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Transcriptional differences between triploid and diploid Chinook salmon (Oncorhynchus tshawytscha) during live pathogen challenge.

Benjamin Ching
University of Windsor

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TRANSCRIPTIONAL DIFFERENCES BETWEEN TRIPLOID AND DIPLOID CHINOOK SALMON (*Oncorhynchus tshawytscha*) DURING LIVE PATHOGEN CHALLENGE

by

Benjamin Ching

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

2006

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ABSTRACT

The immuno-competence of triploid salmon has been in contention for some time. Numerous studies have focused on performance without attention to the fundamental genetic difference between triploid and diploids. In this study, triploid and diploid Chinook salmon (*Oncorhynchus tshawytscha*) were used to examine the effects of polyploidy on specific and genome-wide gene expression response to a severe immune challenge using quantitative real time PCR (qRT-PCR) and microarray technology. Although triploid and diploid fish had significant differences in mortality, qRT-PCR revealed no differences in cytokine gene expression response (interleukin-8, interleukin-1, interleukin-8 receptor and tumor necrosis factor), while differences were observed in constitutively expressed genes, (IgM, MHC-II and β-actin). Genome-wide microarray analysis has revealed that, overall, triploid gene expression is similar to diploids as observed in their similar phenotypes. This balance, however, can be disrupted during stressful events such as immune challenges as shown at a select few assayed genes.
ACKNOWLEDGEMENTS

This work wouldn’t be possible without the support and guidance of my supervisors Dr. Daniel Heath and Dr. Andrew Hubberstey. I would like to thank John and Ann Heath for their enthusiasm and solutions to obstacles encountered in the field. I would also like to thank Dr. R. Devlin and Ben Goh for providing lab access in Vancouver. Furthermore, there would not be fish to sample without the help of all the workers at Yellow Island Aquaculture Ltd. whom have also put up with my presence. This Study was supported by a Collaborative Research and Development grant form the Natrual Sciences and Engineering Research Council of Canada (NSERC) to D. Heath and A. Hubberstey. Field and rearing support was provided by Yellow Island Aquaculture Ltd. Finally, I would like to thank my parents, Irene and Bill Ching, for their support and Margaret Chong for being a source of inspiration and woe. I’m still not sure which.
STATEMENT OF ORIGINALITY

Chapter 2 of this thesis incorporates the outcome of research undertaken in collaboration with D.D. Heath, A.V. Hubberstey, and T. Aykanat and development of qRT-PCR assays in Chinook salmon (*Oncorhynchus tshawytscha*). Both D.D. Heath and A.V. Hubberstey provided helpful input and gave feedback and guidance during the writing process. T. Aykanat aided in design of one of the assays. This chapter has been submitted to *Molecular Ecology Notes* for publication. Chapter 3 of this thesis incorporates the outcome of research undertaken in collaboration with D.D. Heath, A.V. Hubberstey, and T. Aykanat relating to investigation of triploid Chinook salmon gene expression during an immune challenge using quantitative real-time polymerase chain reaction. Both, D.D. Heath and A.V. Hubberstey gave helpful input throughout, and, again, provided feedback and guidance during the writing process. T. Aykanat aided in the sampling. Chapter 4 consists of research in genome-wide gene expression of triploid versus diploid Chinook salmon undertaken in collaboration with D.D. Heath, A.V. Hubberstey, and T. Aykanat. Both, D.D. Heath and A.V. Hubberstey gave helpful input throughout, and, again, provided feedback and guidance during the writing process. T. Aykanat aided in the sampling. All collaborations were undertaken as part of a research project under the supervision of Dr. D. Heath and Dr. A. Hubberstey.

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 supervisors, Dr. D. Heath and Dr. A. Hubberstey.
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CHAPTER 1
INTRODUCTION

Polyploidy can be found throughout nature. Although the majority of organisms that exhibit polyploidy are plants, there are still many examples of polyploidy amongst certain groups of animals (Otto et al. 2000); one such group are fishes. Varying incidence of polyploidy can be found in fish, with high instances in lower teleosts (low specialization) to relatively low instances among higher teleosts (high specialization) (Leggatt et al. 2003).

Salmonids are an intermediate teleost with a recent tetraploid past (Allendorf et al. 1984) and are still going through diploidization (Young et al. 1998). In Chinook salmon (Oncorhynchus tshawytscha), triploidy can be readily induced by the application of a pressure shock to fertilized eggs. The shock disrupts the spindle fibres during meiosis II and allows the retention of the second polar body. This diploid maternal chromosome set and haploid paternal chromosome set then form the triploid embryo (Benfey 2001; Felip et al. 2001).

The triploid salmon produced by this process have several features. They have increased nuclear content and size (Susan A. Small et al. 1987; Johnson et al. 2004). This increase is associated with increased overall cell size, but the total size of the organism is maintained at the cost of cell number (Benfey 1999). The increase in nuclear material also means an increase in allelic diversity, potentially aiding in masking deleterious alleles (Benfey 1999). However, the property of most interest to aquaculture is that triploid females are sterile and do not undergo sexual maturation.

Triploid females typically do not have oocytes which develop to a point where specialized cells secrete steroids. This lack of development results in females which
show no sign of endocrine sexual maturation. Furthermore, low estrogen levels result in much lower plasma vitellogenin in triploid salmon than normally maturing diploid salmon. Vitellogenin is essential for the rapid growth of oocytes prior to ovulation (Piferrer et al. 1994; Benfey 1999; Tiwary et al. 2004).

This lack of sexual maturation serves two purposes in aquaculture. Sexual maturation in salmon involves a decrease in flesh quality and eventual death, which results in economic loss during times of maturation each year. There is also the possibility of increased growth in non-maturing triploid fish (due to the conservation of the energy normally invested into oocyte development); however, triploid growth rates have been reported as quite variable, both within and among species (Benfey 2001; Tiwary et al. 2004). Another potential benefit is genetic containment, which has gained increasing interest. Many farmed species are not native to the local area and domestication can rapidly and inadvertently select for traits that are not beneficial to the local population (Youngson et al. 2001; Heath et al. 2003). Also, as the size of fish farms increase they will eventually make up of a significant percentage of the local biomass increasing the impact of potential escapes (Youngson et al. 2001). Genetic containment by application of triploidy will render the fish sterile and thus inhibit gene flow between domesticated and wild stocks or colonization of foreign species.

While both of these are significant benefits, conflicting reports as to the viability and resilience of triploids has made the industry hesitant to adopt the technology. Some studies have found that there is little or no difference in triploid survival after exposure to bacterial and viral pathogens (Bruno et al. 1990; Dorson et al. 1991). However, Ojolick et al. (1995) found that there were increased mortality rates in triploids infected with bacterial gill disease. Furthermore, seawater survival has been typically poor in triploids.
compared to diploids (O’Flynn et al. 1997; Benfey 2001; Cotter et al. 2002). It has been suggested that the poor seawater performance may be due to the triploid’s inability to handle chronic stress associated with increased time of exposure to pathogens and temperature fluctuations in the water (Ojolick et al. 1995; O’Flynn et al. 1997; Benfey 2001).

