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Transcription Profiling in Environmental Diagnostics: Health Assessments in Columbia River Basin Steelhead (*Oncorhynchus mykiss*)

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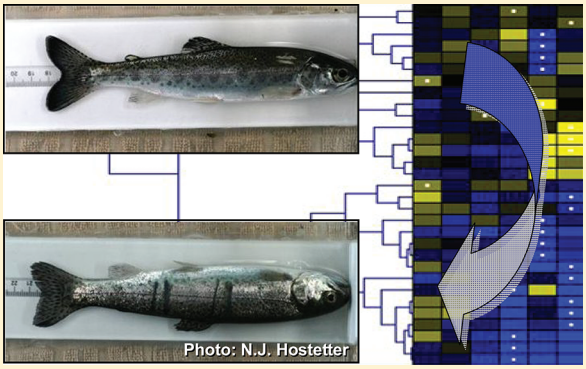
ABSTRACT: The health condition of out-migrating juvenile salmonids can influence migration success. Physical damage, pathogenic infection, contaminant exposure, and immune system status can affect survival probability. The present study is part of a wider investigation of out-migration success in juvenile steelhead (*Oncorhynchus mykiss*) and focuses on the application of molecular profiling to assess sublethal effects of environmental stressors in field-collected fish. We used a suite of genes in *O. mykiss* to specifically assess responses that could be directly related to steelhead health condition during out-migration. These biomarkers were used on juvenile steelhead captured in the Snake River, a tributary of the Columbia River, in Washington, USA, and were applied on gill and anterior head kidney tissue to assess immune system responses, pathogen-defense (NRAMP, Mx, CXC), general stress (HSP70), metal-binding (metallothionein-A), and xenobiotic metabolism (Cyp1a1) utilizing quantitative polymerase chain reaction (PCR) technology. Upon capture, fish were ranked according to visual external physical conditions into good, fair, poor, and bad categories; gills and kidney tissues were then dissected and preserved for gene analyses. Transcription responses were tissue-specific for gill and anterior head kidney with less significant responses in gill tissue than in kidney. Significant differences between the condition ranks were attributed to NRAMP, MX, CXC, and Cyp1a1 responses. Gene profiling correlated gene expression with pathogen presence, and results indicated that gene profiling can be a useful tool for identifying specific pathogen types responsible for disease. Principal component analysis (PCA) further correlated these responses with specific health condition categories, strongly differentiating good, poor, and bad condition ranks. We conclude that molecular profiling is an informative and useful tool that could be applied to indicate and monitor numerous population-level parameters of management interest.



INTRODUCTION

Populations of steelhead trout (*Oncorhynchus mykiss*) in the Columbia River basin have declined in number over the past decades.[1] Recovery of these populations has been limited by a number of environmental stressors, including, but not limited to, hydroelectric development, habitat loss and degradation, poor water quality, disease, and predation.[2−5] Consequently, throughout the West coast of the United States, steelhead trout are listed as either endangered or threatened under the U.S. Endangered Species Act (ESA),[6] dependent on geographic locations.

Pathogenic infections in juvenile steelhead are of particular concern. Bacterial kidney disease caused by *Renibacterium salmoninarum* infections, for example, has reportedly contributed to salmonid mortality in hatcheries in the Columbia River basin.[7] Infections and subsequent effects on the immune system can hinder the ability of fish to contend with other environmental stressors and thus survival and migration success. Moreover, there is growing concern that contaminant exposure may affect salmonid populations either directly or by further increasing susceptibility to disease.[8,9]

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Table 1. Primer and Probe Sequences of Genes Used As Molecular Biomarkers to Determine Health Status in Juvenile Steelhead (*Oncorhynchus mykiss*)[a]

