



# Temporal expression patterns of rainbow trout immune-related genes in response to *Myxobolus cerebralis* exposure



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## ABSTRACT

Infection of salmonids by the myxozoan parasite *Myxobolus cerebralis* can cause whirling disease, which is responsible for high mortalities in rainbow trout hatcheries and natural populations in the United States. Although considerable research has provided insight into disease pathology, host invasion, and inheritance patterns of resistance, the causal genetic variants and molecular mechanisms underlying host resistance or susceptibility remain elusive. A previous study found that expression changes of specific metallothionein genes following *M. cerebralis* infection are implicated in whirling disease resistance. The present study examines the dynamic transcriptional response to infection of several upstream regulators of the metallothionein gene family (IL-1 $\beta$ , KLF2, STAT3, STAT5), along with innate immune response genes (IFN- $\gamma$ , IRF1 and iNOS). Pathogen loads and gene expression were compared across multiple time points after *M. cerebralis* exposure to elucidate how resistant and susceptible rainbow trout strains transcriptionally respond to early invasion. IL-1 $\beta$ , IFN- $\gamma$ , IRF1, and iNOS all showed increased expression following *M. cerebralis* exposure for one or both strains across multiple time points. The interferon-related genes IFN- $\gamma$  and IRF1 had consistently increased expression in the susceptible strain in comparison to the resistant strain, likely due to a less effective initial immune response. STAT3 was the only gene with consistently increased expression in the resistant strain following infection while remaining unchanged in the susceptible strain. Given its pleiotropic effects on immune response, STAT3 is an excellent candidate for future research of whirling disease resistance mechanisms.

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## 1. Introduction

*Myxobolus cerebralis*, which can cause whirling disease, is a myxozoan parasite that infects Salmonidae family members. Rainbow trout (*Oncorhynchus mykiss*) are particularly susceptible to clinical signs of the disease [1,2], which include erratic or “whirling” swimming behavior, black tail, and skeletal deformities [3]. New outbreaks are detected annually and substantial mortalities from the disease have occurred in both hatchery and natural populations [1,2,4,5]. While most tested U.S. rainbow trout strains are highly susceptible to whirling disease, the German Rainbow (GR) (or Hofer) hatchery strain displays resistance at consistently high levels across individuals [6].

Controlled laboratory studies comparing the GR strain and the largely susceptible Colorado River Rainbow (CRR) strain, along with their crossed progeny, have revealed that whirling disease resistance has a genetic component [7]. Broad-sense heritability

estimates range from 0.34 for the GR strain up to 0.89 for the CRR strain [8]. A quantitative trait loci (QTL) mapping study, using an F<sub>2</sub> cross between GR and CRR, identified a single QTL region on chromosome Omy9 [9]. This region explained 50–86% of the phenotypic variation across multiple families; however, the causative gene(s) within the 1.85 cM QTL region remains unknown.

Gene expression studies have proven invaluable for gaining a better understanding of the molecular mechanisms relating to both disease progression and resistance for many fish diseases [10]. Whirling disease gene expression studies for *O. mykiss* have been conducted using both candidate [11,12] and global [13] gene expression techniques to examine host changes in response to infection. These studies provided insight into potential genes and pathways that may be differentially regulated between resistant and susceptible strains. In a previous microarray study two members of the metallothionein gene family were identified, metallothionein A and metallothionein B (MT-A and MT-B, respectively), which were up to 5-fold up-regulated in the resistant GR strain but unchanged in the susceptible Trout Lodge (TL) strain following pathogen exposure [13]. Metallothionein has been shown to mediate leukocyte chemotaxis and has been hypothesized to serve

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as an early “danger signal” during times of stress or infection to activate an immune response [14]. MT-A and MT-B have been mapped and are found on chromosomes Omy1 and Omy2, respectively [15] and do not lie within major effect QTL region on Omy9 [9]. It is possible that upstream regulators of metallothionein expression are the true underlying cause of whirling disease resistance/susceptibility since an expression study alone cannot determine if a gene is directly contributing to a phenotype.

In this study, I sought to determine if genes upstream of MT-A and MT-B in signaling cascades are differentially expressed in resistant and susceptible rainbow trout strains. Expression patterns of four genes capable of modulating metallothionein expression were examined, along with three other immune-relevant genes, at eight early time points after *M. cerebralis* exposure. All genes examined in the present study are part of the innate immune system, though some also have dual roles in the adaptive immune system. Temporal expression patterns were evaluated in conjunction with absolute pathogen levels in the skin at all time points to provide appropriate context for examining host–pathogen interplay.

## 2. Material and methods

### 2.1. Fish rearing and pathogen exposures

GR and TL strains were reared separately in 132.5 L aquaria with 10–15 °C single pass well water until 2–3 months post-hatch (890–899 d). Individuals ( $n = 100$  for each strain) were exposed to 2000 TAMs (triacinomyxons) per fish for one hour by immersion. Additional fish ( $n = 100$  for each strain) served as unexposed controls, which were treated identically to exposed fish at all experimental stages other than their lack of pathogen exposure. Fish were then kept in aquaria receiving 15 °C well water with a daily ration of commercial trout diet until euthanized with an overdose of benzocaine (500 mg/L). The caudal fin, which is comprised mostly of skin tissue, was removed from nine individuals of each of the four experimental groups (i.e., exposed and unexposed fish from both GR and TL strains) at the following early time points after *M. cerebralis* exposure: 2 h, 4 h, 8 h, 12 h, 24 h, 2 d, 3 d, 5 d, 7 d, 10 d.

