Heredity (2010) 104, 224–234

www.nature.com/hdy

ORIGINAL ARTICLE

Transcriptional differences between triploid and diploid Chinook salmon (*Oncorhynchus tshawytscha*) during live *Vibrio anguillarum* challenge

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Understanding how organisms function at the level of gene expression is becoming increasingly important for both ecological and evolutionary studies. It is evident that the diversity and complexity of organisms are not dependent solely on their number of genes, but also the variability in gene expression and gene interactions. Furthermore, slight differences in transcription control can fundamentally affect the fitness of the organism in a variable environment or during development. 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 using quantitative real-time PCR (qRT-PCR) and microarray technology after an immune challenge with the pathogen *Vibrio anguillarum*. 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), whereas differences were observed in constitutively expressed genes, (immunoglobulin (Ig) M, major histocompatibility complex (MHC) -II and β-actin) upon live *Vibrio anguillarum* exposure. Genome-wide microarray analysis revealed that, overall, triploid gene expression is similar to diploids, consistent with their similar phenotypes. This pattern, however, can subtly be altered under stress (for example, handling, *V. anguillarum* challenge) as we have observed at some housekeeping genes. Our results are the first report of dosage effect on gene transcription in a vertebrate, and they support the observation that diploid and triploid salmon are generally phenotypically indistinguishable, except under stress, when triploids show reduced performance.
Heredity (2010) **104,** 224–234; doi:10.1038/hdy.2009.108; published online 26 August 2009

Keywords: chinook salmon; triploid; gene expression; microarray; real-time PCR; *Vibrio anguillarum*

Introduction

Polyploidy has been recognized as a potentially powerful factor in the evolution of species diversity (Otto and Whitton, 2000), yet little is known about the effects of recent polyploidization on vertebrates. 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 evidence of an ancestral tetraploidization event in the salmon genome (Allendorf and Thorgaard, 1984), and they still appear to be going through re-diploidization (Young *et al*., 1998). Thus salmon's genomic plasticity has been a long-standing feature of their genetic make-up. Spontaneous polyploidy in salmonids is observed in natural populations (Leggatt and Iwama, 2003) 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 (Small and Benfey, 1987; Benfey, 1999). Thus, both organ and organism size is maintained, resulting in few obvious morphological differences between diploid and triploid salmon (although triploid salmon are sterile; Johnson *et al*., 2004). 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).

Although 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 after the induction of polyploidy. Studies of nascent plant allopolyploids have shown evidence of massive genomic rearrangements, such as sequence deletion, and reciprocal translocations and transpositions between homologous chromosomes (reviewed by Osborn *et al*., 2003; Chen and Ni, 2006).

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Received 16 September 2008; revised 20 July 2009; accepted 23 July 2009; published online 26 August 2009

Changes to the transcriptome are thought to result mostly from epigenetic effects owing to processes such as DNA methylation, histone alterations, positional effects due to higher order chromatin structural changes and the effects of small RNAs and RNA interference (reviewed by Osborn *et al*., 2003; Chen and Ni, 2006). In autopolyploids, most of the gene loci appear to be governed by a simple, positive dosage effect, where gene expression increases with ploidy (Guo *et al*., 1996; Galitski *et al*., 1999; Birchler *et al*., 2001; Martelotto *et al*., 2005). However, in some cases more complex dosage effects have been reported, in which a few loci are up- or downregulated beyond what could be accounted for by a simple dosage effect, suggestive of epigenetic or complex epistatic mechanisms (Suzuki *et al*., 1999; Adams and Wendel, 2004). Owing 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, 2005).

In salmon, triploidy can be readily induced by preventing maternal meiosis II through the application of pressure or heat shock (Johnson *et al*., 2004). This interferes with the normal movement of chromosomes during meiosis, preventing the second polar body from being extruded properly (Benfey, 2001). Triploid Chinook salmon generally exhibit a normal phenotype with few differences from diploid salmon at resting-state (reviewed by Benfey, 1999), although, in seawater trials, survivability of triploid salmonids has generally been lower than that of diploids (O'Flynn *et al*., 1997; Benfey, 2001; Cotter *et al*., 2002), suggesting a difference in disease resistance. However, studies on the immunocompetence of triploid salmonids have proven equivocal (reviewed by Bruno and Johnstone, 1990; Yamamoto and Iida, 1995; Benfey, 1999), but the inconsistent results may be because of individual and familial variation or genotype-by-environmental interactions, not ploidy differences (Johnson *et al*., 2004, 2007; Miller *et al*., 2004). Immune challenge studies with a variety of pathogens resulted in either equal competence of triploid and diploid fish, or triploids faring poorly relative to diploids (Bruno and Johnstone, 1990; Dorson *et al*., 1991; Ojolick *et al*., 1995; Johnson *et al*., 2004). Although the use of triploid salmon in commercial aquaculture has been proposed, it has not been widely adopted, largely because of contradictory findings about the impact of triploidy on performance and survival. Detailed phenotypic and genetic studies of the effects of triploidy in salmon will improve our knowledge of dosage effects in polyploid vertebrates, as well as provide an understanding of the mechanisms affecting triploid performance and allow the development of improved commercial lineages for triploid production (Johnson *et al*., 2004).

Here, we develop and apply quantitative real-time polymerase chain reaction (qRT-PCR) to study the transcriptional response of selected immune-related genes to a disease challenge in diploid versus triploid Chinook salmon. Based on salmon farm data for diploid and triploid survival through natural disease outbreaks, we predicted that triploid salmon would exhibit lower or delayed gene transcription at immune-related loci. We also use a salmon cDNA microarray to scan the Chinook salmon transcriptome to test whether the pattern of transcriptional differences between diploid and triploid fish observed using qRT-PCR was general. In our study, triploid and diploid Chinook salmon were challenged with live *Vibrio anguillarum*, survival was monitored over a 10-day period, and transcription at seven gene loci (qRT-PCR) and 3700 gene loci (cDNA microarray) was analyzed over the first 3 days of the trial. Our results indicate that triploid salmon typically experience positive dosage effects; however, some genes exhibit dosage compensation. The net effect of these differences is a reduced disease resistance and lower survival in the triploid Chinook salmon.