gene	accession no.	primer sequences	amplicon size (bp)	Roche probe no. and sequence
NRAMP	AF054808	F: GACATGGACAGCGACACGAT	96	25
		R: TGTGAATTCTCTCTAGCTACGCGATG		TGGAGGAG
MX	U30253	F: GACAGCACCTACAGCCACAGTC	60	144
		R: AGGGTTGGTCTTCGTCCTCC		GGAAGAGG
CXC	AF396869	F: ATGACATCAACGGTCCTCATCA	64	68
		R: CACCTGGCCTTCAACATTGG		CTGCTCCT
HSP70	AB176854	F: GGAGATCGCTGAGGCTTACCT	60	163
		R: AGGCAGGGACTGTGATGACTG		GGTGTCCA
Met-A	M18103	F: GCGCATGCACCAGTTGTAAG	73	56
		R: CAGCCTGAGGCACACTTGC		TGCTGTCC
Cyp1a1	U62796	F: TCAACCCGTGGCAGGTCAAC	67	12
		R: AAAAGGTCAGGGTTGAATGAAG		GGAAGGAG
TAF12[b]	NM_001160631	F: GACCTGGTCCTTACGACGACAG	63	115
		R: GACCTAGGATGTCAGATGGTGGT		GACCCAGA

[a]NRAMP (natural resistance-associated macrophage protein), MX (myxovirus resistance), Met-A (metallothionein-A), CXC (chemokine), HSP70 (heat-shock protein 70KDa), Cyp1a1 (Cytochrome P450, family 1, subfamily A, polypeptide 1), TAF12 (TAF12 RNA polymerase II, TATA box binding protein (TBP)-associated factor). [b]Reference gene.

Determining the sublethal effects of environmental stressors in field-collected fish is a major challenge. Molecular biomarkers are becoming increasingly powerful tools that can be used to assess both infection and exposure to contaminants,[10] along with the effects of numerous environmental stressors upon organisms at risk.[11,12] Gene expression profiling has received much attention in the medical arena as a therapeutic predictive tool.[13] Functional classifications have enabled identification of affected biochemical pathways and the modes of action of chemicals[14] and, in recent years, such approaches have been successfully applied in ecological contexts, for example to assess effects of exposure and identify contaminants involved through known modes of action.[15,16] These studies have provided promising outcomes toward health status assessments in ecosystems with some studies linking gene expression with population level effects.[17−20]

The application of genome-wide technologies, such as microarrays, has permitted comprehensive approaches to understanding changes in physiological status.[14,18,21] Such studies can be time-consuming and laborious, especially when classifying molecular functions of thousands of responding genes. Furthermore, microarray data are subjected to global normalization processes that reduce statistical robustness requiring that expression differences be validated through the use of quantitative PCR (q-PCR) techniques.[22] Thus, microarrays are primarily used as screening tools; generating preliminary data for subsequent assessments to be verified and conducted using a relatively small subset of genes, selected to represent the ascertained genome-wide functional classifications. Study-specific approaches can effectively be performed using carefully selected genes to assess the health status and stress responses in individuals. Responses can be extrapolated to population levels by integrating multiple levels of biological organization such as histopathological, visual annotation of exterior physiological features, and probabilistic survival.

We present in this study the use of a suite of genes developed in *O. mykiss*, preselected toward a small-scale and preliminary assessment of the effects of pathogen infection and contaminant exposure. Genes indicating immune system responses, pathogen defense, metal sequestration, xenobiotic metabolism, and general stress, expressed in gill and anterior head kidney,[23] were assessed in tissue samples of out-migrating juvenile steelhead from the Snake River currently listed as threatened under the U.S. Endangered Species Act.[24] Kidney and gill samples were used to compare lethal and possible nonlethal (e.g., gill biopsies) sampling approaches, respectively. Data from this study provides new information and options for the future design of monitoring programs and recovery efforts for this Snake River steelhead.

EXPERIMENTAL SECTION

Fish Collection and Tissue Sampling. Fish collection and tissue sampling are described in detail in Hostetter et al.,[25] hereon referred to as the parent study. In brief, run-of-the-river juvenile steelhead out-migrating from the Snake River basin, were captured at the Lower Monumental Dam fish collection facility, Washington, USA. Collected fish were tagged with passive integrated transponders (PIT), weighed, measured, and classified by physical appearance into good, fair, poor, and bad health status. The PIT-tagged fish were released back into the river system through the juvenile bypass facility out-flow pipe. Survival probabilities of PIT-tagged steelhead were estimated using data collected at sites downstream of Lower Monumental Dam; Ice Harbor Dam, McNary Dam, John Day Dam, Bonneville Dam. Heavy metal analyses were conducted on select fish samples, however, concentrations were either below the limits of detection or were not determined to be significantly different between ranks, thus results are not shown.