### 2.2. Nucleic acid extraction and pathogen load

Each caudal fin was immediately placed into 2X Nucleic Acid Purification Lysis Solution supplied with ABI's TransPrep Chemistry kit (Applied Biosystems, Foster City, CA) and kept at 4 °C overnight. Samples were then transferred to –20 °C, where they remained until extraction. Both genomic DNA and total RNA were extracted using the ABI Prism™ TransPrep system with the ABI Prism™ 6100 Nucleic Acid PrepStation according to manufacturer's instructions. RNA quality was assessed by agarose gel electrophoresis.

The severity of *M. cerebralis* infection at all time points was evaluated by a TaqMan quantitative polymerase chain reaction (qPCR) assay using genomic DNA extracted from caudal fin tissue. The TaqMan assay was modified from Kelley et al. [16] to amplify the 18S rDNA (*M. cerebralis*) and insulin growth factor-I (*O. mykiss*) genes in a single reaction by fluorescently labeling their specific probes with 6-FAM and VIC dyes, respectively. Other than multiplexing and reducing the template DNA to 1 µl and the total reaction volume to 12 µl, all conditions are as described in Kelley et al. [16]. Pathogen load for each individual represents the number of *M. cerebralis* 18S rDNA gene copies per  $10^6$  rainbow trout cells. Significance between GR and TL at each time point was evaluated using a two-tailed *t*-test.

### 2.3. Candidate gene selection and primer design

The keyword “metallothionein” was inputted into the Your Favorite Gene online research tool [17] powered by Ingenuity to identify genes that are known to regulate its expression. Four candidate genes were chosen for relative expression comparisons: interleukin-1 beta 1 (IL-1β1), Krueppel-like factor 2 (KLF2), and signal transducer and activator of transcription 3 and 5 (STAT3 and STAT5, respectively). These genes were selected based on their potential metallothionein regulatory effects and their sequence availability in Genbank for designing salmonid-specific intron-spanning primers. Additional genes, not known to regulate metallothionein, that were analyzed due to their frequent association with innate immune response are: inducible nitric oxide synthase (iNOS), interferon-gamma (IFN-γ), and interferon regulatory factor 1 (IRF1). Beta-actin (β-actin) served as an internal control for normalization. Primer sequences and Genbank accession numbers for all analyzed genes can be found in Table 1.

### 2.4. cDNA synthesis and qPCR analysis

Total RNA was converted to cDNA using ABI's High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's protocol. The cDNA was diluted 1:2 with RNase-free water prior to quantitative real-time PCR (qPCR). The Quantitect™ SYBR® Green RT-PCR kit (Qiagen) was used according to the manufacturer's instructions except the final PCR volume was reduced to 6 µl. The cycling conditions used on a Chromo4 Real-Time PCR Detection System (Bio-Rad) were as follows: HotStarTaq DNA polymerase activation at 95 °C for 15 min, 45 cycles of 15 s denaturation at 94 °C, 15 s annealing at 58 °C, 15 s extension at 72 °C, followed by a melting curve analysis (55–95 °C) to check that a single product peak was produced for each reaction.

For each gene, the relative amount of expression changes was calculated using the comparative  $C_T$  method (i.e.,  $2^{-\Delta\Delta C_T}$ ) [18]. A nonparametric Mann–Whitney *U*-test was used to identify

**Table 1**  
Primers of genes analyzed by RT-qPCR.

Gene name	Primer ID	Sequence (5'–3')	PCR product size (bp)	Genbank accession
Beta-actin	β-actin F	CAGGCATCAGGGAGTGATG	127	AB196465
	β-actin R	GTCCAGTTGGTGACGATG		
Krueppel-like transcription factor 2	KLF2 F	GAACGCACACTGGTAAAAAG	136	BT044789
	KLF2 R	CGATCACACAGGTGGCAC		
Inducible nitric oxide synthase	iNOS F	GCATCACAGCACTTTCAGC	98	AJ295231
	iNOS R	GGTGGACCGCTTGAATG		
Interferon-gamma	IFN-γ F	CCTTGGAGATCTTCATAGTG	101	FJ222819
	IFN-γ R	GGTGGATGACTTTAGGATG		
Interferon regulatory factor 1	IRF1 F	CCATGCACACAGGGAATTC	109	AF332147
	IRF1 R	TTCACITCTCTATGTCAGG		
Interleukin-1 beta 1	IL-1β1 F	GCACCTAGAGGAGGTTCG	148	AJ298294
	IL-1β1 R	CGTGCTGATGAACCAAGTTC		
Signal transducer and activator of transcription 3	STAT3 F	GAATGAAGGGTATATTCTGG	152	U60333
	STAT3 R	TCCCACTGATGCTCTTTCC		
Signal transducer and activator of transcription 5	STAT5 F	GTGGTACCAGAGTTCACCAC	132	AF147499
	STAT5 R	CTAATATGGAGTCACTCATAGG		

differentially expressed genes between exposed and unexposed control fish for each rainbow trout strain. Gene expression significantly differing between exposed and unexposed fish for at least one strain were tested for significant differences between strains, also using a Mann–Whitney *U*-test. For both within and between strain comparisons, *P* values less than 0.05 were considered significant.

