APPLICATION OF MOLECULAR METHODS FOR DETERMINING IMMUNE RESPONSES AND DISEASE STATUS IN BLUEFIN TUNA

Mark Polinski (MSc, BSc, AApSc)

National Centre of Marine Conservation and Resource Sustainability

Australian Maritime College

University of Tasmania

Submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy

February, 2014

Declarations by the Author

Statement of Originality

The work presented in this thesis is, to the best of my knowledge and belief, original and my own work, except where due reference is made. I hereby declare that I have not submitted this material, either in whole or in part, for a degree at this or any other university, nor does the thesis contain any material that infringes copyright.

Authority of Access

The publishers of the papers comprising Chapters 2 thru 5 hold the copyright for that content, and access to the material should be sought from the respective journals. The remainder of this thesis may be made available for loan and limited copying in accordance with the Copyright Act of 1968.

Statement of Ethical Conduct

The research associated with this thesis abides by the international and Australia codes on animal experimentation. Animal ethics permits were obtained from the University of Tasmania Animal Ethics Committee for all aspects of the project where experiments involved live animals (AEC project numbers A0010593 and A0012145).

Signed:  Date: 11/12/13

Mark Polinski

Statement of Co-Authorship and Thesis Contributions

The following people and institutions contributed to the publication of the work undertaken as part of this thesis:

Mark Polinski (Candidate)	University of Tasmania, Launceston, Australia
Dylan Belworthy Hamilton (1)	University of Tasmania, Launceston, Australia
Lukas Neumann (2)	University of Tasmania, Launceston, Australia
Sho Shirakashi (3)	Kinki University, Nishimuro, Wakayama, Japan
Andrew Bridle (4)	University of Tasmania, Launceston, Australia
Barbara Nowak (5)	University of Tasmania, Launceston, Australia

Paper 1 (Chapter 2)

Polinski M, Bridle AR, Nowak B (2013) Temperature-induced transcription of inflammatory mediators and the influence of Hsp70 following LPS stimulation of southern bluefin tuna peripheral blood leukocytes and kidney homogenates. Fish and Shellfish Immunology, 34: 1147-1157.

Candidate was the primary author and performed all laboratory analyses. Author 4 and author 5 contributed to the idea, its formalisation and development, refinement, and presentation.

Paper 2 (Chapter 3)

Polinski M, Belworthy Hamilton D, Nowak B, Bridle A (2013) SYBR, TaqMan, or both: Highly sensitive, non-invasive detection of *Cardicola* blood fluke species in Southern Bluefin Tuna (*Thunnus maccoyii*). Molecular and Biochemical Parasitology 191: 7-15.

Candidate was the primary author and performed all analysis concerning technique development and assessment. Author 4 contributed to qPCR primer design and along with author 5 contributed to the study idea, its formalisation, development, refinement and presentation. Author 1 offered general laboratory assistance with analaysing field samples by qPCR and histology.

Paper 3 (Chapter 4)

Polinski M, Shirakashi S, Bridle A, Nowak B (*in press*) Transcriptional immune response of cage-cultured Pacific bluefin tuna during infection by two *Cardicola* blood fluke species. Fish and Shellfish Immunology, doi: j.fsi.2013.10.008.

Candidate was the primary author and performed all molecular analyses. Author 3 provided assistance in collecting samples and completed microscopic pathogen detection in addition to aiding in the refinement and presentation of the manuscript. Authors 4 and author 5 contributed to the development, refinement, and presentation of the study.

Paper 4 (Chapter 5)

Polinski M, Neumann L, Bridle A, Nowak B (*Submitted*) Preliminary evidence for transcriptional immunomodulation by praziquantel in bluefin tuna and Atlantic salmon *in vitro* cultures. Fish and Shellfish Immunology.

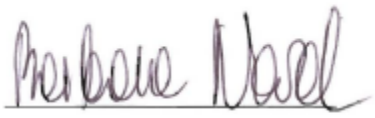
Candidate was the primary author and performed all molecular analyses. Author 4 and author 5 contributed to the formalization, development, refinement, and final presentation of the manuscript. Author 2 offered general laboratory assistance regarding *in vitro* stimulations.

Declaration of agreement

We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

Candidate:  Date: 11/12/13

Mark Polinski

Primary Supervisor:  Date: 11.12.13

Barbara Nowak

Head of School: 

~~John Purser~~

Date: 12/12/13

Abstract

Bluefin tuna (*Thunnus* spp.) are a globally threatened fish that currently constitutes one of the most economically important food fisheries in the world. Culture efforts have intensified in recent years to maximise fishery profitability and mitigate market dependency on wild stock; yet little is currently known concerning health and immune functions and best culture practices continue to be investigated. In an attempt to aid future aquaculture interests and to provide general tools for identifying immune responses and disease status for this group of fishes, this thesis investigated a number of immune and disease-identifying aspects of bluefin tuna through the use of molecular laboratory techniques centering on quantitative PCR.

In the course of this research, six full length and thirteen partial mRNA transcripts were newly identified for two bluefin species [Southern bluefin tuna *Thunnus maccoyii* (SBT) and Pacific bluefin tuna *Thunnus orientalis* (PBT)] which represent a suite of genetic markers involved in immune, stress, growth, and inflammatory functions. As temperature is known to influence inflammatory signalling in mammals, *in vitro* stimulations and subsequent gene expression analyses were conducted on cells of SBT to identify the effects temperature might play during an immune response in these uniquely heterothermic fish. Results from these experiments suggested that temperature exerts influence in the timing but not the degree of an innate inflammatory response in bluefin tuna and also that different cell populations have differential responsiveness to heat shock in this heterothermic species. Additionally, heat shock co-activation of the IL-8 chemokine previously identified in mammals is also likely present in bluefin and suggests that increased immune cell trafficking has long been incorporated into the stress responses of vertebrates.

Infections by three species of blood fluke from the genus *Cardicola – C. forsteri*, *C. orientalis*, and *C. opisthorchis* – represent the greatest disease concern for the sea-cage culture of bluefin tuna. Using real-time qPCR and novel applications of SYBR green nucleic acid dye in combination with a TaqMan probe common reporter system, sensitive quantitative detection of parasite species-specific DNA was achieved and was effective for identifying parasite in samples of host blood, gill, and heart of both SBT and PBT species. This identified infections earlier and more sensitively than either histology or microscopy methods previously employed. These detection techniques were used in conjunction with gene expression analyses to demonstrate that organ-specific transcriptive immune responses occurred in PBT during natural infection which mirrored the relative quantity of pathogenic load. Although ineffective at combating infection during primary exposure, this research demonstrates a cellular immune response is mounted in PBT as a potential rejoinder to future *Cardicola* exposure and/or mitigation against tissue destruction caused by the parasite. Further, IgM transcription was highly correlated to the relative abundance of *C. orientalis* but not *C. opisthorchis* DNA in gill samples, suggesting that host IgM transcription may be targeted to species or life stage specific antigen.

Lastly, Praziquantel (PZQ), a drug long used in veterinary and human medicine for the treatment of helminth parasites and currently applied to treat *Cardicola* infections in tuna, has been shown to enhance humoral and cellular immune response in mammals. Here, PZQ was shown to induce gene transcriptional changes in immune-competent primary tissue cultures of both SBT and Atlantic salmon *Salmo salar*. Although expression profiles varied between species and tissue type, PZQ was observed to significantly induce both T cell receptor and IL-8 transcriptional expression in all cultures, as well as creating a general pattern of heightened antiviral signalling that included elevated transcription of both Type I (IFNα) and Type II (IFNγ) interferon along with elevated expression of MHC class I. These

findings identify a direct immunomodulatory ability of PZQ in immune cells of fish and provide support for further investigation into the immunostimulant/adjuvant capabilities of this drug in fish culture. This work also aids in understanding the mode of action for this drug in the treatment of *Cardicola* infection in bluefin tuna.