Of the total fish caught, 60 individuals from each condition rank were euthanized and samples of steelhead's gills, kidney, liver, spleen, heart, and gastrointestinal tract were stored in RNAlater solution (Qiagen, Valencia, California, USA) and analyzed for the presence and absence of pathogens. Two pathogens were identified by PCR: *Renibacterium salmoninarum* (Rb) and infectious hematopoietic necrosis virus (IHNV). Histopathology analyses were used to identify six clinical signs of disease: hepatic sanguinicoliasis (HS), renal sanguinicoliasis (RS), branchial amebiasis (BA), branchial sanguinicoliasis (BS), mycotic bronchitis (MB), and mycotic dermatitis (MD).

Gill and anterior head kidney tissue samples from eight fish randomly selected from each condition rank, thus not

necessarily from the same individual, were subsequently used in gene transcription analysis as described below.

RNA Extraction and cDNA Synthesis. Total RNA from steelhead anterior head kidney and gill samples was extracted using a Qiagen RNeasy Mini kit following the manufacturer's protocols, with on-column DNase digestion to remove any traces of genomic DNA. RNA concentrations were determined using a NanoDrop ND1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA), total RNA 260/280 and 260/230 ratios ranged between 1.82 and 2.21 and 1.79 and 2.15, respectively. Total RNA integrity was verified through electrophoresis on a 1% agarose gel. Complementary DNA (cDNA) was synthesized using 1 μg total RNA, with 50 units of Superscript III (Superscript III Reverse Transcriptase – Invitrogen, Carlsbad, CA, USA), 600 ng random primers, 10 units of RNaseOut, and 1 mM dNTPs (Invitrogen) to a final volume of 20 μL. Reactions were incubated for 50 min at 50 °C followed by a 5 min denaturation step at 95 °C. Samples were diluted 3 fold with the addition of 40 μL nuclease-free water to a total volume of 60 μL for subsequent real-time PCR assessments.

Real-Time TaqMan PCR. Selected genes are shown in Table 1. Anterior head kidney plays an important role in immune function of fish, thus genes were chosen due to their involvement in immune system functioning and pathogen-defense (MX protein and NRAMP), virus infection signaling/immune surveillance (CXC chemokine). Additional genes known to be expressed in both gill and head kidney[23] involving metal sequestration (metallothionein), xenobiotic metabolism (Cyp1a), and general stress (HSP70) were selected to enhance transcription profiling and to investigate the likelihood of other stressors contributing to decreased health. TAF12 RNA polymerase II, a TATA box binding protein (TBP)-associated factor, was identified using GeNorm[26] as a suitable reference gene for this assessment. Primer pairs and fluorescent probes for real-time TaqMan PCR were designed using Roche Applied Science Universal Probe Library Assay Design, and melting temperatures were verified on *Primer Express* software v2.0 (Applied Biosystems, Foster City, CA, USA). The efficiency of the primers used ranged between 89 and 108%. Real-time TaqMan PCR mixes contained 400nM of each of two primers and 80nM of the appropriate TaqMan probe. We used TaqMan Universal PCR Mastermix (Applied Biosystems, Foster City, CA, USA) containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphates, 0.625U AmpliTaq Gold DNA polymerase per reaction, 0.25U AmpErase UNG per reaction, and 5 μL of the diluted cDNA sample in a final volume of 12 μL. Samples were placed in 384 well plates and cDNA was amplified in an automated fluorometer (ABI HT 7900 A FAST Sequence Detection System, Applied Biosystems). Amplification conditions were 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 60 s at 60 °C. Fluorescence signals were collected during annealing temperature and were considered positive if fluorescence intensity exceeded 10 times the standard deviation of the baseline fluorescence. *SDS 2.2.1* software (Applied Biosystems) was used to quantify transcription.