Materials and methods

Net cage mortality

In the summer of 2003, staff at Yellow Island Aquaculture Ltd. monitored the mortality of 2-year-old diploid and triploid Chinook salmon during a natural outbreak of bacterial kidney disease (*Renibacterium salmoninarum*). Dead fish in saltwater net cages were collected by self-contained underwater breathing apparatus and tallied weekly (eight times over a period of 2 months). Three net cages were monitored, two holding triploid fish, and one holding diploid fish. The initial numbers of fish in the three net cages, before the outbreak, were 5303 and 7175 (triploid) and 8539 (diploid). Mortality was calculated as a percentage of live fish at the beginning of the sample period. We tested for differences in the mortality between the triploid and diploid fish using χ^2 analysis.

Salmon breeding program and immune challenge

The Chinook salmon used in this study for the live pathogen challenge experiments were spawned in fall of 2004 at Yellow Island Aquaculture Ltd. *V. anguillarum* was chosen as the challenge pathogen, as it is a very common marine pathogen that causes high mortality in aquaculture salmon stocks and because it causes rapid infection and an acute pathogen challenge within days of infection. Two females were each mated to three males to produce six families. Each family was split into two wherein one half was incubated without treatment, whereas the other was subjected to hydrostatic pressure shock to induce triploid formation (Benfey and Sutterlin, 1984; Johnson *et al*., 2004). All fertilized eggs were kept in separate compartments in vertical flow-through incubation stacks following standard incubation procedures. The fish were then transferred to 1201 flow-through tanks upon yolk-sac absorption. Before 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. The fish were allowed to acclimatize for 2 weeks before *V. anguillarum* challenge.

Vibrio anguillarum (provided by D Kieser, DFO Pacific Biological Station, Vancouver, BC, Canada) were streaked onto blood agar plates and allowed to grow for 20 h at room temperature (25–27 °C). Just before the

challenge, the *V. anguillarum* culture was suspended in phosphate-buffer saline (PBS) to produce an optical density (OD) of 1.0 at 540 nm. The solution was further diluted to produce a final concentration of approximately 5×10^5 colony forming units ml^{-1}. The suspension was kept on ice and loaded into 1 cc syringes before intraperitoneal injection. The actual dose was determined by standard plate count method and was $7.3 \pm 0.3 \times 10^5$ colony forming units ml^{-1}.

The fish were anaesthetized with 0.1 g l^{-1} MS-222 (2:1 sodium bicarbonate) before handling. The challenged group was injected intraperitoneally with approximately 7×10^5 colony forming units of *V. anguillarum* and the sham group was injected with 0.1 ml of phosphate-PBS. After injection, the fish were allowed to recover in an aerated tank before being returned to their original tanks. The mortality was monitored every 6 h for the first 5 days and every 24 h thereafter for a total of 10 days. Cumulative mortality was calculated for the diploid and triploid sham-injected and challenged fish over the 10-day trial. We tested for differences in diploid and triploid mortality at each time period using a cross-tab analysis. Water temperature was determined with digital data loggers (Onset Computer, Yarmouth, MA, USA) and the average temperature throughout the challenge was 11.3 °C.

Triploid determination

The fish were sampled at seven time points post-injection: baseline (not injected), 6 h, 12 h, 18 h, 24 h, 48 h, 72 h and 10 days. A sample of 10 triploid and diploid fish was taken before the 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 killed with MS-222 and weighed. Blood was collected by capillary tubes from a diagonal cut at the tail to open the caudal vein. The blood was preserved for ploidy confirmation using blood smears and flow cytometry. The samples were briefly spun in a hematocrit centrifuge for 5 min. Blood cells (which are nucleated in salmon) were added dropwise approximately 3 µl–2 ml of ice cold 70% alcohol, gently vortexed and stored at −20 °C The flow cytometry method followed previous published protocols (Shapiro, 1988; Darzynkiewicz, 1997). Briefly, 1.0 ml of collected cells was washed twice with cold PBS. The cells were then re-suspended in 500 µl of 0.1% (v/v) Triton X-100 in PBS with 20 µg ml^{-1} propidium iodide. Stained cells were then diluted five times in PBS and sampled on a flow cytometer (Beckman Coulter Cytomics FC 500, Mississauga, ON, Canada). All confirmed triploid salmon exhibited a shifted DNA profile indicative of 3N ploidy. As additional confirmation, nuclear diameter was determined using blood smears. In any case where a sample did not meet both conditions for triploidy they were excluded from further analysis.

RNA extraction and cDNA synthesis

At each sampling time, 15 challenged and 10 sham-injected fish were humanely killed, and head kidney samples were taken and preserved in RNAlater at −20 °C. Total RNA was extracted from the head kidney samples using Trizol (Invitrogen, Burlington, ON, Canada) as per manufacturer's instructions. Genomic DNA (gDNA) was extracted by Wizard Genomic DNA purification kit (Promega, Madison, WI, USA), whereas cDNA was generated with SuperScript II from 500 ng of RNA samples as per manufacturer's instructions. A subset of the RNA samples (approximately 20%) was initially tested for quality to ensure that the 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 nm measurements.

