AN ABSTRACT OF THE THESIS OF

Tracey S. Momoda for the degree of Master of Science in Fisheries Science on October 9, 2006.
Title: Gene Expression in the Liver of Rainbow Trout, *Oncorhynchus mykiss*, During the Stress Response

Abstract approved:_____
Carl B. Schreck

The extent to which stressful events are maladaptive or adaptive to the long-term survival of fish remains to be better understood. The aim of this study was to identify differentially expressed genes in the livers of rainbow trout, *Oncorhynchus mykiss,* responding to an experimental stressor. Gene expression responses were measured using an oligonucleotide microarray specific for *Oncorhynchus mykiss*, to highlight genes responding to a stressor and to serve as a basis for hypothesis development. We conducted replicate experiments at two different times. In both experiments, fish exposed to a three-hour stressor were compared to control (unstressed) fish. In the second experiment, there were additional treatments of fish that were exposed to only a half-hour of stress and of fish sampled 21 hours after experiencing a three-hour stressor. This 21 hour post-stress treatment was a means to study gene expression during recovery from stress. Plasma cortisol was measured to document the physiological stress response of the fish. Real-time PCR (qPCR) of candidate genes was used to validate the microarray findings. In both experiments the microarray revealed many genes with

differential expression after three hours of stress. The genes we report as differentially expressed met a criteria of at least a 1.4 fold change and a statistical difference ($p \leq 0.05$) from control levels of expression. Among these, five genes responded similarly in both experiments, suggesting that they are robust indicators of stress. These genes are a *major histocompatibility complex class I* molecule (*MHCI*), *JunB, glucose 6-phosphatase* (*G6Pase*), *nuclear protein 1* (*Nupr1*) and *tumor necrosis factor decoy receptor* (*TNFDR*). Interestingly, transcripts of *Nupr1* did not return to control levels in the 21 hours after stress. In fact, the transcripts continued to increase during recovery.

GENE EXPRESSION IN THE LIVER OF RAINBOW TROUT, *ONCORHYNCHUS MYKISS*, DURING THE STRESS RESPONSE

by
Tracey S. Momoda

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APPROVED:

_____

Major Professor, representing Fisheries Science

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Head of the Department of Fisheries and Wildlife

_____

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader on request.

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Tracey S. Momoda, Author

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CONTRIBUTION OF AUTHORS

Dr. Lena Gerwick provided expertise on microarray and qPCR experimental design and analysis. Dr. Christopher J. Bayne and Dr. Carl B. Schreck provided expertise on the interpretation of the microarray and qPCR results, as well as, advise on experimental design.

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DEDICATION

This thesis is dedicated to my parents,

George and Betty Momoda

For always ensuring me that

“No question is a stupid question.”

INTRODUCTION:

The stress response has been extensively studied in fish. There is an immediate cascade of hormonal events through the hypothalamo-pituitary- interrenal (HPI) axis and subsequent osmoregulatory and metabolic compensation thereafter (Wendalaar Bonga, 1997). The extents to which these are adaptive or maladaptive qualities of the stress response are not fully understood (Barton and Iwama, 1991). Although an organism may appear to compensate physiologically and to recover from a stressful experience, its ability to survive may be reduced (Schreck et al., 1989). For example, stress is known to have adverse effects on fish through suppression of growth (Pickering et al., 1991), immune function (Maule et al., 1989), and reproduction (Barton and Iwama 1991 for review; Schreck 2000). Furthermore, environmental stressors are linked with delayed mortality in fish populations through increased susceptibility to predators and disease (Wedemeyer et al., 1990; Budy et al., 2005). Efforts to understand how fish respond to stress are complicated by the facts that physiological parameters may oscillate following removal of a stressor (see Maule, 1989 for an example) and that physiological measurements may not correlate with mortality (Davis et al., 2001).

Understanding of the stress response and subsequent recovery at the transcriptional level can enhance understanding at the physiological level. Thus, in order to better appreciate the mechanisms underlying the physiology of the stress response, we used molecular tools to study gene expression changes in response to stress. Microarrays are a powerful tool for monitoring the expression of multiple genes at one time. The particular microarray that we used was a 70-mer

oligonucleotide microarray specific for *Oncorhynchus mykiss* (Tilton et al., 2005; Gerwick et al., in press; and http://www.science.oregonstate.edu/mfbsc/facility/micro.htm).

At the cellular level, the response to stress begins with the binding of glucocorticosteriods to a glucocorticoid receptor/heat shock protein 90 complex within the cytoplasm of the cell. After the ligand binds its receptor, hsp 90 is released and the ligand/receptor complex translocates as a dimer to the nucleus of the cell. These function as a transcription factor that can activate or repress target genes, thereby influencing a variety of physiological functions (Adcock, 2000). The increase in beta-adrenoceptors with increase in stress has been observed in the fish liver (Reid et al., 1992). In the teleost liver, enzyme activities that are known to be altered as a result of stress include tyrosine aminotransferase, glutamate dehydrogenase and glutamine synthetase (see Mommsen et al., 1999 for review). Also, enzymes of glycogen metabolism including glycogen phosphorylase (GPase) and glycogen synthase (GSase) have been shown to change with stress (Vijayan and Moon, 1992).

Little is known of the effects of handling stress on gene transcription in fish liver. For instance, we know that cortisol production modulates *gluccocorticoid receptor* mRNA production (Vijaayan and Sathiyaa, 2003) as well as *estrogen* and *vitellogenin receptor* mRNA production (Lethmonier et al., 2000). Although these studies have provided insight into the mechanisms involved with the stress response, they assessed the expression of one gene at a time in response to cortisol treatments, neglecting other physiological changes occurring in the stressed fish that may effect gene expression in the liver. A recent study analyzed the transcriptome response to a

repeated stressor in rainbow trout, using a cDNA microarray, however, this study focused on gene expression in the brain and head kidney (Krasnov et al., 2005).

The goal of our research was to identify genes in rainbow trout livers that respond to a stressor. We examined the effects of a confinement stressor on differential gene expression. In order to increase confidence in the discovery of candidate genes, two separate experiments using fish from different year classes were used and microarray gene expression data were validated with quantitative PCR (qPCR). The transcript levels of candidate genes were also observed 21 hours post-stress.

METHODS:

Fish Sampling:

Fish in both experiments were acquired from the Oregon Department of Fish and Wildlife's Roaring River hatchery (Scio, Oregon) then transferred to Oregon State University's Fish Performance and Genetics Laboratory (Corvallis, OR). Here, fish were housed in circular stock tanks with 12-13°C aerated, pathogen-free well water in a flow-through system. Fish were fed twice per day, but once on weekends, with Bio-Oregon® (Warrenton, OR) semi-moist pellet at ~2% body-weight/day. All fish were eating vigorously and growing well before the start of the experiments. Food was withheld 24 hours prior to the stress experiments. Stress consisted of netting, exposure to the air and holding in small, shallow tanks as described subsequently. The Institutional Animal Care and Use Committee at Oregon State University approved this study (IACUC permit #3092).

First Experiment:

In August 2004, three circular tanks (1m diameter; 400L volume) were stocked with about 80 juvenile rainbow trout each. The fish were allowed to acclimate to the laboratory for approximately two months before the onset of the experiment in October 2004. More fish were stocked in the tanks than were sampled in the experiment in order to ensure access to the intended numbers. The mean length was 136mm ±1.01 s.e.m and weight was 31g ±0.85 s.e.m. for all fish (n=60) sampled in this experiment. First, 10 fish were removed from each tank and immediately lethally dosed with buffered tricaine methanesulfonate (MS-222; 200mg/L). These 30 fish

were the non-stressed controls and were processed immediately (see Tissue Sampling below). Then, another 10 fish were netted from each tank and held out of water for 30 seconds, then placed in a different (0.6 m diameter) circular tank that had well-aerated, shallow flow –through water such that the dorsal fins and backs were just above the water level. After three hours, these fish were placed in MS-222 (200mg/L) and processed (see below).

Second Experiment:

In April 2005, five circular (1 m diameter; 400L) tanks were stocked with about 55 juvenile rainbow trout each. As in the first experiment, more fish were stocked in the tanks than were sampled in the experiment in order to ensure access to the intended numbers. The fish were allowed to acclimate to the laboratory for approximately four months before onset of the experiment in August 2005, at which time the mean length of the 200 sampled fish was 114mm ±0.79 s.e.m and weight was 20g ±1.22 s.e.m. First, 10 fish from one tank were lethally dosed with MS-222 (200mg/L); these were the non-stressed controls. Then 10 more fish, from the same tank, were subjected to a 30-second handling stressor followed by a half-hour of low-water stress treatment (1/2 hr stress), as described for Experiment 1. Immediately after placement of fish in the half-hour treatment, another 10 fish from the original tank were subjected to a 30-second handling stressor, followed by three hours of low-water stress treatment (3 hr stress). A fourth set of 10 fish were then subjected to a 30-second handling stressor, followed by three hours of low-water stress treatment then given 21 hours to recover from the stressor (21 hr post-stress); immediately following

the 3hrs of stress, the water in the tank was allowed to rise to the level at which the fish were normally held. After 21 hours these fish were sampled. All fish were lethally dosed with MS-222 (200mg/L) at end of their assigned treatments, and then processed immediately (see below). The experiment was repeated four times, over the course of 4 days using fish from a different stock tank each day, for a total of five replicates per treatment.