Acknowledgements

Funding for this work was provided in part by the Australian Department of Education, Employment, and Workplace Relations via the Endeavour International Postgraduate Award and the University of Tasmania Australian Maritime College John Bicknell Scholarship. I want to thank my supervisors, Dr. Andrew Bridle and Prof. Barbara Nowak, for the opportunity to conduct this research and for the guidance and support they have provided. It would not have been possible without their assistance. I would like to thank all those people who provided me with valuable constructive advice; specifically Sho Shirakashi, Phillip Crosbie, and Kenneth Cain. I would also like to extend my deepest appreciation for the technical support given to me by Karine Cadoret, Victoria Valdenegro, Melanie Leef, Lukas Neumann, Dylan Belwothy Hamilton, and Nichole Kirchhoff. Finally, I would like to thank my partner, Leanne, who has provided assistance and support to the very best of her ability throughout my time spent in attaining this educational goal. I could not have asked for a better companion and am deeply grateful to have her be part of my life.

Explanatory note concerning thesis structure

Chapters 2 through to 5 of this thesis have independently been published (or submitted for publication) as journal articles. As a consequence, some textual and reference overlap occurs between these chapters, and with the introductory material. The first chapter of this thesis is written as a general introduction and review of relevant topics needed to establish the experimental rationale of the subsequent chapters. Although this material is separated into discrete topics, its organisation has been orchestrated to read as a single rational argument culminating in the aims of this PhD project. This material also provides some reference to literature that was not available at the beginning of this PhD project, but nevertheless was critical in shaping the ongoing work. Chapter 6 provides an integrating discussion for the discrete research chapters and general conclusions in this context. The referencing style used by the Public Library of Science (PLoS) has been adopted for this thesis; however, the orthography is consistent with the commonwealth countries of Britain. As chapters 2 through to 5 are presented in their published entirety, discrete bibliography sections are presented at the end of each chapter throughout the thesis.

Table of Contents

Chapter One

General Introduction

1.1. Bluefin tuna

There are three species of fish commonly referred to as bluefin tuna – Atlantic bluefin tuna *Thunnus thynnus* (ABT) found in the Atlantic Ocean and the Mediterranean Sea, Pacific bluefin tua *Thunnus orientalis* (PBT) present in the northern Pacific Ocean as well as in some southern waters, and Southern bluefin tuna *Thunnus maccoyii* (SBT) distributed throughout the south Atlantic, Pacific, and Indian oceans (Table 1.1.). Bluefins represent the largest and longest lived of the tunas, and epitomise the exceptional physiology unique to this genus of fish. Namely, a specialised swimming style, high cardiac output, and partial endothermy which has allowed them to exploit a range of latitudes and ocean depths beyond the physiological capacities of other fish [1].

The physical culmination of such extraordinary evolutionary characteristics has resulted in proportionally large amounts of red muscle relative to other fish, and for more than 3000 years human civilisations have devised techniques to catch these highly prized and energy rich animals. In recent decades however, increases in fishing technology and high consumer demand have led to over-exploitation [2]. Currently the International Union for Conservation of Nature (IUCN) considers all three species of bluefin tuna threatened by extinction, of which SBT and ABT are both believed to be highly in danger [3]. International efforts toward the conservation of this fishery have been initiated, and currently strict regulatory quotas have been set by all five tuna regional fisheries management organizations to protect and sustainably manage this global resource [4]. Nevertheless, there continues to be high demand for bluefin tuna flesh, and even under the limited annual international catch limits, the combined harvest of PBT, ABT and SBT can conservatively be estimated at over $600 million USD per annum [5]. Indeed, the annual harvest of ABT alone has recently been speculated to be worth over $7 billion USD to the global economy [6], and on the opening of

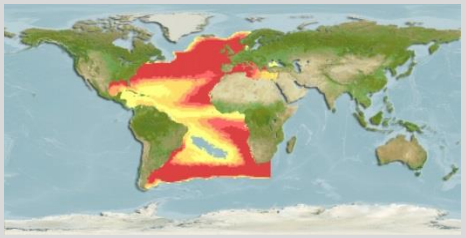
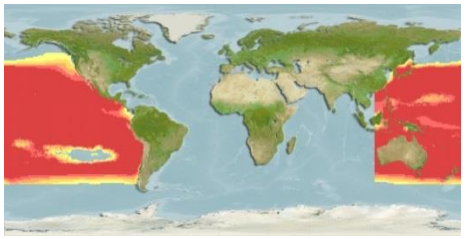
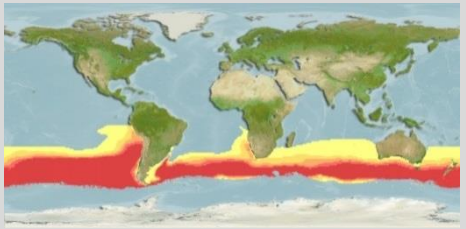
the Japanese bluefin tuna auction in January, 2013, a single 221 kg PBT sold for a record $1.76 million USD [7].

In response to the conservation efforts enacted for this fishery, interest has intensified toward developing and utilising intensive culture techniques. In the past two decades, many fishing companies have adopted the practice of transferring wild caught bluefin to sea-cage environments for a period of months or years prior to harvest. Such 'ranching' is now well established in waters around Japan, Australia, North America, and the Mediterranean Sea, for which more than 20% of the total annual harvest of wild bluefin tuna is now conscribed [5]. Although still dependent on wild stocks, the control over timing, flesh quality and fish size afforded by these practices has maximised profitability for the industry currently restricted by catch limits. Even more important in a conservation context, has been the recent advances in captive breeding and early life cycle rearing of Pacific bluefin. Research through Kinki University, Japan, has culminated in the full lifecycle production of PBT which is now becoming a viable commercial enterprise [8-13]. This provides the first alternative for alleviating the current pressures on wild fish while continuing a market supply of bluefin, and investigation into expanding these practices has already begun in Australia and other parts of the world [14].

One unfortunate consequence of the intensive culture of fish is the increased potential for disease [15,16]. Although bluefin tuna are considered to have a well-developed immune system relative to other marine teleosts, both in the context of early development of immune organs [17] and resilience to situations that cause immune suppression in other fish species [18], disease has nevertheless manifested during the culture of these organisms. Young PBT have been shown to be susceptible to both iridoviral [12] and viral nervous necrosis virus [19,20]. Well over 50 species of metazoan internal and external parasites have been identified from cultured tunas [21-24], and at least 18 bacteria species have also been isolated from

these species [25] (Table 1.1). Currently, eight of these potential pathogens are believed to cause disease or mortality – two viruses: Red sea bream iridvirus, viral nervous necrosis virus, and five species of parasites: *Uronema nigricans*, *Caligus chiastos*, *Cardicola forsteri*, *Cardicola orientalis*, and *Cardicola opishthorchis*. Nevertheless, investigations into disease susceptibility of bluefin tuna, particularly in cultured environments, have been limited and the potential immune response to infection is largely unknown. As the culture of bluefin tuna is likely to expand, a greater understanding of disease susceptibility and immune responsiveness in these organisms will be needed. Specifically, investigations into the impact of *Cardicola* parasites on host immune function and further characterization of their distribution within tuna populations may provide a greater understanding of disease transference in fish from wild to cultured environments and how to best ensure effective immune responses of tuna to such infections under culture conditions to maximise health and growth during aquaculture production.

Table 1.1. Summary of geographic range, physical characteristics, conservation status, and known pathogens of bluefin tuna. Both geographic range and physical characteristics were compiled from FishBase (www.fishbase.org).

