastic genome, and are amenable to extensive chromosomal manipulation, including genome duplication (polyploidy; reviewed by PANDIAN and KOTEESWARAN 1998). There is strong evidence of ancestral polyploidization events in the salmon genome, indicating that this plasticity has been a long-standing feature of the salmon's genetic character (ALLENDORF and THORGAARD 1984). Spontaneous polyploidy in salmonids continues to this day, and there are several methods (chemical, heat or pressure shock) of inducing polyploidy (reviewed by PANDIAN and KOTEESWARAN 1998). In polyploid salmon, the nucleus and cell volumes expand to accommodate the increase in genetic content at the same time that the total cell number decreases (BENFEY 1999; SMALL and BENFEY 1987). Thus, both organ and organism size is maintained, resulting in few obvious morphological differences between diploid and polyploid salmon. The changes in cell and nucleus volume may have more subtle consequences, however, as they alter surface area to volume ratios, which may affect cellular processes involving the plasma membrane (BENFEY 1999). Also, depending on the shape of the triploid cell, the intracellular distance may be increased, possibly affecting processes such as signal transduction from the cell surface to the nucleus (BENFEY 1999).

While polyploid salmon apparently maintain homeostasis on a whole animal level, on a subcellular level there can be dramatic and immediate changes to both the genome and transcriptome following induction of polyploidy. Studies of nascent plant allopolyploids (polyploids arising from hybridization) have shown evidence of massive genomic rearrangements, such as sequence deletion, and reciprocal translocations and

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transpositions between homeologous chromosomes (reviewed by CHEN and NI 2006; OSBORN *et al.* 2003). Changes to the transcriptome are thought to result mostly from epigenetic effects due to processes such as DNA cytosine methylation, histone alterations, positional effects due to higher order chromatin structural changes and the effects of small RNAs and RNA interference (reviewed by CHEN and NI 2006; OSBORN *et al.* 2003). In autopolyploids (polyploids arising from chromosome doubling within an individual), most of the gene loci appear to be governed by a simple, positive dosage effect, where gene expression increases with ploidy (BIRCHLER *et al.* 2001; GALITSKI *et al.* 1999; GUO *et al.* 1996; MARTELOTTO *et al.* 2005); but see (ADAMS and WENDEL 2004; SUZUKI *et al.* 1999). In these organisms, there are usually a few loci that are up- or down-regulated beyond what could be accounted for by a simple dosage effect, suggestive of epigenetic or complex epistatic mechanisms. Due to chromosome homology, autopolyploidy is much less disruptive than allopolyploidy. Similarly, genome duplications are generally not as disruptive as duplications of only a portion of the genome (aneuploidy) because the overall stoichiometry of cellular components is preserved (BIRCHLER *et al.* 2001). In addition, eukaryotic cells are accustomed to regular changes in ploidy number during meiosis and mitosis, and the very fact that salmon have the ability to alter their cell number in response to polyploidy suggests that they possess processes well adapted to managing ploidy changes.

There may be an immediate advantage to new polyploids if their extra genetic material more efficiently masks deleterious alleles and increases the chances of inheriting beneficial alleles (OTTO and WHITTON 2000). Extra gene copies may be especially beneficial for polymorphic genes like the major histocompatibility complex (MHC),

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where high levels of allelic variation are generally thought to result in elevated whole organism fitness. The MHC classical class I gene products are pivotal in the cytotoxic immune response to viral infections and are also important in the nonspecific immune response. As in mammals, salmonid MHC class I genes are codominantly expressed on all somatic cells (KOPPANG *et al.* 1998). MHC gene dosage varies widely among species. For instance, teleosts normally have only one to three classical class I MHC genes, but Atlantic cod are thought to have as many as 42 different MHC loci (MILLER *et al.* 2002). The number of MHC alleles varies considerably, as well, both among species and among populations within a species. Although an individual with a greater diversity of MHC alleles is expected to have a more responsive immune function and, thus, higher fitness (WEGNER *et al.* 2003; ZIEGLER *et al.* 2005), there appears to be an intermediate, optimal number of MHC alleles beyond which the fitness of the organism actually decreases due to depletion of the T-cell repertoire of the individual (LAWLOR *et al.* 1990).

Anecdotal evidence suggests that – far from gaining added protection from their extra MHC alleles – on the whole, triploid fish are sicklier than their diploid counterparts (personal communication John W. Heath, YIAL, Campbell River, BC, Canada). Published evidence is equivocal (reviewed by BENFEY 1999; BRUNO and JOHNSTONE 1990; YAMAMOTO and IIDA 1995), but the inconsistent results could be due to individual or familial variation, not ploidy differences (JOHNSON *et al.* 2006; MILLER *et al.* 2004). The use of triploid salmon in commercial aquaculture has been proposed but not widely adopted largely because of contradictory findings about the impact of triploidy on performance. The advantages to aquaculture of using triploid stock center on their sterility. Due to gonadal impairment, female triploid salmon do not undergo the stress of

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sexual maturation and the resulting reduced flesh quality and mortality (BENFEY 1999; PANDIAN and KOTEESWARAN 1998). More recently, the use of triploid stock has been proposed in order to prevent genetic contamination of local wild populations (BENFEY 2001). Detailed physiological and genetic studies on triploid salmon can help expand our knowledge of variation among triploids in order to make performance and survival more predictable and, thus, improve commercial lineages. Finally, the plasticity of their genome permits investigations of the effects of ancient genome duplications as well as the consequences of new polyploid events on the genome, transcriptome and phenotype.

