Fish & Shellfish Immunology 58 (2016) 490–499

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

# Immune reactivity in early life stages of sea-cage cultured Pacific bluefin tuna naturally infected with blood flukes from genus *Cardicola* (Trematoda: Aporocotylidae)

Ylenia Pennacchi [a, *], Sho Shirakashi [b], Barbara F. Nowak [a], Andrew R. Bridle [a]

[a] IMAS Launceston, University of Tasmania, Australia
[b] Aquaculture Research Institute, Kindai University, Nishimuro, Wakayama, Japan

ARTICLE INFO

*Article history:*
Received 25 May 2016
Received in revised form
28 September 2016
Accepted 30 September 2016
Available online 1 October 2016

*Keywords:*
Pacific bluefin tuna
*Cardicola*
Immune gene expression
Real-time qPCR
Quantitative detection
Praziquantel
Digenea

ABSTRACT

Pacific bluefin tuna (PBT), *Thunnus orientalis*, due to its high average price on the market is an economically valuable fish species. Infections by blood flukes from the genus *Cardicola* (Trematoda: Aporocotylidae) represent a growing concern for the cage culture of bluefin tuna in Japan, Australia and Southern Europe. The accumulation of numerous *Cardicola* eggs in the fish gills causes severe pathology that has been linked to mortality in PBT juveniles up to one year old. The only effective treatment used to mitigate the infection is the oral administration of the antihelminthic drug praziquantel (PZQ) to the affected fish. However, with the need to minimise therapeutic drug use in aquaculture it is hoped that immunoprophylaxis can provide a future alternative to protect the PBT juveniles against *Cardicola* infection. Currently, little is known of the host immune response to these parasites and of their infection dynamics. In this study, using real-time qPCR we aimed to quantitatively detect *C. orientalis* and *C. opisthorchis* DNA within the gills and heart of cultured PBT juveniles and to investigate the host immune response at the transcriptional level in the gills. The research focused mainly during early stages of infection soon after young PBT were transferred to culture cages (from 14 to 77 days post-transfer). An increase (up to 11-fold) of immune-related genes, namely IgM, MHC-I, TCR-β and IL-1β was observed in the PBT gills infected with *Cardicola* spp. (28–77 days post-transfer). Furthermore, IgM (19-fold increase) and MHC-I (11.5-fold increase) transcription was strongly up-regulated in gill samples of PBT infected with *C. orientalis* relative to uninfected fish but not in fish infected with *C. opisthorchis*. *Cardicola*-specific DNA was first detected in the host 14 days post-transfer (DPT) to sea-cages which was 55 days earlier than the first detection of parasite eggs and adults by microscopy. Oral administration of PZQ did not have an immediate effect on parasite DNA presence in the host and the DNA presence started to reduce after 24 days only in the host heart. The results provide evidence of an immune response in early age sea-cage cultured juveniles of PBT naturally infected with *C. orientalis* and *C. opisthorchis*. This response, whilst not protective against primary infection, provides evidence that immunisation at an early age may have potential as a health strategy.



## 1. Introduction

The Pacific bluefin tuna (PBT) (*Thunnus orientalis*) is one of the most popular tuna species and it has a high economic value. As with other commercially relevant bluefin tuna species PBT aquaculture is mainly based on a capture and grow-out approach since its farming relies on the stocking of wild-caught individuals. In 2002, after 32 years of study, researchers at Kindai University, Japan, closed the PBT life cycle in captivity to face the increasing demand for PBT in the Japanese market and to minimise the exploitation of wild stock [1]. Continued improvements in rearing techniques have allowed increased production and in 2012 the Japanese PBT production was 9592 metric tons, of which 244 metric tons were derived from hatchery-reared juveniles [2]. One of the most critical phases during PBT farming is the transfer of fingerlings (approximately 30 days old) from land-based facilities to sea cages [3].

Following the transfer to sea cages, fish are exposed for the first

* Corresponding author. University of Tasmania, Locked bag 1370, Launceston, TAS, 7250, Australia.
*E-mail address:* Ylenia.Pennacchi@utas.edu.au (Y. Pennacchi).

http://dx.doi.org/10.1016/j.fsi.2016.09.060
1050-4648/

time to different pathogens of which two blood fluke species ascribed to the genus *Cardicola* (Trematoda: Aporocotylidae), *C. orientalis* and *C. opisthorchis*, represent the greatest concern for the Japanese tuna farming industry [4]. *C. orientalis* is known to infect mainly the gill arteries whereas *C. opisthorchis* is predominant in the heart [5,6]. Co-infections by *C. orientalis* and *C. opisthorchis* are common, however the eggs of these two species are distinguishable as *C. orientalis* have smaller-ovoid shaped eggs while *C. opisthorchis* eggs are a larger-crescent shape [7]. Eggs can accumulate in the gills and eventually cause the fish to die of suffocation [8]. Juvenile PBT up to one year old are the most threatened by *Cardicola* spp. and to mitigate the recurrent infection outbreaks an anthelmintic drug, praziquantel (PZQ), is orally administered to fish [8]. Immunomodulatory abilities and the potential capacity to act as an immune stimulant/adjuvant have been attributed to PZQ [9]. Recently, a third *Cardicola* species, *C. forsteri*, was reported in farmed PBT in Japan [10]. *Cardicola* spp. represent a threat not only to the PBT farming in Japan but also to the cage culture of other bluefin tuna species worldwide [5,6,11,12]. In the Mediterranean, four species of *Cardicola* (*C. forsteri*, *C. orientalis*, *C. opisthorchis* and a not yet described species) are known to infect Atlantic bluefin tuna (ABT) (*Thunnus thynnus*) cage-reared adult individuals [11] and infection outbreaks by *C. forsteri* and *C. orientalis* represent a recurrent issue for the cage-culture of the Southern bluefin tuna (SBT) (*Thunnus maccoyii*) in Australia [13–15].