Tissue Sampling:

In both experiments, dead fish were immediately submerged in crushed ice to prevent degradation of RNA. Blood was collected into ammonium-heparinized capillary tubes by severing the caudal peduncle. Livers were then removed and frozen in liquid nitrogen. Plasma was separated by centrifugation. Plasma and liver samples were stored at -80º C until later analysis

Cortisol Assay

In order to monitor the stress response of the fish used in both experiments, plasma cortisol was measured. Plasma cortisol was quantified using the radioimmunoassay described in Redding et al. (1984). %CV between sample replicates were <5% and <15% between assays. For statistical analysis, possible tank, size and sex effects (as well as their interactions) were originally included in the analysis and then removed if found to be non-significant. One-way ANOVA followed by a Bonferroni post hoc test was used for analyzing differences between treatments.

Plasma Glucose

In the second experiment, plasma glucose levels were obtained for the same 60 individuals used in qPCR analysis. Plasma glucose was quantified following the protocol described in Wedemeyer and Yasutake (1989). Plasma samples diluted in σ-toluidine reagent (Sigma-Aldrich, St. Louis, MO) were measured using a Beckman DU-64 spectrophotometer (Beckman Coulter, Fullerton, CA) at visible light 635nm. %CV between sample replicates were <5% and <7% between assays. For statistical analysis of glucose data, possible tank, size and sex effects (as well as their interactions) were originally included in the analysis and then removed if found to be non-significant. One-way ANOVA followed by a Bonferroni post hoc test was used for analyzing differences between all treatments.

RNA isolation:

Total RNA was extracted from livers using TRIzol® reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. In the first experiment, five livers were pooled from each replicate, and then RNA was extracted. These pools were used for microarray as well as qPCR analysis. The remaining five livers in each of the three replicates had RNA extracted from them individually for qPCR analysis, as well. In the second experiment, RNA was extracted from five individual livers in each treatment. Then, for each of the five replicates for both the control and three-hour stress group, RNA was pooled from the individuals for microarray analysis. Aliquots of RNA from the individual livers of all treatments were used for qPCR analysis. In both experiments, RNA was quantified using spectrophotometric

absorbance at 260/280 nm and quality was assessed on the 2100 Bioanalyzer™ (Agilent Technologies, Palo Alto, CA). All samples used in analysis met a criterion of a 260/280 >1.8, and a clean, non-degraded state of the RNA was confirmed by means of the Bioanalyzer™.

Reference design:

A reference sample for each experiment was made by pooling RNAs from all samples in the control and stress treatments, for microarray analyses. To make the reference pools, 667μg of control RNA, which was made by pooling equal amounts of RNA from the control replicates, was added to 333μg of stress RNA, which was made by pooling equal amounts of RNA from each stress replicate.

Microarray hybridization and analysis:

The development and fabrication of this rainbow trout oligonucleotide microarray (OSUrbt ver. 2.0) has been previously described (Tilton 2005; Gerwick et al., in press). Briefly, it is a 70-mer oligonucleotide microarray that contains 1672 features known to be associated with physiological processes including carcinogenesis, immunology, endocrinology, toxicology, and other stress responses. These features represent approximately 1400 genes; therefore, while each gene is represented by at least one 70-mer oligonucleotide, several genes are represented by more than one distinct 70-mer oligonucleotide. Each feature is spotted in duplicate within the array. Slides were printed by OSU Central Services lab and stored under desiccation for no more than six months prior to use.

Tagged cDNA preparations and microarray slide hybridizations were performed using the Genisphere Array 50™(Genisphere, Hatfield, PA) kit following the manufacturer's instructions. In brief, for each treatment or reference pool, 15µg of total RNA, spiked with 1µl Alien Oligos® 1-10 (0.0049-2.5ng/µl) (Stratagene, LaJolla, CA), was reverse transcribed with Superscript III (200U/µl)(Invitrogen) using the Genisphere oligo d(T) primers that contained 5' unique sequence overhangs for either the Cy3 or Cy5 labeling reagents. Each treatment (control and stress, separately) cDNA labeled with Cy3 or Cy5 tags was combined with an equal amount of reference cDNA tagged for the other label. Dyeswap replicates were also used for each treatment-reference cDNA comparison to control for technical variation. The tagged cDNAs were concentrated using Millipore Microcon® YM-30 Centrifugal Filter Devices (Millipore, Billerica, MA).

Prior to hybridization, the microarray slides were pre-washed in 2X SSC/0.2% SDS at 55º C for 20 minutes, 0.2X SSC at room temperature for 5 minutes, DEPC-treated water at room temperature for 3 minutes then dried by centrifugation. The concentrated cDNA was hybridized to the microarray in 2X Formamide-Based Hybridization Buffer using 22x25 Lifterslips™ slide covers (Erie Scientific, Portsmouth, NH). They were kept at 47º C for 24 hours in a humidified incubator. Slides were then washed in 2X SSC, 0.2% SDS at 47º C for 20 minutes, 2X SSC at room temperature for 20 minutes, 0.2X at room temperature for 20 minutes, 95% ethanol at room temperature for 2 minutes and then dried by centrifugation.

Protected from the light, the Cy3 and Cy5 fluorescent markers (3DNA capture reagent, Genisphere) were then dispensed on to the slides in the 2X Formamide-based

hybridization buffer and covered with Lifterslips™ slide covers. Hybridization was then allowed to occur in a dark, humidified incubator at 49º C for 3 hours. The slides were then washed (in the dark) in 2X SSC, 0.2%SDS at 47°C for 20 minutes, 2X SSC at room temperature for 20 minutes, 0.2X SSC at room temperature for 20 minutes then dried by centrifugation. All post hybridization solutions contained 0.1 mM diothiothreitol to prevent oxidation to Cy3 and Cy5 fluorophores. As a protective coat, DyeSaver™2 (Genisphere) was applied by dipping the slides in the solution for 30 seconds then drying them by centrifugation.

A digital image of each slide was obtained using a GenePix Professional 4200A (Molecular Devices, Union City, CA) scanner. The photomultiplier tubes (PMT) and power settings of the lasers were adjusted so that the Alien Oligo (Stratagene) control spots on the array had a ratio of medians of approximately one and the overall Cy5/Cy3 ratio for the slide was approximately one. By visually assessing all features on each slide, any saturated or otherwise flawed spots were identified and flagged for later removal from the dataset. Data was then imported into Acuity 4.0 (Molecular Devices, Union City, CA) for normalization and statistical analysis. The images were normalized using the Lowess print-tip method, then the datasets were filtered to eliminate low intensity (Sums of Medians <300) features and those previously flagged.

Twelve slides (3 control with dyeswaps and 3 stress with dyeswaps=12 slides) were hybridized and analyzed in the first experiment and a total of 20 slides (5 control with dyeswaps and 5 stress with dyeswaps) were analyzed in the second experiment. In both experiments, data from the control fish were compared with data from stressed

fish using the Mann-Whitney advanced U-test. Differential fold values for each feature were calculated by dividing the median for the control slides by the median of the treatment slides. Using 1.4 fold change and $p < 0.05$ as our criteria, we identified candidate genes of interest.

Quantitative PCR (qPCR)

Candidate genes were analyzed by qPCR to assess the validity of the microarray data. The absolute quantification method was used to quantify transcript amounts (see Bustin 2000 for review). In short, a standard curve of known amounts of transcript copies was used to extrapolate the quantity of mRNA targets in unknown samples. cDNAs made from the pools of stressed RNA was used as template to create the standard curves. Copy numbers were calculated based on the concentration of the PCR product measured at 260nm using the NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and the molecular weight of that particular gene product. Standards of known copy amounts are then diluted from these products. For each gene of interest a serial dilution of 10^6-10^2 copies was made.

For cDNA synthesis, 5µg aliquots of total RNA from the pools and individuals were reverse transcribed with Superscript III and oligo $d(T)_{18}$ primer (Invitrogen) following the manufacturer's instructions. These cDNAs were then used as templates in the qPCR reactions which were set up at follows: 10 µL of SYBR® Premix Ex Taq™ (Takara Mirus Bio Inc., Madison, WI) with primers at 200 nM final concentration, 0.4 µL of a 50x ROX™ solution (Takara Mirus Bio), 1 µL of cDNA template (made from 5µg total RNA) at 1:200 dilution, and water for a final reaction

volume of 20 μL. Gene specific primers were developed using Primer Express™ (Applied Biosystems, Foster City, CA). Table 1 is a list of the forward and reverse primers designed for each gene. An ABI 7000 (Applied Biosystems) was programmed to run at 95º C for 30s, 40 cycles of 95º C for 5s, with annealing and extension at 60-64º C (depending on the primers) for 32s.