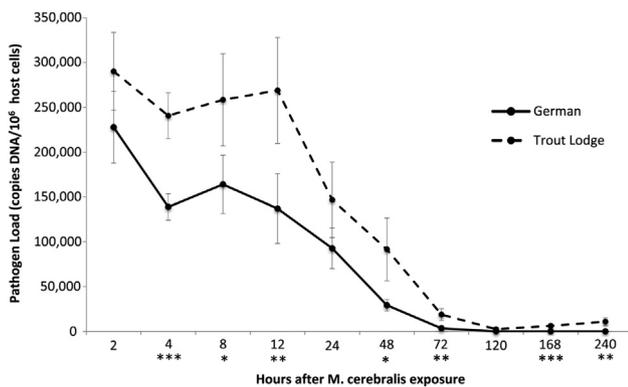
### 2.5. Chromosome location of STAT3

Based on gene expression results, I attempted to determine the chromosomal location of STAT3 using the draft rainbow trout genome (provided courtesy of Dr. Michael Miller). The scaffold containing the first 60 bp of the STAT3 coding sequence (Genbank Accession U60333) was identified using the grep command in Unix. A nucleotide BLAST was conducted for this sequence to ensure that it has similarity to only *O. mykiss* STAT3 and other orthologous sequences. The chromosome of the identified scaffold sequence was determined using previously constructed genetic maps of restriction-site associated DNA (RAD) tags [19,20] and microsatellite markers (see Appendices S3 and S4 in Ref. [19]). These markers were aligned to the draft rainbow trout genome using Bowtie [21].

## 3. Results

### 3.1. Pathogen load in caudal fin during early disease progression

At the initial 2 h PE (post-exposure) time point there was not a significant difference in the caudal fin pathogen loads of GR and TL, with both strains experiencing their highest pathogen loads (Fig. 1). By 4 h PE, GR's pathogen load dropped dramatically to 61% relative to the 2 h amount. TL also experienced a drop in pathogen load between the 2 and 4 h time points, though it was a more modest drop, with 83% remaining. At all time points between 4 and 72 h PE, with the exception of 24 h, the GR strain had a significantly lower pathogen load than the TL strain. Between 3 and 5 d (72–120 h) PE most remaining *M. cerebralis* sporoplasm cells have entered nervous tissue for both strains and are no longer detectable in the caudal fin, consistent with previous reports [22]. Unexposed controls for each strain had no detectable pathogen loads at any time point (data not shown).



**Fig. 1.** Pathogen loads in skin tissue of resistant GR (German) and susceptible TL (Trout Lodge) rainbow trout strains following *Myxobolus cerebralis* exposure. Four hours after exposure the GR strain has substantially reduced pathogen levels compared to the TL strain. Pathogen loads continue to decline in the skin tissue of both strains and between 3 and 5 days post-exposure most *M. cerebralis* has entered nervous tissue and is no longer detectable in skin. Asterisks below each time point indicate when pathogen loads significantly differ between strains: \* <0.05, \*\* <0.01, \*\*\* <0.001. Standard deviation error bars are included.