Species	Geographic Range	Maximum physical characteristics			IUCN status [3]	Confirmed potential pathogens
		Length	Weight	Lifespan		
Atlantic bluefin tuna *Thunnus thynnus* (Linnaeus, 1758)		4.6 m (15 ft)	684 kg (1,508 lbs)	35-50 yrs	EN Endangered	50+ Metazoan parasite species [22] *Photobacterium damsela* [22]
Pacific bluefin tuna *Thunnus orientalis* (Temminch & Schlegel, 1844)		3.0 m (9.8 ft)	450 kg (990 lbs)	15-26 yrs	LC Least concern	Viral nervous necrosis virus [20] Red seabream iridovirus [12] Unknown Scuticociliate [22] 30+ Metazoan parasite species [22-24]
Southern bluefin tuna *Thunnus maccoyii* (Castelnau, 1872)		2.45 m (8 ft)	260 kg (570 lbs)	20-40 yrs	CR Critically endangered	*Uronema nigrican* [26] 20+ Metazoan parasite species [22] 18+ bacteria species [25]

1.2. Blood flukes of the genus *Cardicola*

Of the pathogenic organisms which have been isolated from bluefin tuna, blood flukes in the genus *Cardicola* have emerged as the most serious threat during intensive culture. Three species have now been identified to infect bluefin – *C. orientalis* [24], *C. opisthorchis* [23], and *C. forsteri* [27]. In Japan, *C. orientalis* has been shown to cause high mortality in young PBT shortly following their transfer to sea cages [24,28]. In Australia, *C. forsteri* has been linked with mortality events in ranched populations of juvenile (2-3 year old) SBT [29,30]. *Cardicola opisthorchis* has also been identified to cause pathology in both PBT and ABT cultured stocks, and although not yet directly linked to mortality, is suspected to contribute significantly toward an unhealthy status [23,31].

Most digenean organisms possess high host specificity and it was initially believed that each *Cardicola* spp. was likely present only in a single host species. Continued research however has revealed that each bluefin species can be parasitised by at least two species of *Cardicola*, sometimes in concurrent infections [23,32]. PBT have now been shown to be susceptible to both *C. orientalis* and *C. opisthorchis* [see 23,24], ABT to *C. forsteri* and *C. opisthorchis* [see 31,33], and SBT to *C. forsteri* and *C. orientalis* [see 27,32,34].

The recent discovery of a benthic intermediate host for *C. forsteri*, the marine polychaete *Longicarpus modestus*, has linked at least a portion of the life cycle of this parasite to the sea floor [35]. A polychaet has also been linked to the lifecycle of *C. opisthorchis* in Japan [64] and aids in explaining the increased prevalence and intensity of parasitic infection during the introduction of wild or hatchery raised bluefin to sea-cage environments typically maintained in relatively shallow (<50 m), locations [36,37]. The importance of this benthic reservoir for causing disease in cultured fish is further supported by the reduction in parasitic infections which has been observed by moving SBT sea-cages

further off shore and presumably away from the source of infection [38]. Nevertheless, the dispersal potential of these parasites, as well as other possible sources of infection remains unclear. Once inside the tuna host, *C. orientalis* adults and eggs have a tropism for the gill [24,28]. *C. opisthorchis* adults are found in the heart [23], while their eggs accumulate in the afferent arteries of the gill [28]. Adult *C. forsteri* also have a tropism for the heart, but their eggs have been found in both the heart and gill [29,33,39]. Concurrent infections of two parasite species can often occur in both PBT and SBT; however, it remains uncertain if these species separately or cumulatively contribute to disease and mortality [28,37]. Indeed, little is known about the mechanisms for disease, host immune response, or the full extent of distribution within the host for these parasites.

Current methods for the detection of *Cardicola* have exclusively relied on microscopic observation for primary diagnosis. This technique has been applied *in situ* [23,24], following saline heart flushes [36], or from formalin-fixed tissue specimens [39]. In many instances, PCR has been used as a confirmatory tool for species identification; but as yet has not been applied in the initial detection of *Cardicola* infection. Nevertheless, given the potentially severity of infection caused by blood fluke parasites in conjunction with the difficult and time consuming methodology for detecting and identifying species by microscopy, a rapid method for sensitively detecting, differentiating, and potentially quantifying *Cardicola* infections in bluefin tuna would be highly advantageous. Further, the ability for non-lethal sampling is also highly desirable due to the high market value of each individual fish.

In recent years, farmers of both SBT in Australia and PBT in Japan have attempted to treat *Cardicola* spp. infections through the use of praziquantel (PZQ) [40,41], a drug commonly used to treat Platyhelminth-associated diseases in veterinary medicine for both mammals and fish [42,43]. Although dosages and treatment duration are still under

investigation, this drug appears to be effective at killing adult parasites but is much less effective at eliminating eggs from infected fish [37]. Early investigations into the anti-*Schistosoma* properties of praziquantel identified the drug to cause paralysis of the worm musculature [44], presumably due to a rapid influx of calcium ions [45], and to induce morphological alterations of the worm tegument including vacuolization and blebbing [46]. The drug has also been observed to increase antigen exposure of the *Schistosoma* parasite [47], promoting a heightened immune responses of the mammalian host which has been hypothesised to contribute to the increased effectiveness observed for PZQ *in vivo* [48,49]. Interestingly, PZQ has recently been identified to directly induce a $CD8^+$ T-cell response associated with intracellular pathogen recognition and cytotoxicity, and Th1 responses [50], and has shown strong efficacy as an adjuvant during administration of influenza and hepatitis DNA vaccines to mice [51,52]. At least part of the adjuvant capabilities of PZQ appear to stem from creating a suppressed state of immune regulation through the down-regulation of TGF-β/Smad2,3 signalling and decrease in T-regulatory cell function in mammals [52,53]. Nevertheless, a comprehensive understanding of the influences PZQ has on host immune signalling is far from complete. This is particularly evident with regard to administration of PZQ to bluefin tuna, as there has been limited investigations into the immunomodulatory ability of PZQ in teleost fish despite its long use in an aquaculture context [42].

1.3. Real-time qPCR

Real-time quantitative PCR (qPCR) has emerged as a powerful and rapid tool for detection of pathogens [54] and presents a logical choice for development with regard to *Cardicola* detection in tuna. Although a variety of other nucleic acid amplification methods

have been developed, none have been implemented to the extent of qPCR due to the highly sensitive and rapid 'real-time' quantitative ability of this technique [55]. Comprehensive review of qPCR techniques and principles can be found elsewhere [55,56], but in summary there are two main methods for nucleic acid detection upon which the qPCR operates. One is based on fluorescent dyes which bind indiscriminately to double stranded DNA, such as SYBR® Green 1 [57], the other is on a target-specific hybridization of a fluorescent-tagged sequence to single-stranded DNA, such as a TaqMan probe [58]. Each method has potential advantages depending on the desired application and outcome. Probe-based detection is relatively expensive, but can be multiplexed to detect several targets of interest in a single reaction. Double stranded DNA binding dyes are cheaper and allow for melt curve specificity analysis, but are precluded from quantitative multiplex analysis due to indiscriminate binding.

Historically, dsDNA fluorescent dyes and probes have been implemented separately; however, recent data suggested they may be combined to incorporate quantitative multiplexed detection of a target sequence while ensuring specificity against non-specific amplification by melt curve analyses [59]. Additionally, the utilization of a common-reporter (CR) on a tailed sequence-specific primer has also been shown to add flexibility and decrease costs involved in some hybridization-based detection methods by allowing the relatively expensive TaqMan reporting probe to be designed against a common sequence for multiple use, rather than to a specific target [60]. In the current context for identifying and quantifying multiple *Cardicola* spp. from within a single host organism or sample, it is interesting to consider applying a combined SYBR/TaqMan system, which may further enhance the flexibility and decrease the costs associated with multiplexed detection of these parasites.