Although many physiological-based performance studies have been performed, experiments into the specific gene expression differences between triploid and diploids have been largely ignored. Current experiments involving other species polyploids have revealed some interesting gene transcription differences and patterns. Mechanisms for multiple gene copy regulation are mainly examined using artificially-induced aneuploids. Regardless of the source of the multiple gene copies, several types of effects are generally described: 1) dosage effects, 2) dosage compensation, and 3) co-suppression. Dosage effects result from the proportional increase of gene product relative to increases in copy number, or ploidy (Timko et al. 1980; Birchler et al. 1981; Galitski et al. 1999b). Dosage compensation occurs when extra gene copies are either silenced or regulated to near normal (diploid) levels (Devlin et al. 1982; Birchler et al. 2005). Finally, co-suppression results when an extra copy or copies of a gene cause the suppression of gene expression in homologues genes (Jorgensen 1995; Pal-Bhadra et al. 1999). Even though these effects have been documented primarily in aneuploid and transgenic experiments, they are expected to act in a similar manner in polyploid situations.

Although both natural and induced polyploidy are well known phenomenon in plants and animals, studies of gene regulation in polyploids relative to diploids are rare and spread amongst widely different organisms (Guo et al. 1996; Galitski et al. 1999a; Osborn et al. 2003; Comai 2005). Several interesting patterns of expression have been
described in those studies. Studies in maize have found that overall, polyploids exhibit dosage effects mimicking the relative expression levels in diploids (Guo et al. 1996; Galitski et al. 1999a). Also, an interesting odd/even chromosome number expression pattern was observed in one of the studies, where odd ploidy (1N,3N) had increased levels of gene expression compared to the normal expression levels in even ploidy cells (2N,4N) (Guo et al. 1996). A study of triploid silkworm gene expression, however, found that triploidy induced varying degrees of up-regulation and down-regulation in triploids relative to diploids in a number of genes which were sampled (Suzuki et al. 1999). Additionally, these studies and others have found that increased levels of gene expression do not always proportionally match the increases in ploidy (Guo et al. 1996; Suzuki et al. 1999; Galitski et al. 1999a).

Triploid Chinook salmon were used in this study to better understand the workings of polyploidy and gene expression. Specifically, the expression of immune related genes were studied to determine their comparative functionality between triploids and diploids in a disease challenge. Two separate approaches were taken to assay gene transcription levels in diploid and triploid Chinook salmon: 1) quantitative real time polymerase chain reaction (qRT-PCR), and 2) cDNA microarrays. QRT-PCR allows a sensitive and targeted look into specific mRNA transcript levels in triploids versus diploids. QRT-PCR offers good sensitivity and specificity for each individually developed assay. Each assay, however, requires detailed sequencing information in order to develop the primer and probe sets. The time invested into obtaining such sequencing information and developing qRT-PCR assays is well spent, since the developed assays become a valuable resource for other researchers who wish to study the same genes.
In contrast, the microarray approach allows a wide range of gene expression to be analyzed, but comes at the cost of decreased sensitivity. Many individuals can be sampled easily and readily for a specific gene using qRT-PCR. Each microarray, however, only allows the comparison of two samples per slide but over thousands of genes. Samples may be pooled to increase the number of individuals, but at a loss of sensitivity.

The development of the qRT-PCR assays used in this study are described in Chapter 2. Degenerate primers were designed for several genes based on the alignments of a variety of species. These primers were then used to amplify cDNAs and genomic DNA which were then sequenced. The sequencing data was used to design the specific qRT-PCR primer and probe sets at intron/exon boundaries required for the assays.

The assays were put to use in Chapter 3, where qRT-PCR was utilized to assay transcription of several cytokine and immune related genes in disease-challenged diploid and triploid Chinook salmon. Gene expression profiles were generated for interleukin-8, interleukin-8 receptor, interleukin-1, tumor necrosis factor, major histocompatibility complex class II, immunoglobulin M heavy chain, and β-actin in diploid vs. triploid salmon over a three day period subsequent to *Vibrio anguillarum* exposure. After pathogen challenge, some immunologically induced genes responded to disease challenge similarly between diploid and triploid salmon while constitutively expressed genes show significant differences.

In the fourth chapter, genome-wide analysis of gene expression differences between triploid and diploid Chinook salmon exposed to a severe pathogen challenge was conducted. Using Atlantic salmon microarrays, the overwhelming majority of genes
showed no difference in response between diploid and triploid salmon. However, some genes did show a consistent transcriptional difference in response to the disease challenge.

Using both qRT-PCR and microarrays I was able to determine that slight differences in gene expression do occur in triploids compared to diploids after a severe pathogen challenge. This did not include highly up-regulated genes which were responding to the immune challenge and showed a net dosage effect. The differences which were detected in a few genes, though, suggest that possible downstream effects may be the cause of poor triploid Chinook salmon immuno-competence. By using both qRT-PCR and microarrays I was able to provide both a general and focused view on issues revolving gene expression and polyploidy in Chinook salmon.


CHAPTER 2

Immune Function-Related Quantitative Real Time PCR Assays for Chinook Salmon

(Oncorhynchus tshawytscha)

\[1\] This chapter has been submitted to Molecular Ecology Notes for publication. It follows Molecular Ecology Notes format.
Understanding how organisms function at the gene-transcription level is becoming increasingly important for both ecological and evolutionary studies (Gibson 2002). It is evident that the diversity and complexity of organisms are not dependent solely on the number of genes their genome contains, but also the variability in gene expression and gene interactions (Levine et al. 2003). Furthermore, slight differences in transcription control can fundamentally affect the fitness of the organism in a variable environment or during development (Matoba et al. 2000; Giulietti et al. 2001). One critical component of fitness in feral animals is the immune response to pathogen challenges which can be monitored with quantitative real time polymerase chain reaction (qRT-PCR) to reflect transcriptional effects. By quantifying transcription and determining how environmental and epigenetic effects can impact transcription, we may ultimately be better able to manage our wild and domestic stocks.

QRT-PCR can provide fast and accurate quantification of the transcription levels of selected genes. QRT-PCR, however, requires accurate sequencing information and annotation of intron/exon boundaries to help minimize the effect of possible genomic DNA (gDNA) contamination. We designed degenerate and specific PCR primer sets to obtain gDNA sequence data for six immune-related genes: interleukin-8 (IL-8), interleukin-8 receptor (IL-8R), interleukin-1 (IL-1), tumor necrosis factor (TNF), major histocompatibility complex class II (MHC-II), and immunoglobulin M heavy chain (IgM) in Chinook salmon (Oncorhynchus tshawytscha). These specific genes were selected for their probable relevance to resistance to bacterial infection, although their functions were inferred from mammalian and other teleost fishes based on sequence homology. Investigations into the role of IL-1, TNF, IgM and MHC II in fish have suggested they may have similar functions as in mammals. In mammalian systems, interleukin 1 has
many target cells: mature T and B cells, monocytes, neutrophils, fibroblasts and endothelial cells, regulating immune and inflammatory response (Dinarello 1997), and it is likely these functions are largely conserved in teleost fishes, including salmon. In fish, IL-1 appears to have chemoattractant properties (Peddie et al. 2001), but expression in different species suggests that, in fish, IL-1 may have additional functions (Engelsma 2002). IL-8 has also been characterized through sequence homology and expression studies in fish (Laing et al. 2002; Chen et al. 2005) and is thought to function similar to mammalian IL-8, a chemoattractant for neutrophils produced by cells upon an inflammatory response (Oppenheim et al. 1991). TNF in fish shares homology with mammalian TNF-α and TNF-β, but expression patterns suggest it is closer to TNF-α (Hirono et al. 2000). TNF is also produced by macrophages upon activation by lipopolysaccharides (LPS) (Laing et al. 2001) suggesting they have similar inflammatory and bactericidal properties as in mammals (Vassalli 1992). The major immunoglobulin in fish, IgM, appears to have comparable antigen affinity to mammalian IgM (Kaattari et al. 2002). MHC, while being one of the most studied immune function genes, still lacks detailed understanding of its specific interactions due to the unavailability of fish homologues of cytokines (Dixon et al. 2001). The accumulated evidence does suggest that fish MHC II functions as an extra-cellular antigen-presenting protein, similar to mammalian MHC (Klein et al. 2000). Here, we describe the development and validation of qRT-PCR assays for these genes in Chinook salmon.