### 3.2. Host gene expression changes during early disease progression

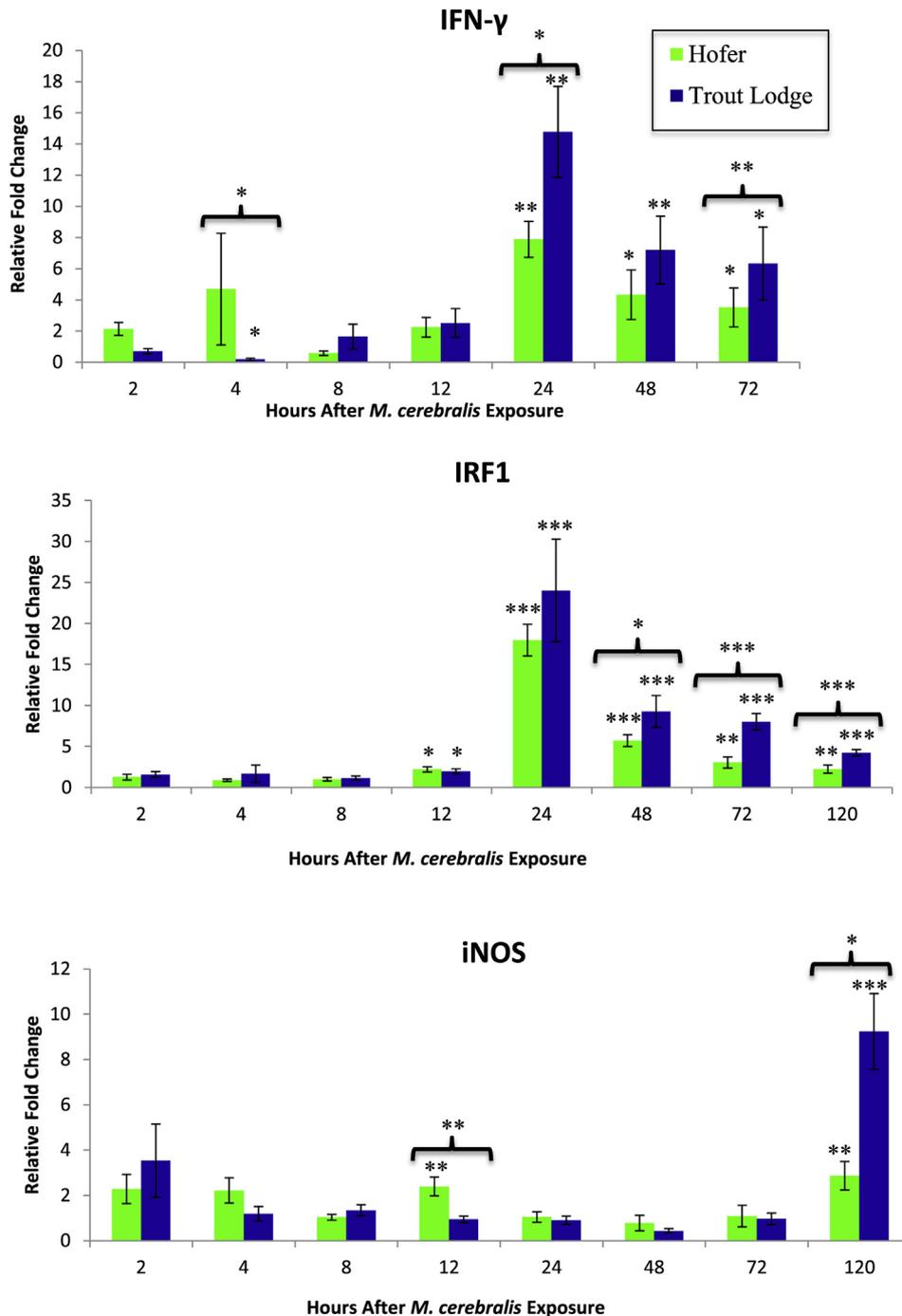
Expression changes for candidate genes were examined up to 3–5 d after *M. cerebralis* exposure in the caudal fin. Fig. 2 shows expression levels for three immune-related genes commonly associated with early host pathogen response: IFN- $\gamma$ , IRF1, and iNOS. For both strains, the interferon pathway genes IFN- $\gamma$  and IRF1 showed significant up-regulation when compared to unexposed controls at later studied time points. Up to 24 h PE, the two strains do not significantly differ in the studied IFN expression profiles, with the exception of a modestly significant up-regulation of IFN- $\gamma$  expression in GR relative to TL at 4 h PE. However, during the later time points (24–120 h PE) there is a general pattern of increased expression of the studied IFN pathway genes in TL relative to GR individuals. At 12 h PE, iNOS had up-regulated expression for the GR strain (2.4-fold) while the TL strain was unchanged. At 120 h PE, iNOS is up-regulated for both strains, though TL is significantly more up-regulated (9.2-fold) than GR (2.9-fold).

Genes selected based on the Ingenuity pathway analysis search (keyword: metallothionein) with significant gene expression changes for at least one time point are shown in Fig. 3. KLF2 and STAT5 were not differentially expressed at any of the studied times in caudal fin tissue of either strain in response to *M. cerebralis* infection. IL-1 $\beta$  is modestly up-regulated for one or both strains for the majority of time points. There is not a significant difference in expression between the two strains for any time points, partially due to the large within-strain expression variance found at some of the time points. STAT3 had the most differential expression changes between the two strains of all examined genes. GR's STAT3 expression is increased for 5/8 time points, with the remaining 3 times showing increased but non-significant expression changes. In contrast, TL expression remains unchanged for all time points. The greatest GR up-regulation occurred at 8 h PE, with a 3.9-fold increase. In comparison, TL had a 1.8-fold decrease in expression at that time point. Based on sequence alignment results, STAT3 is positioned on chromosome Omy13 of the rainbow trout draft genome.

## 4. Discussion

The expression results suggest that the innate immune system is being activated in both of the studied rainbow trout strains during the early stages of *M. cerebralis* infection. Innate immune mediators are the first line of defense against pathogens. In fish, it is believed that innate immunity is generally stronger than adaptive immunity and serves to activate and instruct the adaptive immune response [23]. Fish mucus is the initial barrier that *M. cerebralis* encounters upon epidermal attachment. Since the mucus does not impair sporoplasm penetration [24], *M. cerebralis* enters the skin equally well in resistant and susceptible *O. mykiss* strains. Based on pathogen load and gene expression data, it appears that the GR strain is eliciting an innate immune response that is more effective than TL. This initial time period corresponds with *M. cerebralis* sporoplasm cells multiplying and migrating from the epidermis to the dermis during the first 2 h of infection and then progressively moving into peripheral nerves [25]. The trend of reduced pathogen load for the GR strain in comparison to TL (Fig. 1) largely continues until most parasitic cells have either degenerated or entered nervous tissue by 2–4 d PE [22].