Data Analyses. Quantitative PCR data was analyzed using the relative quantification $2^{(-\Delta\Delta Ct)}$ method.[27] Data are reported as the $\log_2$ gene transcription relative to TAF12 and normalized to the mean transcription of each gene corresponding to the good status classification, on the assumption that this rank was indicative of the best health status. Eight gill and eight kidney samples from each condition were assessed. To fully assess, interpret, and validate the applicability of transcription data, we present the data in three different formats. First, we used conventional mean responses per classification group, with $\log_2$ data assessed for statistical significance between classification ranks using Kruskal–Wallis with Dunns' Multiple Comparison Test relative to the mean of good status fish (*Prism 4.0*, GraphPad Software, CA, USA). Second, we used gene profiling based on per gene and per fish response correlation, using Log_2 normalized qPCR data obtained for either gill or anterior head kidney from each individual fish, which were subjected to agglomerative hierarchical clustering using Genesis software version 1.7.5.[28] Average dot product metric, with complete linkage clustering, was used to generate a heatmap profile of gene expression and physiological health status. Third, principal component analysis (PCA) was conducted on the ΔCt data set, relative to TAF12, (i.e., not normalized to good status fish), using IBM *PASW Statistics 18.0* (SPSS Inc., 1993–2007) to further assess, confirm, and interpret gene profiling differences between the attributed physiological ranks.

RESULTS AND DISCUSSION

Transcription responses were tissue-specific for gill and anterior head kidney (Figure 1). Gene responses in anterior head kidney

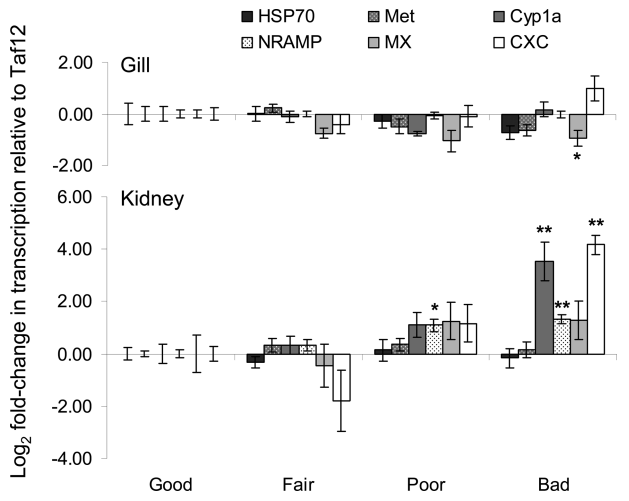

Figure 1. Mean $\log_2$ fold-change in gene transcription, per visual physical appearance classification. HSP70 (heat-shock protein 70KDa), MT (metallothionein-A), Cyp1a1 (Cytochrome P450, family 1, subfamily A, polypeptide 1), NRAMP (natural resistance-associated macrophage protein), MX (myxovirus resistance), CXC (chemokine). Bars indicate standard errors. Levels of significance ($p < 0.05$ and $p < 0.01$) are represented by * and ** respectively, comparable to good rank classification.

were distinctly different among health classification ranks, identifying xenobiotic detoxification (upregulation of Cyp1a) and immune system responses (upregulation of NRAMP and CXC) as the primary responses compared to those in fish classified in good condition. Responses in gill differentiated between good and bad conditions with a significant down-regulation of MX. Responses to infection are suggested not only by the changes in transcription of NRAMP, MX, and CXC but also in combination with the upregulation of Cyp1a1, which is involved in xenobiotic biotransformation and detoxification of many polycyclic aromatic hydrocarbons (PAHs) polychlorinated biphenyls (PCBs) and dioxins, which have been shown to