The Chinook salmon used in this study were spawned in the fall of 2004 at Yellow Island Aquaculture Ltd (YIAL; Quadra Island, BC, Canada), and the offspring were raised in flow-through tanks under standard commercial rearing conditions. On August 10, 2005, 95 fish were anaesthetized, and injected intraperitoneally with $5 \times 10^5$
colony forming units of *Vibrio anguillarum*. An equal number of fish were sham injected with Phosphate-buffered saline (PBS). Twelve hours post-challenge, 15 challenged and 10 sham-injected fish were humanely euthanized and head kidney samples were taken and preserved in RNAlater at -20 °C. Total RNA (RNA) was extracted from the head kidney samples using Trizol (Invitrogen, Burlington, Canada) as per manufacture’s instructions. Genomic DNA (gDNA) was extracted by Wizard Genomic DNA purification kit (Promega, Madison, USA), while cDNA was generated with SuperScript II (Invitrogen, Burlington, Canada) as per manufacture’s instructions using an oligo dT<sub>20</sub> anchor and random hexamers.

Degenerate PCR primers designed from GenBank sequences for various species (see Table 1) were used to amplify gene fragments from both gDNA and cDNA in a 25 µL reaction with 10-50 ng of gDNA or cDNA, 0.5 U *Taq* DNA polymerase (Sigma-Aldrich, Oakville, Canada), 2.5 mM MgCl<sub>2</sub>, 0.2 mM each dNTP, 150 nM of each primer, and 10X PCR buffer. The cycling parameters were 94 °C for 2 mins, 30 cycles of 30s at 94 °C, 30s gradient at 52-62 °C, and 1 min at 72 °C, with a final extension for 5 mins at 72 °C. Individual bands were extracted from gels with multiple bands using GenElute gel extraction kit (Sigma-Aldrich, Oakville, Canada) and then re-amplified to produce a single fragment. PCR products were sequenced on an ABI 3700 (Genome Quebec Innovation Centre, McGill University, Montreal, Que., Canada). Sequence identity was confirmed using BLAST, and the gDNA and cDNA sequences were aligned using Dialign (Morgenstern *et al.* 1998) to identify intron/exon boundaries. TaqMan probes and primer sets were designed using Primer Express 3.0 software (Applied Biosystems, Foster City, USA). Final primer and probe sequences are listed in Table 2.1. Each qRT-PCR primer and probe set was designed to anneal at 60 °C to work under default ABI
7500 Real-Time thermocycler conditions of 50 °C 2 mins., 95 °C 10 mins., and 40 cycles of 95 °C 15 secs. and 60 °C 1 min. All reactions were run in 20 μL consisting of 2X TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, USA), 150 nM TaqMan probe, and 900 nM forward/reverse primer.
Table 2.1 Summary of the degenerate primer sequences used to amplify putative gene fragments from Chinook salmon gDNA and cDNA, with approximate fragment sizes and the resulting QRT-PCR primer sequences, fragment size and Taqman probe sequence for six immune-related genes

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Degenerate primer sequences</th>
<th>PCR fragment sizes (gDNA/cDNA)</th>
<th>GeneBank Accession numbers</th>
<th>QRT-PCR primer sequences</th>
<th>QRT-PCR fragment size</th>
<th>QRT-PCR probe sequence</th>
</tr>
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<tbody>
<tr>
<td>IL-8</td>
<td>STTGTSRTKGTGCTCCTGG ATGACYYTCTTSSACCCAMG</td>
<td>800/200 bp</td>
<td>DQ778949</td>
<td>CGCACTGCAGAGACACTGA ACAAATCTCTTGACCCTGTCTTG</td>
<td>58 bp</td>
<td>FAM-TCAGAGTGCCA ATC-MGB</td>
</tr>
<tr>
<td>IL-8 receptor</td>
<td>GAYGTCTACCTGTTTACCTGAC GACGAAGGCTASAGSAC</td>
<td>700/700 bp</td>
<td>DQ778948</td>
<td>GCGGGGTTTACAGAAG ACAGGAGGAAGGCGAACAC</td>
<td>64 bp</td>
<td>FAM-ACGGCAATGAT CC-MGB</td>
</tr>
<tr>
<td>IL-1</td>
<td>GACCTGATAGCATTAGCATGC AGGGTGGATCTTCTTGCATG</td>
<td>400/150 bp</td>
<td>DQ778946</td>
<td>CCAGGGAGGCAGACGCTA CGGGCTACGTACGAA</td>
<td>59 bp</td>
<td>FAM-AACAAATGAT AAC-MGB</td>
</tr>
<tr>
<td>TNF</td>
<td>TGGTGTCAGCAGTGGACAC GACGAAGGACAGGCCAG</td>
<td>800/300 bp</td>
<td>DQ778945</td>
<td>CCCACCATACATTTGGAACGCTATT GGATTGTAATCTAGCCTGAA</td>
<td>70 bp</td>
<td>FAM-CGGCAATGCA -MGB</td>
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<tr>
<td>MHC class II</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>AAGCTTCTCAGCTGGCTCAAA GCTCAACTCTGTTCTCCAGTATGG</td>
<td>99 bp</td>
<td>FAM-TCTCTAGCTGCTG-MGB</td>
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<tr>
<td>IgM heavy chain</td>
<td>ATGAGGACCTGGAGCAATGGG CTCCTCTCCTCCACCGCT</td>
<td>700/250 bp</td>
<td>DQ778947</td>
<td>CGCTGTAGATACCTGGGAACACC TCTCCACGTCTCCCTCTTG</td>
<td>69 bp</td>
<td>FAM-ACCTTGGTAAA AGC-MGB</td>
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All qRT-PCR assays were normalized to elongation factor 1 alpha (EF1A) (FWD primer 5'-AATACCCTCCTCTTGGTCTGTTTC-3', REV primer 5'-CATCAAGGCCGTCGACAAG-3', and probe 5'-VIC-TGCGTGACATGAGGC-3' run with the same conditions as above) using the 2^-ΔΔCt method (Livak et al. 2001). Serial dilutions of total RNA were used to generate calibration curves for each of the probes to ensure similar efficiency and validity of the 2^-ΔΔCt method (Applied Biosystems User Bulletin No. 2 (P/N 4303859)). Disease challenged fish showed significant transcriptional up-regulation (P < 0.01, T-test) in IL-1, TNF and IL-8 (Figure 2.1). This was expected since all cytokines used in the assay were part of the early innate immune response (Secombes et al. 1999; Goetz et al. 2004; Chen et al. 2005). Significant up-regulation was also detected in IgM (P<0.05 T-test), but not in MHC-II (P = 0.21) and IL-8R (P=0.06). This is interesting since MHC II is generally up-regulated when stimulated with TNF and LPS and our study clearly shows TNF up-regulation. The lack of MHC-II response may be due to the timing of the sampling (i.e. 12 hours post-challenge). There also appears to be a higher degree of variation in the interleukin and TNF transcription compared to MHC-II and IgM (Figure 2.1).
Figure 2.1 Gene transcription levels at six immune-related loci relative to elongation factor 1-alpha transcription measured using qRT-PCR on Chinook salmon prior to, and 12 hours after, a severe immune challenge. *P < 0.05; **P < 0.01; ***P < 0.001.