qRT-PCR primer design

Degenerate and specific PCR primer sets to obtain gDNA sequence data for six immune-related genes were designed for Chinook salmon (*Oncorhynchus tshawytscha)*: interleukin (IL)-8, IL-8R, IL-1, tumor necrosis factor (TNF), major histocompatibility complex class II (MHC-II), and immunoglobulin (Ig) M heavy chain (Table 1). Degenerate PCR primers designed from GenBank sequences for various species 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, ON, Canada), 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 150 nM of each primer and 2.5 µl of 10X PCR buffer. PCR products were sequenced on an ABI 3700 (Genome Quebec Innovation Centre, McGill University, Montreal, QC, Canada). Sequence identity was confirmed using BLAST, and the gDNA and cDNA sequences were aligned using Dialign to identify intron/exon boundaries. TaqMan probes and primer sets were designed using Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA). Final primer and probe sequences are listed in Table 1. Note that MHC-II probe is situated in the β1 peptide-binding region (PBR), and thus may be affected by the known high levels of sequence variation in that region. We chose the MHC-II qRT-PCR probe sequence based on a multiple species and individual sequence alignment to reflect minimal sequence variation, and thus our assay is likely to be robust to MHC-II β1-PBR sequence variation among individuals in our study. Each qRT-PCR primer and probe set was designed to anneal at 60 °C to work under default ABI 7500 Real-Time thermocycler conditions.

qRT-PCR assays

A total of eight 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 elongation factor 1a (EF-1a) as an endogenous control. The β-actin Taqman assay was supplied by Dr Robert Devlin, DFO, West Vancouver, BC, Canada. In all, 10-challenged and 6-sham injected fish from each time sample were used for analysis. All qRT-PCR assays were run in duplicate and the mean value was used for analyses.

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 six 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 as per manufacturer's

Table 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

Gene	*Degenerate primer sequences*	*PCR size (gDNA/cDNA)*	*GeneBank accession numbers*	*qRT-PCR primer sequences*	*qRT-PCR size*	*qRT-PCR probe sequence*
IL-8	STTGTSRTKGTGCTCCTGG ATGACYYTCTTSACCCAMG	800/200 bp	DQ778949	CGCACTGCAGAGACACTGA ACAAATCTCCTGACCGCTCTTG	58 bp	FAM-TCAGAGTGGCAATGATC-MGB
IL-8Receptor	GAYGTCTACCTGTTTCACCTG CACGAAGGCRTASAGSAC	700/700 bp	DQ778948	GCGCGGCTTCCAGAAG ACAGGAGGAAGGCGAACAC	64 bp	FAM-ACGGCAATGATGACCC-MGB
IL-1	GAGCTGCATGCCATGATGC AGGTTGGATCCCTTGATGCC	400/150 bp	DQ778946	CCAGGGAGGCAGCAGCTA CGGGCGTGACGTACGAA	59 bp	FAM-ACAAAGTGCATTTGAAC-MGB
TNF	TGGTGTCAGCATGGAAGAC GTAAACGAAGAAGAAGAGCCCAG	800/300 bp	DQ778945	CCCACCATACATTGAAGCAGATT GGATTGTATTCACCCTCTAAATGGA	70 bp	FAM-CCGGCAATGCAAAA-MGB
MHC-II	NA	NA	NA	AAGGTCCTCAGCTGGGTCAA GCTCAACTGTCTTGTCCAGTATGG	99 bp	FAM-TCTGTAAGCCTAACGCTG-MGB
IgM heavy chain	ATGAGGACTGGAGCAATGGG CTCGTTCTCTCCACCGGCT	700/250 bp	DQ778947	CGCTGTAGATCACTTGGAAAACC TCTCCTCCAGTCTCCCTCTTGT	69 bp	FAM-ACCTTGGTAAAGAAAGC-MGB

Abbreviations: gDNA, genomic DNA; Ig, immunoglobulin; IL, interleukin; MHC, major histocompatibility complex; qRT-PCR, quantitative real-time PCR.

instructions. Results were exported from SDS 1.2 (Applied Biosystems) and analyzed in Excel using the $2^{-\Delta\Delta Ct}$ method (Livak *et al*., 2001) in two ways. In the equation;

$$\Delta\Delta C_T = (C_{T,target} - C_{EF1a})_{Timex} - (C_{T,target} - C_{EF1a})_{calibrator}$$

the calibrator is used as a relative reference point to which the samples are compared. We calibrated the data using two approaches. The effect of handling stress on the fish was measured by using the baseline unchallenged time zero diploid as the calibrator relative to the sham-injected fish. Second, disease response was measured by using 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.

Microarray hybridizations and analysis

Seven GRASP (Genomic Research on Atlantic Salmon Project) genomic DNA microarrays (3.7 K expression sequence tags) (Rise *et al*., 2004a,b) were hybridized with labeled cDNA produced from RNA extracted from triploid and a diploid *V. anguillarum* challenged Chinook salmon. An annotated list of the genes present on the microarray is available at http://web.uvic.ca/grasp/microarray/array.html (see Rise *et al*., 2004a,b). Eighteen hours post-challenge, seven-challenged fish (diploid and triploid) were humanely killed and head kidney samples were taken and preserved in RNAlater at $-20\,^{\circ}C$. Slide hybridization and cDNA generation was done using GRASP's Genisphere Array 50 Protocol Revised version 4 (web.uvic.ca/cbr/grasp/).

Four microarray slides were hybridized with diploid cDNA labelled with Cy3 (triploid cDNA with Cy5), whereas three microarray slides had the reverse (diploid cDNA with Cy5 and triploid cDNA with Cy3). Hybridized GRASP chips were scanned using ScanArray Express v 3.01.0001 (Perkin-Elmer, Waltham, MA, USA) using a ScanArray Express Microarray Scanner (Perkin-Elmer). Images were exported and quantified in Spotfinder (www.tigr.org). Raw intensities were normalized in MIDAS (www.tigr.org) using lowess and s.d. regularization (Quackenbush, 2002; Yang *et al*., 2002). Normalized intensities were exported into Excel (Microsoft) for replicate analysis and intensity dependent *Z*-score calculations. Genes of interest were identified using a standard *Z*-score with a cut off at $Z > 2.0$ (approximately 95.5% confidence interval) (Quackenbush, 2002; Yang *et al*., 2002). Only genes with at least one corresponding equal expression value on another chip were included in the analysis.