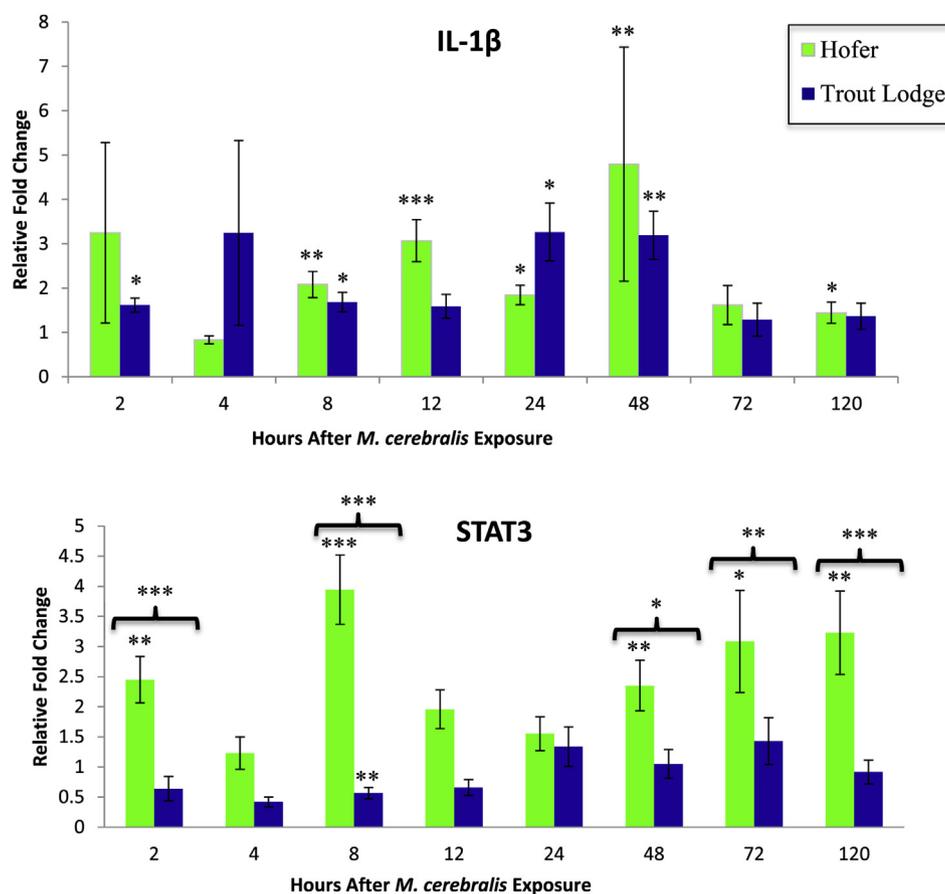
Interferon pathways frequently play a pivotal role in host defense against invading pathogens. In this study, expression profiles of two interferon-related genes, IFN- $\gamma$  and IRF1, were examined. IFN- $\gamma$  is a pro-inflammatory cytokine capable of mediating a diverse range of host immune responses, including inflammation, antigen processing, cell death, and other anti-



**Fig. 2.** Relative fold change of IFN- $\gamma$ , IRF1, and iNOS in rainbow trout caudal fin tissue following *Myxobolus cerebralis* exposure. Relative fold change in caudal fin represents the change in expression of infected fish compared to uninfected controls and normalized with  $\beta$ -actin expression levels. Error bars are standard deviations that represent variability across nine biological replicates. Asterisks indicate when expression levels significantly differ within strains (infected vs. controls) and between strains (GR vs. TL): \*  $< 0.05$ , \*\*  $< 0.01$ , \*\*\*  $< 0.001$ . IL-1 $\beta$  expression is up-regulated for both strains at the majority of time points but expression is not significantly different between the strains. Both IFN- $\gamma$  and IRF1 show increased expression for both strains at later time points, with TL have great up-regulation than the GR strain. The iNOS expression was up-regulated for the GR strain at 12 h post-exposure and for both strains at 5 days post-exposure, with TL expression considerably greater.

pathogen responses (reviewed in Ref. [26]). IRF1 is a transcription factor that regulates expression of a variety of genes involved in host defense, including those involved in IFN- $\gamma$  signaling [26]. The two rainbow trout strains show similar interferon pathway expression patterns during the examined time points after *M. cerebralis* infection, with dramatic changes between exposed and unexposed controls occurring at 24 h PE and later (Fig. 2). It is clear that the innate immune systems of both strains had been

activated by this point, with both interferon pathway genes up-regulated to their greatest levels (GR: 8-fold and TL: 15-fold). At 24 h and later time points, the susceptible TL strain trends toward having greater up-regulation of these interferon pathway genes than the resistant GR strain. This increased transcription may be detrimental to the TL strain since IFN- $\gamma$  expression must strike a balance between anti-pathogenic effects and host inflammatory tissue damage [27].



**Fig. 3.** Relative fold change of IL-1 $\beta$  and STAT3 in rainbow trout caudal fin tissue following *Myxobolus cerebralis* exposure. Relative fold change in caudal fin represents the change in expression of infected fish compared to uninfected controls and normalized with  $\beta$ -actin expression levels. Error bars are standard deviations that represent variability across nine biological replicates. Asterisks indicate when expression levels significantly differ within strains (infected vs. controls) and between strains (GR vs. TL): \* <0.05, \*\* <0.01, \*\*\* <0.001. IL-1 $\beta$  expression is up-regulated for both strains at the majority of time points but expression is not significantly different between the strains. STAT3 shows a general pattern of up-regulation for the resistant GR strain while the susceptible TL strain's expression remains unchanged following pathogen infection.