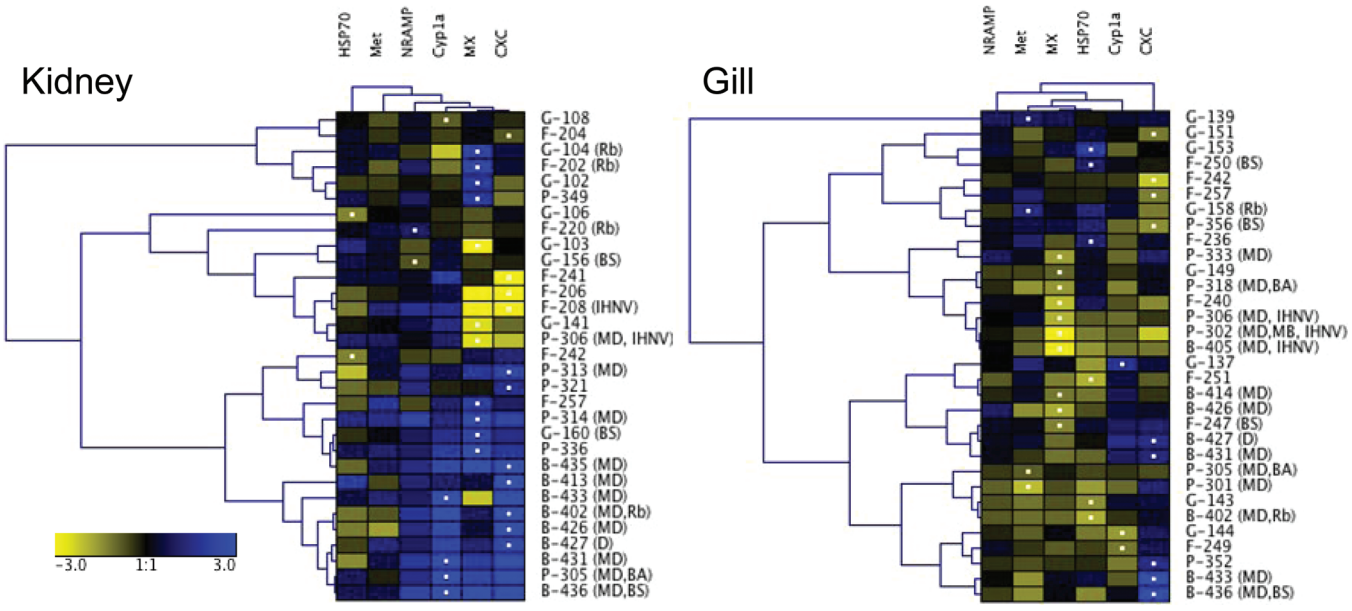

Figure 2. Gene profiling: Hierarchical clustering based on average dot product of $\log_2$ gene transcription gill and anterior head kidney gene expression data with individual juvenile steelhead labeled correspondingly to visually assigned health status. HSP70 (heat-shock protein 70KDa), MT (metallothionein-A), Cyp1a1 (Cytochrome P450, family 1, subfamily A, polypeptide 1), NRAMP (natural resistance-associated macrophage protein), MX (myxovirus resistance), CXC (chemokine). Red indicates up-regulation and green down-regulation, relative to the control group mean (good condition steelhead). Fish labels are presented as condition (G, good; F, fair; P, poor; B, bad), numbers as attributed to fish upon capture and classification. Pathogen detection is depicted as follows: Rb, *Renibacterium salmoninarum*; IHNV, infectious hematopoietic necrosis virus; BS, branchial sanguinicoliasis; BA, branchial amebiasis; MD, mycotic dermatitis; MB, mycotic bronchitis. White dots indicate absolute maximum value of respective genes.

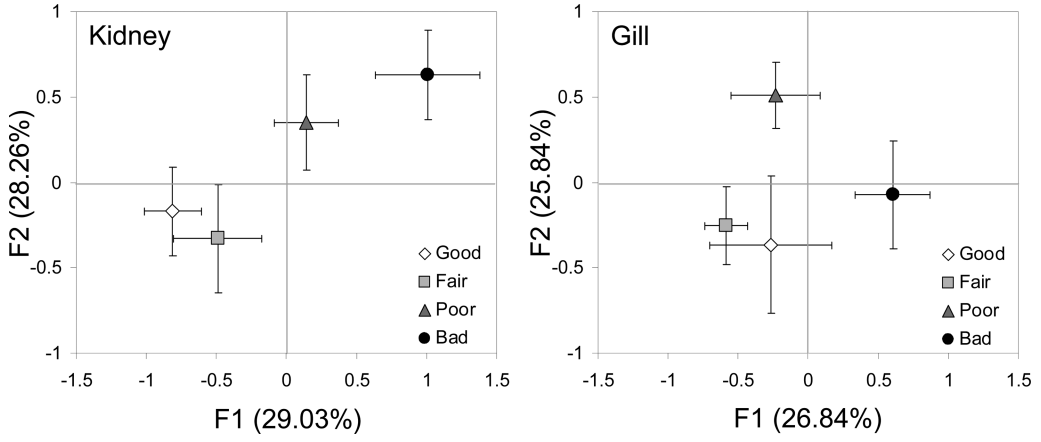

Figure 3. Principal component analysis: Percentage contribution of factors F1 and F2 to variability attributed per juvenile steelhead trout, per visual physical appearance classification.