We quantified the number of transcripts of a gene in each treatment pool then divided the average for the stress pools by the average of the control pools to determine the fold differences, which were calculated for each gene. For the statistical analysis in the first experiment, the Wilcoxon rank test (non-parametric *t*-test) was used to assess the differences between control and stressed pools of RNA. For the individual RNA, Student's *t*-test was first used, then, if results were non-significant, the Wilcoxon rank test was applied.

In the second experiment, differences between the groups were calculated for each candidate gene as mentioned above. Student's *t*-test was used for comparison between control and 3hr stress groups, using the qPCR data from the pools of RNA (for microarray validation). For the individual data, possible tank, size and sex effects (as well as their interactions) were originally included in the analysis and then removed if found to be non-significant. One-way ANOVA followed by a Bonferroni post hoc test was used for analyzing the individual data between all treatments for each gene.

Table 1. List of primers used for qPCR analysis of candidate genes.

Feature	**Gene Name**	**Primer sets**
OSUrbt2_625_JUNB	Transcription factor JunB	forward: 5'-CTACACGCACAGCGATATTCG-3' reverse: 5'-TCGTCGCTGCTCTGCATGT-3'
OSUrbt2_717_MHC1AF287488	MHC class I heavy chain precursor, Onmy-UBA*501 allele	forward: 5'-CCCAACATTGTCCTCATCATTG-3' reverse: 5'-CCCCA ACAACAGCAACGAT-3'
OSUrbt2_123_C1qASP	C1q-like adipose specific protein	forward: 5'-AAGAATGGACAGCGCATGGT-3' reverse:5'-CCCGTTGTCAGCTCCATCA-3'
OSUrbt2_571_IIP	Interferon inducible protein 1	forward: 5'-ACAGCCACAGCCCGTTGTA-3' reverse: 5'-GCTGCAAAGTGACCAGATAATG-3'
G6PC_Omy_C4589	Glucose 6 phosphatase	forward: 5'-TCTACGTGCTGCTGAAGGCTCTAG-3' reverse: 5'-CGGCTCTCACACACCACTTCTG-3'
OSUrbt2_1248_TC8456 and OSUrbt2_1249_TC8457	Nuclear Protein p8	forward: 5'-GACAAATCGGACGGCTAATCCT-3' reverse: 5'-CTGCCTGCCCATTTGGTTTT-3'
MHC_made from conserved alpha-3 domain of allele Onmy-UBA*501	MHC class I heavy chain precursor, Onmy-UBA*501 allele	forward: 5'-TGCCACGCGACAGGTTTCT-3' reverse: 5'-CATGCTGATCTTGTCCGTCTTTC-3'

RESULTS:

Cortisol

First Experiment:

The treatment used in this experiment was effective at inducing stress responses in handled fish compared to the control fish. Plasma cortisol levels were significantly elevated in the stressed fish compared to the control fish ($P<0.001$). There were no differences between control replicates ($P>0.1$), however, one of the stress replicates was significantly lower, though markedly higher than all of the controls, than the other two stress replicates ($P<0.05$). Regardless, all stress replicates were significantly higher than the control replicates (Figure 1).

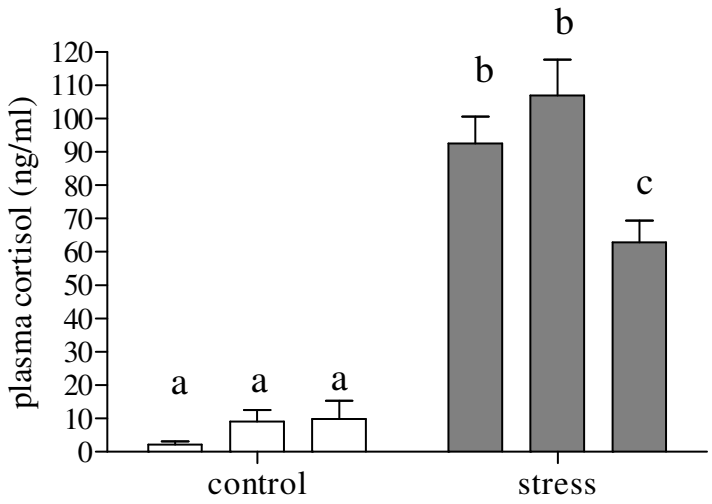

Figure 1. Plasma cortisol concentrations for all treatment replicates in the 1st experiment. Bars are the mean for each replicate with standard error lines (n =10). The overall mean cortisol level for the control group was 7.0 ng/ml ±0.77 s.em. (n=30) and the overall mean cortisol level for stress groups was 87.4 ng/ml ±4.10 s.em. (n=30). Replicates with the same letter are not significantly different at P< 0.05.

Second Experiment:

In the second experiment, plasma cortisol concentrations increased after ½ hour of stress and were highest in the 3 hr stress treatment group. Fish in the 21 hour post-stress group had lowered levels compared to those in the ½ hr and 3 hr stress groups, however, this recovery time was insufficient to bring concentrations down to control levels. There were slight tank effects (P =0.04) but this did not change the overall results of the comparisons between treatments, therefore, for ease of visualization of the data, it is shown as pooled (Figure 2).

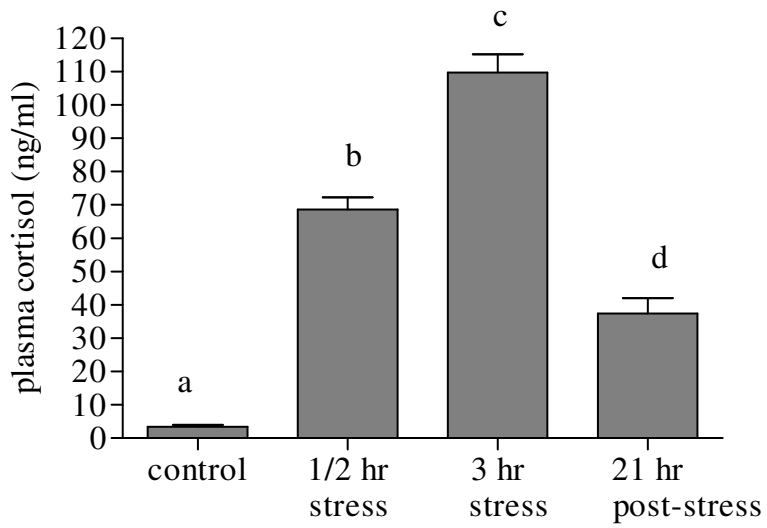

Figure 2. Plasma cortisol levels for all treatments in the 2nd experiment; replicates were pooled. Bars are the means for each treatment ±s.e.m (n=50). Plasma cortisol concentrations in the 1/2 hour (68.7 ng/ml±3.7) and three-hour (110.5 ng/ml ±5.5) stress treatments were statistically higher than the control (3.4 ng/ml ±0.56) and 24 hour treatments (37.5±4.5 ng/ml). The 24-hour treatment was also significantly different from the control fish (ANOVA P<0.001 Bonferroni post hoc test). Treatments with different letters are significantly different at P <0.05. There were slight tank effects (P =0.04) but this did not change the differences between treatments.

Glucose

Second Experiment:

There were no significant effects of tank, sex or size on the plasma glucose results; therefore the data were pooled (ANOVA $P > 0.1$). Plasma glucose increased after ½ hour of stress and was highest in the 3 hr stress treatment group. Fish in the 21 hour post-stress group had lowered levels compared to those in the ½ hr and 3 hr stress groups, however, this recovery time was insufficient to bring concentrations down to control levels (Figure 3).