Here we describe an experiment involving ploidy manipulation of the genome of Chinook salmon, *Oncorhynchus tshawytscha*, to investigate the occurrence of segregation distortion in allelic inheritance by tracking the transmission of MHC classical class I alleles from a single mated pair into diploid and triploid offspring. Once the MHC genotype of each individual has been ascertained, we can then determine if ploidy changes are altering expression levels of MHC class I alleles (e.g. dosage effects).

METHODS AND MATERIALS

Monitoring mortality

In the summer of 2003, researchers at Yellow Island Aquaculture Ltd. (YIAL) monitored mortality of 2-year-old diploid and triploid Chinook salmon during a natural outbreak of bacterial kidney disease (BKD). Dead fish in saltwater netcages were collected by SCUBA and tallied approximately weekly (eight times over a two month period). Three netcages were monitored, two holding triploid fish, and one holding diploid fish. The initial numbers of fish in the three netcages, prior to the outbreak, were

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5,303 and 7,175 (triploid) and 8,539 (diploid). Mortality was calculated as a percentage of the live fish at the beginning of the sample period.

Breeding and sampling

Salmon eggs proceed only partially through meiosis before fertilization, so applying a hydrostatic shock within an hour of fertilization will result in a triploid embryo by preventing the completion of the meiotic cycle (BENFEY and SUTTERLIN 1984). In November 2002, half the fertilized eggs from a breeding pair of Chinook salmon were pressure-shocked 20 min after fertilization (69 MPa for 8 to 10 min), inducing triploidization by retention of the polar body (BENFEY and SUTTERLIN 1984). The remaining half of the fertilized eggs were permitted to complete meiosis and left untreated as a diploid control group. Both triploid and diploid eggs were incubated in vertical stack incubation trays at 7.8°C (average) and approximately 12 $L \cdot min^{-1}$ at Yellow Island Aquaculture Ltd. (YIAL) on Quadra Island, British Columbia, Canada following standard hatchery practice. Parental adipose fin tissue was sampled and stored in 95% ethanol for later DNA extraction.

At seven months post-fertilization, the diploid and triploid fish were vaccinated with a commercial *Vibrio* vaccine (Alpha-Dip 2100, *V. anuillarum,* serotype 01 and *V. ordalii* bacterin; Alpharma NW Inc.), differentially fin clipped, pooled, and then transferred to a saltwater netcage.

The following summer, the 19 month old diploid (N=41) and triploid (N =90) adult offspring were sampled. On average, the diploid fish were 18.5% heavier than their triploid siblings (diploid average weight: 192.9 g; triploid average weight: 157.3 g). A

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blood smear was made on a microscope slide and gill tissue was stored in RNA*later* (Ambion, Inc., Austin TX; KOPPANG *et al.* 1998) for later mRNA and DNA extraction.

Confirmation of ploidy

The ploidy status of the offspring was confirmed by examining the blood smears after staining with Giemsa stain. The mean length of the major axis of the erythrocyte nucleus was determined for each sample (BENFEY *et al.* 1984) and those samples with average major axes longer than or equal to 9.5 μM were classified as triploid (JOHNSON *et al.* 2004). The final sample sizes were 27 diploid and 59 triploid offspring.

DNA extraction and MHC sequencing

DNA was extracted from parental and offspring fin tissue using the Wizard Genomic Purification Kit (Promega, Madison, WI) following manufacturer's protocol for DNA extraction from animal tissue. Parental DNA and DNA from two diploid offspring were used as amplification templates and the results were cloned and sequenced. The peptide binding region (PBR) of the classical class I MHC loci was amplified through polymerase chain reaction (PCR) with appropriate primers (5'-TGACTCACGCCCTGAAGTA-3', 5'-CTCCACTTTGGTTAAAACG-3'; MILLER *et al.* 1997). The reactions were comprised of: 10X *PfuUltra* HF reaction buffer, 2.5 U *PfuUltra* High-Fidelity DNA Polymerase (Stratagene, La Jolla, CA), 25 mM dNTPs, 100 ng of each primer and 50-100 ng of genomic template DNA (50 μL total reaction volume). PCRs were performed with the following reaction profile: 2 min initial denaturation (95°); followed by 30 cycles of 30 sec denaturation (95°), 30 sec annealing (52°), 1 min extension (72°); 10 min concluding extension cycle (72°).

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The amplicons were purified with QIAquick PCR Purification Kits (QIAGEN Inc., Venlo, The Netherlands) then cloned using TOPO Cloning Kits (Invitrogen, Carlsbad, CA) both following manufacturer's protocols. Colonies were grown in LB broth, then equal parts broth and ddH_2O were combined, vortexed and boiled for 2 min. The M13 forward primer (5'-GTAAAACGACGGCCAGT-3') and M13 reverse primer (5'-AAACAGCTATGACCATG-3') were used to amplify the insert. The reactions were comprised of: 10X PCR Buffer (10 mM Tris-HCl (pH-8.4) 50mM KCl), 2.5 mM $MgCl_2$, 200 μM dNTPs, 0.05 μg of each primer, 0.5 units DNA *Taq* polymerase (Sigma, St. Louis, MO), and 50-100 ng of genomic template DNA (25 μL total reaction volume). PCRs were performed with the following reaction profile: 2 min initial denaturation (94°); followed by 35 cycles of 1 min denaturation (94°), 1 min annealing (55°), 1 min extension (72°); 3 min concluding extension cycle (72°). Finally the inserted class I MHC PBRs were sequenced using a $1/8^{th}$ reaction of a CEQ DTCS Quick Start Kit and analyzed on a CEQ 8000 automated DNA sequencer (Beckman Coulter, Fullerton, CA). Sequencing of each sample continued until a saturation curve confirmed that all of the MHC alleles present had been detected.