Lastly, although an excellent tool in diagnostic and quantitative pathogen detection, real-time qPCR techniques were initially developed for quantitative gene expression [61] and

this has continued to be its most common application [55]. Over the past decade, this technique has provided remarkable insight into cellular signalling pathways and their response to environmental pressures in a variety of eukaryotic and prokaryotic organisms. These techniques have been particularly valuable in elucidating stress and immune functions in humans and other mammals, as well as in a limited number of fish species for which genomes have been extensively annotated. However, the use of qPCR or indeed any other gene expression analysis technique has seen minimal application in bluefin tuna which at least in part has been due to a lack of specifically identified gene sequences. At the beginning of this PhD project, only two full length and six partial immune or stress related mRNA transcripts had been identified in bluefin tuna (Table 1.2.), for which interspecies heterogeneity was unknown. These transcripts were used to partially characterise cytokine signalling of PBT in response to antigen and temperature related stress [19,62,63]; however, the role of these cytokines in response to parasitisation by *Cardicola* spp. or the impact of heterothermic physiology on immune response signalling was not fully explored. Therefore, for effective utilisation of qPCR gene expression analyses in bluefin tuna, identification of additional immune, stress, or growth associated gene sequences will be needed to provide the valuable insight required for efficient culture and conservation of these important fish.

Table 1.2. A summary of partial and full length mRNA sequences relating to immune, stress or growth related functions published for bluefin tuna prior to the commencement of this study

mRNA target	Species	Length (bp)	GenBank	Reference
B actin	PBT	196 (partial)	EU300941	[62]
EF1 α	ABT	811 (partial)	FM995222	[19]
IL-1β	PBT	74 (partial)	EU300945	[62]
TNF1	PBT	1,385 (complete)	AB354733	[19]
TNF2	PBT	1,059 (complete)	AB354734	[19]
Na^+/K^+ ATPase	PBT	283 (partial)	EU300940	[62]
Hsp70	PBT	539 (partial)	EU300943	[62]
HIF-1α	PBT	677 (partial)	EU300942	[62]

.

1.4. Research Objectives

Given the seriousness and prevalence of *Cardicola* spp. infection in bluefin tuna, coupled with the relatively unknown immune signalling pathways and response to *Cardicola* infection or its treatment in this genus of fish, investigation into identifying immune signalling pathways and the effects of *Cardicola* on such signalling will be paramount for the further development of sea-cage culture of bluefin tuna. Therefore, research in the following chapters has been focused toward completing the following objectives:

- *Develop a real-time PCR technique for sensitive and specific identification of C. forsteri, C. orientalis, and C. opisthorchis.*
    - Identify applicability for the use of qPCR for a variety of sample matrices, particularly those which can be obtained non-lethally such as blood or blood components (Chapters 3 & 4).
    - Investigate the effectiveness for possible multiplexed qPCR analyses (Chapter 3).
    - Explored the possibility for combining the SYBR Green 1 nucleic acid dye with a TaqMan probe-based in the context of a CR real-time detection system (Chapter 3).
- *Identify genetic coding sequence for a suite of immune, stress and growth-associated molecules in bluefin tuna.*
    - Assess the utility of newly identified sequences as biological markers of immune function *in vitro* (Chapters 2, 4, & 5).
    - Explore the relationships between the cellular stress induced signalling and the innate immune response, particularly with regard to temperature (Chapter 2).

- *Combine the qPCR techniques for differential* Cardicola *spp. detection with host immune gene transcription to determine relative prevalence and identify host responsiveness to* Cardicola *infection.*
    - Articulate the time course of natural infection following the transfer of bluefin tuna to sea-cage environments (Chapter 4).
    - Determine relative parasite prevalence and identify host response to natural infection by *Cardicola* spp (Chapters 3 & 4).
- *Assess the direct immunomodulatory ability of PZQ in fish* in vitro.
    - Assess the possible use of PZQ as an adjuvant and/or immunostimulatory compound in fish (Chapter 5).
    - Identify potential immunomodulatory roles in anthelminthic capabilities of drug as applied to bluefin tuna (Chapters 4 & 5).

1.5. References

1. Graham JB, Dickson KA (2004) Tuna comparative physiology. Journal of Experimental Biology 207: 4015-4024.

2. Collette BB, Carpenter KE, Polidoro BA, Juan-Jorda MJ, Boustany A, et al. (2011) High value and long life - double jeopardy for tunas and billfishes. Science 333: 291-292.

3. International Union for Conservation of Nature (2013) IUCN Red List of Threatened Species. Version 2013.1. Available at www.iucnredlist.org. Accessed November 2013.

4. Allen R (2010) International management of tuna fisheries: arrangements, challenges and a way forward. FAO Fisheries and Aquaculture Technical Paper 536: 45p.

5. Food and Agriculture Organization of the United Nations (2010) FAO Yearbook of Fishery Statistics 2010. Rome. FAO 107p.

6. Sea Shepherd Conservation Society (2013) Facts about the Atlantic bluefin tuna. Available at www.seashepherd.org. Accessed November, 2013.

7. Fackler M (2013) New high for tuna at Tokyo fish sale. The New York Times. New York: The New York Times Company. A10.

8. Biswas BK, Ji S-C, Biswas AK, Seoka M, Kim Y-S, et al. (2009) Dietary protein and lipid requirements for the Pacific bluefin tuna *Thunnus orientalis* juvenile. Aquaculture 288: 114-119.

9. Gwo H-H, Weng T-S, Fan L-S, Lee Y-H (2005) Development of cryopreservation procedures for semen of Pacific bluefin tuna *Thunnus orientalis*. Aquaculture 249: 205-211.

10. Honryo T, Okada T, Kurata M, Tamura T, Ishibashi Y (2012) Optimal periods of night-time lighting in the sea cage culture of Pacific bluefin tuna, *Thunnus orientalis*, juvenile (Temminck and Schlegel). Aquaculture Research doi: 10.1111/are.12059.

11. Mourente G, Tocher DR (2009) Tuna nutrition and feeds: Current status and future perspectives. Reviews in Fisheries Science 17: 373-390.

12. Sawada Y, Okada T, Miyashita S, Murata O, Kumai H (2005) Completion of the Pacific bluefin tuna *Thunnus orientalis* (Temminck et Schlegel) life cycle. Aquaculture Research 36: 413-421.

13. Tsuda Y, Sakamoto W, Yamamoto S, Murata O (2012) Effect of environmental fluctuations on mortality of juvenile Pacific bluefin tuna, *Thunnus orientalis*, in closed life-cycle aquaculture. Aquaculture 330–333: 142-147.

14. Hughes T (2010) Clean Seas doubles SBT spawning period. Hughes Public Relations and Clean Seas Tuna Limited, SA, Australia. Available www.cleanseas.com.au. Accessed November 2013.

15. Snieszko SF (1974) The effects of environmental stress on outbreaks of infectious disease of fishes. Journal of Fish Biology 6: 197-208.

16. Winton JR (2001) Fish Health Management. In: Wedemeyer GA, editor. Fish Hatchery Mangement; 2nd Edition. Bethesda: American Fisheries Society.

17. Watts M, Kato K, Munday BL, Burke CM (2003) Ontogeny of immune system organs in northern bluefin tuna (*Thunnus orientalis*, Temminck and Schlegel 1844). Aquaculture Research 34: 13-21.

18. Watts M, Munday BL, Burke CM (2002) Investigation of humoral immune factors from selected groups of southern bluefin tuna, *Thunnus maccoyii* (Castelnau): implications for aquaculture. Journal of Fish Diseases 25: 191-200.

19. Kadowaki T, Harada H, Sawada Y, Kohchi C, Soma G-I, et al. (2009) Two types of tumor necrosis factor-α in bluefin tuna (*Thunnus orientalis*) genes: Molecular cloning and expression profile in response to several immunological stimulants. Fish and Shellfish Immunology 27: 585-594.

20. Sugaya T, Mori K, Nishioka T, Masuma S, Oka M, et al. (2009) Genetic heterogeneity of betanodaviruses in juvenile production trials of Pacific bluefin tuna, *Thunnus orientalis* (Temminck & Schlegel). Journal of Fish Diseases 32: 815-823.

21. Deveney MR, Bayly TJ, Johnston CJ, Nowak BF (2005) A parasite survey of farmed Southern bluefin tuna, *Thunnus maccoyii* (Castelnau). Journal of Fish Diseases 28: 279-284.

22. Munday BL, Sawada Y, Cribb T, Hayward CJ (2003) Diseases of tunas *Thunnus spp*. Journal of Fish Diseases 26: 187-206.