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The qRT-PCR assays for immune function-related genes described here generally showed a strong transcriptional response to a severe disease challenge. Thus they will likely have valuable applications in salmon management, since levels of expression and timing of expression of immune function genes may reflect immune competence, or for evolutionary studies since immune function is clearly a critical fitness-related parameter.


CHAPTER 3

Chapter 3: Gene expression analysis in triploid and diploid salmon subjected to pathogen exposure.

1Chapters 3 and 4 are formatted to be jointly submitted to Genetics for publication.
INTRODUCTION

While polyploidy in animals does occur, the effects of polyploidy on animals has not been as thoroughly explored as plants (Osborn et al. 2003; Comai 2005). However, studies on the effect of polyploidy on gene expression are still rare in any organism. A study looking at maize euploidy series illustrated several interesting effects in relation to ploidy: dosage compensation, dosage effect and increases beyond dosage effect (Guo et al. 1996). As a whole, however, maize polyploidy was found to be relatively undistruptive. In contrast, a triploid silkworm study found many of the genes sampled had expression profiles that deviated from those of diploids (Suzuki et al. 1999). The effects found in those studies have also been mirrored in other studies (Timko et al. 1980; Devlin et al. 1982; Jorgensen 1995). Although few studies utilize euploid polyploidy as a model, the effects of aneuploidy do exhibit some interesting regulatory effects. Dosage effects are the proportional increase of gene product in relation to increases in gene copy number (Timko et al. 1980; Birchler et al. 1981; Galitski et al. 1999). Dosage compensation occurs when extra copies of the gene or genes are silenced or regulated to near normal levels (Devlin et al. 1982; Birchler et al. 2005). Co-suppression is the down-regulation of homologous genes in response to increases in the dosage of a gene (Jorgensen 1995; Pal-Bhadra et al. 1999). Finally, the maize ploidy series study also provided examples of expression surpassing that of a simple gene copy dependent dosage effect, as well as a complex expression pattern that was dependent on whether ploidy was odd or even (Guo et al. 1996).

Because of the rare occurrence of viable polyploids among animals (Otto et al. 2000), there is relatively little detailed research on dosage effects in animals, hence polyploid fish provide a valuable model system. Many fish species have had a polyploidy
ancestor, though this occurrence is more common among lower than higher teleosts (Leggatt et al. 2003). Salmoniformes, an intermediate teleost, appear to have undergone tetraploidization approximately 25-100 million years ago (Allendorf et al. 1984) and still appear to be going through re-diploidization (Young et al. 1998). This probably allows salmonids to be more resistant to the potential lethal effects of polyploidy common among animals. Because of the ease of genetic manipulation and extensive animal husbandry information (Powers 1989), salmon are a unique and valuable model system for ploidy experiments.

In this study I have chosen to investigate gene expression differences between triploid and diploid Chinook salmon (Oncorhynchus tshawytscha) in response to an immune challenge. Most previous published studies concerning gene expression and polyploidy have mainly focused on resting-state experiments (Comai 2005). Triploid Chinook salmon, generally exhibit a normal phenotype with few differences from diploid salmon at resting-state. Studies into the immuno-competence of triploids have provided varying results, and immune challenge studies with a variety of pathogens have come up with either equal competence of triploids versus diploids or triploids faring poorly relative to diploids (Bruno et al. 1990; Dorson et al. 1991; Ojolick et al. 1995; Johnson et al. 2004). However, in seawater trials, survivability of triploids has generally been lower compared to that of diploids (O'Flynn et al. 1997; Benfey 2001; Cotter et al. 2002). In order to investigate the discrepancy in those results a more in-depth genetic approach was taken.

While in mammalian systems many immune function related genes have been characterized, in most cases the function of the fish immune related genes are discerned from homology to mammalian genes based on sequence. Here, four putative innate
immune related genes: interleukin-1 (IL-1), interleukin-8 (IL-8), interleukin-8 receptor (IL-8R), and tumor necrosis factor (TNF), were selected to monitor innate immune responses (Secombes et al. 2001). IL-1 has suspected functions as a chemoattractant for leukocytes (Peddie et al. 2001) and possess similar functions as mammalian IL-1: namely, targeting mature T and B cells, monocytes, neutrophils, fibroblasts and endothelial cells, regulating immune and inflammatory response (Dinarello 1997). Mammalian IL-8 is a chemoattractant for neutrophils produced by cells upon an inflammatory response (Oppenheim et al. 1991); expression and homology studies in fish suggest similar function (Laing et al. 2002; Chen et al. 2005). TNF is produced by macrophages upon activation by lipopolysaccharides (LPS) in fish (Laing et al. 2001), and based on gene homology and expression data (Hirono et al. 2000), TNF probably has similar inflammatory and bactericidal properties as in mammals (Vassalli 1992). Two additional immune related genes, immunoglobulin M heavy chain (IgM) and major histocompatibility complex class II (MHC-II), and one housekeeping gene, β-actin, were selected for use in this study. IgM is the major immunoglobulin in fish and appears to have comparable antigen affinity as mammalian IgM (Kaattari et al. 2002). MHC-II appears to have extra cellular antigen presenting functions similar to the mammalian MHC (Klein et al. 2000). β-actin has a highly conserved role as a housekeeping gene; it is expressed at high levels, and has a role in supporting cell motility and structure (Jae-Seong, 2000)

To study the transcriptional response of the selected genes to a disease challenge in diploid and triploid Chinook salmon, quantitative real-time polymerase chain reaction (qRT-PCR) was used. QRT-PCR has the advantage of being sensitive to the low copy number typical of cytokines (Giulietti et al. 2001) and hence should be able to detect the

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possible small differences between triploid and diploid Chinook salmon transcription responses. The development of the qRT-PCR assays used in this study is described in Chapter 2. The endogenous internal standard gene used was elongation factor 1 alpha which appears to be primarily developmentally regulated (Gao et al. 1997) and was validated using geNorm (Vandesompele et al. 2002).

In our study, triploid and diploid Chinook salmon were challenged with *Vibrio anguillarum*. Survival was monitored over a ten day period, and mRNA samples were analyzed using qRT-PCR over the first 3 days of the trial. The results show that after challenge very different expression patterns between triploids and diploids can occur which are markedly different than unchallenged patterns.

**MATERIALS AND METHODS**

**Breeding Program**

The Chinook salmon used in this study were spawned in fall of 2004 at Yellow Island Aquaculture Ltd (YIAL, Campbell River, B.C.). Two females were each mated to three males to produce six families. Each family was split into two where one half was incubated without treatment, while one was subjected to hydrostatic pressure shock to induce triploidy (Benfey et al. 1984; Johnson et al. 2004). All fertilized eggs were held in separate compartments in vertical flow-through incubation stacks following standard incubation procedures. Fish were then transferred to 120 L flow-through tanks upon yolk sac absorption. Each group was then randomly selected to produce two tanks of 120 fish.

