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Transcriptomic screening of the innate immune response in delta smelt during an *Ichthyophthirius multifiliis* infection

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ABSTRACT

Fish health can be affected by many environmental factors including infection with pathogens. Infectious disease can lead to high mortality in wild populations and in aquaculture, resulting in significant economic losses. Teleost fishes share a highly conserved response to pathogen infections, starting with an immediate activation of the innate immune response before shifting to an adaptive immune response. Fishes also show altered transcriptomic expression in stress-related genes during acute and chronic infections. The ciliate *Ichthyophthirius multifiliis* (*Ich*) is one of the most widely distributed ectoparasites for freshwater fish, providing the possibility to identify infection severity via phenotypical assessments. Molecular markers are increasingly incorporated in health assessments and to determine sublethal physiological responses to stressors. We evaluated the effects of an infection with *Ich* using the pelagic fish species delta smelt (*Hypomesus transpacificus*) as a model. We investigated transcriptional changes of ten genes belonging to the innate immune response and five heat shock genes related to thermal and general stress response. Molecular assessments were conducted in three tissues (gill, spleen and kidney), and collected from fish with different infection intensities. The combination of molecular markers, including immune and stress-related genes, resulted in significant differentiation between infection levels in all tissue types. The strongest results were observed in gill and kidney, however, the detection of infection severity was most effective when combining the transcriptomic results from all tissues. Chemokine *cxcb* was the most responsive gene in all tissues and provided significant upregulation in fish with visible *Ich*-infection. The identification of molecular markers targeting fundamental host responses associated with infections is contributing to the establishment of techniques to assess fish health in natural habitats and in aquaculture.

1. Introduction

Fish health can be affected by many environmental factors such as water quality, predation, food limitation, temperature stress, contaminants and pathogens (Baxter et al., 2008; Geist, 2011). Pathogens can contribute to mass mortalities both in wild fish populations and aquaculture, resulting in significant economic losses (Austin and Austin, 2007; Gozlan et al., 2005; Pike and Wadsworth, 2000; Wurtsbaugh and Alfaro Tapia, 1988). Pathogens further elicit sublethal responses that can alter behavior, decrease fertility or swimming performance, ultimately limiting reproductive success and increasing risk of predation (Barber et al., 2000). Such effects can be exacerbated by simultaneous exposure to multiple environmental stressors (Barber et al., 2000).

A first response of fishes to pathogens is the activation of the innate immune system, subsequently followed by a shift to an adaptive immune response (Alvarez-Pellitero, 2008; Magnadóttir, 2006). During the innate immune response, pathogens are detected via pathogen recognition receptors (PRRs) which elicit specific responses to various pathogen-associated molecular patterns (PAMPs) in the infected animal (Janeway and Medzhitov, 2002; Medzhitov and Janeway, 2002). Indicators of an activated innate immune system as well as direct response mechanisms within an innate immune response include members of the complement system, toll-like receptors, inflammation-inducing cytokines, immune cell migration-directing chemokines, anti-proteases, members of the mitogen activated pathway and major histocompatibility complex components (Alvarez-Pellitero, 2008; Dalmo et al., 1997; Dixon and Stet, 2001; Gasque, 2004; Sitjà-Bobadilla et al., 2006; Umasuthan et al., 2015). Furthermore, heat shock proteins (HSPs) are also known to respond to a wide range of stressors, including pathogen

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infections (Ackerman and Iwama, 2001; Cho et al., 1997; Forsyth et al., 1997), and temperature is known to govern both the infection susceptibility as well as immune defense mechanisms in fishes (Bly and Clem, 1992; Matthews, 2005; Tort et al., 2003).

One of the most widespread ectoparasite for fishes is the ciliate *Ichthyophthirius multifiliis* (*Ich*) (Maki and Dickerson, 2003), which appears as white spots on the skin or gills. Fish may also show signs of irritation, loss of appetite, and decreased activity. The *Ich* life cycle comprises three life stages: a parasitic feeding stage, a reproductive stage, and a free-swimming stage. In the parasitic stage, the trophont lives in, and feeds on, the fish epithelia. It then leaves the host and initiates the reproductive tomont stage resulting in a tomocyst, releasing free swimming theronts which infect new hosts (Matthews, 2005). Growth and development rates of the parasite are positively correlated to temperature (Traxler et al., 1998). *Ich* is known to have adverse effects on fishes and can cause severe defects in gills and skin, where the feeding trophont resides (Matthews, 2005; Xu et al., 2005). Mortality due to *Ich* infection is high, and significant losses in fish farming as well as in wild populations have been reported (Jessop, 1995; Martins et al., 2011; Traxler et al., 1998). Fish mortality is mainly attributed to extensive feeding of the parasite during the trophont stage prior to tomonts leaving the host, resulting in damage to the gill and skin epithelia of fish, and often in secondary infections (Athanassopoulou et al., 2004; Ewing and Kocan, 1992; Matthews, 2005).