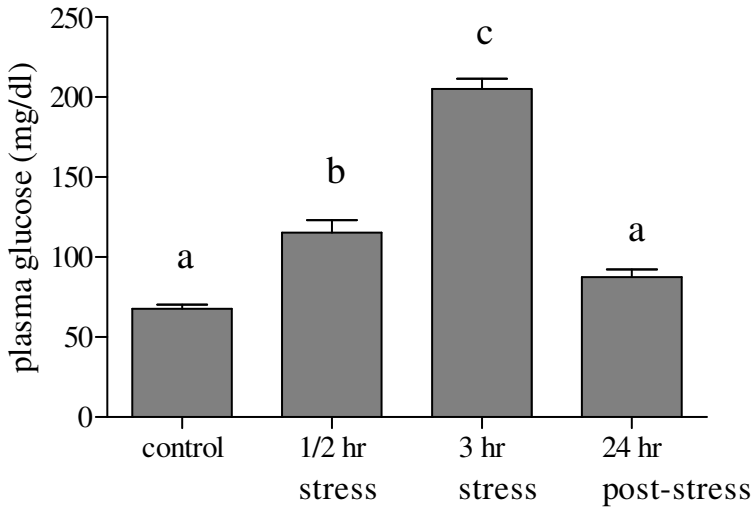

Figure 3. Plasma glucose levels for all treatments in the 2nd experiment. Bars are the means for each replicate ±s.e.m (*n*=15). Plasma glucose concentrations in the 1/2 hour (115.2 mg/dl±7.8) and three-hour (205.2 mg/dl ±6.3) stress treatments were statistically higher than the control (67.6 mg/dl ±2.7) and 24 hour treatments (87.3 mg/dl ±4.9). Treatments with different letters are significantly different at $P<0.05$.

Microarray

On this array, some genes are represented by two or more distinct oligonucleotide probes. Therefore, when discussing the raw data, we refer to the individual oligonucleotide probes as 'features' to avoid confusion over the fact that some 'features' represent the same gene. In order to confirm that gene annotation is current, the TIGR (www.tigr.org) and BLASTx databases (www.ncbi.org) were reexamined sequence by sequence.

First Experiment:

Twenty-seven features on the microarray showed increased expression in the stressed fish based on the criterion of a minimum of 1.4-fold change and a significant *p*-value. One additional feature was significantly different between control and stressed groups although its difference was below 1.4-fold (Appendix 1). Most interestingly, one third of the features upregulated were for the *major histocompatibility complex class I heavy chain precursor (MHC I).* Three genes were down-regulated by at least 1.4-fold and were significantly different from the control group (Appendix 2).

Second Experiment:

Twelve features were expressed differently in stressed and non-stressed fish (Appendix 3). Furthermore, another 25 differed significantly in the stress and the control pools (Appendix 4). Only one feature met both criteria for down-regulation,

although 17 more genes were identified as down-regulated by statistical significance only (Appendix 5).

Similarities between experiments:

In seeking to determine which genes were responding consistently to stress in the liver of rainbow trout, we found that ten features yielded similar results in both experiments (Table 2). Of these, eight features were upregulated in both experiments; the other features (both *MHCI*) were statistically different in both experiments but not upregulated by at least 1.4-fold. Of the eight features, three were for *MHC I* and two were for *Nupr1*. *Features for glucose 6 phosphatase (G6Pase), tumor necrosis factor decoy receptor (TNFDR),* and *JunB* also yielded similar results between experiments. No features were down-regulated in both experiments.

Table 2. Microarray data representing fold change and statistical *p*-value for features up-regulated after three hours of stress in both the 1st and 2nd experiments.

TIGR ID [a]	Feature ID	1st Expt: Fold Change	1st Expt: p-val [c]	2nd Expt: Fold Change	2nd Expt: p-val [c]	Gene Name (accession #, species) [b]
TC63296	OmyOSU86	1.96	0.0260	1.74	0.0000	Glucose-6-phosphatase (**AF120150**; *O. mykiss*)
TC46690	OmyOSU1662	1.59	0.0325	1.42	0.0002	Nuclear protein 1 (protein p8) (**NM_053611**; *R. norvegicus*)
TC46690	OmyOSU1663	1.77	0.0043	1.49	0.0004	Nuclear protein 1 (protein p8) (**NM_053611**; *R. norvegicus*)
TC57168	OmyOSU1622	3.74	0.0040	4.06	0.0022	Tumor necrosis decoy factor receptor (**AF401631**; *O. mykiss*)
CA387113	OmyOSU1486	3.70	0.0119	3.26	0.0002	MHC class I a region (**AB162342**; *O. mykiss*)
TC65531	OmyOSU804	8.84	0.0022	7.64	0.0357	Transcription factor JunB (**AY614595**; *O. mykiss)*
TC81254	OmyOSU796	7.95	0.0011	2.08	0.0111	MHC class I heavy chain precursor, (**AF287490**; *O. mykiss*)
TC65784	OmyOSU880	2.16	0.0476	2.83	0.0079	MHC class I heavy chain (**AF296366**; *O. mykiss*)
TC81254	OmyOSU824	6.25	0.0159	1.28	0.0093	MHC class I heavy chain precursor (**AF287490**; *O. mykiss*)
TC61811	OmyOSU826	12.10	0.0286	1.22	0.0315	MHC class I antigen (**AY278455**; *O. mykiss*)

[a]TIGR ID number of the tentative consensus or singleton expressed sequence tag (EST) sequence corresponding to OSUrbt ver. 2 microarray feature. (http://www.science.oregonstate.edu/mfbsc/facility/micro.htm)

[b]Mann-Whitney Advanced U-test p-values

[c]Information on putative functions of the genes was obtained through the TIGR database (www.tigr.org) for each particular TIGR ID, which was re-examined through the BLASTX (www.ncbi.org) database.

qPCR

First Experiment:

As a means to independently assess the findings of the microarray study, we elected to analyze the expression of some candidate genes using qPCR. We chose to measure transcripts for the following genes: *JunB*, *C1q-like adipose specific protein* and *interferon inducible protein 1 (IIP).* These genes were chosen based on their variation in differential fold values on the microarray, as well as the fact that all were significantly differentially expressed. We used the same pools of RNA analyzed with the microarray for developing the cDNA template used for qPCR. The qPCR data replicated the microarray data in regards to the differential fold values (Table 3). However, of these three genes, none were found to be statistically different between treatments (Wilcoxon Rank sum $P > 0.05$). The remaining five individual fish from each replicate of the experiment were also assayed for gene expression of these candidate genes using qPCR. *JunB* was found to be statistically different between treatments (Wilcoxon rank $P = 0.001$), however, neither *IIP* or *C1q* were different (Wilcoxon rank $P > 0.1$)(Table 3).

Second Experiment:

As in the first experiment, candidate genes were selected from the microarray data to be assayed using qPCR. Data from the same pools of RNA replicated the findings of the microarray analysis (Table 3). We were also able to replicate the statistical significance determined by the microarray (*t*-test $P < 0.005$) for all genes, except for *MHCI* (*t*-test $P > 0.05$).

Table 3. Differential fold values for candidate genes in 1st and 2nd experiments from both qPCR and microarray data

Feature	Gene Name	**Microarray: Pools** Fold change (±s.e.m)	**qPCR: Pools** Fold change (±s.e.m)	**qPCR: individuals** Fold change (±s.e.m)
Experiment 1				
OmyOSU804	Transcription factor JunB	8.84 (±7.37)[a]	9.09 (±0.76)	3.13 (±0.18)[b]
OmyOSU371	C1q-like adipose specific protein	4.00 (±2.85)[a]	2.61 (±0.02)	1.88 (±0.01)
OmyOSU983	Interferon inducible protein 1	1.74 (±0.69)[a]	1.70 (±0.02)	1.26 (±0.01)
Experiment 2				
OmyOSU804	Transcription factor JunB	7.64 (±6.39)[a]	8.14 (±0.18)[c]	7.08 (±0.001)[c]
OmyOSU796	MHC class I heavy chain precursor	2.08 (±0.92)[a]	1.80 (±0.02)	1.18 (±0.005)
OmyOSU86	Glucose 6 phosphatase	1.74 (±0.41)[a]	3.14 (±0.01)[c]	1.97 (±0.001)[c]
OmyOSU1662	Nuclear protein 1	1.42 (±0.31)[a]	2.37 (±0.01)[c]	1.73 (±0.003)[c]

[a] Mann-Whitney Advanced U-test $P < 0.05$
[b] Wilcoxon Rank test $P = 0.001$
[c] Student's *t*-test $P < 0.05$

After observing upregulation of candidate genes by both microarray and qPCR analysis, we elected to further analyze the expression of four candidate genes. This was done in order to better understand how their expression changed in response to stress and during subsequent recovery. We analyzed three individual's RNAs from each of the five replicates in all treatments for *JunB, MHCI, G6Pase* and *Nupr1* gene expression using qPCR (Figure 4).