**Immune Challenge**

Prior to the immune challenge, equal numbers of fish from each of the six families were combined into four groups: triploid challenge, triploid sham, diploid challenge and
diploid sham. Each challenge group consisted of three tanks which contained 95 fish each and both sham groups consisted of one tank which contained 95 fish. Fish were allowed to acclimatize for two weeks prior to pathogen challenge. *Vibrio anguillarum* (Dorothee Kieser, DFO Pacific Biological Station) was streaked on to blood agar plates and allowed to grow for 20 hours at room temperature (25-27 °C). Just prior to challenge, the *Vibrio* culture was suspended in PBS to produce an O.D. of 1 at 540nm. The neat solution was further diluted to produce a final concentration of approximately $5 \times 10^5$ CFU/mL. The suspension was kept on ice and loaded into 1 cc 26 gauge syringes prior to intraperitoneal injection. The actual dose was determined by standard plate count method (Harley and Prescott 1996) and was found to be $7.3 \pm 0.3 \times 10^5$ CFU/mL.

Fish from each tank were anaesthetized with 0.1 g/L MS-222 with 2:1 sodium bicarbonate. The challenged group was injected with 0.1 mL of the *Vibrio* suspension and the sham group was injected with 0.1 mL of PBS. After injection, the fish were allowed to recover in an aerated tank before being returned to their original tanks.

The challenge lasted for ten days. Mortalities were monitored every 6 hours for the first 5 days and every 24 hours thereafter. Water temperature was determined with digital data loggers (Onset Computer Corp.) and the average temperature throughout the challenge was 11.32 °C.

**Sampling**

Fish were sampled at 7 time points post-injection: baseline (not injected), 6 hrs, 12 hrs, 18 hrs, 24 hrs, 48 hrs, 72 hrs, and 10 days. A sample of 10 triploid and diploid fish was taken before first injection (baseline). After baseline, each sampling point consisted of 15 challenged fish from both triploid and diploid groups and 9 sham fish from both triploid and diploid groups.
At each sampling point, fish were humanely euthanized with MS-222 and weighed. Blood was taken with capillary tubes by first making a diagonal cut at the tail opening up the caudal vein. Blood was then used for blood smears and preserved for ploidy confirmation by flow cytometry. Briefly, samples were spun in a hematocrit centrifuge for 5 minutes. Blood cells were added dropwise ~3μL to 2 mL of ice cold 70% alcohol (85% ethanol, 15% methanol). Samples were gently vortexed and stored at -20°C. The flow cytometry method followed previous published protocols (Shapiro 1988; Darzynkiewicz 1997). Briefly, 1 mL of collected cells were washed 2 times with cold PBS. Cells were then resuspended in 500 μL of 0.1% (v/v) Triton X-100 in PBS with 20 μg/mL propidium iodide (PI). Stained cells were then diluted 5 times in PBS to be run on the flow cytometer. Head kidney was taken and preserved in RNAlater. Samples in RNAlater were placed at 4 °C for 24 hrs before going into -20 °C storage.

RNA extraction and cDNA synthesis

RNA extraction was done using Trizol reagent (Invitrogen, Burlington, Canada) according to the manufacture’s protocol. A subset of the RNA samples (~20%) were initially tested for quality until sample processing was consistent. Samples were run on an Agilent 2100 bioanalyzer to confirm RNA integrity and a Perkin Elmer Victor 3 for 260/280 measurements. Trials revealed that qRT-PCR for our samples were robust to an acceptable range of degradation and concentration. cDNA was generated using Superscript II (Invitrogen, Burlington, Canada) from 500 ng of RNA samples using manufacturer’s protocol using oligo dT anchors and random hexamers.

Quantitative Real-time PCR

A total of 8 sets of Taqman probes were used for this experiment. The targeted genes were IL-1, IL-8, IL-8R, MHC-II, TNF, IgM, and β-actin with EF1A as an
endogenous control. The design process has been discussed in chapter 2. The β-actin assay was supplied by Dr. Robert Devlin, DFO, West Vancouver, B.C. Ten challenged and 6 sham samples were used for each analysis.

Two tests were run before using the $2^{-\Delta\Delta Ct}$ method; geNorm was used to check for endogenous gene stability (Vandesompele et al. 2002) and the amplification efficiency and effective detection range of the probes was checked by 6 ten fold serial dilutions of cDNA (Applied Biosystems User Bulletin No. 2 (P/N 4303859)).

All probes and primer sets were run on an ABI 7500 Real-Time PCR System. Twenty µL reactions contained 10 µL 2X Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, USA), 150 nM Taqman probe, 900 nM forward/reverse primer and ddH₂O to 20 µL. All Taqman assays were run under the following cycling condition: 50 °C 2 mins., 95 °C 10 mins., and 40 cycles of 95 °C 15 secs. and 60 °C 1 min. Results were exported from SDS 1.2 (Applied Biosystems, Foster City, USA) and analyzed in Excel using the $2^{-\Delta\Delta Ct}$ method (Livak et al. 2001) in two ways. In the equation $\Delta\Delta C_T = (C_T,\text{target} - C_{EF1\alpha})_{\text{Time}} - (C_T,\text{target} - C_{EF1\alpha})_{\text{calibrator}}$ the calibrator is used as a relative reference point from which to compare samples from. In my analysis, the data was calibrated two ways: One uses the baseline unchallenged time zero diploid as the calibrator. This allows for a better perspective on differences compared to a normal diploid Chinook salmon at resting-state. The other analysis used the respective sham of each challenged sample as the calibrator. The sham calibrator perspective better describes the stress subtracted response at each time point and better describes differential expression patterns between triploid and diploid Chinook salmon. All statistical analyses were done in Statistica v 6.0.
RESULTS

Ploidy determination

Triploidy success was found to be 98% by flow-cytometry. Any ambiguous samples were further confirmed using microscopy by determining red blood cell nuclear length. All samples used in the final analysis were categorised to the correct ploidy.

Survival

To quantify differences in mortality between the diploids and triploids, mortality rates were calculated after pathogen exposure. Total mortality rates for both triploids and diploids were approximately the same after the 10 day trial; however, challenged fish (from a total number of 207 triploid, 218 diploid) showed a significant increase in initial mortality rates compared to sham injected fish (Fig. 3.1). Pearson Chi-square test of day 5 revealed a significant difference between triploid and diploid survival (P < 0.05; Fig. 3.1), while no significant differences were identified at any other time point.
Figure 3.1: Cumulative mortality for live pathogen challenged and sham injected triploid and diploid Chinook salmon over a 10 day period. No change was observed prior to day 3. Differences in mortality rates between challenged triploid (n=207) and diploid (n=218) groups were tested for statistical significance by Chi-square: *P < 0.05.
Gene expression analysis