In macrophages, iNOS produces large amounts of nitric oxide as a host defense mechanism and plays a major role in inflammation [28]. In a previous study, which did not quantify pathogen load, increased iNOS expression (2.5- to 5.6-fold) was observed for TL from 24 h to 4 d after *M. cerebralis* exposure, along with even greater up-regulation at two additional later time points (8 and 20 d) [11]. The GR strain exhibited increased iNOS expression (4.2-fold) only at the 8 d PE time point. Contrastingly, in the current study TL had increased iNOS expression only at the 5 d PE time point while GR had increased expression at both 12 h and 5 d PE. The different results from these studies may be due to differing experimental conditions, with factors such as water temperature, age, and TAM dosage known to influence *M. cerebralis* infections and disease severity [29]. The two studies used different pathogen exposure concentrations (2000 vs 5000 TAMs/fish). Additionally, caudal fin is known to be a primary site of initial *M. cerebralis* invasion [22] and was the sole tissue source for the current study while Severin et al. [11] used a pooled mixture of skin, muscle, and cartilage. Changes in experimental design can have profound effects on gene expression and, along with typical experimental expression variability, likely contributed to the observed differences. Both studies show increased iNOS transcription of TL and GR at some of the later time points during early *M. cerebralis* infection. Additionally, both studies show that during the later time points of early disease progression TL has increased up-regulation in comparison to GR. It seems plausible that increased transcription of

some immune response genes, such as IFN- $\gamma$ , IRF1, and iNOS, is due to TL's inability to wage an effective early immune response. Specifically, these transcript levels likely reflect TL's increased pathogen load rather than a protective effect against whirling disease. A similar example of this is the greater interferon response of the most susceptible rainbow trout when exposed to the Viral Hemorrhagic Septicemia virus (VHSV) [30]. When comparing pathogen loads and expression patterns, it is apparent that increased mRNA expression does not always correlate with increased resistance.

A chief objective of this study was to evaluate the expression of several immune-relevant candidate genes found upstream of metallothionein in signaling cascades. One of these genes, IL-1 $\beta$ , is a cytokine commonly associated with the innate immune response and inflammation. It has been implicated in rainbow trout's initiation of an innate immune response against *Yersinia ruckeri* [31] and resistance to *Aeromonas salmonicida* [32], which are both bacterial infections. In this study, both strains show increased IL-1 $\beta$  expression for the majority of early time points relative to unexposed controls. However, there is not a significant difference in the degree of up-regulation between the strains and it seems likely that this gene is not directly involved in conferring resistance during the early stages of infection in caudal fin tissue.

In contrast, STAT3 was differentially expressed between resistant and susceptible strains. The STAT protein family transduces cytoplasmic signals from extracellular stimuli and activates transcription of genes involved in multiple pathways, including

apoptosis, cell proliferation, inflammation and immune response [33]. STAT3 induces metallothionein gene expression [34] as well as other genes associated with immunity and inflammation. In the current study, the resistant GR strain has significantly increased STAT3 expression relative to unexposed controls at five of eight time points, and increased but non-significant increased expression at the three remaining times. TL expression remains consistently unchanged, except for slight down-regulation at 8 h PE.

Higher STAT3 in the GR strain might promote resistance via generation of a specific class of T helper cells. STAT3 is critical for the differentiation of T helper 17 cells (Th17) from naive CD4<sup>+</sup> T cells [35]. Th17 cells produce cytokines that aid in the reduction of pathogen loads at epithelial/mucosal barriers [36] and serve as a bridge between innate and adaptive immunity [37,38]. Connecting the dots, it may be that the induction of STAT3 in the GR strain is causing an increase in Th17 differentiation that increases cytokine production in the epithelial tissue in order to decrease *M. cerebralis* load. In support of this hypothesis, a previous study found greater TGF- $\beta$  expression for the GR strain in comparison to the TL strain in response to *M. cerebralis* exposure [12]. TGF- $\beta$  is a cytokine with pleiotropic effects on the immune system, including playing a key role in Th17 differentiation [39]. It will be informative to assess expression patterns for other genes critical for Th17 differentiation (e.g., ROR $\gamma$ t [40], which is regulated by STAT3 [41]) as well as strongly associated cytokines (e.g., IL-17 [36]) to determine if this avenue of whirling disease resistance research is worth further pursuit.

The divergence between GR and TL STAT3 expression indicates that it is a good candidate for being involved in early whirling disease resistance. However, it is unlikely that variation in this gene is the sole factor leading to whirling disease resistance since it is not positioned in the discovered major effect whirling disease QTL region [9]. It is possible that the causative variant is upstream of STAT3 and induces its expression. Additionally, not being in the single known QTL region found to date does not preclude the possibility that a candidate gene may be playing a causative role in host response to infection. It has been estimated that  $9 \pm 5$  genes may be involved in conferring whirling disease resistance in rainbow trout [8]. Other genetic regions outside the identified QTL region may have a minor effect on resistance or are more critical to host response at alternative life stages. Additionally, mechanisms of resistance may be variable across rainbow trout strains and families so further confirmation of mapping and expression results will be of benefit.

STAT5 and KLF2 were not differentially expressed for either strain at any of the examined early time points. This indicates that these genes are not involved in rainbow trout's early immune response to whirling disease or that experimental conditions (tissue type, time points, etc.) were not conducive to demonstrate their expression changes. Although both STAT3 and STAT5 are part of JAK/STAT signaling pathways, it is not uncommon for STAT family members to display differential induction. As a perhaps relevant example, STAT3 and STAT5 bind to multiple common regulatory sites on the IL-17 gene and serve to induce or repress IL-17 expression, respectively [35,42]. IL-17 is a key cytokine produced by Th17 cells and contributes to inflammation during host defense. Divergent IL-17 expression levels could have substantial consequences when a host is initiating an immune response since IL-17 induces a number of cytokines and chemokines (reviewed by Korn et al. [43]).