23. Ogawa K, Ishimaru K, Shirakashi S, Takami I, Grabner D (2011) *Cardicola opisthorchis* n. sp. (Trematoda: Aporocotylidae) from the Pacific bluefin tuna, *Thunnus orientalis* (Temminck & Schlegel, 1844), cultured in Japan. Parasitology International 60: 307-312.

24. Ogawa K, Tanaka S, Sugihara Y, Takami I (2010) A new blood fluke of the genus *Cardicola* (Trematoda: Sanguinicolidae) from Pacific bluefin tuna *Thunnus orientalis* (Temminck & Schlegel, 1844) cultured in Japan. Parasitology International 59: 44-48.

25. Valdenegro-Vega V, Naeem S, Carson J, Bowman JP, Tejedor Del Real JL, et al. (2013) Culturable microbiota of ranched southern bluefin tuna (*Thunnus maccoyii* Castelnau). Journal of Applied Microbiology 115: 923-932.

26. Munday BL, O' Donoghue P, Watts M, Rough K, Hawkesford (1997) Fatal encephalitis due to the scuticociliate *Uronema nigricans* in sea-caged, southern bluefin tuna *Thunnus maccoyii*. Diseases of Aquatic Organisms 30: 17-25.

27. Cribb TH, Daintith M, Munday BL (2000) A new blood-fluke, *Cardicola forsteri*, (Digenea : Sanguinicolidae) of southern blue-fin tuna (*Thunnus maccoyii*) in aquaculture. Transactions of the Royal Society of South Australia 124: 117-120.

28. Shirakashi S, Kishimoto Y, Kinami R, Katano H, Ishimaru K, et al. (2012) Morphology and distribution of blood fluke eggs and associated pathology in the gills of cultured Pacific bluefin tuna, *Thunnus orientalis*. Parasitology International 61: 242-249.

29. Colquitt SE, Munday BL, Daintith M (2001) Pathological findings in southern bluefin tuna, *Thunnus maccoyii* (Castelnau), infected with *Cardicola forsteri* (Cribb, Daintith & Munday, 2000) (Digenea: Sanguinicolidae), a blood fluke. Journal of Fish Diseases 24: 225-229.

30. Dennis MM, Landos M, D'Antignana T (2011) Case–control study of epidemic mortality and *Cardicola forsteri*–associated disease in farmed Southern bluefin tuna (*Thunnus maccoyii*) of South Australia. Veterinary Pathology Online 48: 846-855.

31. de Ybañez RR, Peñalver J, Martínez-Carrasco C, del Río L, Dolores EM, et al. (2011) Blood fluke infection of cage reared Atlantic bluefin tuna *Thunnus thynnus* in West Mediterranean. Fish Pathology 46: 87-90.

32. Shirakashi S, Tsunemoto K, Webber C, Rough K, Ellis D, et al. (2013) Two species of *Cardicola* (Trematoda: Aporocotylidae) found in Southern Bluefin Tuna *Thunnus maccoyii* ranched in South Australia. Fish Pathology 48: 1-4.

33. Bullard SA, Goldstein RJ, Goodwin RH, Overstreet RM (2004) *Cardicola forsteri* (Digenea: Sanguinicolidae) from the heart of a Northern Bluefin Tuna, *Thunnus thynnus* (Scombridae), in the Northwest Atlantic Ocean. Comparative Parasitology 71: 245-246.

34. Aiken HM, Bott NJ, Mladineo I, Montero FE, Nowak BF, et al. (2007) Molecular evidence for cosmopolitan distribution of platyhelminth parasites of tunas (*Thunnus* spp.). Fish and Fisheries 8: 167-180.

35. Cribb TH, Adlard RD, Hayward CJ, Bott NJ, Ellis D, et al. (2011) The life cycle of *Cardicola forsteri* (Trematoda: Aporocotylidae), a pathogen of ranched southern bluefin tuna, *Thunnus maccoyi*. International Journal of Parasitology 41: 861-870.

36. Aiken HM, Hayward CJ, Nowak BF (2006) An epizootic and its decline of a blood fluke, *Cardicola fosteri*, in farmed souther bluefin tuna, *Thunnus maccoyii*. Aquaculture 254: 40-45.

37. Shirakashi S, Andrews M, Kishimoto Y, Ishimaru K, Okada T, et al. (2012) Oral treatment of praziquantel as an effective control measure against blood fluke infection in Pacific bluefin tuna (*Thunnus orientalis*). Aquaculture 326–329: 15-19.

38. Kirchhoff NT, Rough KM, Nowak BF (2011) Moving Cages Further Offshore: Effects on Southern Bluefin Tuna, *T. maccoyii*, Parasites, Health and Performance. PLoS One 6: e23705.

39. Norte dos Santos C, Leef M, Jones B, Bott N, Giblot-Ducray D, et al. (2012) Distribution of *Cardicola forsteri* eggs in the gills of southern bluefin tuna (*Thunnus maccoyii*) (Castelnau, 1872). Aquaculture 344–349: 54-57.

40. Hardy-Smith P, Ellis D, Humphrey J, Evans M, Evans D, et al. (2012) *In vitro* and *in vivo* efficacy of anthelmintic compounds against blood fluke (*Cardicola forsteri*). Aquaculture 334–337: 39-44.

41. Ishimaru K, Mine R, Shirakashi S, Kaneko E, Kubono K, et al. (2013) Praziquantel treatment against *Cardicola* blood flukes: Determination of the minimal effective dose and pharmacokinetics in juvenile Pacific bluefin tuna. Aquaculture 402–403: 24-27.

42. Schmahl G, Taraschewski H (1987) Treatment of fish parasites. 2. Effects of praziquantel, niclosamide, levamisole-HCl, and metrifonate on monogenea (*Gyrodactylus aculeati*, *Diplozoon paradoxum*). Parasitology Research 73: 341-351.

43. Reich MR, Govindaraj R, Dumbaugh K, Yang B-m, Brinkmann A, et al. (1998) International Strategies for Tropical Disease Treatments - Experiences with Praziquantel - EDM Research Series No. 026. In: Reich MR, editor. Action Programme of Essential Drugs Division of Control of Tropical Diseases. Cambridge: Harvard University Press.

44. Gönnert R, Andrews P (1977) Praziquantel, a new broad-spectrum antischistosomal agent. Zeitschrift für Parasitenkunde 52: 129-150.

45. Pax R, Bennett JL, Fetterer R (1978) A benzodiazepine derivative and praziquantel: effects on musculature of *Schistosoma mansoni* and *Schistosoma japonicum*. Naunyn Schmiedebergs Arch Pharmacology 304: 309-315.

46. Becker B, Mehlhorn H, Andrews P, Thomas H, Eckert J (1980) Light and electron microscopic studies on the effect of praziquantel on *Schistosoma mansoni*, *Dicrocoelium dendriticum*, and *Fasciola hepatica* (Trematoda) *in vitro*. Z Parasitenkd 63: 113-128.

47. Harnett W, Kusel JR (1986) Increased exposure of parasite antigens at the surface of adult male *Schistosoma mansoni* exposed to praziquantel *in vitro*. Parasitology 93: 401-405.

48. Brindley PJ, Sher A (1987) The chemotherapeutic effect of praziquantel against *Schistosoma mansoni* is dependent on host antibody response. The Journal of Immunology 139: 215-220.

49. Joseph S, Jones FM, Walter K, Fulford AJ, Kimani G, et al. (2004) Increases in human T helper 2 cytokine responses to *Schistosoma mansoni* worm and worm-tegument antigens are induced by treatment with praziquantel. Journal of Infectious Diseases 190: 835-842.

50. Zou Q, Yao X, Feng J, Yin Z, Flavell R, et al. (2011) Praziquantel Facilitates IFN-γ-Producing CD8+ T Cells (Tc1) and IL-17-Producing CD8+ T Cells (Tc17) Responses to DNA Vaccination in Mice. PLoS One e25525.