All genes chosen for this study showed a significant response between sham PBS injected and challenged *Vibrio* injected Chinook salmon during at least one time point over the three day period (Fig. 3.2, †=P<0.05). Large expected increases in relative cytokine expression, IL-1, IL-8 and TNF, were observed in both triploids and diploids in response to immune challenge while smaller variations were exhibited in constitutively expressed genes, IgM, MHC-II, and β-actin.
Figure 3.2: Relative gene expression of seven genes using qRT-PCR on triploid and diploid sham, PBS injected, and challenged, Vibrio injected, Chinook salmon over a three day period. Expression levels are calibrated to diploid Chinook salmon at baseline, time zero, before injection. Significant differences between sham and challenged diploid salmon and sham and challenged triploid salmon are shown with † P < 0.05 t-test. Significant differences between challenged triploid and diploid salmon are shown with * P < 0.05 t-test.
The pre-challenge resting-state levels shown in Figure 3.3 allow insight into gene expression differences between triploids and diploids prior to challenge. No differences were observed in constitutively expressed genes MHC-II, β-actin and IgM between triploid and diploids. IgM expression, though, was close to significance (P=0.057, Fig 3.3). Significantly lower expression was found in IL-1 and IL-8 (P < 0.001 IL-8, P < 0.05 IL-1, t-test, Fig 3.3) at resting-state for triploids relative to diploids. When these differences are compared to expression levels after injection (Fig. 3.2), one finds that the initial expression differences are not consistent over time. The diploid baseline calibrated Figure 3.2 shows relative expression levels compared to that of normal (diploid) resting-state Chinook salmon. This allows the comparison of levels of expression between triploid and diploid samples from a common calibrated point. This, in turn, shows that triploid and diploid differences at baseline, IL-1, and IL-8, are no longer present after challenge, but differences are found in IgM, β-actin and MHC-II (P <0.05 t-test, Fig. 3.2) which were not present before challenge (Fig. 3.3)
Figure 3.3: Relative gene expression of seven genes using qRT-PCR on triploid and diploid sham and challenged of baseline levels prior to any treatment. Expression levels are calibrated to diploid Chinook salmon at baseline, time zero, before injection. Significance between triploids and diploids calculated by t-test shown as *P < 0.05.
The difference between triploid and diploid expression is further accentuated when viewed with a sham calibrator (Fig. 3.4). The sham calibrator shows differences in response pattern in relation to stress response. This reiterates that triploid response in the cytokines, IL-8, IL-1 and TNF, are just as timely and strong as those found in diploids. The inverted expression pattern between the diploids and triploids of IgM, MHC-II, and β-actin are even more prominent in this graph. At several time points triploid and diploid expression of these genes showed an opposite responses which were statistically significantly different (P < 0.05, t-test, Fig. 3.4).
Figure 3.4: Relative gene expression of seven genes using qRT-PCR on triploid and diploid sham and challenged over a three day period using average respective sham as calibrator. Significant differences between challenged triploid and diploid salmon are shown with * P < 0.05 t-test.
DISCUSSION

In this study I investigated phenotypic (survival) and genetic (gene transcription) responses to an immune challenge in triploid and diploid Chinook salmon. Due to increased mortality rates in sea trials (O'Flynn et al. 1997; Benfey 2001; Cotter et al. 2002), triploidy likely has an adverse effect on gene expression of immuno-related genes. Although there was no difference in total mortality rates at the end of ten days between diploid and triploid groups, there was a difference between the challenged fish earlier during the experiment. Because the challenge was quite severe, approximately LD 50 in both triploids and diploids, the final similarity is not surprising. This suggests that early during infection, diploids have higher survivability which has also been suggested from the survival rates of pen stocks (O'Flynn,F.M. et al. 1997). This difference will likely be magnified given a lower and more natural challenge level of pathogen. The diploid Chinook salmon were likely overcome eventually by the high doses of pathogen even though a more effective initial response was observed.

QRT-PCR was used in this study to analysis the gene expression differences resulting from euploid polyploidy. Cells from triploid salmon are on average 1.5 times larger in volume than their diploid counterparts (Susan A.Small et al. 1987; Benfey 1999), therefore the internal control standard gene transcript (EF1A) will be diluted in the triploid cells. Secondly, since the internal control standard EF1A is only regulated during development (Gao et al. 1997) and subsequently transcribed in high abundance, it is likely that the dosage effect at the EF1A gene will compensate directly for the increased volume in the triploid cells. Due to the relative nature of the quantification used, a dosage effect on the target genes in the triploid salmon will be reflected as equal concentrations
of the transcribed mRNA in the triploid relative to diploid cells because of the relative increases in EF1A and cell volume. Likewise, any reduction in expression in triploid relative to diploid cells would likely reflect dosage compensation. While there may be debate as to the type of effect observed in each instance this, however, does not affect the overall gene expression patterns observed due to triploidy.

My results can be classified into two categories: the first includes genes that have similar levels of expression in triploids and diploids after challenge (e.g. cytokines) and the second which show a general decrease in expression in the triploids relative to the diploids (e.g. IgM, MHC-II and β-actin), possibly due to co-suppression, dosage compensation, or other complex regulatory mechanisms.

Two of the cytokines sampled, IL-8 and IL-1 were significantly down-regulated in triploid salmon at baseline compared to diploid salmon (Figure 3.2). This effect, however, was not carried over to when the Chinook salmon were challenged. It has been speculated that trans-regulatory elements may be responsible for the enhanced or inhibited effects of polyploidy (Guo et al. 1996; Suzuki et al. 1999; Birchler et al. 2001). It is possible that these regulatory elements may be turned off when the cytokines are activated in the event of an immune response resulting in the dosage effects observed after challenge.

In contrast, a general suppression at several time points of gene expression in triploid Chinook salmon was observed in IgM, MHC-II, and β-actin. The decrease in triploid expression may be an “across the board” compensation back to diploid levels upon immune response activation. Though co-suppression is an attractive explanation, it is unlikely since co-suppression involves the suppression of homologous gene products (Pal-Bhadra et al. 1999) whereas in this case there would be little homology between β-
actin and IgM. One possibility is that because of the tetraploid past of salmonids, the system may recognize four doses of a gene as the norm. Therefore, if only three chromosomes are present, the system would function as if one set was missing instead of responding to an extra set. This may result in tightly regulated systems producing insufficient product from the three copies of the gene available when four may be necessary to reach normal levels. This odd/even effect has been reported in maize (Guo et al. 1996), but in reverse, where odd ploidy had remarkable up-regulated expression and even ploidy maintained normal levels.

The above expression patterns may explain some of the performance limitations in triploid Chinook salmon. The gene expression balance in triploids appears to be at its limits and any additional stress may be lead to loss of control. The timely expression of the cytokines suggests a normally functioning innate immune system at least in the early stages of response. Gene regulation of constitutively expressed genes, however, responded abnormally compared to diploids after challenge. This inability to maintain normal function during an immune response may result in the decreased triploid performance observed in the increased mortality rates shown in Figure 3.1.

Current research has mainly dealt with manipulating the genome to observe the results of genomic regulatory regions at a resting state (Birchler et al. 1990; Bhadra et al. 1997). However, our results suggest that some ploidy effects may not be observed except under response to stressful conditions. Interestingly, while there were expression differences, down-regulation, in IL-8 and IL-1 the patterns observed after challenge were characterized by either simple dosage effects, or clear compensation in the triploid salmon. Our study suggests that while observations at resting state is important,
additional stresses applied to polyploidy could reveal further insight to regulatory elements.

This study identified a phenotypic difference in the survival response of diploid and triploid Chinook salmon to a severe live pathogen challenge. The gene transcription profiles reveal that there are imbalances in gene expression that likely are contributing to compromised immune function in triploid salmon. Although the triploids respond in a timely fashion for cytokine activity, their overall physiology and biochemistry does not appear to be responding properly, as suggested by the MHC-II, IgM, and β-actin transcription levels. However, this study does not address the specific mechanism behind the reduced immunity of the triploid fish. A larger sample of genes will likely reveal additional breakdown of gene expression similar to that observed for IgM, β-actin and MHC-II. Microarray technology would be an excellent means to test large numbers of genes. Although microarrays are less sensitive than qRT-PCR and the slight changes observed in IgM, β-actin and MHC-II will likely be reflected as being normal, the slight changes observed here does suggest that there may be larger discrepancies in triploid Chinook salmon gene expression which could be picked up on a microarray.
LITERATURE CITED


Benfey, T. J., 2001 Use of Sterile triploid Atlantic Salmon (Salmo salar L.) for Aquaculture in New Brunswick, Canada. ICES Journal of Marine Science 58: 525-529.