Immune-relevant proteins secreted by macrophages (e.g., arginase-1 [11], iNOS [11], IL-1 $\beta$ ), along with interferon proteins (IFN- $\gamma$  and IRF1), display increased transcription following *M. cerebralis* infection. This up-regulation, however, is found in both the resistant and susceptible strains at similar levels. Therefore, while these genes appear to be involved in host response to

infection, they are unlikely to be involved in a resistance mechanism. Contrastingly, STAT3 is up-regulated in the resistant strain but remains transcriptionally unchanged in the susceptible strain compared to unexposed controls.

In conclusion, results from a previous microarray study served to guide this more directed and detailed analysis of specific candidate genes. Gene expression and pathogen load were quantified in parallel in order to better understand host/pathogen interplay. STAT3 was up-regulated at most studied time points for the GR strain but unchanged for the TL strain in comparison to unexposed controls. STAT3 was differentially expressed between GR and TL following *M. cerebralis* infection and plays a key role in immune response signaling and transcriptional regulation. STAT3 is a critical component of Th17 cell differentiation, which may be particularly relevant to host response to initial *M. cerebralis* invasion since Th17 secreted proteins decrease pathogen loads at epithelial and mucosal barriers. Continued study is warranted on the role that STAT3 has in conferring whirling disease resistance as well as upstream and downstream immunoregulatory genes. Resolution of the mechanistic differences between resistant and susceptible rainbow trout strains should pave the way to develop effective strategies to control the spread and severity of whirling disease. Hopefully by combining complementary approaches (e.g., linkage mapping and gene expression) the genetic variation and mechanisms that underlie whirling disease resistance in rainbow trout may be discovered.

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## References

- [1] O'Gradnick JJ. Susceptibility of various salmonids to whirling disease (*Myxosoma cerebralis*). Transactions of the American Fisheries Society 1979;108: 187–90.
- [2] Hedrick RP, McDowell TS, Mukkatira K, Georgiadis MP, MacConnell E. Susceptibility of selected inland salmonids to experimentally induced infections with *Myxobolus cerebralis*, the causative agent of whirling disease. Journal of Aquatic Animal Health 1999;11:330–9.
- [3] MacConnell E, Vincent ER. The effects of *Myxobolus cerebralis* on the salmonid host. In: Whirling Disease: Reviews and Current Topics, Symposium, vol. 29. American Fisheries Society; 2002. p. 95–107.
- [4] Nehring RB, Walker PG. Whirling disease in the wild: the new reality in the intermountain West. Fisheries 1996;21:28–30.
- [5] Bartholomew JL, Reno PW. The history and dissemination of whirling disease. In: Whirling Disease: Reviews and Current Topics, Symposium, vol. 29. American Fisheries Society; 2002. p. 3–24.
- [6] Hedrick RP, McDowell TS, Marty GD, Fosgate GT, Mukkatira K, Myklebust KA, et al. Susceptibility of two strains of rainbow trout (one with suspected resistance to whirling disease) to *Myxobolus cerebralis* infection. Diseases of Aquatic Organisms 2003;55:37–44.
- [7] Schisler GJ, Myklebust KA, Hedrick RP. Inheritance of *Myxobolus cerebralis* resistance among F<sub>1</sub>-generation crosses of whirling disease resistant and susceptible rainbow trout strains. Journal of Aquatic Animal Health 2006;18: 109–15.
- [8] Fetherman ER, Winkelman DL, Schisler GJ, Antolin MF. Genetic basis of differences in myxospore count between whirling disease-resistant and -susceptible strains of rainbow trout. Diseases of Aquatic Organisms 2012;102: 97–106.
- [9] Baerwald MR, Petersen JL, Hedrick RP, Schisler GJ, May B. A major effect quantitative trait locus for whirling disease resistance identified in rainbow trout (*Oncorhynchus mykiss*). Heredity 2011;106:920–6.
- [10] Alvarez-Pellitero P. Fish immunity and parasite infections: from innate immunity to immunoprophylactic prospects. Veterinary Immunology and Immunopathology 2008;126:171–98.