51. Zou Q, Hu Y, Xue J, Fan X, Jin Y, et al. (2012) Use of Praziquantel as an Adjuvant Enhances Protection and Tc-17 Responses to Killed H5N1 Virus Vaccine in Mice. PLoS One 7: e34865.

52. Zou Q, Zhong Y, Su H, Kang Y, Jin J, et al. (2010) Enhancement of humoral and cellular responses to HBsAg DNA vaccination by immunization with praziquantel through inhibition TGF-β/Smad2,3 signaling. Vaccine 28: 2032-2038.

53. Watanabe K, Mwinzi PN, Black CL, Muok EM, Karanja DM, et al. (2007) T regulatory cell levels decrease in people infected with *Schistosoma mansoni* on effective treatment. American Journal of Tropical Medicine and Hygiene 77: 676-682.

54. Johnson M, Brzoska P, Petrauskene O, Melancon C (2005) Using Real-Time PCR for Pathogen Detection. Genetic Engineering News 25: www.genengnews.com.

55. VanGuilder HD, Vrana KE, Freeman WM (2008) Twenty-five years of quantitative PCR for gene expression analysis. BioTechniques 44: 619-626.

56. Logan J, Edwards K, Saunders N (2009) Real-Time PCR: Current Technology and Applications. Norfolk, UK: Caister Academic Press.

57. Morrison TB, Weis JJ, Wittwer CT (1998) Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. Biotechniques 24: 954-962.

58. Livak KJ, Flood SJ, Marmaro J, Giusti W, Deetz K (1995) Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods and Applications 4: 357-362.

59. Van Poucke M, Van Zeveren A, Peelman LJ (2012) Combined FAM-labeled TaqMan probe detection and SYBR green 1 melting curve analysis in multiprobe qPCR genotyping assays. BioTechniques 52: 81-86.

60. Rickert AM, Lehrach H, Sperling S (2004) Multiplexed Real-Time PCR Using Universal Reporters. Clinical Chemistry 50: 1680-1683.

61. Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real time quantitative PCR. Genome research 6: 986-994.

62. Mladineo I, Block BA (2009) Expression of Hsp70, Na+/K+ ATP-ase, HIF-1α, IL-1β and TNF-α in captive Pacific bluefin tuna (*Thunnus orientalis*) after chronic warm and cold exposure. Journal of Experimental Marine Biology and Ecology 374: 51-57.

63. Mladineo I, Block BA (2010) Expression of cytokines IL-1β and TNF-α in tissues and cysts surrounding *Didymocystis wedli* (Digenea, Didymozoidae) in the Pacific bluefin tuna (*Thunnus orientalis*). Fish and Shellfish Immunology 29: 487-493.

64. Sugihara Y, Yamada T, Tamaki A, Yamanishi R, Kanai K (*in press*) Larval stages of the bluefin tuna blood fluke *Cardicola opisthorchis* (Trematoda: Aporocotylidae) found from Terebella sp. (Polychaeta: Terebellidae). Parasitology International, doi: j.parint.2013.11.010

Chapter Two

Temperature-induced transcription of inflammatory mediators and the influence of Hsp70 following LPS stimulation of southern bluefin tuna peripheral blood leukocytes and kidney homogenates

Published in:

Polinski M, Bridle AR, Nowak B (2013) Temperature-induced transcription of inflammatory mediators and the influence of Hsp70 following LPS stimulation of southern bluefin tuna peripheral blood leukocytes and kidney homogenates. Fish and Shellfish Immunology, 34: 1147-1157.

2.1. Abstract

Temperature is known to influence inflammatory signalling in mammals, but far less understood in fish. The aim of the present study was to explore the potential effects of temperature on innate immune signalling in head kidney and leukocyte populations of the economically important southern bluefin tuna through the identification and utilisation of gene expression targets *in vitro*. Here, we identified the mRNA sequences of five potential inflammatory mediators – TNFα (1 and 2), IL-1β, IL-8, and Cox2 – and demonstrate induction of four – TNFα (2), IL-1β, IL-8, and Cox2 – following LPS stimulation of both peripheral blood leukocytes and head kidney homogenates *in vitro* by real-time quantitative PCR. Comparison of transcriptional expression in cultures held at 18 and 25°C (both within the presumed natural temperature range of this heterothermic species) showed accelerated transcription of cytokines TNFα, IL-1β and IL-8 following LPS stimulation at 25°C in both tissue types. Peak induction reached comparable levels for each transcript at both temperatures during the 24 h test period with only limited (if any) protraction in expression resulting from cold temperature (18°C) incubation. Partial mRNA sequences were also identified for both the constitutively expressed and heat inducible chaperone proteins Hsc70 and Hsp70, and 24h incubation at 25°C was sufficient to induce Hsp70 transcription in leukocyte but not in head kidney cell populations. Taken together these findings suggest that temperature exerts influence in the timing but not the degree of an innate inflammatory response in bluefin tuna and that different cell populations have differential responsiveness to heat shock in this heterothermic species. Further, LPS stimulation failed to induce Hsp70 at either incubation temperature in leukocytes; whereas 25°C incubation caused Hsp70 up-regulation in leukocytes with or without the presence of LPS. This suggests that Hsp70 does not play a direct role in immune responsiveness for this species and that an environmental

temperature of 25°C in excess of 24 h initiates a cellular stress response in blood cells of this organism. Lastly, a strong correlation between Hsp70 and IL-8 transcriptional expression was observed following LPS/heat shock stimulation of leukocytes and five potential heat shock response elements were subsequently identified on the gene promoter region of IL-8 indicating that heat shock co-activation of this chemokine previously identified in mammals is also likely present in fish.

2.2. Introduction

Inflammation is an integral component of the vertebrate innate immune response and critical for the repair of damaged or infected tissue. Biological control and initiation of inflammation is complex, involving a variety of molecular signalling pathways which allows for a large degree of variability in shaping a response. As such, signalling mediators have become critical in understanding the inflammatory process and present attractive biological markers for indicating and assessing innate immune function. Among the most prominent and well-studied mediators of inflammation are the signalling cytokines tumour necrosis factor-α (TNFα) and interleukin-1β (IL-1β), the chemokine interleukin-8 (IL-8), as well as eicosanoid prostaglandins produced via the cyclooxygenase-2 (Cox2) pathway.

Temperature is known to have an important influence on inflammatory signalling in mammals; for example, mild hypothermia has been shown to delay the onset but prolong and enhance inflammatory cytokine generation in human monocytes and macrophages [1-3] and in conditions of febrile hyperthermia, suppressed cytokine signalling has been observed [1]. Further, the induction of heat shock factors and inducible heat shock proteins such as Hsp70 are known to antagonise the pro-inflammatory transcription factor NF-κβ [4]. Although

temperature would be expected to have an equal if not elevated importance on inflammatory signalling in poikilothermic fish, available evidence is limited. Zou and colleagues observed suppressed short-term IL-1β signalling in rainbow trout *Oncorhynchus mykiss* leukocytes following low (4°C) temperature incubation for 4 h [5]; however, the potential longer-term effects of delayed, prolonged, and heightened signalling as seen by Fairchild *et al.* [1,2] in mammalian cells was not investigated, leaving potential questions regarding delayed or enhanced temperature related signalling for immune function in fish unanswered. Additionally, transcription and protein expression of the heat-inducible Hsp70 were observed to be suppressed in kidneys of silver sea bream *Sparus sarba* following *in vivo* bacterial infection [6], whereas in contrast the addition of LPS to carp phagocytes and lymphocytes *in vitro* resulted in elevation of Hsp70 transcription [7,8]; an *in vitro – in vivo* contradiction regarding the biological significance of Hsp70 in the immune signalling of fish which has yet to be explained.