suppress immune responses.[29] Though not assessed in the present study, these and other contaminants have been associated with the decline in stocks of salmon and trout in the Snake River.[30]

Hierarchical cluster analysis using individual fish gene transcription data (Figure 2) were conducted separately for each tissue type. Profile responses from kidney were successful in identifying transcriptional differences between bad status and the remaining classifications, and by and large gene profiles between poor status and good or fair were differentiated. There was a strong overlap between good and fair, which corroborate lack of significant differences observed in the mean responses of each classification rank (Figure 1). Profile responses in gills however, were not as successful in discriminating between the groups, although there is trend from good to bad.

Corresponding pathogen presence-absence assessments from Hostetter et al.[25] with transcription responses indicate that organisms displaying mycotic dermatitis correlated strongly with fish in bad condition. Renibacterium was detected in fish classified as good and fair (G-104, G-158F-202, F-220) and a single fish classified as bad (B-402), yet the presence of this pathogen did not appear to alter the gene response with respect to their visual condition. Fish in which IHNV was detected clustered together (both for kidney and gill samples) suggesting that different infection types may yield specific gene response patterns and that these profiles could be used to distinguish between these two pathogens. Interestingly, the gene encoding for MX protein, which was significantly downregulated in fish where IHNV was detected, has been postulated as a reliable indicator of viral exposure.[10,23]

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The presence of internal pathogens and clinical signs of disease was assessed in the parent study, in which Hostetter et al.[25] indicate that 48% of the sampled fish tested positive for internal pathogens or disease, and the odds of poor internal condition were 5.6 times greater for steelhead classified visually as in poor or bad condition compared to steelhead classified as in good condition. The parent study also indicated that probability of in-river survival was dependent on health status as attributed by visual rank classification, and in-river survival data indicated that the odds of survival for juvenile steelhead classified as in poor or bad condition were 1.7 times less than those ranked as in good condition. Fish classified as in good condition were not in their entirety free of pathogen infection. PCR analysis identified the presence of *R. salmoninarum* in approximately 25% of the sampled fish. Histopathology assessments determined a predominance of mycotic dermatitis approximating 80% among fish in bad condition.

Principal component analysis of gene expression data was successful in differentiating health classifications status according to Hostetter et al.[25] (Figure 3). Both PCA factors 1 and 2 on kidney transcription data successfully differentiated between bad and poor status separating them from the overlapping fair and good groups. For gill transcription data, PCA factor 1 separated bad status from the other three, and PCA factor 2 separated poor status from the rest.

Both PCA and hierarchical clustering suggest that gene assessments support the visual classification system devised in the parent study[25] with mean transcription differences corresponding to each classification. However, hierarchical clustering of individual fish responses indicate that some fish that were visually classified as in good and fair condition represent statistical outliers to this classification. Several of these outliers, though not all, were demonstrated by both PCR and histopathology techniques to be infected fish. Others represent organisms whose visual external appearance did not directly correspond to internal condition, or were infected by pathogens or conditions not determined in this study. These results are further supported by the PCA assessments that were not only successful in separating the different health conditions but also highlighted overlaps and response variation among the different visual classification ranks. Furthermore, in the parent study, classifying bad fish as compromised was relatively straightforward. Classifying good fish as uncompromised was not as simple due to the likelihood of the visual examination technique missing an alternative and important metric. Thus, transcription data helps toward a better understanding of why some differences (e.g., survival or internal condition) that were observed in Hostetter et al.[25] were not always as large as expected when comparing compromised with uncompromised groups. This outcome suggests that inclusion of transcription profiling assessments can effectively complement the visual health status prediction under circumstances where lethal sampling is a permissible approach, or likely through nonlethal, biopsy, sampling.