- [11] Severin VIC, Soliman H, El-Matbouli M. Expression of immune-regulatory genes, arginase-2 and inducible nitric oxide synthase (iNOS), in two rainbow trout (*Oncorhynchus mykiss*) strains following exposure to *Myxobolus cerebralis*. *Parasitology Research* 2010;106:325–34.
- [12] Severin VIC, El-Matbouli M. Relative quantification of immune-regulatory genes in two rainbow trout strains, *Oncorhynchus mykiss*, after exposure to *Myxobolus cerebralis*, the causative agent of whirling disease. *Parasitology Research* 2007;101:1019–27.
- [13] Baerwald MR, Welsh AB, Hedrick RP, May B. Discovery of genes implicated in whirling disease infection and resistance in rainbow trout using genome-wide expression profiling. *BMC Genomics* 2008;9:37.
- [14] Yin X, Knecht DA, Lynes MA. Metallothionein mediates leukocyte chemotaxis. *BMC Immunology* 2005;6:21.
- [15] Phillips RB, Nichols KM, DeKoning JJ, Morasch MR, Keatley KA, Rexroad C, et al. Assignment of rainbow trout linkage groups to specific chromosomes. *Genetics* 2006;174:1661–70.
- [16] Kelley GO, Zagmutt-Vergara FJ, Leutenegger CM, Myklebust KA, Adkison MA, McDowell TS, et al. Evaluation of five diagnostic methods for the detection and quantification of *Myxobolus cerebralis*. *Journal of Veterinary Diagnostic Investigation* 2004;16:202–11.
- [17] <http://www.sigmaldrich.com/life-science/your-favorite-gene-search.html>. 2012.
- [18] Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. *Nature Protocols* 2008;3:1101–8.
- [19] Miller MR, Brunelli JP, Wheeler PA, Liu S, Rexroad CE, Palti Y, et al. A conserved haplotype controls parallel adaptation in geographically distant salmonid populations. *Molecular Ecology* 2011;21:237–49.
- [20] Hecht BC, Thresher FP, Hale MC, Miller MR, Nichols KM. Genetic architecture of migration-related traits in rainbow and steelhead trout, *Oncorhynchus mykiss*. *G3: Genes/Genomes/Genetics* 2012;2:1113–27.
- [21] Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology* 2009;10:R25.
- [22] El-Matbouli M, Hoffman RW, Mandok C. Light and electron microscopic observations on the route of the triactinomyxon-sporoplasm of *Myxobolus cerebralis* from epidermis into rainbow trout cartilage. *Journal of Fish Biology* 1995;46:919–35.
- [23] Rauta PR, Nayak B, Das S. Immune system and immune responses in fish and their role in comparative immunology study: a model for higher organisms. *Immunology Letters* 2012;148:23–33.
- [24] Kallert DM, Borrelli J, Haas W. Biostatic activity of piscine serum and mucus on myxozoan fish infective stages. *Fish and Shellfish Immunology* 2012;33:969–76.
- [25] Hedrick RP, Adkison MA, El-Matbouli M, MacConnell E. Whirling disease: re-emergence among wild trout. *Immunological Reviews* 1998;166:365–76.
- [26] Schroder K. Interferon- $\gamma$ : an overview of signals, mechanisms and functions. *Journal of Leukocyte Biology* 2003;75:163–89.
- [27] Hu X, Ivashkiv LB. Cross-regulation of signaling pathways by interferon-gamma: implications for immune responses and autoimmune diseases. *Immunology* 2009;31:539–50.
- [28] Secombes CJ, Wang T, Hong S, Peddie S, Crampe M, Laing KJ, et al. Cytokines and innate immunity of fish. *Developmental & Comparative Immunology* 2001;25:713–23.
- [29] Markiw ME. Experimentally induced whirling disease I. Dose response of fry and adults of rainbow trout exposed to the triactinomyxon stage of *Myxobolus cerebralis*. *Journal of Aquatic Animal Health* 1992;4:40–3.
- [30] Dorson M, Torhy C, De Kinkelin P. Viral haemorrhagic septicaemia virus multiplication and interferon production in rainbow trout and in rainbow trout  $\times$  brook trout hybrids. *Fish and Shellfish Immunology* 1994;4:369–81.
- [31] Raida MK, Holten-Andersen L, Buchmann K. Association between *Yersinia ruckeri* infection, cytokine expression and survival in rainbow trout (*Oncorhynchus mykiss*). *Fish and Shellfish Immunology* 2011;30:1257–64.
- [32] Hong S, Peddie S, Campos-Pérez JJ, Zou J, Secombes CJ. The effect of intraperitoneally administered recombinant IL-1 $\alpha$  on immune parameters and resistance to *Aeromonas salmonicida* in the rainbow trout (*Oncorhynchus mykiss*). *Developmental & Comparative Immunology* 2003;27:801–12.
- [33] Ihle JN. The Stat family in cytokine signaling. *Current Opinion in Cell Biology* 2001;13:211–7.
- [34] Oshima Y, Fujio Y, Nakanishi T, Itoh N, Yamamoto Y, Negoro S, et al. STAT3 mediates cardioprotection against ischemia/reperfusion injury through metallothionein induction in the heart. *Cardiovascular Research* 2005;65:428–35.
- [35] Durant L, Watford WT, Ramos HL, Laurence A, Vahedi G, Wei L, et al. Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. *Immunity* 2010;32:605–15.
- [36] Dubin PJ, Kolls JK. Th17 cytokines and mucosal immunity. *Immunological Reviews* 2008;226:160–71.
- [37] Stockinger B, Veldhoen M, Martin B. Th17 T cells: linking innate and adaptive immunity. *Seminars in Immunology* 2007;19:353–61.
- [38] Kolls JK, Khader SA. The role of Th17 cytokines in primary mucosal immunity. *Cytokine and Growth Factor Reviews* 2010;21:443–8.
- [39] Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 2006;441:235–8.
- [40] Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, Chung Y, et al. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR $\alpha$  and ROR $\gamma$ . *Immunity* 2008;28:29–39.
- [41] Yang XO, Panopoulos AD, Nurieva R, Chang SH, Wang D, Watowich SS, et al. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *Journal of Biological Chemistry* 2007;282:9358–63.
- [42] Yang X-P, Ghoreschi K, Steward-Tharp SM, Rodriguez-Canales J, Zhu J, Grainger JR, et al. Opposing regulation of the locus encoding IL-17 through direct, reciprocal actions of STAT3 and STAT5. *Nature Immunology* 2011;12:247–54.
- [43] Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 cells. *Annual Review of Immunology* 2009;27:485–517.