Class I MHC clones from the parents showed several unique sequences which were not transmitted to the two offspring cloned and sequenced. Sequences from clones of the two offspring showed a similar distribution. The pattern was confirmed when outsourced sequencing duplicated the results for both parental and offspring clones. We concluded that these unique sequences were artifacts and they were removed from further analysis.

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RNA extraction and MHC allele determination in offspring

RNA was extracted from offspring gill tissue samples using Trizol Reagent. Following extraction, 2 μL of total RNA was treated with DNAse I, Amplification Grade and first strand synthesis was performed using SuperScript II Reverse Transcriptase and Oligo(dT)12-18 Primer (all Invitrogen, following manufacturer's protocols).

Offspring cDNA and gDNA were then used as amplification templates with the MHC class I primers following a procedure identical to the M13 PCR, above. The samples were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (ABI, Foster City, CA) following manufacturer's protocols. Sequences were visualized on ABI's 3130*xl* Genetic Analyzer using their POP-7 Polymer. Single nucleotide differences at 11 sites in the MHC class I sequence were examined to distinguish among the three parental MHC alleles.

RESULTS

Survival

Triploid mortality rates in the two saltwater net pens were consistently and significantly higher (chi-square; $P < 0.0001$) than diploid mortality rates following a natural outbreak of BKD (Figure 1).

MHC allele characterization

The majority of the male parent's clones (N=50) had class I MHC PBR sequences identical to GenBank submission *U80284* (MILLER *et al.* 1997). The remaining clones (N=13) produced 11 sequences that showed up once, or – at most – twice only, none of which were identical to previous GenBank submissions (Figure 2). A similar situation existed in the class I MHC clones from the female parent; the two most common

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FIGURE 1

Variable responses of triploid and diploid Chinook salmon (*Oncorhynchus tshawytscha*) to a natural outbreak of bacterial kidney disease (BKD) at Yellow Island Aquaculture Ltd.. The initial numbers of fish were 5,303 (Pen 1), 7,175 (Pen 2) and 8,539 (Pen 3). Mortality was calculated as a percentage of the live fish at the beginning of the sample period.

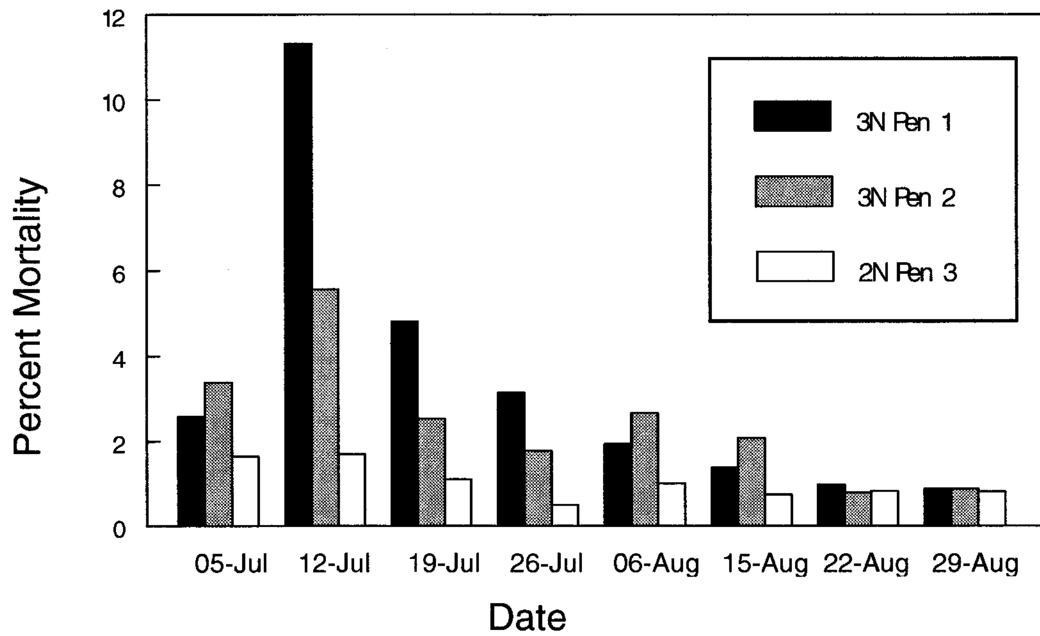

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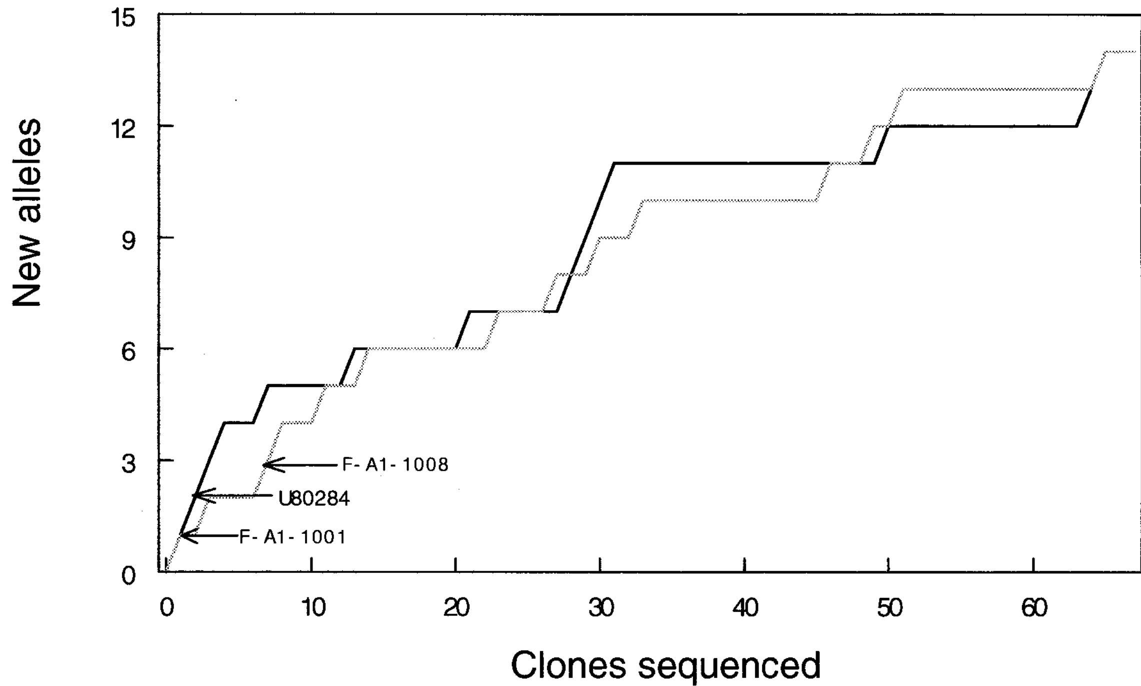
FIGURE 2