Gene profiling approaches have successfully been carried out using molecular techniques, identifying specific signatures to chemical contaminants.[20,31−34] These studies have utilized genome-wide responses (microarrays) to generate profiles, rather than a selected suite of genes, but all have subsequently verified responses of a subset of genes with quantitative PCR techniques. The present study suggests that the health status of organisms can be assessed using a small suite of genes. Although the present research was somewhat limited in the number of genes, and tissue samples assessed, strong correlations were found between transcription responses and pathogenic infection, which in turn were linked to survival probabilities in the parent study.

Genes were selected to generate transcription profiles, developing approaches to assess differences between health classifications, as opposed to individual biomarker assessments. MX protein, NRAMP, and CXC are known to express in response to effects on the immune system and are found in both kidney and gill tissues. Metallothionein, cyp1a1 and HSP70 were added to evaluate effects besides those upon the immune system. Whereas the individual responses of each of the assessed genes were taken into account from a functional perspective, utilizing biomarkers as single entities can reduce the power of conducting overall health studies. Analyzing transcription data using both PCA and hierarchical clustering offers a broader perspective for the assessment of changes or differences between populations that individual biomarkers cannot. The selection of a broad range of genes that are not specific to a single response type (e.g.: immune system or contaminant specific) permits a greater assessment of health parameter responses, which is essential for field based assessments. Genes selected in this study were applied to both gill and kidney regardless of tissue specificity, constituitive expression, or response type to generate profiles, demonstrate, and highlight the potential applicability of transcription profiling.

The selection of genes can thus be focused on specific monitoring needs. A number of informative molecular biomarkers of specific and general effects have been validated in many studies that warrant their application in field studies, for example vitellogenin, used to measure exposure of oviparous animals to estrogen or estrogen mimics[35] and heat shock proteins, used to measure sublethal cellular stress.[36] Molecular profiling, and specifically gene expression hierarchical clustering along with PCAs, could prove to be an informative approach to defining health status and should be applied where possible to complement other monitoring parameters. Gene profiling and PCA, when used in conjunction, can greatly assist in determining overall effects on populations giving statistical weight to each individual fish.

The overall aim of our investigations is to develop a suite of genes that expands on immune system and pathogenic defense systems through increases in the number of informative genes investigated and tissue types assessed. Further, these genes have the potential to be focused on other functional responses such as neuromuscular assessments, which can be linked with swimming performance[21,37] and endocrine systems that influence development and reproduction. The objective is to generate a library of functionally informative tissue-specific, gene expression profiles representing different health status categorizations in *O. mykiss*. Baseline levels of transcription, beyond that at which the health status of an organism is known to be compromised, must be established for this to be achievable. The PCA investigation conducted in the present study is a promising tool in setting this baseline. By comparing survival probability data with classification and transcription data, it may be possible to extrapolate the clustered responses to overall out-migration success. However, such an approach could only be tested and validated through conducting a series of similar studies.

This research supports the use of gene profiling as a tool for monitoring the health status of field-caught fish. Not only

genome-wide profiling but also a surprisingly small-but specific suite of genes can be used to assess pathogenic infections and health classification in juvenile steelhead; an approach that is transferable to many other species. Where possible, these tools should be incorporated into monitoring programs to assess the health status of fish populations. Where specific organ assessments are not possible, such as with species listed under endangered or threatened species acts, nonlethal biomarker approaches are available,[20,38] by obtaining tissue samples through biopsies. However, genomic responses are tissue specific, as substantiated in this study (gill vs kidney), thus gene suites should be carefully selected for specific nonlethal assessments. Furthermore, as emphasized by this and other recent studies and reviews,[18,19,21,38−40] for the application of molecular biomarkers and profiling to be successful, concurrent response measurements at higher levels of biological organization are required, and representative tissue-specific transcription baselines also need to be established. Furthermore, by increasing the number of assessed genes, and their specificity, and incorporating them into carefully designed multilevel of biological organizations studies, it is possible to identify metrics linking cell responses to tissue specific responses, whole organism, survival probability, and population dynamics. This is further supported by research conducted by Miller et al.[20] on the sockeye salmon (*Oncorhynchus nerka*), who identified specific genomic profiles directly associated with pathogen infection that predict migration and spawning success. We conclude that molecular profiling is a significantly informative and useful tool that could be applied for the prediction of adverse outcomes and extrapolated to population dynamics.

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Notes

The authors declare no competing financial interest.

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