Southern bluefin tuna (SBT) *Thunnus maccoyii* are a highly valued food fish with global economic importance. They also belong to a unique family of fishes that possess exceptional physiological characteristics, including heterothermy and high cardiac output, that in many regards parallel the physiology of aquatic mammals [9]. As such, they present an attractive target for immunological investigations, particularly concerning the influence of temperature. In the present study, our aims were to identify genetic coding sequence for a suite of inflammatory signalling molecules in southern bluefin tuna and analyse their relative expression within immune cell populations in *vitro* in response to lipopolysaccharide (LPS) stimulation and temperature manipulation. Further, we hoped to explore what relationships if any, were present between the cellular stress induced signalling pathway involving Hsp70 and the innate immune response with regard to temperature and potentially clarify unstressed physiological limits for this heterothermic fish species.

2.3. Materials and Methods

2.3.1. Ethics Statement

All work with animals and methods for recovering samples were approved by the University of Tasmania Animal Ethics Committee, project number A0010593.

2.3.2. Field Collection

Peripheral blood and visceral organs were collected from healthy SBT weighing 35-40 kg (approximately 2-3 years of age) during commercial harvest of ranched stocks from off-shore sea cages near Port Lincoln, South Australia, with capture and sea conditions as those previously described [10]. Visceral organs and PBLs were collected from individuals representing three separate wild capture events held in three discrete sea cages by two different commercial companies. Blood was obtained from the severed lateral artery of three fish in the pectoral recess and collected into 50 mL tubes pre-treated with 100 IU heparin. Similarly, visceral organs were removed from three fish, sealed in waterproof plastic bags, and immediately placed on ice. Once collected, samples were held on ice during transit to a laboratory (approximately 1-2 h).

2.3.3. Establishment of primary cultures

Peripheral blood leukocytes were isolated from heparinised blood by centrifugation at 800 x *g* for 10 min at 4°C. The buffy coat was harvested, diluted 1:1 with phosphate buffered saline (PBS), and further purified over a 34/49% Percoll-PBS discontinuous density gradient to remove residual erythrocytes. Purified cells were washed twice with L-15 culture media supplemented with GlutaMAX™ and 4x penicillin-streptomycin-neomycin antibiotic

mixture (0.2, 0.2, 0.4 mg/ml, respectively), pH to 7.4 (Invitrogen, VIC, Australia). A small portion of cells was visualised during the second wash by hemocytometer following trypan blue staining to confirm the identity of a PBL population and ensure viability greater than 95%. Cells were then resuspended to a final concentration of 10^8 cells/mL in L-15 without antibiotic supplemented with 10% fetal bovine serum (FBS). Aliquots of 100 µL were seeded to each of the 96 wells in flat-bottom tissue culture plates, yielding final cell concentrations of 10^7 cells/well.

Head kidney homogenates were prepared from approximately 10g of tissue. Tissue was minced with scissors, washed four times with L-15 antibiotic media to remove residual blood and non- adherent cells, then enzymatically digested for 1h with 0.25% trypsin in EDTA (Invitrogen, Vic, Australia) at room temperature. Following digestion, cells were gently forced through a 100 µm steel mesh using a rubber syringe plunger and washed two times with L-15 antibiotic media to remove lipid and cellular debris. Cells were recovered from the wash solution by gentle (400 x *g*) 15 min centrifugation and a small portion of cells was visualised during the second wash by hemocytometer following trypan blue staining to confirm the single cell separation and ensure viability greater than 95%. Cells were then resuspended to a final concentration of 10^8 cells/mL in L-15 with 10% FBS and aliquots of 100 µL were seeded to each of the 96 wells in flat-bottom tissue culture plates, yielding final cell concentrations of 10^7 cells/well. All cultures were held for a total period (following initial matrix isolation) of approximately 6 h at 21°C prior to subsequent stimulation and temperature manipulation. In our experience this has resulted in relatively stable and comparable expression of target and reference genes of interest. Tissues from individual fish were assayed separately to inhibit ‘non-self’ recognition in triplicate, yielding 9x biological replication for each subsequent stimulation time point.

2.3.4. Stimulation and temperature manipulation of primary cell cultures

Cell cultures prepared above were stimulated with 10 µg/mL LPS from *E. coli* 026:B6 (Sigma-Aldrich, NSW, Australia) by addition of 10 µL stimulant eluted in PBS (11 times final concentration) to each culture well. Cultures were immediately placed at either 18 or 25°C and incubated for 3, 6, 12, or 24 h in triplicate. Non-stimulation controls were included for each time point and temperature by addition of stimulant-free PBS. Following the appropriate incubation period, cultures were gently triturated to resuspend settled cells and the entire volume (110 µL) was removed and diluted 1:7 in RNA preservation solution (4 M ammonium sulphate, 25 mM sodium citrate, 10 mM EDTA; pH 5.5). Preserved suspensions were held overnight at 4°C and then stored at -20°C until RNA extraction. Unfortunately, it was determined that one of the three PBL population sets used for these experiments became bacterially contaminated and had to be eliminated from all subsequent data analyses, thus biological replication was reduced to N=6.

2.3.5. RNA extraction and cDNA synthesis

Cells were recovered from the preservation solution by centrifugation at 5,000 x g for 5 min at room temperature. Total nucleic acid was extracted from the cell pellet in Extraction buffer (0.5% SDS, 0.2M NaCl, 10% glycerol) supplemented with 60 µg Proteinase K (Bioline, NSW, Australia) and incubated at 65°C for 10 min with occasional vortexing. Protein was removed by precipitation with the addition of one volume 5 M ammonium acetate and centrifugation at 14,000 x *g* for 5 min. Nucleic acids were then precipitated from the supernatant with the addition of one volume isopropanol and centrifugation at 16,000 x *g* for 10 min. The nucleic acid pellet was rinsed with 75% ethanol and resuspended in 100 µL TE buffer (10 mM Tris, 1 mM EDTA, pH 8).

RNA was phase separated from eluted total nucleic acids by addition of 700 µL RNAzol® and 50 µL BAN® phase separation reagent (Molecular Research Center, OH, USA) as outlined by the manufacturer. Complete removal of DNA was ensured by treatment of the purified RNA with 4 units Baseline- Zero DNAse (Epicentre, WI, USA) for 30 min at 37°C as per manufacturer's instructions. RNA was quantified using a Qubit fluorometer (Invitrogen, VIC, Australia), and quality was visualised on a 2% formaldehyde denaturing agarose gel using RNA EZvision dye (Amresco, Sydney, Australia) as per manufacturer's instructions.

For expression analysis, a portion of RNA (1 µg) from each sample 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 for sequencing analysis using a BD SMART™ RACE cDNA Amplification Kit (BD Biosciences, NJ, USA) according to manufactures instructions.

2.3.6. Identification of SBT mRNA target sequences

Previously published sequences from Pacific bluefin tuna *Thunnus orientalis* were used as a template to generate RACE gene-specific primers for two homologues of TNFα (designated TNF1 and TNF2) [11] as well as Hsp70 [12]. Specific bluefin sequence was unavailable for all other genes of interest, and thus gene sequences from available perciform species in GenBank were aligned using Geneious 4.8.2 software [13], and multiple degenerate and non-degenerate primers pairs were designed using the consensus nucleotide identify for each target gene. Pooled cDNA template from all HK and PBL replicates described above was used in a 35-cycle PCR using a 2X MyTaq™ Red master mix with reaction and cycling conditions as suggested by the polymerase manufacturer (Bioline, NSW, Australia). Highly amplified products of (or near) anticipated size were excised from a 1%

agarose gel and submitted directly to Macrogen Inc. (Seoul, Korea) for sequencing analysis. The Basic Local Alignment Search Tool (BLAST) [14] was used to identify putative SBT target sequences, from which gene-specific RACE primers were designed using Primer Premier 5.0 software (Biosoft International, CA, USA) (Table 1). RACE-ready cDNA was used with the newly designed primers in a Touchdown-RACE PCR as suggested by the manufacturer (BD Biosciences) to amplify overlapping 5' and 3' mRNA partial transcripts. Products of anticipated size were gel-excised, directly sequenced with gene-specific RACE primers, and then assembled using Geneious software.