Cumulative number of novel major histocompatibility complex (MHC) class I sequences identified in Chinook salmon parents. Parental class I MHC peptide binding region (PBR) amplicons were cloned and sequenced until the number of new alleles found in the clones had reached saturation. The black line indicates the male clones and the gray represents the female clones. Arrows indicate where the 3 common alleles were first sequenced. For instance, the maternal-origin allele *F-A1-1008* was first discovered in the seventh clone sequenced.



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sequences were designated *F-A1-1001* (N=22) and *F-A1-1008* (N=20) and submitted to GenBank, and the remaining 12 sequences were unique and rare (Figure 2, Appendix C). The unique parental sequences differed from the common by two base pairs, at most; in a neighbor-joining tree all the parental class I MHC sequences separated into three branches grouped by the three common parental alleles (Appendix D). Transitions occurred almost twice as often as transversions, and coding alterations were fairly evenly distributed between synonymous and non-synonymous changes; one substitution resulted in a nonsense mutation. The rare unique parental alleles were not transmitted to either diploid offspring tested. The class I MHC sequences from the clones of the two diploid offspring samples were similarly distributed between common and rare unique alleles. The majority of the sequences was identical to the common parental alleles, while the remainder was unique to each offspring.

The segregation of the male parental allele into both the diploid and triploid offspring was anomalous: approximately 50% of the diploid offspring had no detectable paternal allele. The most likely explanation for this is that the male parent carried a null allele (i.e. one that consistently fails to detectably amplify in a PCR). Although we could not confirm this possibility, we restricted our analyses to the maternal-origin alleles in order to eliminate the potential problems caused by the distribution of the male allele.

Segregation Analysis

Both the frequency of the maternal-origin alleles and their distribution into genotypes were not significantly different from Mendelian expectations in the diploid offspring. The triploid samples, however, showed significant departure from Mendelian expectations in maternal-origin allele frequencies and marginal significance levels in the

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maternal-origin genotype frequencies (Table 1). There was no apparent paternal bias (i.e. all three maternal-origin allele combinations segregated with the single paternal-origin allele; data not shown).

Expression Analysis

The parameters of this study were limited to determining if individual alleles were being expressed; it did not extend to measuring those expression levels. Consequently, a dosage effect could only be detected in those triploid offspring heterozygotic for maternal alleles. The six triploid samples that inherited both female alleles also expressed both alleles, indicating that a dosage effect was occurring in response to the ploidy increase.

DISCUSSION

Because of their economic and ecological potential to aquaculture, triploid fish have been the subject of numerous performance trials, including disease challenges, in order to compare their results to those of normal, diploid fish. Except under conditions of oxygen stress, where triploid salmon perform consistently worse than diploid, the results of these performance trials have been equivocal, perhaps at least partially due to the confounding effects of age, size, family and behavioral differences between the diploid and triploid subjects (BENFEY 1999). The BKD outbreak at the YIAL salmon farm clearly showed that the triploid fish were more susceptible to the disease than their diploid counterparts (Figure 1). This is in direct contrast to both commercial and laboratory trials of all female diploid and triploid Atlantic salmon in Scotland (BRUNO and JOHNSTONE 1990). Immune responses to vaccination are affected by family more than ploidy in Chinook salmon, and poor performance in diploid fish appears to become exaggerated in their triploid relatives (JOHNSON *et al.* 2004). It is unlikely, however, that

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TABLE 1

Maternal-origin (M) allele and genotype frequencies found in diploid (2N) and triploid (3N) Chinook salmon offspring from a single cross. M_1 corresponds to allele *F-A1-1001*; M_2 to allele *F-A1-1008*.