CHAPTER 43
Microarray analysis of gene expression differences between diploid and triploid
Chinook salmon in response to an immune challenge

3 Chapters 3 and 4 are formatted to be jointly submitted to Genetics for publication
INTRODUCTION

The aquaculture industry has debated the advantages and disadvantages of commercial rearing of triploid salmon for some time; in general the industry is reluctant to adopt the technology primarily due to perceived lower performance of the triploid fish. Although considerable work has been published on physiological comparisons between diploid and triploid salmonids (Bruno et al. 1990; O'Flynn et al. 1997; Benfey 1999; Johnson et al. 2004), surprisingly little research on the gene expression differences has been published. Polyploidy can be surprisingly undisruptive for organisms which tolerate it, presumably along with the proportional increases in gene dosage (Guo et al. 1996; Galitski et al. 1999; Birchler et al. 2001). In higher order animals, such as mammals, the polyploidy usually results in severe deleterious phenotypes, primarily due to disruptions in development (Otto et al. 2000). In fish, however, polyploidy lies on a gradient ranging from little to severe deleterious effects. There is a much higher incidence of viable polyploids amongst lower orders of fish with decreasing incidences in higher taxa (Leggatt et al. 2003). Chinook salmon (Oncorhynchus tshawytscha) is an intermediate teleost that can produce viable triploids (Allendorf et al. 1984), which are morphologically identical to diploids but are sterile, and females do not sexually mature.

While triploid salmon would be beneficial for the Aquaculture industry because of prospects for genetic containment due to sterility, comparative performance analyses between triploids and diploids is essential (Benfey 2001). Conflicting studies have hinted at problems in terms of immuno-competence (Bruno et al. 1990; Ojolick et al. 1995; O'Flynn et al. 1997; Cotter et al. 2002), but stress was also a contributing factor (Benfey 1999). In order to examine the causative nature of possible performance differences between diploid and triploid salmon, gene transcription quantification was used in this
study. Quantitative real-time polymerase chain reaction (qRT-PCR) data (Chapter 3) showed that although several selected innate immune response genes (e.g. tumor necrosis factor, interleukin-1, and interleukin-8) function normally after a severe disease challenge, other seemingly unrelated genes (e.g. immunoglobulin M, major histocompatibility complex class II, and β-actin) appeared to fluctuate from their initial norm. In order to further investigate the relationship between triploids and diploids over a large range of genes, an Atlantic salmon gene microarray containing 3700 genes from the Genomic Research on All Salmon Project (GRASP)(Rise et al. 2004) was used on Chinook salmon challenged with live Vibrio anguillarum.

MATERIALS AND METHODS

7 GRASP chips were hybridized with a triploid and a diploid Vibrio anguillarum challenged Chinook salmon. The Chinook salmon were spawned in the fall of 2004 at Yellow Island Aquaculture Ltd (YIAL; Quadra Island, BC, Canada), and the offspring were raised in flow-through tanks under standard commercial rearing conditions. On August 10, 2005, 95 fish were anaesthetized, and injected intraperitoneally with 5 x 10^5 colony forming units of Vibrio anguillarum. An equal number of fish were sham injected with Phosphate-buffered saline (PBS). Eighteen hours post-challenge, 7 challenged fish (diploid and triploid) were humanely euthanized and head kidney samples were taken and preserved in RNAlater at -20 °C. Total RNA was extracted from the head kidney samples using GenElute™ Mammalian Total RNA (Invitrogen, Burlington, Canada) as per manufacture’s instructions. Excess volume from total RNA extractions was removed by NaOAc / Isopropanol precipitation. Slide hybridization and cDNA generation was done using GRASP’s Genisphere Array 50 Protocol Revised version 4 (web.uvic.ca/cbr/grasp/).
Four arrays were hybridized with diploid cDNA labelled with Cy3 (triploid cDNA with Cy5), while three arrays had the reverse (diploid cDNA with Cy5 and triploid cDNA with Cy3). GRASP chips were scanned using ScanArray Express v 3.01.0001 (PerkinElmer) using a ScanArray Express Microarray Scanner (PerkinElmer). Images were exported and quantified in Spotfinder (www.tigr.org) using Otsu. Raw intensities were normalized in MIDAS (www.tigr.org) using lowess and standard deviation regularization (Yang et al. 2002; Quackenbush 2002). Normalized intensities were exported into Excel (Microsoft) for replicate analysis and intensity dependent Z-score calculations (Yang et al. 2002; Quackenbush 2002). Genes of interest were identified using a standard Z-score with a cut off at Z > 2.0 (approximately 95.5% confidence) (Yang et al. 2002; Quackenbush 2002). Only genes with at least one corresponding equal expression value on another chip was included in the analysis.

RESULTS

Several genes (34 - 84) were identified as presenting significantly different transcription levels between the diploid and triploid challenged salmon on the seven arrays using the intensity dependent Z-score calculations (Yang et al. 2002). Of those, 27 genes had signal agreement on at least two arrays. After eliminating all genes that had conflicting direction of expression differences amongst the 7 arrays, 12 genes remained that were deemed to have the most consistent pattern of differential transcription between the triploid and diploid salmon. The relative intensity (RI) plot (Fig. 4.1) represents relative intensities which were first lowess normalized (Cleveland et al. 1988) and replicate filtered (Yang et al. 2002; Quackenbush 2002). The Y axis represents a ratio of the intensity of Cy3/Cy5 of each gene and the X axis represents the product of the intensity of Cy3*Cy5 of each gene. The figure reveals a general tadpole shape.
characteristic of highly similar transcriptomes. This is not surprising since diploid and triploid salmon are phenotypically virtually identical (Benfey 2001), and since previous qRT-PCR results showed that gene expression levels of triploids and diploids were the same or only very slight different (Chapter 3) after immune challenge, which would be unlikely to be detected using microarray analysis.
Figure 4.1: Intensity dependent calculation of Z-scores based on a sliding window. Z-scores of above 2 (95.5% confidence) are in red, from 1 to 2 in green and from 0 to 1 in blue. Crosses represent differentially expressed genes with at least two concurring arrays (see Table 4.1). A characteristic tadpole pattern is observed for highly similar samples.
The 12 genes conservatively identified as showing gene expression differences between the ploidy groups (Table 4.1) included several genes that are consistent with our understanding of triploid versus diploid performance differences. The putative functions of the genes were originally identified by GRASP using Blast-X (Rise et al. 2004).
Table 4.1: Differentially expressed genes between Triploid and Diploid *Vibrio* challenged salmon

<table>
<thead>
<tr>
<th>Accession Number</th>
<th>Gene Name/suspected</th>
<th>Gene Ontology</th>
<th>3N fold change (+/-)</th>
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<td>Immune response</td>
<td>1.626</td>
</tr>
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<td>low molecular mass protein 7</td>
<td>Immune response</td>
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<tr>
<td>AF180488</td>
<td>β-2 microglobulin</td>
<td>Cellular defense response</td>
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<td>phospholipase A2-activating protein</td>
<td>Inflammatory response</td>
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<td>CA051685</td>
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<td>unknown</td>
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Collected differentially expressed genes between triploid and diploid vibrio challenged Chinook salmon are shown. Putative functions have been assigned to each gene. Triploid up regulation in comparison to diploid is denoted by positive values and a down regulation is denoted by negative values. Gene ontology listed was obtained from [http://www.geneontology.org](http://www.geneontology.org).
DISCUSSION

Two functional types of genes on the list of differentially expressed genes (Table 4.1) are of particular interest; the inflammatory function genes and the immune function or major histocompatibility complex class I (MHC-I) related genes. Pyrin and phospholipaseA2-activating protein (PLAP), both inflammatory function genes, were down-regulated in triploid salmon. Pyrin is a caspase-1 regulator (Chae et al. 2006) and PLAP aids in the production of eicosanoids after IL-1 or TNF stimulation (Clark et al. 1988; Bomalaski et al. 1992). Since the normal (diploid) expression levels of these genes are higher than observed in the triploids, the triploids likely suffer from an inadequate inflammatory response. This suggests that while initial innate response was similar in triploids compared to diploids, inadequate control or signaling to downstream effectors may be a cause of increased mortalities in triploids (Chapter 3).