MHC-I inheritance and transcription

To evaluate the possibility of dosage compensation in triploid Chinook salmon by whole chromosome silencing, we used the highly polymorphic MHC-I PBR as a chromosome marker: the high level of polymorphisms allowed us to breed triploid fish with three different MHC-I PBR alleles at their single locus. Subsequent cDNA characterization would thus allow us to determine if there was a consistent silencing of one of the

three loci, and hence chromosomes. We selected a female and male parent such that the female was heterozygous at the MHC-I locus, whereas the male was homozygous for a different allele. The parental PBR of the classical MHC-I locus was amplified through PCR with appropriate primers (5′-TGACTCACGCCCTGAAGTA-3′, 5′-CTCCACTTTGGTTAAAACG-3′ (Miller *et al*., 1997)) followed by sub-cloning and sequencing of the sub-clones for the PBR. Both diploid and triploid offspring of the selected male and female fish were reared to 19 months post-fertilization at Yellow Island Aquaculture Ltd., when the fish were humanely killed and the gill arch tissue was collected and stored for subsequent gDNA and RNA extraction. Offspring were genotyped at MHC-I by PCR amplification of the PBR from gDNA, followed by cloning and sequencing (see above). We selected 10 diploid and 12 triploid offspring with a variety of gDNA MHC-I PBR genotypes for further RNA characterization. RNA was extracted from offspring gill-tissue samples and cDNA was synthesized as mentioned above. We genotyped the offspring cDNA at the MHC-I PBR by PCR amplification (as above), and then identified the parental MHC-I PBR alleles by direct PCR product sequencing.

Results

Disease mortality

Triploid salmon mortality rates in the two saltwater net pens were consistently and significantly higher (χ^2; $P<0.0001$) than diploid mortality rates after a natural outbreak of BKD (Figure 1a). In the Vibrio anguillarum challenge, total mortality for the triploid and diploid fish were approximately the same after the 10-day trial; however, challenged fish (from a total number of 207 triploid and 218 diploid) showed a dramatic increase in mortality after day 4, compared with sham-injected fish (Figure 1b). Cross-tab analyses revealed a significant difference between challenged triploid and diploid mortality at day 6 ($P<0.05$; Figure 1b), whereas no significant differences were identified at any other time point.

Gene expression analysis (sham injected)

Little information exists on gene expression level differences between diploids and triploids of the same species. In this study, no differences in the resting (pre-challenge) state were observed in constitutively expressed genes MHC-II, β-actin and IgM between triploid and diploids. IgM expression, though, was close to significance ($P=0.057$, Figure 2). Significantly lower expression was found in IL-1 and IL-8 ($P<0.001$ IL-8, $P<0.05$ IL-1, *t*-test, Figure 2) at resting-state for triploids relative to diploids. However, when these differences are compared with expression levels after injection (Figure 3), we found that the initial expression differences are not consistent through time. Triploid and diploid differences at baseline (that is, IL-1 and IL-8) are no longer present post-sham injection, but differences are seen in IgM, β-actin and MHC-II ($P<0.05$ *t*-test), which were not present before injection (Figure 2).

Gene expression analysis (*V. anguillarum* challenge)

All genes showed a significant response to the challenge during at least one time point over the 3-day period ($P<0.05$; data not shown). 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, whereas smaller changes were exhibited in constitutively expressed genes (IgM, MHC-II and β-actin). The triploid gene transcription response in the cytokines (IL-8, IL-1 and TNF) was as timely and strong as those found in diploids (Figure 3). However, the expression patterns of the diploid and triploid fish at IgM, MHC-II and β-actin are inverted, where the diploid fish show elevated expression, whereas the triploid fish show reduced expression (Figure 3). At several time points this difference is statistically significant ($P<0.05$, Figure 3).

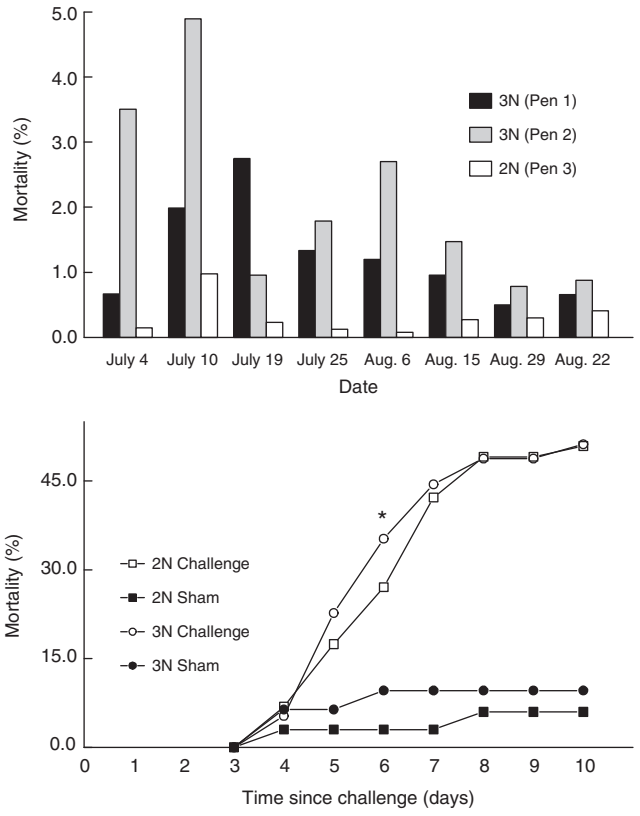

Figure 1 Differential mortality in diploid and triploid Chinook salmon. (**a**) percent mortality in three replicate net cages holding triploid and diploid salmon in response to a natural outbreak of bacterial kidney disease (*Renibacterium salmoninarum*). Diploid mortality rates were significantly lower than either triploid group ($P<0.0001$). (**b**) Mortality rates for live *Vibrio anguillarum* challenged and sham-injected triploid and diploid salmon over a 10-day period (significant differences are marked by $^*P<0.05$).

