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Serum IgM, MH class IIβ genotype and respiratory burst activity do not differ between rainbow trout families displaying resistance or susceptibility to the coldwater pathogen, *Flavobacterium psychrophilum*

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ABSTRACT

Flavobacterium psychrophilum, the causative agent of bacterial coldwater disease (BCWD) is a significant threat to global aquaculture. True to its name, BCWD tends to occur at temperatures between 8 and 12 °C and presents as a systemic disease with characteristic skin ulcerations. Juvenile rainbow trout are particularly susceptible and in these fish the condition is referred to as rainbow trout fry syndrome (RTFS). Resistance to *F. psychrophilum* is heritable and is not adversely correlated with the growth of fish, thus selective breeding appears to be an achievable approach to its control. The current study explores the connection between resistance to BCWD and several immunological markers. After determining resistance/susceptibility to *F. psychrophilum* following experimental infection in 40 full-sibling families of rainbow trout, selected families were experimentally infected with *F. psychrophilum* and differences in antibody production, major histocompatibility (MH) class IIβ genotype and respiratory burst activity (RBA) throughout infection were compared. Serum IgM production increased over time but significant differences between resistant and susceptible families were not observed at either 28 days or 120 days. Of the six families that were genotyped for MH class IIβ, there did not appear to be specific genotypes that conferred resistance or susceptibility to *F. psychrophilum*. Further, the RBA of both head kidney leukocytes and whole blood was not significantly different between the resistant and susceptible rainbow trout families. Although the selected immune markers did not differ based on resistance status, the RBA of head kidney leukocytes in all families studied dramatically decreased seven days after infection while total blood RBA remained constant. Day seven was also when severe symptoms and/or mortality due to BCWD was first observed, thus these results may reveal information regarding the pathogenesis of the organism. A better understanding of appropriate immune defenses could provide the basis for breeding programs to effectively combat this costly pathogen, but further study of functional immune markers particularly during the fry stage of development is required.

1. Introduction

A large obstacle to overcome when dealing with aquaculture production of any type is the vulnerability to disease outbreaks. Like other forms of agriculture, aquaculture places animals in a setting that deviates significantly from that of their natural environment. The stress induced by conditions such as overcrowding, temperature fluctuations and handling can result in normally benign microorganisms becoming opportunistic pathogens (Meyer, 1991). One such pathogen causing significant losses in the farming of rainbow trout (*Oncorhynchus mykiss*) is *Flavobacterium psychrophilum*, the etiological agent of bacterial coldwater disease (BCWD). Rainbow trout, a coldwater salmonid, is an important species of choice in global aquaculture, which has enabled BCWD to become a worldwide concern.

F. psychrophilum is widespread in freshwater aquatic environments but typically only causes infection in intensively reared salmonids at temperatures lower than 16 °C (Holt, 1987). Although *F. psychrophilum* can infect many salmonid species and some non-salmonid species, juveniles of coho salmon (*Oncorhynchus kisutch*) and rainbow trout are particularly susceptible (Nematollahi et al., 2003; Starliper, 2011).

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Clinical signs and manifestations vary depending on location as there appears to be geographic variability between isolates (Lumsden et al., 2004). In North America, the most common presentation of BCWD in fish larger than fingerling is an ulcerative dermatitis known as peduncle disease (Lumsden et al., 2004). The nature of this presentation means that the financial losses associated with BCWD are not due to mortalities alone as surviving fish often produce fillets that are less valuable or not marketable (Lumsden et al., 2004). In fry, the most important presentation is a systemic disease, often with exopthalmia and anaemia, known as rainbow trout fry syndrome (RTFS, Bebak et al., 2007). With no commercial vaccine available and mortality, although variable, has been reported to be as high as 50–85% (Brown et al., 1997, Cipriano and Holt, 2005), the financial losses due to *F. psychrophilum* infection can be devastating.

To combat the significant losses due to infectious disease, there has been increasing interest in selectively breeding aquaculture stocks for disease resistance. As has been demonstrated with many other aquatic diseases (Gjedrem et al., 1991; Henryon et al., 2005; Kjoglum et al., 2006; Kjoglum et al., 2008), resistance to BCWD does appear to be heritable (Silverstein et al., 2009), but there has been little research to identify the basis for this resistance. In addition, a method to consistently produce resistant crosses is yet to be developed for the vast majority of aquatic organisms. Creating and challenging families of rainbow trout takes a great deal of time and finances, thus identifying a marker for the observed resistance to BCWD would decrease costs and expedite this process immensely. With the immune system playing a vital role in the prevention and clearance of infection, it is reasonable to hypothesise that the heritable resistance to BCWD may be associated with teleostean immune function.

Like mammals, teleosts have both innate and adaptive immune systems. The innate immune response constitutes the first line of defense against infection and is activated through recognition of conserved molecular patterns common to pathogenic organisms (Uribe et al., 2011). Phagocytosis, or the engulfment of particles, is a major component of immunity in fish (Uribe et al., 2011). Circulating phagocytes will take up foreign bodies such as bacteria and eradicate them through the production of reactive oxygen species (ROS) during a respiratory burst (Nikoskelainen et al., 2004). Phagocytosis, and the killing activity associated with it, is reported to be the mechanism of innate immunity that is the least influenced by low temperatures (Callazos et al., 1994a; Callazos et al., 1994b; Nikoskelainen et al., 2004), thus this process may be critical when dealing with a cold-water pathogen such as *F. psychrophilum*.