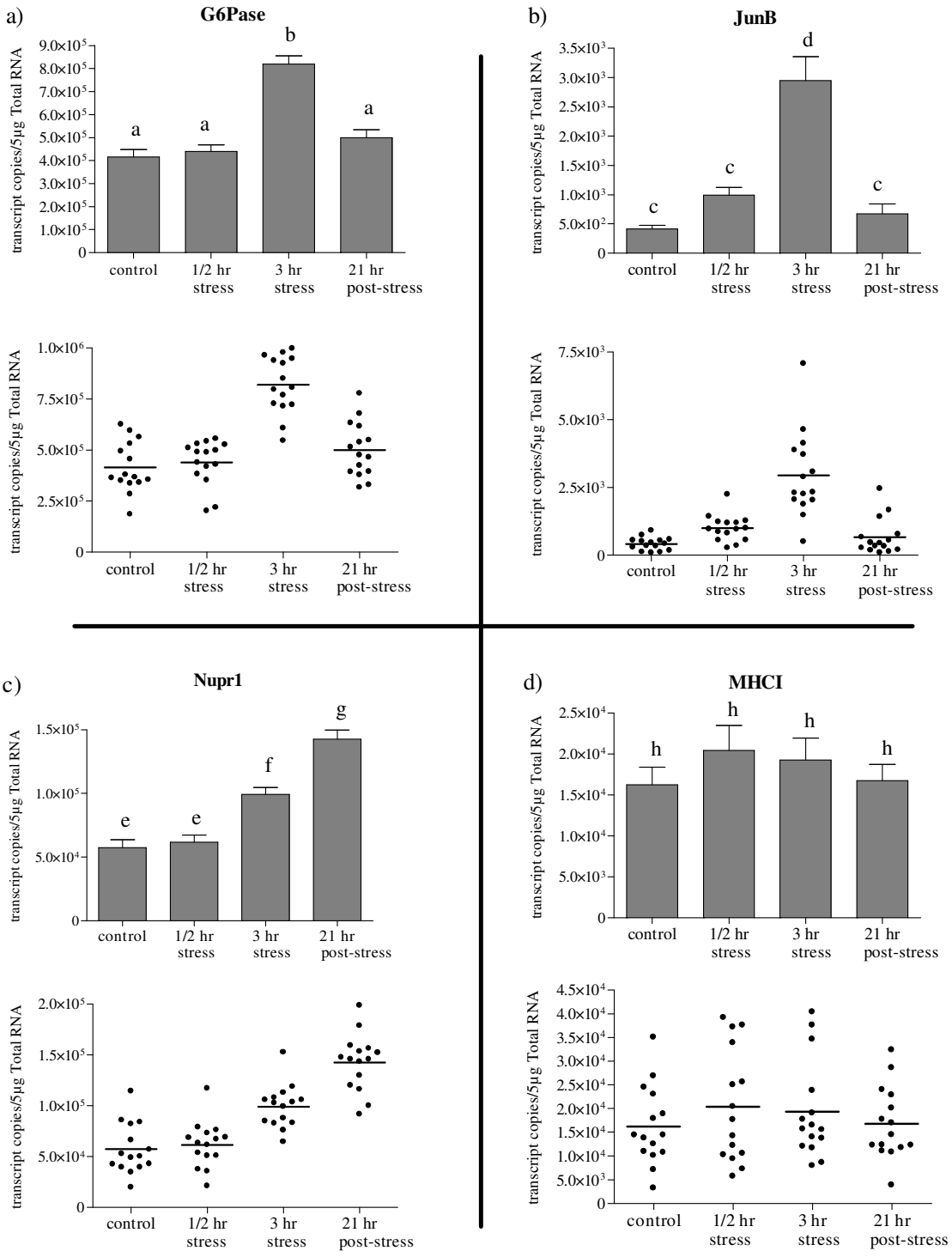
a)
G6Pase
transcript copies/5µg Total RNA
9.0x10^5
8.0x10^5
7.0x10^5
6.0x10^5
5.0x10^5
4.0x10^5
3.0x10^5
2.0x10^5
1.0x10^5
0
a
a
b
a
control
1/2 hr stress
3 hr stress
21 hr post-stress
1.0x10^6
7.5x10^5
5.0x10^5
2.5x10^5
b)
JunB
3.5x10^3
3.0x10^3
2.5x10^3
2.0x10^3
1.5x10^3
1.0x10^3
5.0x10^2
c
c
d
c
7.5x10^3
5.0x10^3
2.5x10^3
c)
Nupr1
1.5x10^5
1.0x10^5
5.0x10^4
e
e
f
g
2.0x10^5
d)
MHCI
2.5x10^4
2.0x10^4
1.5x10^4
1.0x10^4
5.0x10^3
h
h
h
h
4.5x10^4
4.0x10^4
3.5x10^4
3.0x10^4
2.5x10^4
2.0x10^4
1.5x10^4
1.0x10^4
5.0x10^3

Figure 4. Gene expression for each candidate gene for all treatments in the 2nd experiment. For each bargraph, the treatments that have different symbols are statistically different from each other (P <0.05). The bars in the bargraphs are the means for each treatment ±s.e.m (n=15) for each gene. The individual data for each fish are shown in the scatterplots for each gene below its given bargraph. The horizontal lines in the scatterplots are representative of the treatment means as represented by the bars in their respective bargraph.

The effect of stress on *G6Pase* expression was significant in this experiment (ANOVA $P < 0.001$). *G6Pase* transcripts were most abundant after 3 hours of stress with a 1.97-fold (±0.001 s.e.m) increase from control levels. There were no differences between the control and the ½ hr stress and 21-hour post-stress treatments ($P > 0.05$) (Figure 4a).

In this experiment, the *G6Pase* expression appears to have been different for males and females (Figure 5a). Indeed, when sex was included as variable in the ANOVA model, the interaction between sex and the stress treatments was significant ($P < 0.001$), although across all time points sex alone was non-significant statistically (ANOVA $P > 0.1$), because the sex with the greater expression in each treatment changed. The females in the control group had higher *G6Pase* expression than the males; this pattern was the same 21-hour post-stress. Interestingly, *G6Pase* expression in the 3 hr group was higher in males than in the females.

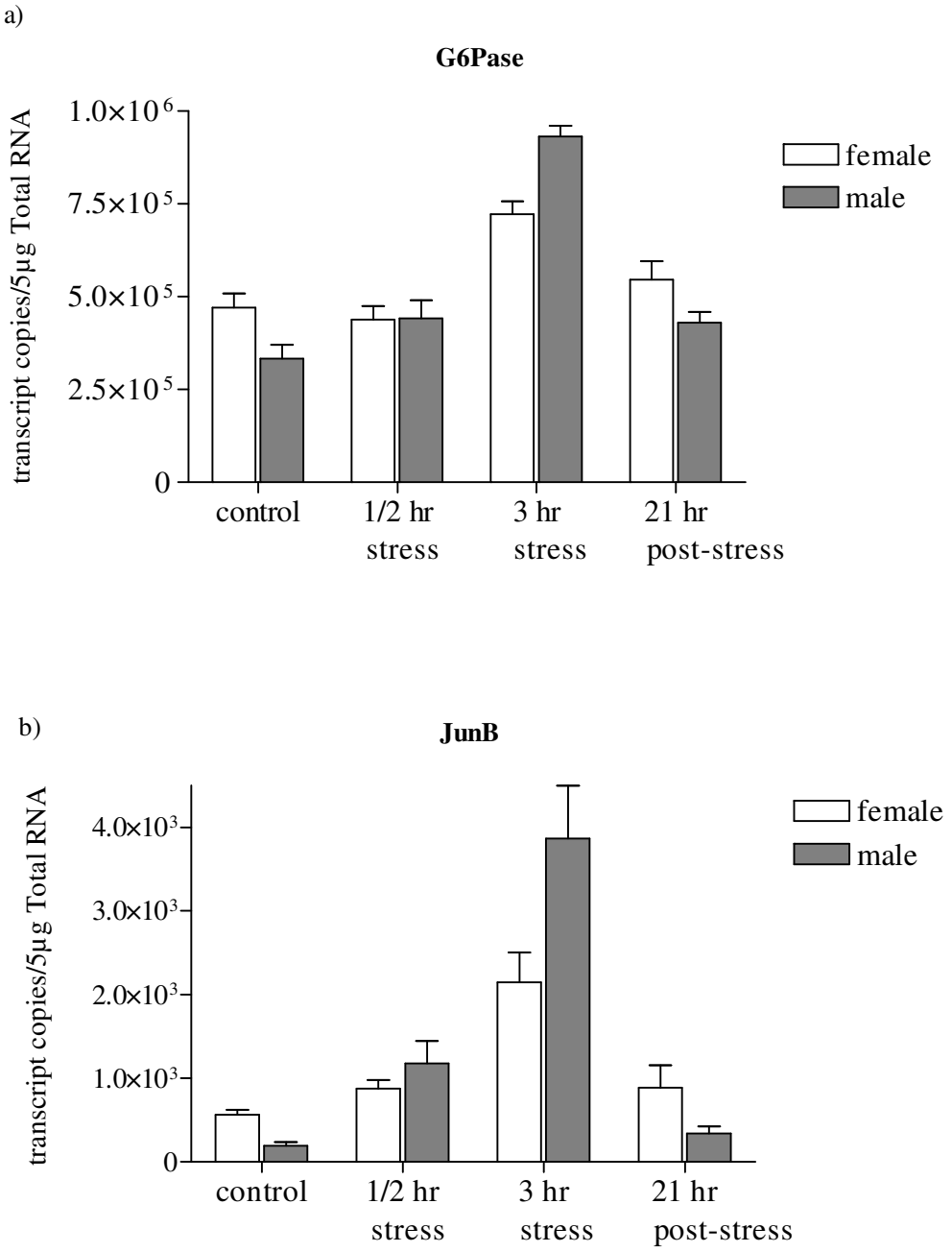

Figure 5. *G6Pase* and *JunB* expression in the 2nd experiment, grouped by sex. Bars are the means for each group ± s.e.m. For both *G6Pase* and *JunB* the effect of the treatments as well as the interaction variable of sex*treatment was significant (ANOVA P <0.001). For both *G6Pase* and *JunB* data: in the control, ½ hr stress and 21-hr post stress groups the female n=9 and the male n=6; in the 3 hr stress group the female n=8 and the male n=7.