Until recently, the only method to determine a *Cardicola* spp. infection in bluefin tuna was through the use of gill microscopy and heart flushes, respectively to identify the parasite's eggs and adults. A recent study showed the usefulness of qPCR techniques as a diagnostic method to differentially and quantitatively identify the presence of *Cardicola* spp. DNA in bluefin tuna organs [16]. Furthermore, compared to microscopy, the qPCR diagnostic method allows the farmers to determine the onset of infection at earlier developmental stages [15–17]. In fact, the qPCR is able to detect (but not distinguish) the DNA of any *Cardicola* life cycle stage [15].

In the last decade, the parasitic infections of blood flukes belonging to the genus *Cardicola* became of worldwide interest due to the high commercial value of the fish host. Although extensive research has been carried out on the bluefin tuna, little is known about the mechanisms involved in the host immune response to *Cardicola* spp. Nevertheless, there is evidence of a serum antibody response in SBT [18] and resistance to recurrent infection during the first year of cage culture in PBT [8], which suggest a partially protective *anti-Cardicola* spp. antibody response is developed. Recently, investigations of the PBT immune response to *Cardicola* spp. infection have shown an increased transcription of selected immune genes in the gill (IgM) and heart (IgM, MHC-II, TCR-β, and IL8) of infected juveniles relative to non-infected fish during long-term infection [17]. A highly significant positive correlation between the relative quantity of IgM transcription and *C. orientalis* DNA relative abundance in the gills was shown for the same fish [17]. Altogether these previous results on the PBT immunity against *Cardicola* suggest that host immunisation may represent a future possible prophylactic health strategy.

The aims of this study were to investigate the PBT early life stages immune response to *Cardicola* spp. infection in the main affected organ – the gills, determine the relative prevalence of *C. orientalis* and *C. opisthorchis* in PBT gill and heart and investigate the relationship between the immune response and the relative prevalence of each *Cardicola* spp. To achieve these aims, qPCR techniques were used to differentially detect *Cardicola* spp. DNA in the host gills and heart and to investigate the host immune gene expression at the mRNA level. Furthermore, routine histology was used to describe the pathology associated with *Cardicola* spp. eggs in the host gills. The current study was focused on early rearing of PBT, which is the farming stage with the highest recorded mortality during cage culture due to *Cardicola* spp. infection. Given the recent finding of a third *Cardicola* species (*C. forsteri*) found to infect PBT, we also aimed to describe its presence and relative prevalence.

## 2. Materials and methods

### 2.1. Juvenile PBT farm history

The use of juvenile PBT and the experimental procedures were approved by the University of Tasmania (Animal Ethics Committee Permit No. A0013248). PBT came from a broodstock of farm raised fish at Oshima Station, Aquaculture Research Institute, Kindai University, Wakayama prefecture, Japan. The spawning occurred around the 19$^{th}$-20th of June 2013 and fertilised eggs were collected from sea cages and transferred to Oshima land based facilities where the hatching occurred on the 21st of June. On the 24th of July, fish were transferred into sea cages. Fish were fed a commercial diet and frozen sand lance (*Ammodytes* spp.) to satiation throughout the duration of the study. Mortality in the sea cages was checked and recorded daily and any dead fish were removed by technical personnel to prevent the spread of diseases. In the individuals used in this study, infection by *Cardicola* spp. eggs and adults was first detected by microscopy in the gills and heart at 69 days post transfer (DPT) to sea cages. The antihelminthic praziquantel (PZQ) (150 mg/kg) was orally administered to fish 50–53 DPT after first detection of the *Cardicola* spp. infection in the PBT juveniles stocked in nearby cage facilities.

### 2.2. Sampling strategy and samples collection

Juvenile PBT ($n = 45$), five fish each sampling, were collected at 14, 21, 28, 35, 42, 49, 56, 69 and 77 DPT from the same sea cage. At the first sampling (14 DPT) the fish average weight was 1.2 g and length 4.2 cm and at the last sampling (77 DPT) the average weight was 233.3 g and lenght 20.9 cm. Fish were caught from the cage using a small dip net, immediately killed following standard industrial practice, and put on ice until dissection. For histological analysis the second left gill arch was dissected, rinsed with filtered seawater and immediately fixed in 10% neutral buffered formalin. After 24 h, histological samples were transferred to 70% ethanol. For molecular analysis the second right gill arch and the ventricle of the heart were dissected, rinsed with filtered seawater and immediately placed in an RNA preservation reagent (4 M ammonium sulphate, 25 mM sodium citrate, 10 mM EDTA; pH 5.2). The samples collected in RNA preservation reagent were temporarily stored at 4 °C overnight and then at −20 °C for long term storage. Following each sampling 5–10 gill filaments from the first gill arch of each fish were placed on a slide and examined under a light microscope for the presence of *Cardicola* spp. eggs and the heart checked for the presence of *Cardicola* spp. adults using a dissecting microscope. PBT (originated from the same spawning event) stocked in nearby sea cages were routinely sampled (same time points of the fish used in this study) from the fish farm staff as industrial practice to monitor the fish health and check for signs of *Cardicola* infection.

### 2.3. Gill histology

Samples were decalcified for 45 min using the Rapid decalcifying fluid (Australian Biostain Pty. Ltd.) then processed for routine histology in a Leica TP1050 tissue processor by being dehydrated in a sequence of increasing concentration of ethanol (80%, 95% and 100%), then immersed in xylene and finally in paraffin. Samples were embedded in clean paraffin, sectioned