MHC-I inheritance and transcription

The male parent's class I MHC PBR α1 sequence was identical to GenBank submission *U80284* (Miller *et al*., 1997). The class I MHC α1 clones from the female parent yielded two common sequences, designated *F-A1–1001* ($N=22$) and *F-A1–1008* ($N=20$); submitted to GenBank as DQ647922 and DQ647923, respectively. We were thus able to unambiguously identify all of the alleles in diploid and triploid offspring, and determine dosage effects for this one highly polymorphic locus. Three triploid offspring inherited both maternal alleles (this occurs only when recombination occurs between the

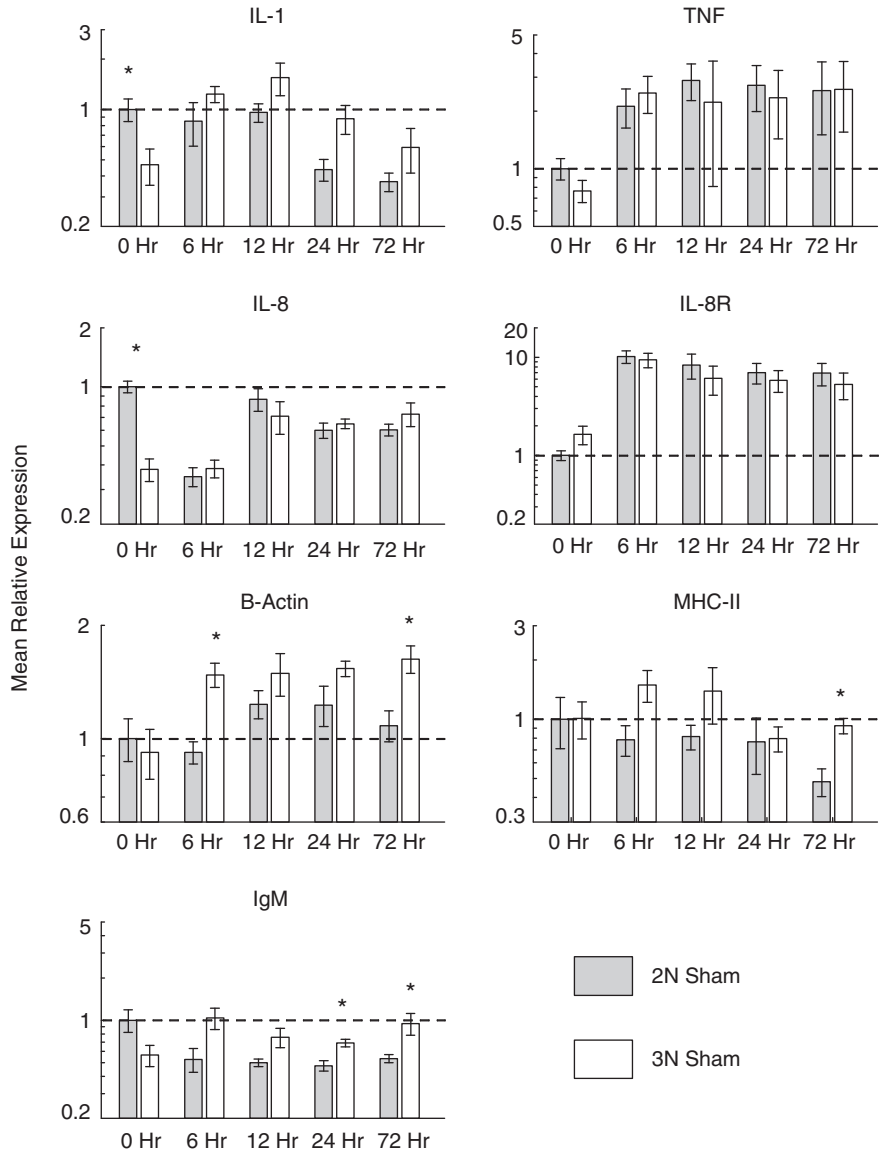

Figure 2 Mean relative gene transcription (±1 s.e.m.) for seven genes using quantitative real-time PCR (qRT-PCR) in triploid and diploid Chinook salmon sham-injected with saline solution. Time zero fish were sampled before injection. All values are normalized to an endogenous control (EF-1a) and calibrated to time zero diploid control values. Significant differences ($P<0.05$) between diploid and triploid values at each time point are indicated by asterisks. Six fish (assayed in duplicate) were used for time zero and sham-injected qRT-PCR assays.

MHC-I locus and the centromere in maternal meiosis I) and the male parent's allele such that they had three different alleles. In those three triploid offspring, we could unambiguously track the expression of the alleles on all three chromosomes. In all three of these triploid offspring, both maternal and paternal alleles were present in the cDNA, indicating a dosage effect resulting from the ploidy increase. In diploid offspring and the triploid offspring, which inherited only one maternal allele (due to a lack of centromere-MHC-I locus recombination), the single maternal and paternal alleles were both identified in the cDNA.