The pattern of *JunB* expression was similar to that of *G6Pase* ($P < 0.001$). Transcripts were most abundant in the 3 hr samples with a 7.08-fold increase (±0.142 s.e.m) from control values. The control, ½ hr stress and 21 hr post-stress treatments were not different from each other ($P > 0.05$) (Figure 4b).

As with *G6Pase, JunB* expression appears to have been different between control males and control females (Figure 5b). Again, when sex was included as a variable in the ANOVA model, the interaction between sex and the stress treatments was significant ($P < 0.001$), although across all time points sex alone was non-significant statistically (ANOVA $P > 0.1$). This is because the sex with the greater expression in each treatment changed. The females in the control group had higher *JunB* expression than the males; this pattern was the same 21-hour post-stress. *JunB* expression in the 3 hr group was higher in males than in the females.

Nupr1 expression was 1.73-fold (±0.003 s.e.m) higher after three hours of stress compared to control levels. There was no difference between the control and the ½ hr treatments. The expression of *Nupr1* was 1.43-fold (±0.001 s.e.m) higher 21 hours post-stress than in fish that had experienced three hours of stress and 2.48-fold (±0.004 s.e.m) higher than control levels (See Figure 4c).

The expression of *MHCI* was not significantly different between any of the treatments in the second experiment ($P > 0.1$) (Figure 4d). These results are not consistent with what was found with the pooled RNA data; explanations are offered subsequently.

DISCUSSION:

Using an oligonucleotide microarray and pools of RNA from livers of control and stressed rainbow trout, we identified five genes that were consistently up-regulate during the stress response (Table 2): *G6Pase, JunB, Nupr1, MHCI* and *TNFDR*. Transcripts of *G6Pase*, *JunB*, *Nupr1* and *MHCI* were then measured using qPCR in individuals exposed to half-hour and three hours of stress as well as fish allowed to recover for 21 hours. Transcripts *G6Pase* and *JunB* were elevated in only the three-hour stress groups. However, *Nupr1* was upregulated after three hours of stress and even more so 21 hours post-stress. It was a surprise to observe this persistence of transcriptional activation as long as 21 hours after cessation of the stressor. As this protein acts is a transcription factor, its continued up-regulation suggests that other cellular/ physiological pathways are still affected at least this long after removal of the stressor. Elevated cortisol levels are indicative of stress (Barton and Iwama 1991), and the elevated cortisol levels evident in both experiments confirm that the fish were undergoing a stress response during the three hours of stress treatment. The fact that levels had not returned to resting, albeit considerably decreased from high levels, also suggests that the fish had not yet fully recovered from the stress 21 hours afterwards. The co-occurrence of elevated plasma cortisol and Nupr1 mRNA 21 hours after removal of a stressor may indicate a causative or interdependent relationship.

The transcription factor Nupr1 regulates the expression of genes related to cell growth and apoptosis (Mallo et al., 1997). This protein is expressed in several different tissues and has been found to respond to different stressors. In the liver, a deficiency of this protein has been linked to higher sensitivity to CCl_4 (Taieb et al.,

2005) and LPS (Vasseur et al., 2003) suggesting Nupr1 production contributes to the ability to tolerate such toxins. In the pancreas, increased Nupr1 has been correlated with increased glucose production (Päth et al., 2004), which increases the cell's resistance to stress, possibly through an anti-inflammatory pathway (Vasseur et al., 2004). However, mouse embryonic fibroblasts expressing Nupr1 had increased apoptosis and arrested growth in response to DNA damage compared to cells deficient in Nupr1 expression (Vasseur et al., 2002). Since the effect of this protein on the fate of the cell is context dependent (similar to *JunB*), we cannot be conclusive as to whether the increased expression of the protein in the trout liver was detrimental or beneficial. Furthermore, this protein has only recently been identified and is known only in mammals (Mallo et al., 1997 and Vasseur et al., 1999); accordingly, little is yet known of the downstream targets of this transcription factor (Malicet 2006).

G6Pase is an enzyme in the gluconeogenic/ glycolytic pathways and catalyzes the hydrolysis of glucose-6-phosphate into glucose, thus allowing glucose to be released into the blood. Therefore, this enzyme plays a critical role in glucose homeostasis (Mithieux, 1997). The finding that *G6Pase* was upregulated in fish exposed to stress for three hours was not surprising given what is known about stress effects on metabolic responses in fish. In the present study, *G6Pase*, cortisol and glucose all had similar trends in response to stress. The cortisol and glucose data are similar to that which has been previously reported for rainbow trout (Barton 2000). It is known that cortisol plays a direct role in stimulating gluconeogenesis in the fish liver (Mommsen et al., 1999). This observation is consistent with recent studies in both *Cyprinus carpio* L. and *Oreochromis mossambicus* (Dziewulska-Szwajkowska et

al. 2003 and Sunny et al., 2003, respectively) in which G6Pase protein increased in the liver following cortisol injections. Additionally, the decrease in *G6Pase* transcription 21 hours post stress is in accordance with the decreased blood glucose and cortisol levels.

The sex-specific differences we observed in the expression of *G6Pase* and *JunB* are suggestive of differential responses to stress in males and females, at the transcriptional level. This was not observed in the other two genes we studied. Our statistical analysis revealed the interaction between sex and treatment significantly affected the expression of both *JunB* and *G6Pase* in the second experiment. This suggests that the males responded differently to the stress treatments compared to the females. For both genes, females expressed more of each transcript than males in both the control and 21-hr post stress treatments. However, the reverse pattern existed in the 3 hr stress treatment, as males seem to have increased their transcription of more so than females. With little research done on this subject in fish, our results deserve more attention with follow up studies. Nevertheless, this highlights the need to take sex differences into account when measuring transcriptional responses.

Like *G6Pase*, the expression of *JunB* in the second experiment was unchanged after ½ hour of stress, was at its highest in the three hour stress group and had returned to its baseline level by 21 hours post stress. Whether or not the upregulation of this gene is beneficial or detrimental to the cell is unclear. JunB is a proto-oncogene that belongs to the Jun family of proteins that heterodimerize with members of Fos family of proteins to form the AP-1 transcription factor. JunB seems to inhibit the activity of AP-1, causing an arrest in cell growth and increased

apoptosis. However, JunB has different effects depending on which combinations of proteins dimerize with each other (see Shaulin and Karin 2002 for review) and its response is dependent on tissue and stressor (Pryzbyla-Zawislak et al 2005). In mice, the function of JunB is dependent on the concentrations of other Jun proteins within the cells (Passegue et al., 2002). The function of this protein in teleosts has not been confirmed, however its open reading frame (ORF) has been sequenced for several species, including rainbow trout (Goetz, F.W., Iliev, D.B., Liarte, C.Q., Planas, J.V. and MacKenzie, S., unpublished; GenBank accession # AY614595). Investigation of known downstream targets of JunB (Shaulin and Karin, 2001) might help illuminate other cellular/ physiological pathways that are affected by the stressor and during recovery.

MHCI molecules are members of the immunoglobulin superfamily of proteins. Their functions include the presentation to cells in the immune system of self antigen or antigens produced by intracellular pathogens. These molecules are produced by most cell types throughout the body and play an important role in the immunological identity of cells (York and Rock 1996). Little is known regarding the functions of these molecules in teleosts (Dixon and Stet 2001). However, increased expression has been seen following response to viral injections in rainbow trout (Hansen and La Patra, 2002).