Ploidy	Allele Frequency		Genotype Frequency		
status	M_1	M_2	M_1/M_2	M_1/M_1	M_2/M_2
2N	0.40	0.60	N/A	N/A	N/A
3N	0.39*	0.61*	0.17	0.31	0.53

Values with asterisks (*) indicate significant departures from expected 1:1 segregation (P < 0.05)

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the differential mortality observed at YIAL was simply a result of familial traits, as the percent mortality in the triploid netcages was, at one point, six times higher than that in the diploid netcage (Figure 1). This would mean that either the triploid netcages had a far higher proportion of poor performance family members, or that the inferior response of the poor performance families was extremely exaggerated by the increase in ploidy number. Conclusively separating the effects of family versus ploidy could be done through disease challenge of several different families with full-sib diploid and triploid offspring. Alternatively, if response to BKD could be attributed solely to the possession of a specific MHC allele or to MHC heterozygosity in general, then, on average, triploid fish should experience fewer losses than diploid in disease challenge studies; as triploid fish have an extra chromosome copy, triploid organisms have both increased heterozygosity and an increased chance of possessing the beneficial allele. Finally, there is clear evidence that stress, such as handling, transport, and variable water quality, has an impact on the immune system of salmon (BARTON and IWAMA 1991; VAN MUISWINKEL *et al.* 1999). Furthermore, certain kinds of stress affect disease resistance of triploid salmon more severely than that of diploid. In a study with rainbow trout, triploids exposed to vibriosis had a higher level of mortality than diploids when under chronic stress due to high temperatures (i.e. oxygen stress; OJOLICK 1995). Accordingly, perhaps the trauma of the triploidization process led to an impaired immune function, although, again, one would expect more consistent results in disease challenge studies. Instead, possibly a stressor more deleterious toward triploids than diploids (e.g. oxygen stress) weakened the immune function of the triploid fish.

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In contrast to our findings in Chinook salmon, induced triploid *Xenopus* hybrids were found to inherit all of the parental MHC alleles (KOBEL and DU PASQUIER 1986). There were two potential sources of segregation distortion in this experiment: the polyploid nature of the subjects, in general, or the function of the MHC loci, specifically. A previous study on this family of Chinook salmon had found clear evidence of segregation distortion at two microsatellite marker loci (Chapter 2). Those results suggested that the bias was driven either by an alteration in the cellular stoichiometry or by an increase in the concentration of a deleterious allele linked to the microsatellite markers. It is possible that the similar distortion pattern seen in this study is simply an artifact of the bias occurring at the microsatellite marker. If the MHC locus is partially linked to the deleterious allele posited in the microsatellite study, the majority of the *F-A1-1001* homozygotes would inherit the deleterious allele, thereby decreasing their fitness, while the remaining *F-A1-1001* homozygotes would be free of the deleterious allele and, thus, maintain their fitness.

Another theory explaining the results centers around functional selection acting on the MHC. One of the key differences between this and Chapter 2 was in the age of the fish. In the neutral marker study, the subjects were Chinook salmon eggs, and had experienced little selection. The salmon in this study had experienced 19 months of intensive selection possibly acting at the MHC and other loci further exacerbated by high fish density and handling stress. This could be selection either for the triploid *F-A1-1008* homozygotes or against *F-A1-1001* homozygotes. It is impossible to comment on the maternal genotype heterozygotes, as their low frequency could be the product of selection or of a distal location on a chromosome arm (THORGAARD *et al.* 1983; Chapter 2). The

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lack of bias in the diploid data indicates that the individual maternal alleles, themselves, do not have a deleterious or beneficial impact, so we can infer that their influence is dosage independent. As all the MHC alleles inherited are expressed (see below), perhaps an increase of *F-A1-1008* expressed on cell surfaces caused an increase in survival.

The unique alleles found when the parental class I MHC PBR was cloned and sequenced (Figure 2) could be either PCR artifacts or the result of somatic mutations. As the outcome from the outsourced sequencing of the clones was identical to that from our instrument, we can conclude that the unique alleles are not a sequencing artifact and, thus, occurred earlier in the experiment, during cloning. An artificially increased number of alleles in MHC sequencing is not uncommon (LANGEFORS *et al.* 2000), and are usually dismissed as PCR artifacts. We did the same in our study for three reasons: (1) the majority of the unique, rare alleles differed from the common by a single base pair, (2) in a neighbor-joining tree, the unique alleles clustered together, branching behind the appropriate common allele (Appendix D), and (3) these unique and rare alleles were not heritable. However, the probability of PCR errors occurring in 20% (female) or 29% (male) of the sequences – as it did in this study – is extremely low. Misincorporation of nucleotides occurs at a rate of 10^{-2} to 10^{-6} for regular *Taq* DNA polymerase, such as that used in the M13 reaction. High-fidelity *Pfu* DNA polymerase, which contains 3'→5' exonuclease-dependent proofreading activity, was used in the initial reaction to produce cloning vector inserts and has a base substitution rate ~10-fold lower than that of regular *Taq* (CLINE *et al.* 1996).

The MHC is one of the genome's mutational "hot spots." This polymorphic gene complex maintains high levels of variability through a number of mechanisms including

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point mutation, duplication, retroelement translocation, and gene conversion and other recombination events (BERNATCHEZ and LANDRY 2003; CULLEN *et al.* 2002; KULSKI *et al.* 2002; MARTINSOHN *et al.* 1999). In addition, somatic hypermutation is a well-established feature of immunoglobulin genes and may well have been the first mutational mechanism developed to promote polymorphism in immune-related genes (LEE *et al.* 2002). To our knowledge, no studies have found evidence of mitotic mutation in MHC *in vivo.* This is unsurprising, as unchecked somatic diversification of MHC alleles *in vivo* could increase the risk of an autoimmune response. Still, it is possible that the mechanism remains in the MHC's mutational repertoire to be activated *in vitro*, perhaps in combination with specific classes of DNA polymerases available only artificially. A somatic mutation would help to explain the surprisingly low number of classical MHC loci found in salmon – a number more characteristic to solitary and/or isolated populations rather than an organism which lives in schools and migrates from fresh- to saltwater (AOYAGI *et al.* 2002; LAWLOR *et al.* 1990; MILLER *et al.* 2004). Perhaps a mitotic mutation mechanism evolved in order to increase the MHC allele complement in the face of limited coding diversity. Whatever the mechanism, the high proportion of unique alleles is specific to the PBR of the MHC, as we routinely execute this and similar protocols with other loci without experiencing this artificial increase in the number of alleles.