Ich is known to affect a wide range of fish species, including the delta smelt (*Hypomesus transpacificus*). This pelagic fish is endemic to the San Francisco Estuary and Watershed (California, USA) and is listed as threatened and endangered under the Federal and California Endangered Species Acts (CESA), respectively (CDFW, 2017; USFWS, 1993). During a series of experiments conducted to investigate thermal stress responses in adult delta smelt, an *Ich* outbreak occurred. We opportunistically utilized this event to evaluate delta smelt transcriptomic response profiles at different severities of phenotypical *Ich* infection. We specifically investigated transcriptomic responses relating to the innate immune response as well as genes associated with thermal stress. Molecular biomarkers are becoming increasingly important tools to assess sublethal physiological responses to stressors (Connon et al., 2012; Miller et al., 2011), and have been specifically used to assess environmental stressors such as salinity, turbidity, contaminant exposure or stocking density (Geist et al., 2007; Hasenbein et al., 2016; Komoroske et al., 2016; Terova et al., 2005; Valavanidis et al., 2006). Recent studies have been highly successful in using transcriptomic approaches in diagnosing pathogen and parasites infection, as well as disease in fishes (Connon et al., 2012; Gonzalez et al., 2007a; Gonzalez et al., 2007b; Jeffries et al., 2014a; Miller et al., 2011). The potential of these markers as early disease indicators has, to the best of our knowledge, not yet been utilized to assess the health status of endangered fishes such as the delta smelt.

In this study we analyzed and compared gene transcription responses in *Ich*-infected delta smelt across three tissue types: gill, kidney and spleen. The gene expression patterns were linked with phenotypic levels of infection. We hypothesized tissue-specific responses dependent on the severity of infection. In addition, we compared these findings to earlier studies that described the effects of *Ich* on the innate immune response in other fish species.

2. Materials and methods

2.1. Test organisms, holding conditions and treatment

All fish were reared at the UC Davis Fish Conservation and Culture Laboratory (FCCL) in Byron, CA, USA, and transferred to the Putah Creek facility (Davis, CA) as adults (320 to 400 days post hatch; dph). At UC Davis, fish were held in circular, flow through tanks (diameter 1.2 m, depth 0.8 m) with water inflow of 8 L min^{-1}, at a density of 130 fish per tank. The system was supplied with non-chlorinated, air-equilibrated, temperature-controlled (12.5 ± 0.5 °C) well water. Tanks were covered with fine screen mesh to minimize illumination and disturbance.

The initial salinity while transferring them between the facilities was 5 PSU to minimize transport stress (Swanson et al., 1996), and reduced to 0.5 PSU at a constant rate over 10 days. After a 18 day adjustment period, fish were acclimated to three different temperatures (low = 10.2 ± 0.5, medium = 13.2 ± 0.5 and high = 18.1 ± 0.5 °C). At this time point no fish displayed any symptoms of disease. Acclimation to the final temperatures was conducted within 7 days and fish were maintained at the final temperatures for 16 days. An observable *Ich* outbreak occurred in the two tanks with higher temperatures on day 11 after acclimation, at which time salinity was increased to 5 PSU in all tanks to assist in fish recovery and to control the *Ich* (Aihua and Buchmann, 2001). No observable signs of infection or any indication of altered health status were detected during daily fish observation prior to day 11. On day 16 all fish were euthanized, using a 50 mg/L dose of buffered (with $NaHCO_3$) MS-222 (Tricaine methanesulfonate, Finquel). At this time, 10 fish from each treatment were sampled and infection levels were quantified. Specimens were weighed to the nearest 0.1 g, measured (fork length to the nearest mm), photographed with a high resolution camera (Canon 5D MK II, fitted with a Canon EF Macro 100 mm f/2.8 lens), the sex of each fish was recorded, and tissues were sampled for molecular assessments (Table 2). There was no evidence for disease other than *Ich* when sampling took place. Gill, kidney and spleen tissues were sampled from 30, 29 and 27 individuals (loss during sampling occurred), respectively, snap frozen in liquid nitrogen and subsequently stored at −80 °C. Phenotypical infection intensity was determined by counting visible trophonts on the whole left side of the fish body using high resolution images. Trophont size was determined using ImageJ (Version 1.48).

During the experiments delta smelt were fed a mixture of formulated 4/6 NRD diet (INVE Aquaculture, Dendermonde, Belgium) and 370 Hikari plankton food (By-Rite Pet Supply, Hayward, California) at a 2:1 volume ratio. Fish care was performed according to standard operating procedures established at FCCL (Lindberg et al., 2013). The pH was measured using a Beckman 240 pH meter and was recorded as 8.6 ± 0.1. Total ammonia concentration was measured with a HACH DR/890 Colorimeter and a HACH AmVer™ Low Range Ammonia Test 'N Tube™ Reagent Set 0–2.5 mg L^{-1} N (HACH Inc.) and ranged between 0 and 0.25 mg/L. Dissolved oxygen (DO) was quantified using a YSI 85 meter and was constant at 11.5 ± 0.8 mg/L before water temperature was altered. All procedures were performed in accordance with UC Davis Institutional Animal Care and Use Committee (IACUC protocol 16,233).

2.2. Detection of molecular stress response

Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Venlo, Limburg, The Netherlands) according to manufacturer's protocols. Extraction efficiency was confirmed using a NanoDrop ND1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Total RNA 260/280 and 260/230 ratios ranged from 1.93 to 2.14 and from 1.71 to 2.40, respectively. Complementary DNA (cDNA) was synthesized using 500 ng 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, Carlsbad, CA, USA) resulting in 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 with the addition of 100 μL nuclease-free water to a total volume of 120 μL for subsequent real-time qPCR assessments.

Targets for transcriptomic analysis were identified and selected using previously conducted microarray studies on delta smelt (Connon et al., 2011b; Connon et al., 2009; Connon et al., 2011a; Hasenbein et al., 2014; Jeffries et al., 2015; Komoroske et al., 2015). Ten genes related to the complement system (*c1*, *c3*, *c5*), toll-like