Microarray analysis

Several genes (34–84 per array) 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, 12 genes had signal and directionality agreement on at least two arrays (Table 2). Those 12 genes conservatively identified as showing gene expression differences between the ploidy groups (Table 2), 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*., 2004a,b). Across all genes, the relative intensity plot (Figure 4) represents relative intensities that were lowess normalized (Cleveland and Devlin, 1988) and replicate filtered (Quackenbush, 2002; Yang *et al*., 2002). The *y* axis represents a ratio of the intensity (Cy3/Cy5) of each gene and the *x* axis represents the product of the intensity (Cy3 × Cy5) of

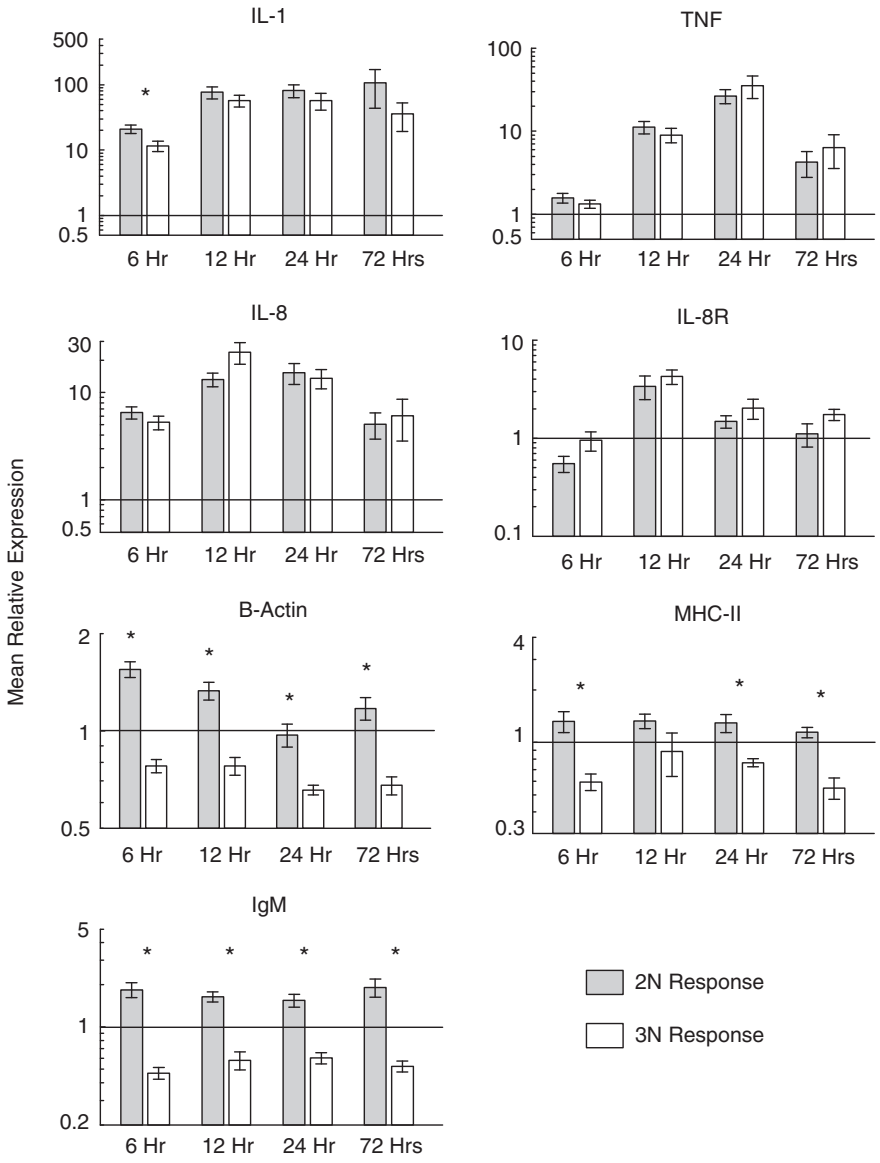

Figure 3 Mean relative gene transcription (±1 s.e.m.) for seven genes using quantitative real-time PCR (qRT-PCR) on triploid and diploid Chinook salmon challenged with live *Vibrio anguillarum* over a 3-day period after injection. All values are normalized to an endogenous control (EF-1a) and calibrated to transcription values for sham-injected salmon sampled at the same time, Asterisks indicate significant differences ($P<0.05$) between diploid and triploid response at each sampling time. Ten fish were used for all challenge qRT-PCRs, whereas the sham-injected qRT-PCR assays included six fish.

each gene (Figure 4). The figure reveals a general tadpole shape characteristic of highly similar transcriptomes (Figure 4). All but one of the genes (TNF) chosen for the qRT-PCR analysis were present on the microarray as well; however, none of those genes showed a significant difference between 3N and 2N transcription on more than one array. Although IgM (BG934806), β-actin (AF330142) and IL-1 (AJ004821) showed a significant 3N:2N transcription ratio on a single array (1.67, 1.71 and 1.34 respectively, see supplementary Table 1), we judge such results to be weak evidence for microarray-based transcriptional differences. The lack of agreement between the qRT-PCR and microarray results for the selected genes likely reflects the difference in sensitivity between qRT-PCR and microarrays.