While the innate immune system is fast-acting, adaptive immunity requires more time to develop and has greater specificity. A key component of vertebrate adaptive immunity is the generation of antigen-specific antibodies from B cells. Antibodies promote the control and clearance of infection through such processes as opsonisation for phagocytes, complement activation and neutralization of microbes and toxins (Forthal, 2014). To date, three classes of immunoglobulins have been discovered in teleosts: IgM, IgT and IgD (Sunyer, 2013). The most abundant immunoglobulin is a tetramer of the IgM class (Fillatreau et al., 2013). IgM is the first antibody class to appear after initial exposure to an antigen and may be part of an effective humoral response against invading pathogens (Fillatreau et al., 2013; Reyes-Cerpa et al., 2013). With these protective properties in mind, there have been numerous attempts to identify antigens capable of inducing an effective antibody response to combat *F. psychrophilum* (LaFrentz et al., 2002; Crump et al., 2005; Dumetz et al., 2006; Sudheesh et al., 2007). Although antibody titres have been observed to increase after immunization with many of these antigens and whole-cell lysates, similar increases were often observed with adjuvant alone (LaFrentz et al., 2002). As there is limited information regarding consistent infection and vaccination models for *F. psychrophilum* (reviewed in Gomez et al., 2014), there is a critical need to better understand the serum IgM response in fish presenting resistance/susceptibility to BCWD.

Although fish farmers can benefit from breeding programs based solely on survival phenotype, identifying genes (markers) correlated with enhanced immune performance could aid in the more rapid development of optimum stocks. One possible marker, and a focus of this study, is the major histocompatibility (MH) class II genes. As the class of molecules that recognize and present antigens from extracellular pathogens to tailor the resulting immune responses (reviewed in Neefjes et al., 2011), there has been a great deal of interest in determining whether specific MH class II genotypes are linked to bacterial resistance/susceptibility in fish species (Xu et al., 2010; Dionne et al., 2009; Rakus et al., 2009; Kjoglum et al., 2008). Conversely, MH class I is associated with recognition and presentation of antigens from intracellular pathogens such as viruses (reviewed in Neefjes et al., 2011). Because *F. psychrophilum* appears to have both intracellular and extracellular tendencies throughout its infection cycle (Wood and Yasutake, 1956; Decostere et al., 2001; Nematollahi et al., 2005; Nilsen et al., 2011), it is not fully understood whether MH class I or MH class II genes would provide more protection towards BCWD. To date there has been some evidence of an association between MH class II variability and resistance to *F. psychrophilum* (Johnson et al., 2008), but there has been minimal research since then to relate specific alleles with BCWD resistance. As such, one component of this study aims to link MH class IIβ alleles with observed resistance to BCWD and determine whether this association goes further.

In the present study, full-sibling families of rainbow trout displaying resistance/susceptibility to BCWD were experimentally infected with *F. psychrophilum* so that differences in MH class IIβ genotype, respiratory burst activity and antibody production could be observed and compared. As there is limited knowledge regarding both the pathogenesis of *F. psychrophilum* and what constitutes an effective immune response against the organism, this study aims to analyze functional immune differences in fish that are resistant/susceptible to provide information that could be used for the development of effective breeding programs.

2. Materials and methods

2.1. Fish

Forty full-sibling families of rainbow trout were produced at Lyndon Fish Hatcheries (Dundee, Ont.). These families were transported to the Hagen Aqua Lab or the Alma Aquaculture Facility at the University of Guelph as eyed eggs. Susceptibility to *F. psychrophilum* experimental infection was compared between the 40 family groups. Six to ten families per experiment were divided into four 60 L tanks containing 40 fish (40 g each) per tank, which were supplied with single pass well-water at 11 °C. Fish were fed a commercial pellet diet (Martin Mills Inc.). All maintenance and procedures were done in accordance with the University of Guelph Animal Care Guidelines.

2.2. Culture and quantification of *F. psychrophilum* strain 101

Frozen stocks of *F. psychrophilum* strain 101 (FPG 101) were subcultured onto cytophaga agar (CA) then following incubation at 15 °C for 72 h, cells were harvested and suspended in cytophaga broth (CB, Hesami et al., 2011). Cell suspensions were adjusted to an optical density of 0.6 at 600 nm using a Novaspec Plus spectrophotometer (GE Healthcare Life Sciences) and the suspension was then diluted to obtain a final concentration of $\sim 1 \times 10^9$ colony forming units (CFU)/mL. The concentration was then adjusted so that the desired CFU/mL for each trial could be obtained. To confirm the cell numbers, suspensions were enumerated by plating 0.1 mL of ten-fold serial dilutions on CA plates in triplicate. After incubation at 15 °C for 4 d, plates containing 25–250 colonies were counted and the number of CFU/mL calculated.