In this study, approximately half of the diploid offspring genotyped had apparently not inherited an allele from the male parent, indicating that the male parent possessed a null allele. For our purposes, null alleles are those that consistently fail to amplify to detectable levels in a PCR. Null alleles can be caused by alterations in the

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primer site, large changes in product size or poor DNA preparation (reviewed by DAKIN and AVISE 2004; O'CONNELL and WRIGHT 1997). It is possible that a mutation in the primer site is responsible for the hypothetical null allele, although the primer is designed to anneal to a very highly conserved region of the MHC. This possibility could be investigated simply by replicating the experiment with redesigned PCR primers. Although the MHC is an area prone to mutation, there is no chance of an insertion large enough to prevent amplification occurring in the ~260 bp sequence of interest. Finally, all DNA templates were prepared identically and the frequency of the supposed null allele matches Mendelian expectations in the diploid salmon. In some cases, however, template quality prevents amplification only at some alleles, while others amplify with ease (DE LEON *et al.* 1998). This possibility could be explored further by replicating the experiment using a different – perhaps even automated – DNA extraction method.

Our investigation into expression patterns of the MHC in diploid and triploid Chinook salmon found a dose effect pattern in the triploid samples; those individuals that inherited both maternal-origin alleles also expressed both of them. Studies of ploidy series in organisms such as yeast, maize and *Drosophila* have found that the majority of the genes monitored show a dosage effect (BIRCHLER *et al.* 2001; GALITSKI *et al.* 1999; GUO *et al.* 1996; MARTELOTTO *et al.* 2005). In addition, the MHC is codominantly expressed in diploid organisms and in induced triploid *Xenopus* hybrids (KOBEL and DU PASQUIER 1986). It would be of interest to quantify the MHC expression in the diploid and triploid salmon to see if a true 1:1 dosage effect is occurring, and also to be able to include those samples homozygous for maternally inherited alleles.

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This investigation was prompted by the differential mortality between diploid and triploid salmon in response to a natural BKD outbreak at an aquaculture facility. By investigating full-sib diploid and triploid fish, we were able to equalize the number of possible MHC alleles in both ploidy groups, while altering the quantity of inherited and expressed alleles. In this way, we could eliminate confusion caused by familial or allelic differences and, instead, focus solely on the effect of ploidy changes. With this technique, we discovered a dose-dependent lethality among the adult triploid fish caused either by MHC functionality, or incomplete linkage to a previously described deleterious allele (Chapter 2). The results of this investigation indicate that a cautious approach to using triploid fish in commercial hatcheries may be warranted due to unforeseen complications arising from their altered ploidy status on their immune function.

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CONCLUSIONS AND FUTURE DIRECTIONS

Diploid and triploid offspring of a single breeding pair of Chinook salmon (*Oncorhynchus tshawytscha*) were amplified with 11 microsatellite primers (Chapter 1) and with primers flanking the peptide binding region (PBR) of the classical class I major histocompatibility complex (MHC; Chapter 2) to determine if segregation distortion was occurring at those loci. Since triploid salmon are created by blocking maternal meiosis II, observed segregation distortion could be attributed to meiosis I or II by comparing diploid and triploid offspring. Maternal-origin alleles at two unlinked microsatellite loci and at the MHC locus in the triploid samples showed evidence of departure from expected Mendelian ratios, hence segregation distortion occurred in meiosis I, but only in triploid offspring. These results indicate that triploid offspring died due to maternal dosage effects; that is, when the maternal allele dosage was doubled, the single paternal allele could no longer mask the effects of the putatively deleterious maternal alleles. There are three possible mechanisms associated with this effect: if the deleterious maternal-origin allele produces a nonfunctional product, dilution of the active paternally-derived product could lead to a debilitating biochemical imbalance; alternatively, the maternal deleterious product could be fully functional and deleterious, and observed segregation distortion results from gene dosage effects of functional deleterious proteins; finally, the distortion seen at the MHC could be the result of selection acting on the immune system of the individual. The MHC mRNA expression was also examined in order to determine if dosage compensation or dose effects were occurring. The triploid offspring showed the expected pattern of a positive dosage effect (i.e. all alleles inherited were expressed; Chapter 2).

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In order to gain further insight into the mechanisms involved in the transmission and expression of alleles in triploid salmon, several potential lines of research should be explored. An investigation to confirm or refute the existence of a dose-dependent deleterious allele is presently being carried out. Such an experiment may also be able to account for the low survival characteristic of specific triploid strains in commercial aquaculture. If the existence of deleterious alleles is corroborated, it would be fascinating to attempt to localize the gene through a mapping experiment and to discover its identity and function. A study into the phenomenon of unique, rare and untransmitted alleles in MHC sequencing seen in this investigation is also being carried out in order to determine if these are the result of somatic mutation or procedural artifacts.

It would be useful to repeat this experiment with several different mated pairs to see if segregation distortion is a common theme in triploid salmon, or if it is relatively rare. Expanding on this study in this manner would also provide an opportunity to explore the inheritance and expression of class II MHC, a path that had to be abandoned in this study, due to the homogeneity of the parental class II MHC alleles. In sampling diploid and triploid offspring from several families, it would be of interest to subject the families to a disease challenge: one intracellular (e.g. bacterial), to examine the response of class I MHC and one intracellular (e.g. viral), to focus on class II MHC. This would further assist in separating family and ploidy effects in disease susceptibility, and perhaps provide a strain with high immunity for further experimentation. It would also be useful to quantify the expression levels of the MHC and, perhaps, of other genes involved in disease resistance, in order to determine if the dosage effect detected in this study displays a strict one to one ratio of allele to mRNA transcript. A relative comparison

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could be performed through a QRT-PCR procedure, while the overall effects of ploidy on the transcriptome could be gained through microarray experiments.