Discussion

In this study we investigated phenotypic (survival) and genetic (transcription) responses to *V. anguillarum* challenges in triploid and diploid Chinook salmon to evaluate the effect of gene dosage. Although a number of studies have investigated the effect of natural or artificial ploidy manipulation on gene expression (Guo *et al*., 1996; Galitski *et al*., 1999; Suzuki *et al*., 1999; Birchler *et al*., 2001; Martelotto *et al*., 2005), this study is one of the first to report such effects in a vertebrate. The fact that triploid salmon are viable, and essentially indistinguishable from their diploid counterparts, would tend to indicate that the majority of their transcriptome should reflect patterns very similar to that of diploid salmon. However, there are important differences between diploid and

Table 2 Differentially expressed genes based on the microarray analysis (see Figure) between triploid and diploid Vibrio challenged salmon are shown. Putative functions have been assigned to each gene

Accession number	*Gene name/suspected*	*Gene ontology*	*3N fold change (+/−)*
BG933901	MHC-I 3′ UTR	Immune response	+1.626
AF184937	Low molecular mass protein 7	Immune response	+2.090
AF180488	β-2 microglobulin	Cellular defense response	+2.195
NP004244	Phospholipase A2-activating protein	Inflammatory response	−1.449
NP000234	Mediterranean fever protein; pyrin	Inflammatory response	−1.587
BG934453	Glutathione peroxidase	Response to oxidative stress	+1.718
NP033292	Selenophosphate synthetase 2	Selenocysteine biosynthesis	−1.659
NP008454	NADH dehydrogenase subunit 4	Electron transport	+1.841
XP010362	Solute carrier family 25 (adenine nucleotide translocator)	Generation of precursor metabolites and energy	+1.559
XP001466	Tyrosine phosphatase, receptor type, f polypeptide	Cell matrix adhesion	+1.626
NP081864	Autoimmune infertility-related protein	DNA metabolism	−1.508
BG934633	Atlantic salmon cDNA clone	Unknown	+2.119

Abbreviation: MHC, major histocompatibility complex.
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.

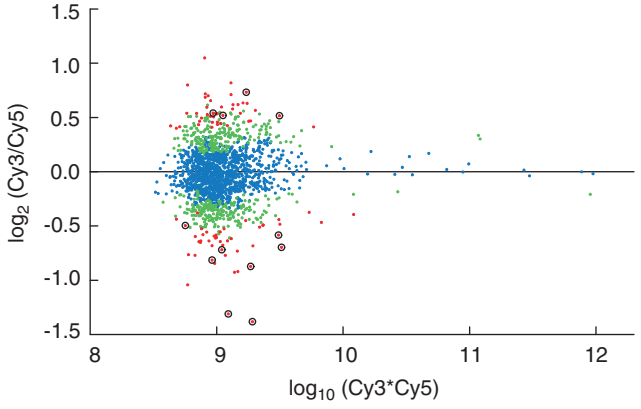

Figure 4 Scatter plot of results of DNA microarray analysis of transcriptional response of diploid versus triploid Chinook salmon 18 h after a live *Vibrio anguillarum* challenge. Intensity dependent *Z*-scores were calculated using a sliding window and *Z*-scores above 2.0 (approximately 95% confidence interval) are in red, from 1.0 to 2.0 in green, and from 0.0 to 1.0 in blue. Circled points are the genes that were consistently differentially expressed in diploid relative to triploid challenged fish across seven microarray replicates. The putative function and 3N:2N transcription ratios for the twelve genes are given in Table 2.

triploid Chinook salmon in both their survival and gene expression at selected immune-related genes that perhaps reflect the breakdown of triploid homeostasis under severe stress.

We found that the triploid fish exhibited elevated mortality in response to natural and controlled disease challenges, as reported elsewhere for other species (O'Flynn *et al*., 1997; Benfey, 2001; Cotter *et al*., 2002; Jhingan *et al*., 2003). Although there was no difference in total mortality between diploid and triploid groups at the end of 10 days following our experimental challenge, there was a significant difference during the peak of the mortality response. Thus, diploid Chinook salmon have transient higher survivability relative to triploid fish, which has also been shown in Atlantic salmon (O'Flynn *et al*., 1997). The approximately equal final mortality rates in the experimentally challenged 2N and 3N fish, likely reflect the severity of the challenge, and may thus not be comparable to the natural outbreak results in the net cages. However, our data generally indicate that triploidy is reducing the efficacy of Chinook salmon's disease resistance. The compromised immune function may result from either a residual effect from the pressure-shock and subsequently disrupted development, or, perhaps more likely, increased gene dosage adversely affecting gene expression and epistatic and dominance interactions (Johnson *et al*., 2007).

Cells in triploid salmon are on average 1.5 times larger in volume than their diploid counterparts (Small and Benfey, 1987; Benfey, 1999), hence the concentration of the endogenous control gene transcript will be diluted in the triploid cells. Second, as the endogenous control gene (*EF-1a*) is transcribed in high abundance (Gao *et al*., 1997), and our MHC-I results indicate that whole-chromosome inactivation is not occurring in triploid salmon, all three copies of the *EF-1a* gene are likely transcribed in triploid cells. Thus, the relative nature of qRT-PCR analysis means that a dosage effect on target genes in triploid salmon will lead to equal concentrations of the transcribed mRNA in the triploid and diploid cells (because *EF-1a* transcript number and cell volume are both increased by 50%). Conversely, a reduction in mRNA concentration (as indicated by lower qRT-PCR values) will reflect dosage compensation for the target gene. Based on this, our qRT-PCR results can be classified into genes that have similar levels of expression in triploids and diploids after sham-injection or live *V. anguillarum* challenge (for example, dosage effects), and those which show a generally elevated expression in the sham-injected triploids relative to the diploids and reduced triploid expression in the challenged group (for example, dosage compensation).

Two of the cytokines assayed, IL-8 and IL-1, were significantly downregulated in triploid salmon compared with diploid salmon before injection. This effect, however, is not detected after sham-injection or challenge. It has been speculated that trans-regulatory elements may be responsible for the enhancing or inhibiting 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 to *V. anguillarum*, resulting in the dosage effects observed after challenge. In contrast, we observed a general enhanced expression of triploid IgM, MHC-II and β-actin relative to diploid after sham-injection, this likely reflects handling stress effects associated with the injection. There is evidence for a reduction in IgM and β-actin transcription resulting from stress in fish (Olsvik *et al*. 2005; Perez-Casanova *et al*., 2008); however, the effect of stress on the transcription of MHC-II is not known. Triploid salmonids are known to physiologically respond to stress more severely than diploids (reviewed in (Ojolick *et al*., 1995; Benfey, 1999); and apparently IgM, MHC-II and β-actin reflect that elevated stress sensitivity.