Our data suggest the possibility of transcriptional activation of *MHCI* genes in response to stress. However, qPCR analysis of individual RNA extracts failed to replicate these findings. Given the outbred nature of the fish in this study, we must consider the possibility that our results are complicated by the high degree of

polymorphism in this gene family. For this reason, we designed PCR primers based on the least variable regions within the ORFs of these genes (Aoyagi et al. 2002), the α-3 domain, in order to increase the likelihood of quantifying all of the transcripts. The qPCR results leave open the possibility that these primers led to unequal amplification efficiencies of different alleles between individuals. The expression of the individuals in all the treatments had one or a few individuals with greater than $3x10^4$ copies and others with less than $1x10^4$ copies, with the remaining individuals containing copy numbers within or close to this range. The potential importance of increased expression of MHCI molecules in response to stress warrant further investigation, and that may be best approached by quantifying the molecules at the level of the proteome.

TNFDR gene expression increased in response to stress. This gene encodes a decoy receptor that is thought to compete with membrane bound tumor necrosis factor receptors in modulating the immune response (Kim et al., 2003). The mRNA for this gene was obtained from phytohaemagglutinin (PHA)-activated haematopoietic cells of rainbow trout, however very little is understood of its function (Liu et al., 2002). In *Salvelinus fontinalis*, the mRNA for this gene was upregulated in ovaries in response to phorbol ester treatments during ovulation (Bobe and Goetz 2000). In mammals, over 20 proteins have been identified in the TNFDR superfamily of receptors. Members of the family participate in a variety of processes such as cell cycle/death, inflammation, cytotoxicity and antiviral activity, as well as modulators of apoptosis (see Baker and Reddy 1998 for review).

Genes that were upregulated in one but not both experiments (Tables 2-6) reflect the biological variation in the stress response between the two different year classes of rainbow trout studied. The genes found to be upregulated in the first but not the second experiment were mostly immune-relevant genes: *C1q-like protein*, *haptoglobin, complement receptor 1, interferon inducible proteins 1&2* and *complement factor H*. Transcripts upregulated in the second but not the first experiment were *class I helical cytokine receptor number 21 (CRFA21)*, *trypsin III precursor* and *ferritin H-3*. Differences in the genes that were downregulated in the two experiments could be due to differences in development, sex ratios and size of fish used between the experiments.

GENERAL CONCLUSIONS:

The microarray has proven to be a useful tool for identifying genes in the rainbow trout liver that are transcriptionally altered during the stress response. Further qPCR analysis of candidate gene expression post-stress proved useful in development of hypotheses relevant to the mechanisms behind the physiology of recovery from a stressor. Although patterns of *G6Pase* and *JunB* gene expression suggested that, by 21 hours post-stress, the fish had recovered from the stressor, *Nupr1* gene expression suggested something quite different. The elevation of *Nupr1* as late as 21 hours after stress indicates that recovery was incomplete at that time. The differential expression of this transcription factor highlights the induction of pathways associated with cell growth and death, however, additional studies are needed in order to profile in greater detail the kinetics of transcriptional changes in response to stress. The continued elevation of *Nupr1* mRNA nearly a day after a relatively short stressor could make this gene a suitable indicator of the time necessary for full recovery following stress. Future work looking at the differential expression of these genes throughout 21 hours and more post-stress (i.e. up to two weeks) will shed light on the spectrum of responses to and recovery from stressors.

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APPENDICES

Appendix 1. Features up-regulated after 3 hours of stress in 1st experiment.

TIGR ID[a]	Feature ID	Fold Change	p-val[b]	Gene Name (accession #, species) [c]
TC61811	OmyOSU826	12.10	0.029	MHC class I antigen (**AY278455**; *Oncorhynchus mykiss*)
TC65531	OmyOSU804	8.84	0.002	Transcription factor JunB (**AJ511783**; *Fugu rubripes*)
TC61800	OmyOSU758	8.32	0.004	MHC class I heavy chain precursor (**AF287492**; *O. mykiss*)
TC81254	OmyOSU796	7.95	0.001	MHC class I heavy chain precursor, (**AF287490**; *O. mykiss*)
TC81254	OmyOSU293	7.27	0.004	MHC class I heavy chain precursor, (**AF287490**; *O. mykiss*)
TC81254	OmyOSU824	6.25	0.016	MHC class I heavy chain precursor (**AF287490**; *O. mykiss*)
TC64777	OmyOSU371	4.00	0.001	C1q-like adipose specific protein *(***AF394686***; Salvelinus fontinalis*)
TC57168	OmyOSU1622	3.74	0.004	Tumor necrosis decoy factor receptor (**AF401631**; *O. mykiss*)
CA387113	OmyOSU1486	3.70	0.012	MHC class I a region (**AB162342**; *O. mykiss*)
TC78878	OmyOSU882	3.57	0.008	MHC class I heavy chain precursor (**AF296371**; *O. mykiss*)
TC78878	OmyOSU816	2.91	0.032	MHC class I heavy chain precursor (**AF296371**; *O. mykiss*)
TC65784	OmyOSU880	2.16	0.048	MHC class I heavy chain (**AF296366**; *O.mykiss*)
TC63296	OmyOSU86	1.96	0.026	Glucose-6-phosphatase (**XM_702785**; *Danio rerio*)
TC62635	OmyOSU454	1.85	0.036	complement receptor-like protein 1 (**AJ620466**; *O.mykiss*)
TC54512	OmyOSU821	1.83	0.021	Haptoglobin (**AF279136**; *O. mykiss*)
TC46690	OmyOSU1663	1.77	0.004	Nuclear protein 1 (protein p8) (**NM_053611**; *Rattus norvegicus*)
TC86967	OmyOSU983	1.74	0.008	Interferon inducible protein 1 (**AJ291989**; *O. mykiss*)
TC69545	OmyOSU1292	1.74	0.041	Growth hormone inducible soluble protein (**XM_686083**; *D. rerio*)
TC46330	OmyOSU1358	1.65	0.021	Antifreeze protein (**AY584595**; *Gadus morhua*)
TC57084	OmyOSU165	1.62	0.047	Interferon Inducible Protein 2 (**AJ320157**; *O. mykiss*)
TC50776	OmyOSU1194	1.60	0.032	Tyrosine aminotransferase (**XM_684816**; *D. rerio*)
TC46690	OmyOSU1662	1.59	0.032	Nuclear protein 1 (protein p8) (**NM_053611**; *R. norvegicus*)
TC68275	OmyOSU1060	1.55	0.029	Signal sequence receptor alpha (**AY115484**; *D. rerio*)
NP544030	OmyOSU792	1.53	0.032	MHC class I heavy chain precursor (**AF115522**; *O. mykiss*)
TC62634	OmyOSU346	1.52	0.048	complement receptor-like protein 1 (**AJ620465**; *O. mykiss*)
TC61950	OmyOSU1426	1.44	0.021	Complement factor H protein (**AJ505940**; *O. mykiss*)
TC61950	OmyOSU1425	1.43	0.032	Complement factor H protein (**AJ505940**; *O. mykiss*)
TC54511	OmyOSU659	1.24	0.021	Haptoglobin fragment 2 (**AF279136**; *O. mykiss*)

Appendix 1

[a]TIGR ID number of the tentative consensus or singleton expressed sequence tag (EST) sequence corresponding to OSUrbt ver. 2 microarray feature (http://www.science.oregonstate.edu/mfbsc/facility/micro.htm).

[b]Mann-Whitney Advanced U-test p-values

[c]Information on putative functions of the genes was obtained through the TIGR database (www.tigr.org) for each particular TIGR ID then was re-examined through the BLASTX (www.ncbi.org) database. The significant BLASTX result is shown (E-value<10^{-6}).

Appendix 2. Features down-regulated after 3 hours of stress in 1st experiment.

TIGR ID[a]	Feature ID	Fold Change	p-val[b]	Gene Name (accession #, species) [c]
TC52976	OmyOSU683	1.70	0.0130	C-type lectin 2-2 (**Q8JJ68;** *O. mykiss*)
TC52976	OmyOSU636	1.76	0.0325	C-type lectin 2-2 (**Q8JJ68;** *O. mykiss*)
TC54970	OmyOSU638	1.60	0.0325	C-type lectin 2-2 (**Q8JJ68;** *O. mykiss*)

[a]TIGR ID number of the tentative consensus or singleton expressed sequence tag (EST) sequence corresponding to OSUrbt ver. 2 microarray feature (http://www.science.oregonstate.edu/mfbsc/facility/micro.htm).

[b]Mann-Whitney Advanced U-test p-values

[c]Information on putative functions of the genes was obtained through the TIGR database (www.tigr.org) for each particular TIGR ID then was re-examined through the BLASTX (www.ncbi.org) database. The significant BLASTX result is shown (E-value<10^{-6}).

Appendix 3. Features up-regulated after 3 hours of stress in 2nd experiment based on our most stringent criteria.