Triploid salmon are of interest in both applied and basic scientific research. High-performance triploid stock would improve the economic outlook for commercial aquaculture, as well as allaying fears for the long term well-being of wild salmon populations in the face of escapes from fish farms. In more fundamental scientific research, triploid fish are of interest in studying both the long term consequences of ancient polyploidization events and the immediate effects of polyploidy on the genotype and phenotype of a complex vertebrate.

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APPENDIX A

Genotypes for diploid Chinook salmon (*Oncorhynchus tshawytscha*) at 11 microsatellite loci. *A* and *B* refer to maternal-origin alleles, *C* and *D* correspond to paternal-origin alleles.

Sample	Ots104	Ots107	OtsG311	OtsG83b	OtsG432	OtsG249	Ssa85	OtsG78b	OtsG68	RT36	One114
1	*AD*	*BC*	*BC*	*AD*	*BC*	*AD*	*AC*	*AD*	*BD*	*AC*	*AD*
2	*AD*	*BC*	*BC*	*AC*	*AD*	*BD*	*AC*	*AD*	*BD*	*AC*	*AC*
3	*BD*	*AD*	*BD*	*BD*	*AC*	*BD*	*AC*	*AC*	*BD*	*BD*	*BD*
4	*BD*	*AD*	*AD*	*AC*	*BC*	*BC*	*BC*	*AD*	*AD*	*AD*	*AC*
5	*AD*	*AD*	*AC*	*BD*	*BD*	*BD*	*BC*		*AD*	*AD*	*BD*
7	*BC*	*BD*	*AC*	*BC*	*BD*	*AD*	*BD*		*BC*	*AD*	*BC*
8	*BD*	*AC*	*AD*	*AD*	*BC*	*AC*	*AD*		*AD*	*AC*	*AD*
9	*AC*	*AD*	*AD*	*BD*	*BC*	*BD*	*AD*	*BC*	*AC*	*AD*	*BD*
11	*BC*	*AD*	*AD*	*BC*	*AC*	*AD*	*BC*	*BD*	*BC*	*AD*	*BC*
12	*BC*	*BD*	*AC*	*BC*	*BD*	*BC*	*AC*	*BC*	*BC*	*BD*	*BC*
13	*AC*	*AC*	*BD*		*AD*		*BC*	*AC*	*BC*	*BC*	*BD*
16	*BC*	*AC*	*AC*	*AC*	*BD*	*AC*	*AC*	*BC*	*AC*	*BC*	*AC*

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17	*BC*	*BC*	*BD*	*BD*	*BC*	*BD*	*BC*	*BD*	*BC*	*AC*	*BD*
18	*AC*	*BC*	*AC*	*BD*	*BC*	*BD*	*AC*	*AC*	*AC*	*AC*	*BD*
19	*AC*	*BD*	*BD*	*AC*	*BC*	*AD*	*AD*	*BD*	*AC*	*BD*	*AC*

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APPENDIX B

Genotypes for triploid Chinook salmon (*Oncorhynchus tshawytscha*) at 11 microsatellite loci. *A* and *B* refer to maternal-origin alleles, *C* and *D* correspond to paternal-origin alleles.

Sample	Ots104	Ots107	OtsG311	OtsG83b	OtsG432	OtsG249	Ssa85	OtsG78b	OtsG68	RT36	One114
21	*ABD*	*AAD*	*ABC*	*ABC*	*AAC*	*ABC*	*BBC*		*ABD*	*AAD*	
22	*ABC*	*AAC*	*ABC*	*ABD*	*AAC*	*AAC*	*BBD*	*ABC*	*BBC*	*AAC*	*ABD*
23	*ABD*	*AAD*	*ABD*	*ABC*	*BBC*	*BBC*	*ABD*	*ABC*	*AAC*	*AAD*	*ABC*
24	*ABD*	*ABC*	*ABC*	*ABD*	*BBC*	*BBD*	*BBC*		*AAD*	*BBC*	*ABD*
25	*ABC*	*ABC*	*ABC*	*ABC*	*AAC*	*BBD*	*BBD*	*ABC*	*ABC*	*AAC*	*ABC*
26	*ABD*	*AAD*	*ABC*	*ABC*	*AAC*	*AAD*	*BBD*	*ABD*	*AAD*	*BBD*	*ABC*
27	*ABC*	*AAD*	*ABD*	*ABC*	*AAD*	*AAC*	*AAC*	*ABD*	*ABC*	*AAD*	*ABC*
28	*ABC*	*AAC*	*ABC*	*ABD*	*AAD*	*ABC*	*BBD*	*ABD*	*ABC*	*AAC*	*ABD*
29	*ABC*	*AAC*	*ABD*	*ABD*	*AAC*	*ABC*	*AAD*	*ABD*	*AAC*	*BBC*	*ABD*
31	*ABD*			*ABD*	*BBD*	*BBD*	*AAC*	*ABD*	*BBD*	*BBC*	*ABD*
32	*ABD*	*ABC*	*ABD*	*ABC*	*BBD*	*BBC*	*BBC*	*ABC*	*ABD*	*BBC*	*ABC*
33	*ABD*	*ABC*	*ABC*		*BBC*	*AAC*	*AAD*	*ABD*	*AAD*	*BBC*	*BBD*