The second functional group of genes showing differential expression was the immune function genes, or the MHC-I related genes (Table 1). While MHC-I is not related to innate immune response, it does possess specific and acquired immunity functions. Even though the main interest of this study was focused on early innate response (i.e. the eighteen hour samples), the significance of abnormalities in acquired immunity may also account for the reported poor performance of triploids. Both low molecular mass protein 7 and β-2 microglobulin showed elevated levels of expression in triploids relative to diploids. Higher levels of expression may suggest superior MHC in triploid salmon; however transcription imbalances within complex multi-gene pathways may result in deleterious phenotypes. In this case there is evidence that increased levels of β-2 microglobulin may inhibit antigen presentation and other immune functions (Xie et al. 2003).
The application of microarray technology in this study provides a unique perspective on the phenotypic effects of Chinook salmon triploidy. Not surprisingly, triploid salmon gene expression generally appears to be very similar to that of diploids, reflecting the remarkable phenotypic similarity of diploid and triploid salmon. Furthermore, this genome-level study has identified significant differences in the expression of a few genes in severely immune challenged diploid and triploid Chinook salmon. While qRT-PCR data (Chapter 3) showed that initial innate immune response was functioning properly at a temporal level, the significantly different early mortality rates indicated that the two ploidy groups were not responding identically. Indeed, both qRT-PCR (non-innate genes) and microarray data revealed that slight but significant fluctuations in several seemingly unrelated genes and some downstream effectors for innate response may be responsible for observed and reported poor performance of triploid salmon under stress (O’Flynn et al. 1997; Benfey 2001; Cotter et al. 2002). This study revealed many interesting genes to pursue. Overall, however, gene expression in triploid Chinook salmon appear to be similar to their diploid counterparts in an immune challenge, but minor differences between them may be enough to account for the discrepancies in performance observed.
LITERATURE CITED


Chapter 5

CONCLUSIONS AND FUTURE DIRECTIONS

Despite the severe deleterious effects of polyploidy in most animals, salmon are surprisingly unaffected by this condition. While triploids are fundamentally different on a cellular scale, with increased nuclear material, larger cell size and fewer cells, the overall phenotypic result is very similar to that of a normal diploid. In order for this to hold true triploids must show proportional increases in gene expression while maintaining similar patterns of expression. This study provides evidence for the genetic basis of the surprisingly undisruptive nature of polyploidy in salmon. However, we also found that during immune challenge, some constitutively expressed genes showed anomalous patterns of expression (relative to diploid controls), while innate response genes showed few or no effects.

Quantitative real time polymerase chain reaction (qRT-PCR) assays developed in Chapter 2 and utilized in Chapter 3 showed very specific responses from each gene to immune challenge in diploids versus triploids. The constitutively expressed genes, IgM, MHC-II, and β-actin, surveyed show that there was no significant difference between triploids and diploids at resting state. These likely accounts for the normal diploid phenotype observed during resting state. The significant variation observed in the cytokines between triploids and diploids at resting state were at low levels and not responding to an immune challenge. This likely reflected the effects of polyploidy but was functionally neutral. When the immune challenge occurred, however, there was a dosage effect probably indicating an adaptive functional response.

During the immune challenge, however, the constitutively expressed genes showed a quite different expression pattern than seen prior to the challenge. These
abnormal expression levels likely cause adverse changes in homeostasis and contribute to the poor survival performance of triploids reported in Chapter 3. Diploid-triploid differences in the innate response genes, however, disappeared after the immune challenge. This suggests that while a comparable response to immune challenge has occurred in triploids something must be breaking down resulting in a loss of balance to the constitutively expressed genes which leads to poor performance.

Given the modest magnitude of expression differences between triploids and diploids, it was unlikely that the microarray would have picked up any of the constitutively expressed gene expression differences. However, a whole genome analysis was still beneficial due to the narrow scope of the qRT-PCR analysis. My selection of genes for the qRT-PCR assays are almost certainly not representative of gene expression occurring in the whole organism. Regardless of the outcome the microarray analysis the increased number of genes assayed would have provide valuable insight into polyploidy gene expression. Indeed, the microarray revealed there were only a few differentially expressed genes. These genes include inflammatory, MHC-I related and immune function related genes. This further indicates that signalling or downstream processes in triploids may breakdown during stress.

Although this study offers insight into some issues concerning gene expression in polyploidy, additional work using qRT-PCR would provide additional clarification. An absolute quantification method would shed light as to the exact number of transcripts being expressed by triploids and diploids as opposed to relative quantification. This would add further resolution to the method and allow us to tell whether certain thresholds are reached before regulating factors are engaged. Additionally, exact transcript numbers will allow us to determine definitively whether dosage compensation or dosage effects are
taking place. In addition, knowledge of transcript numbers may reveal concentration of copy number dependent regulatory systems.

Additional work can also be done by titrating the dose of the pathogen challenge. The mortality rate differences observed between triploids and diploids may be magnified at lower doses which imitate more natural conditions. Previous studies in transgenic fish have also shown that mortality rates can be very different between normal and transgenic fish at varying levels of infection.

Further work can also be done using a Chinook microarray. Not only will this provide additional sensitivity for the genes sampled, but it will provide a basis for comparison between chips of different species. The data generated from the Atlantic salmon array, however, will provide genes of interest to pursue in future immune challenges. Also, because some of the genes found on the array were associated with MHC-I, research should include acquired as well as innate immune responses.

Furthermore, our study has been centered on gene expression at an mRNA level. Studies have demonstrated that gene expression at the mRNA level doesn’t necessarily translate to differences at the protein level. The differences in cell size will likely cause changes in signalling at the protein level. The addition of protein level research will definitely further our understanding of the effects of polyploidy.

For the most part, regulatory elements of polyploidy are still unknown. It has been suggested that at least a portion of the regulatory elements reside in the genomic DNA sequence. One possible method to discover those genomic regulatory elements would be to look for similarities in the genomic sequence in genes which also have similar expression patterns. By clustering genes which show similar expression patterns in polyploidy experiments and then looking at the genomic DNA sequence in proximity
to the gene or within the gene itself, similar sequencing information may be revealed. This can be easily done with the increasing numbers of sequences deposited to databases and with the increased ease and cost effectiveness of sequencing. Pursuing these similar sequences may yield regulatory elements that influence these genes in similar ways leading to potential discovery of new regulation sites.

In this study, immune challenged triploid and diploid Chinook salmon were examined using qRT-PCR and microarrays. Through these methods I was able to gain novel insight into the effect of polyploidy on gene expression. The analysis of polyploidy solely at resting states may not adequately describe the complex regulatory issues surrounding polyploidy. The dichotomy observed between challenged and resting state gene expression suggest that extrapolations made from resting state may not describe active function adequately. Furthermore, this study confirms that, while triploid Chinook salmon may appear normal at rest, this balance is tenuous and can be easily altered by an immune challenge, leading to increased mortalities in triploids.
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