TIGR ID[a]	Feature ID	Fold Change	p-val[b]	Gene Name (accession #, species) [c]
TC65531	OmyOSU804	7.64	0.0357	Transcription factor JunB (**AJ511783**; *F. rubripes*)
TC57168	OmyOSU1622	4.06	0.0022	Tumor necrosis decoy factor receptor (**AF401631**; *O. mykiss*)
CA387113	OmyOSU1486	3.26	0.0002	MHC class I a region (**AB162342**; *O. mykiss*)
TC65784	OmyOSU880	2.83	0.0079	MHC class I heavy chain (**AF296366**; *O.mykiss*)
TC81254	OmyOSU796	2.08	0.0111	MHC class I heavy chain precursor, (**AF287490**; *O. mykiss*)
TC54221	OmyOSU844	1.68	0.0416	Trypsin III precursor (**X70074**; *Salmo salar*)
TC86600	OmyOSU1489	1.46	0.0385	Ferritin H-3 (**D86627**; *O.mykiss*)
TC63296	OmyOSU86	1.74	0.0000	Glucose-6-phosphatase (**XM_702785**; *D. rerio*)
TC69557	OmyOSU1220	1.59	0.0004	Unnamed protein product (**CAAE01007602**; *Tetraodon nigroviridis*)
TC46690	OmyOSU1662	1.42	0.0002	Nuclear protein 1 (protein p8) (**NM_053611**; *R. norvegicus*)
TC46690	OmyOSU1663	1.49	0.0004	Nuclear protein 1 (protein p8) (**NM_053611**; *R. norvegicus*)
TC57859	OmyOSU56	1.42	0.0004	Class I helical cytokine receptor number 21 (CRFA21) (**AY374493**; *T. nigroviridis*)

[a]TIGR ID number of the tentative consensus or singleton expressed sequence tag (EST) sequence corresponding to OSUrbt ver. 2 microarray feature (http://www.science.oregonstate.edu/mfbsc/facility/micro.htm).

[b]Mann-Whitney Advanced U-test p-values

[c]Information on putative functions of the genes was obtained through the TIGR database (www.tigr.org) for each particular TIGR ID then was re-examined through the BLASTX (www.ncbi.org) database. The significant BLASTX result is shown (E-value<10^{-6}).

Appendix 4. Features up-regulated after 3 hours of stress in 2nd experiment, based on statistical significance at $P<0.05$.

TIGR ID[a]	Feature ID	Fold Change	p-val[b]	Gene Name (accession #, species) [c]
TC57859	OmyOSU272	1.38	0.0008	Class I helical cytokine receptor number 21 (**AY374493**; *T. nigroviridis*)
TC69642	OmyOSU1490	1.32	0.0177	Envelope protein (**NC_007654**; *S. salar*)
TC69632	OmyOSU1388	1.32	0.0364	Glutathione peroxidase (**AY216589**; *D.rerio*)
TC78484	OmyOSU333	1.31	0.0206	Adipose differentiation-related protein (**AF234676**; *Sus scrofa*)
TC55460	OmyOSU1552	1.29	0.0315	Vitelline envelope protein gamma (**AF231708**; O.mykiss)
TC86911	OmyOSU579	1.28	0.0144	Beta-2 microglobulin (**L63537**; *O. mykiss*)
TC81254	OmyOSU824	1.28	0.0093	MHC class I heavy chain precursor (**AF287490**; *O. mykiss*)
TC47641	OmyOSU921	1.25	0.0411	Vascular cell adhesion protein 1 (V-CAM 1) (**XM_689331**; *D. rerio*)
TC50468	OmyOSU89	1.24	0.0260	Tetraspanin-7 (**NM_019634**; *Mus musculus*)
TC54503	OmyOSU1447	1.24	0.0137	Immunoglobulin light chain L2 (**U69987**; *O. mykiss*)
TC46942	OmyOSU91	1.23	0.0152	FcRI (Fc receptor 1) (**DQ286290**; *Ictalurus punctatus*)
TC61811	OmyOSU826	1.22	0.0315	MHC class I antigen (**AY278455**; *O. mykiss*)
TC46867	OmyOSU1181	1.22	0.0476	Alpha-1-antiproteinase precursor (**XM_702628**; *D. rerio*)
TC87189	OmyOSU674	1.22	0.0190	Interleukin 18 (**AJ781817**; *O. mykiss*)
TC49431	OmyOSU992	1.21	0.0367	similar to mal, T-cell differentiation protein 2 (**XM_692569**; *D.rerio*)
TC62028	OmyOSU1661	1.19	0.0216	Apolipoprotein E (**AJ132620**; *O. mykiss*)
TC50756	OmyOSU9	1.19	0.0232	Androgen receptor alpha (**AB012095**; *O. mykiss*)
TC78031	OmyOSU1575	1.18	0.0296	Immunoglobulin light chain (**AF104898**; *G. morhua*)
TC70047	OmyOSU603	1.17	0.0357	Chaperonin containing TCP1, subunit 5 (**AY398321**; *D. rerio*)
TC54764	OmyOSU449	1.15	0.0116	Apolipoprotein B (**XM_689735**; *D. rerio*)
TC60092	OmyOSU1059	1.15	0.0082	Mx3 protein (antiviral activity) (**U47946**; *O. mykiss*)
CA358547	OmyOSU588	1.15	0.0325	Unnamed protein product (**CAAE01010490**; *T. nigroviridis*)
TC70092	OmyOSU1652	1.14	0.0116	Apolipoprotein E (**AJ132620**; *O. mykiss*)
TC69319	OmyOSU1250	1.12	0.0406	Transposase (**AJ249085**; *Pleuronectes platessa*)
BX318408	OmyOSU1469	1.11	0.0315	Cathepsin S cysteine protease (**AY950578**; *Paralichthys olivaceus*)

Appendix 4

[a]TIGR ID number of the tentative consensus or singleton expressed sequence tag (EST) sequence corresponding to OSUrbt ver. 2 microarray feature (http://www.science.oregonstate.edu/mfbsc/facility/micro.htm).

[b]Mann-Whitney Advanced U-test p-values

[c]Information on putative functions of the genes was obtained through the TIGR database (www.tigr.org) for each particular TIGR ID then was re-examined through the BLASTX (www.ncbi.org) database. The significant BLASTX result is shown (E-value<10^{-6}).

Appendix 5. Features downregulated in 2nd experiment, based on statistical significance at $P<0.05$.

TIGR ID[a]	Feature ID	Fold Change (down)	p-val[b]	Gene Name (accession #, species) [c]
TC59432	OSUrbt2_749_MMP19	1.60	0.0026	Hypothetical protein
TC61995	OmyOSU46	1.37	0.0215	glutathione peroxidase type 2 (**AY622862**; *O.mykiss*)
CB489832	OmyOSU67	1.35	0.0116	glutathione peroxidase type 2 (**AY622862**; *O.mykiss*)
TC54593	OmyOSU667	1.33	0.0005	Alpha-globin IV (**Q98974**; *O.mykiss*)
TC61798	OmyOSU1507	1.31	0.0045	Glutathione S-transferase (**Q9W647**; *Oncorhynchus nerka*)
TC62174	OmyOSU665	1.29	0.0467	Alpha-globin I (**Q98973**; *O.mykiss*)
TC61798	OmyOSU971	1.27	0.0093	Glutathione S-transferase (**Q9W647**; *Oncorhynchus nerka*)
TC70155	OmyOSU1557	1.23	0.0116	SPARC protein (**Q9YGD9**; *O.mykiss*)
TC86622	OmyOSU1555	1.23	0.0212	CD9 antigen (p24) (**AAH59691**; *D.rerio)*
TC86622	OmyOSU972	1.22	0.0177	Lysozyme type II (**AAL48290**; *O.mykiss*)
TC54300	OmyOSU1599	1.22	0.0034	Calmodulin (**Q6YNX6**; *Ovis aries*)
CA342942	OmyOSU763	1.21	0.0019	unnamed protein product
CA376466	OmyOSU658	1.19	0.0073	Mitogen-activated protein kinase 10 (**XP_883225**; *Bos taurus*)
TC54774	OmyOSU85	1.15	0.0177	Proteasome subunit beta 7 (XP_682902; D. rerio)
TC50138	OmyOSU35	1.15	0.0326	angiotensin converting enzyme (**XP_694336**; *D. rerio*)
??	OmyOSU4	1.14	0.0086	alpha-2-macroglobulin
TC48089	OmyOSU1471	1.11	0.0286	immunoglobulin light chain (**BAB91008**; *Cyprinus carpio*)

Appendix 5

[a]TIGR ID number of the tentative consensus or singleton expressed sequence tag (EST) sequence corresponding to OSUrbt ver. 2 microarray feature (http://www.science.oregonstate.edu/mfbsc/facility/micro.htm).

[b]Mann-Whitney Advanced U-test p-values

[c]Information on putative functions of the genes was obtained through the TIGR database (www.tigr.org) for each particular TIGR ID then was re-examined through the BLASTX (www.ncbi.org) database. The significant BLASTX result is shown (E-value $<10^{-6}$).