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Transcriptional immune response of cage-cultured Pacific bluefin tuna during infection by two *Cardicola* blood fluke species

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ABSTRACT

Infections by two blood fluke species, *Cardicola orientalis* and *Cardicola opisthorchis*, currently present the greatest disease concern for the sea-cage culture of Pacific bluefin tuna (PBT) – a species of high global economic importance and ecological concern. In this study, we aimed to rapidly, quantitatively, and differentially identify infections by these two parasite species in cultured PBT as well as identify potential host immune responses. Using real-time qPCR, we were successful in quantitatively detecting parasite-specific DNA from within host blood, gill, and heart tissues; positively identifying parasitic infections 44 days earlier than microscopy methods previously employed. Both gill and heart became heavily infected by both parasite species in PBT within two months of sea-cage culture, which was only mitigated by the administration of anthelmintic praziquantel. Nevertheless, fish were observed to mount an organ specific transcriptive immune response during infection that mirrored the relative quantity of pathogenic load. In heart, significant (3–6 fold) increases in IgM, MHC2, TCRβ, and IL-8 transcription was observed in infected fish relative to uninfected controls; whereas in the gills only IgM transcription was observed to be induced (11 fold) by infection. Interestingly, the relative quantity of IgM transcription was highly correlated to the relative abundance of *C. orientalis* but not *C. opisthorchis* DNA in the gill samples, even though this organ showed high prevalence of DNA from both parasite species. Taken together, these findings indicate that although ineffective at combating infection during primary exposure, a cellular immune response is mounted in PBT as a potential rejoinder to future *Cardicola* exposure, particularly against *C. orientalis*. Although future investigation into antibody effectiveness will be needed, this work provides valuable preliminary insight into host responsiveness to *Cardicola* infection as well as additional support for the need of anthelmintic treatment following primary parasite exposure during PBT culture.

1. Introduction

Pacific bluefin tuna (PBT) *Thunnus orientalis*, along with its two sibling species (Southern bluefin tuna *Thunnus maccoyii* and Atlantic bluefin tuna *Thunnus thynnus*), comprise one of the most economically important food fisheries in the world. Historically, wild fish were captured and sent directly to market; however, in recent decades this fishery has become increasingly reliant on intensive culture practices to maximize profitability. Currently more than 20% of the total annual wild harvest of bluefin is conscribed to intensive culture practices [1], and full-lifecycle production of these fish has been recently achieved at Kinki University which is quickly becoming a viable means of commercial production in Japan [2–5].

Infections by blood flukes in the genus *Cardicola* represent the most important disease currently influencing the culture of bluefin tuna [6]. Although infection has been linked to mortality through at least the juvenile life stage in all three bluefin tuna species [7], young tank-reared PBT have shown the highest susceptibility and mortality following transfer to sea-cage environments [6]. In such cases, two species of blood fluke from genus *Cardicola* have been attributed to disease in PBT – *Cardicola orientalis* and *Cardicola opisthorchis* [8,9]. Microscopic analysis has revealed that *C. orientalis* adults and eggs have a tropism for the gill [9,10], whereas *C. opisthorchis* adults are found in the heart [8] while their eggs are known to accumulate in the afferent arteries of the gill [10]. Concurrent infections appear to be common, and although the presence of either parasite is thought to contribute to disease and

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cumulatively linked to mortality [10,11], little is known about the mechanisms for disease, host immune response, or the full extent of distribution within the host for these parasites.

Recently, we have developed qPCR techniques to sensitively and quantitatively detect *Cardicola* DNA that were successfully applied to the identification and differentiation of concurrent infections of *C. orientalis* and *Cardicola forsteri* in Southern bluefin tuna heart, gill, and serum [12]. These analyses demonstrated a surprising prevalence and distribution of *C. orientalis* which had only recently been identified in ranched Southern bluefin [13], demonstrating the utility for potentially identifying previously undetectable free living parasite life stages within the host. Further, some immune and stress associated genetic biomarkers have also recently been identified for bluefin tuna which have shown effectiveness in monitoring immune responsiveness during *in vitro* LPS stimulation [14], as well as *in vivo* during parasitic *Didymocystis wedli* gill infections [15]. Immune responsiveness of bluefin tuna to *Cardicola* infection is relatively unknown; however, there is serological evidence that *C. forsteri* can elicit an antibody response in juvenile Southern bluefin tuna [16]. Additionally, there is anecdotal evidence that cultured fish which survive a parasitic outbreak during the first year of cage culture are resistant to further disease [11], which has led us to hypothesise that an antibody response specific to *Cardicola* infection may be effective at combating disease in some circumstances.

In this study, our objectives were to articulate the time course of natural infection following the transfer of young PBT to sea-cage environments, as well as determine relative prevalence and identify host responsiveness to infection by *C. orientalis* and *C. opisthorchis*. To do this, we aimed to combine the recently developed qPCR techniques for differential *Cardicola* spp. detection with host immune gene transcription. This provided the ability to directly link the presence of *Cardicola* with host cell signalling and to potentially differentiate the roles that each of these two *Cardicola* species may play during infection.