It is interesting to note that we observed transcriptional suppression (dosage compensation) at several time points in the response of triploid Chinook salmon to the live *V. anguillarum* challenge for those same three genes. It is not clear why the genes that are more constitutively expressed are exhibiting dosage compensation upon *V. anguillarum* exposure, but not at rest or after sham-injection. Part of the effect results from the elevated expression of these genes in the sham-injected triploid fish (relative to the diploid), and as the live *V. anguillarum* challenge transcription values are normalized to the sham-injected values, they will tend to exhibit lower relative values. However, the downregulation of the triploid challenge response in IgM, MHC-II and β-actin is still significant when normalized to pre-injection diploid control values (data not shown). One possibility would be that the transcriptional response of IgM, MHC-II and β-actin reflect a genome-wide dosage compensation effect that is overridden in the induced cytokine immune response. Alternatively, the large induction of the cytokine genes may sequester basal transcription factors, which would lead to an overall decrease in housekeeping gene expression. However, the results of our microarray analysis indicates that majority of genes in triploid Chinook salmon exhibit dosage effects (that is, the diploid and triploid mRNAs were at similar concentrations), thus 'across the board' dosage compensation does not seem to be a likely explanation. Though co-suppression of IgM, MHC-II and β-actin is an attractive hypothesis, our understanding of the functional relationships among those genes makes it unlikely, as co-suppression generally involves homologous gene products (Pal-Bhadra *et al*., 1999), whereas there is little homology among those genes. Simple odd–even dosage effects on transcription levels have been reported in maize (Guo *et al*., 1996), where odd ploidy had remarkable upregulated expression and even ploidy maintained normal levels, whereas here we observed the opposite. Regardless of the mechanism of transcription suppression, it is clear that IgM, MHC-II and β-actin exhibit dosage compensation in the disease-challenged triploid salmon. Given the reduced survival characteristic of Chinook salmon to pathogen exposure, it would be reasonable to assume that this downregulation is not adaptive.

The vast majority of genes included in our microarray analysis exhibited dosage effects in the triploid Chinook salmon. 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. However, any subtle transcriptional differences are perhaps biased by the insensitivity of microarray technology to small transcriptional change. Published studies have used microarrays to characterize salmon response to immune challenges and have reported strong and consistent effects of pathogen challenge on transcription of genes associated with immune function, as well as a range of other classes of genes, including many of the genes reported here as differing between immune challenged diploid and triploid Chinook salmon (Rise *et al*., 2004a,b; Martin *et al*. 2006; Wynne *et al*., 2008; Young *et al*., 2008). However, the purpose of our study was to characterize dosage effects on the transcriptional response to a challenge with *V. anguillarum*, and our microarray study identified significant differences in the expression of a few genes in the severely immune challenged diploid and triploid Chinook salmon. Two functional categories of genes among those differentially expressed between diploid and triploid salmon are of particular interest; the inflammatory function genes and the immune function, or MHC-I related, genes. Pyrin and phospholipaseA2-activating protein, both inflammatory function genes, were downregulated 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). As the 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 triploid and diploid fish, inadequate control or signaling to downstream effectors may be contributing to the elevated mortality rate in disease-challenged triploid salmon.

The second functional group of genes showing differential expression was the immune function genes, or the MHC-I related genes, which were overexpressed in the triploid relative to the diploid fish. Although MHC-I is not related to innate-immune response, it does possess specific and acquired immunity functions. Even though the main interest of the microarray study was early innate response (that is, 18 h post-challenge), the abnormalities in acquired immunity may also account for the poor immune performance of triploid Chinook salmon. 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 function 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 (Xie *et al*., 2003). The microarray identified genes representing additional potential mechanisms for the phenotypic divergence between diploid and triploid Chinook salmon under disease challenge stress.

While qRT-PCR data showed that initial innate immune response was functioning properly, the significantly higher early mortality rate in triploid fish 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 the poor performance of triploid salmon under stress (O'Flynn *et al*., 1997; Benfey, 2001; Cotter *et al*., 2002).

This study identified a phenotypic difference in the survival response of diploid and triploid Chinook salmon to severe disease challenges. Post-disease challenge gene transcription profiles reveal that there are substantial differences in gene expression that are likely contributing to compromised immune function in triploid salmon. However, our study failed to identify a likely molecular genetic mechanism behind the reduced immune function of the triploid salmon. However, we do show that the majority of the transcriptomes of diploid and triploid Chinook salmon show dosage effects that appear to compensate for cell volume differences. Apparently, Chinook salmon are well adapted to polyploidy, perhaps as a result of their tetraploid ancestry (Allendorf and Thorgaard, 1984). Although most published research on dosage effects on gene expression has dealt with genomic regulatory differences at a resting state (Birchler *et al*., 1990; Bhadra *et al*., 1997), our results show that perhaps the majority of anomalous ploidy effects on transcription may be evident under challenge or stress conditions. This work advances our understanding of the mechanisms and patterns of dosage effects on gene transcription, and will contribute to the evaluation of the role of genome duplication in the evolution of gene diversity (for example, Ohno, 1999).

Acknowledgements

This research was supported by a Natural Science and Engineering Research Council of Canada grant to DH and AH, and by Yellow Island Aquaculture, which provided field and laboratory support. We would also like to thank A Newhook, R Hepburn, T Aykanat and A Heath for their assistance in the laboratory and field.

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Supplementary Information accompanies the paper on Heredity website (http://www.nature.com/hdy)