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35	*ABC*	*ABC*	*ABD*	*ABC*	*AAC*	*AAC*	*AAC*	*ABC*	*AAC*	*BBC*	*ABC*
36	*ABC*	*AAC*	*ABD*	*ABC*	*BBC*	*AAC*	*BBD*	*AAD*	*AAC*	*ABC*	*ABC*
37	*ABC*	*AAD*	*ABD*	*ABC*	*BBD*	*BBC*	*BBD*	*ABD*	*AAC*	*AAD*	*ABC*
38	*ABC*	*AAD*	*ABC*	*ABC*	*AAC*	*BBD*	*AAC*	*AAC*	*AAC*	*AAD*	*ABC*
39	*ABC*	*ABC*	*ABD*	*ABC*	*BBD*	*AAC*	*AAC*	*ABC*	*AAC*	*BBC*	*ABC*
40	*ABC*	*ABC*	*ABD*		*AAD*	*BBC*	*AAC*	*ABC*	*AAC*	*AAC*	*ABC*

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APPENDIX C

Sequences from the peptide binding regions (PBR) of the classical class I major histocompatibility complex (MHC) found most frequently in the Chinook salmon broodstock. *U80284* was found in the male parent, *F-A1-1001* and *F-A1-1008* in the female.

```
U80284      TTCTACACCGCATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGGTTGTGGGGACGG
F-A1-1001   ----T-----------------------------------------------------
F-A1-1008   -------------------------------------------------------T--

U80284      TGGATGGTGTTCAGATGTTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACA
F-A1-1001   ----------------------------------------------------------
F-A1-1008   ----------------------------------------------------------

U80284      GGACTGGATGAACAAGGCAGCAGAAACACTGCCACAGTACTGGGAGAGGGAGACAGGG
F-A1-1001   ---------A------------------------------------------------
F-A1-1008   ---------G---------------------------------------A-C--T---

U80284      ATTGACAAGGGTGCCCAGCAGACTTTCAAAGCCAACATCGATATTGTAAAGCAG
F-A1-1001   ---TG-------A-----------------------------------------
F-A1-1008   -A----------GA----------------------------------------
```


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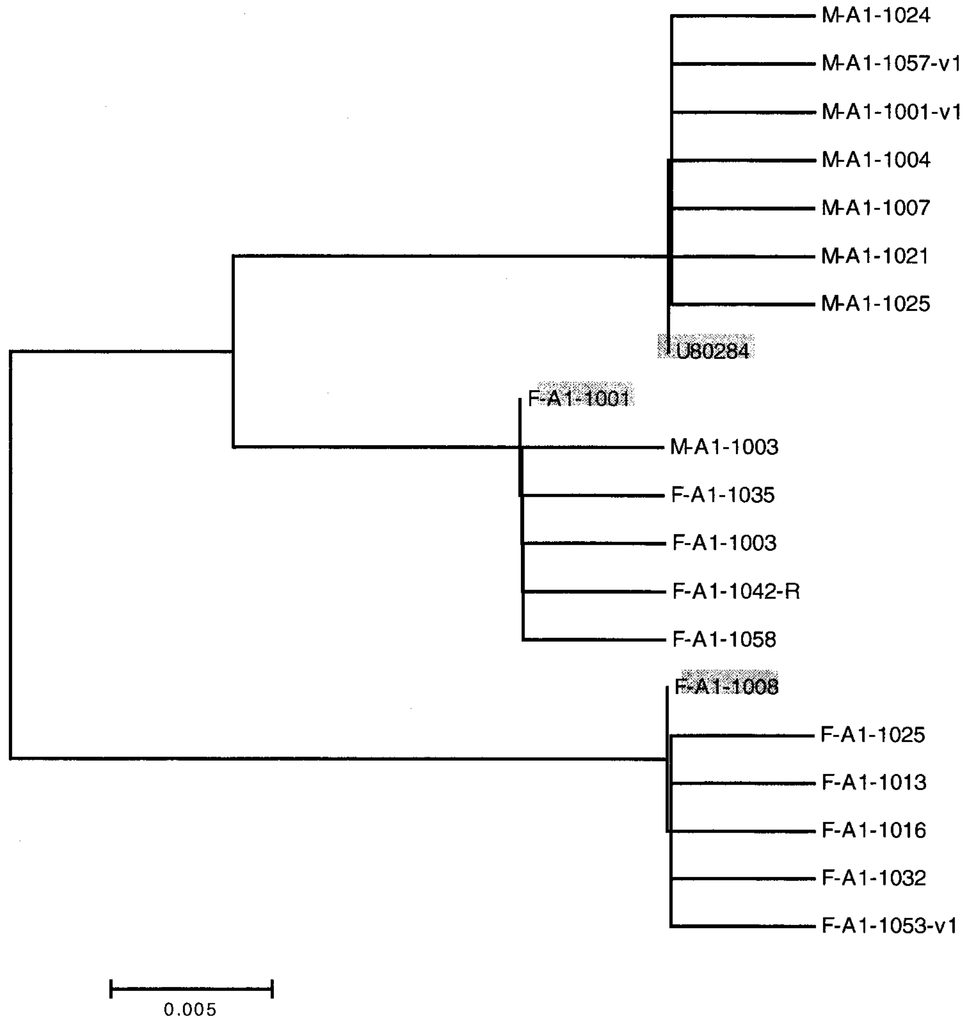
APPENDIX D

Unrooted neighbor-joining tree (2,000 replicates) based on peptide binding region (PBR) sequences from the classical class I major histocompatibility complex (MHC) locus of Chinook salmon (*Oncorhynchus tshawytscha*). Sequences differ at only a single nucleotide from the highlighted alleles behind which they are grouped. Allele *U80284* was originally submitted as a GenBank submission by MILLER *et al.* 1997, other alleles are original to this work.



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