2. Materials and methods

2.1. Subjects and field collection

Approximately 140,000 full-cycle cultured PBT supplied by the Ohshima and Uragami Fish Nursery Centers of Kinki University, Wakayama prefecture, Japan, were transferred to commercial-sized sea-cages (6000 fish/cage) at Kinki University Ohshima Experimental Station approximately 30 days post-hatch. Fish were maintained on commercial pellets and frozen sand lance (*Ammodytes* spp.) to satiation throughout the duration of this study [3,5,11]. Over a period of four months (August–December, 2012), six sets of six fish were lethally subsampled from selected sea-cages to identify the presence of *Cardicola* spp. and to preserve samples for molecular analysis (Fig. 1). In each instance, fish were caught by a dip net or hook and line, stunned by percussive blow to the head, and killed by a spike to the brain as per standard industrial practice with highest consideration for animal welfare. Blood (250 μL) was collected immediately from the heart using a 24 gauge needle and heparinised syringe and preserved in four volumes (1.25 mL) nucleic acid preservation solution (NAPS; 4 M ammonium sulphate, 25 mM sodium citrate, 10 mM EDTA; pH 5.5). An approximate 100 mg sample was removed from the first left gill arch and heart ventricle of each fish, minced with scissors, and stored separately in 1.25 mL of NAPS at 4 °C. Additionally, a portion of the remaining gill was evaluated microscopically for the presence/absence of *Cardicola* as previously described [10]. At 83–87 days post-transfer, cages were individually treated with an oral dose of 150 mg/kg praziquantel (PZQ) to control against *Cardicola* infection [11].

2.2. Nucleic acid extraction and cDNA synthesis

Isolation of *C. orientalis* and *C. opisthorchis* DNA was attempted from all preserved heart, gill, and whole blood samples collected in this study. In each instance, total nucleic acid was extracted from 10 mg organ sample or 10 μL blood. NAPS was first removed from pelleted blood components following centrifugation at 5000 × *g* for 5 min, or from tissues following gentle dabbing on a clean glass plate. Samples were then digested and cells lysed in 400 μL Extraction Buffer (4 M Urea, 0.5% SDS, 0.2 M NaCl, 10% glycerol) supplemented with 20 U Proteinase K (Bioline) at 4 °C. To hasten digestion and cell lysis, both heart and gill samples were immediately homogenized with the aid of a micro pestle (Eppendorf) and blood titurated with a 1 mL pipette following addition of the extraction buffer. Samples were incubated on ice with occasional vortexing for 15 min and then divided into two equal volumes. In one portion, total nucleic acid (TNA) was selectively separated from cellular debris and protein by the addition of 200 μL 7.5 M ammonium acetate and cleaned following alcohol precipitation as previously described [14]. The other portion was used for selective RNA extraction by addition of 1 mL RNAzol® and 50 μL BAN® phase separation reagent (Molecular Research Center, OH, USA) as outlined by the manufacturer. Complete removal of DNA was ensured with 4 units Baseline-Zero DNAse (Epicentre, WI, USA) incubated for 30 min at 37 °C as per manufacturer's instructions. RNA was quantified using a Qubit fluorometer (Invitrogen, VIC, Australia), and RNA integrity was confirmed by visualization on a 1% agarose bleach denaturing gel [17]. A portion of RNA (1 μg) from 36 samples (representing all 6 sampling events) was reverse transcribed using a cDNA Synthesis Kit (Bioline, NSW, Australia) with Oligo $(dT)_{18}$ primer mix. A portion of remaining RNA from each sample was pooled, and 1 μg reverse transcribed in quadruplicate for use in preparing qPCR standards. Three additional 1 μg reactions of pooled RNA without reverse transcriptase provided no-reverse transcription controls.

2.3. Real-time qPCR analyses

All real-time qPCR analyses were conducted on a CFX Connect Real-Time PCR detection system (Bio-Rad, NSW, Australia) with

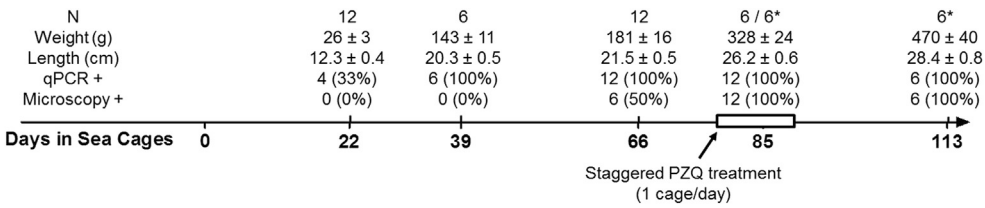

Fig. 1. Linear presentation for the time course and sampling events used in this study. Arrow indicates the time course for early cage culture of juvenile Pacific Bluefin tuna with marked sampling events, for which sample number (*N*), mean weight, and fork length (±SEM) of fish are provided. The number of individuals from which *Cardicola* spp. was positively identified by gill microscopy and qPCR analysis (heart and gill) is also provided, as is the period for single anthelmintic praziquantel treatments (PZQ; 150 mg/kg) of culture cages. (*) indicates sample populations which had received a PZQ treatment.