As it was hypothesised that heat shock response elements may be present in the promoter region of IL-8 gene, a GenomeWalker™ Universal Kit (BD Biosciences) was used to determine adjacent 5' sequence to the IL-8 mRNA coding region once identified. Methods were carried out as indicated by the manufacturer where RNA preserved SBT whole blood was used to obtain genomic DNA and the IL8_GSP5' gene-specific RACE primer (Table 2.1) was used for PCR amplification of digest libraries. Products were gel-excised and submitted directly to Macrogen Inc. for sequencing analysis. Putative HSF-1 binding sites were identified as described by Nagarsekar *et al.* [15].

Table 2.1. Oligonucleotide primers used to amplify SBT specific sequences for sequencing and expression analysis.

Target	Accession #	Objective	Name	Product size	Sequence (5′→3′)
βactin	JX157141	qPCR	q_actin_F	89 bp	TATCCTGACCCTGAAGTA
			q_actin_R		CATTGTAGAAGGTGTGATG
		RACE and sequencing PCR	actin_Gsp5′	871 bp	GGTGGTCTCGTGGATGCCGCAGGACTCC
			actin_Gsp3′	1.14 kb	GCACTGCTGCCTCCTCCTCCCTGGA
EF1α	JX157143	qPCR	q_EF1a_F	86 bp	TTGGTGTCAACAAGATGG
			q_EF1a_R		GATGTAGGTGCTCACTTC
		RACE and sequencing PCR	EF1a_Gsp5′	446 bp	CGTGCTCACGGGTCTGGCCGTTCTTGGA
			EF1a_Gsp3′	1.41 kb	CGCTGTGCTGGTCGTTGCTGCTGGTGTT
IL-1	JX157146	qPCR	q_IL1_F	92 bp	AGCCACAAGATAACCAAG
			q_IL1_R		TTCTCTACAGCGATGATG
		RACE and sequencing PCR	IL1_Gsp5′	845 bp	AGCGGTTGGCGGTCGCCTGGCACATT
			IL1_Gsp3′	880 bp	GCTCCAAGCTGTGATGCTGCAGGGAGGC
IL-8	JX157147	qPCR	q_IL8_F	108 bp	CTACTGTTCGCTTGTCGCTAA
			q_IL8_R		TTGATAGGTTGTCATCGGACTTAC
		RACE and sequencing PCR	IL8_Gsp5′	362 bp	TGTGGCGGCCGATGGGTTTGCTCTCT
			IL8_Gsp3′	667 bp	TGGTGCTCCTGTGCCTTCTGGCCACC
TNF1	JX157148	qPCR	q_TNF1_F	75 bp	CTGGAGTGGAGAGTTGAT
			q_TNF1_R		*GATCACGATCTGGTTATCC*
		RACE and sequencing PCR	TNF1_Gsp5′	742 bp	TGGCACGCCGACCTCACCGCGCTCAT
			TNF1_Gsp3′	805 bp	TGCAGCGATGGCGACGAGCAGGGAGC
TNF2	JX157149	qPCR	q_TNF2_F	76 bp	ATCTGAGCCATACTGTGAA
			q_TNF2_R		AGGATGGTCTGGTAGGAA
		RACE and sequencing PCR	TNF2_Gsp5′	596 bp	GGCGGTGCGGACAGAGTGCAGGATGGT
			TNF2_Gsp3′	836 bp	TGCTGCTGCGGCTGCTACTGCTCTCCTT
Cox2	JX157142	qPCR	q_Cox2_F	85 bp	CCACATTTACGGAGAGAC
			q_Cox2_R		TCCTTCCAGAGTCTGATAT
		RACE and sequencing PCR	Cox2_Gsp5′	940 bp	TGCCTCATGGCCCACAGCGAAGCGGTGA
			Cox2_Gsp3′	1.72 kb	TCACCGCTTCGCTGTGGGCCATGAGGCA
Hsp70	JX157145	qPCR	q_Hsp70_F	116 bp	GACATGAAGCACTGGC
			q_Hsp70_R		AGGACCATGGAGGAG
		RACE and sequencing PCR	Hsp70_Gsp5′	1.25 kb	CGGCACCGTAAGCCACCGCCTCGTCT
			Hsp70_Gsp3′	1.73 kb	ACCTCGGCCAAACGGTGTCCAACGCAGT
Hsc70	JX157144	qPCR	q_Hsc70_F	93 bp	GTCGGATATGAAGCACTG
			q_Hsc70_R		TAGAAGGACTTGGACTCAC
		RACE and sequencing PCR	Hsc70_Gsp5′	1.05 kb	TCTCCACAGGGTCCAGGGTGCCACGG
			Hsc70_Gsp3′	1.79 kb	TCAACGACTCCCAGCGCCAGGCCACT

2.3.7. Phylogenetic analyses

Phylogenetic trees of novel buefin tuna IL-1β and IL-8 were assembled with other bony fish based on mature IL-1β and IL-8 proteins. The phylograms, and nucleic acid translation of bluefin mRNA, were assembled with Geneious software. Branches were assigned based on 1,000 bootstrap replications using the Jukes-Cantor neighbour-joining method following MUSCLE alignment.

2.3.8. Expression analysis

Extracted and reversed transcribed cDNA described above was used to measure the expressions of IL-1β, IL-8, TNFα (TNF1 and TNF2), Cox2, Hsc70 and Hsp70 by SYBR Green chemistry on a CFX Connect Real-time PCR Detection System (Bio-Rad, NSW, Australia). Primers were designed using Primer Premier 5.0 software based on assembled SBT mRNA transcripts identified above (Table 2.1). These targets were analysed against two reference genes - βactin and elongation factor 1α (EF1α), also specifically identified and sequenced for SBT as described above. Each PCR reaction consisted of 2X SensiFast™ +SYBR® mastermix (Bioline, NSW, Australia), forward and reverse primers (400 nM each), and 1 µL cDNA template in molecular grade water to a final volume of 10 µL. Samples were assayed in duplicate with a five-step, four-fold dilution series of pooled cDNA included in each run to calculate amplification efficiencies and for use as inter-run calibrators. Thus, 9x biological replicates of each tissue were assayed in duplicate for each time point and stimulation. Cycling conditions consisted of an initial activation of DNA polymerase at 95°C for 3 min, followed by 40 cycles of 5 sec at 95°C, 20 sec at 55°C, and 10 sec at 72°C. At the end of the cycling protocol melt curve analysis was run to ensure amplification specificity. Runs were only considered for analyses when amplification efficiency (E) of standard dilutions ranged between 85-105% with a linear regression (r^2) of 0.98-1.00.

2.3.9. Statistical analysis

The qPCR data were analysed with qBase software as described by Hellemans et al. [16] where mRNA expression was normalised using the mean expressions of the two reference genes ($M < 1$; $CV < 0.5$) [17]. The normalised relative quantity (NRQ) was calculated for each gene in each assay replicate and scaled to the minimum value. Data from grouped biological replicates were then combined following a $\log_{10}$ transformation as suggested by Hellemans and Vandesompele [18], and used for statistical comparisons. Induction of the potential heat shock associated genes Hsc70 and Hsp70 were compared at each time point by a one-way analysis of variance. The effects of temperature on fold induction of inflammatory mediators following LPS stimulation was compared for each gene at each sample time point by a one-tailed Student's *t*-test and a Welch's correction was incorporated if unequal variance was observed. For fold induction comparisons, data were rescaled relative to mean control values at the time point specified and therefore standard errors were propagated as described by Rieu [19] prior to statistical comparison. Statistical analyses were performed in Graphpad Prism 5.01 (Graphpad Software, CA, USA) with a p-value < 0.05 acknowledging significant elevated expression. Additionally, linear regression was performed using the NRQ values from heat shocked LPS stimulated leukocytes to identify possible correlation between Hsp70 and IL-8 transcription. Correlation of Hsp70 and TNF2 was also performed on the same data set for comparison.

2.4. Results

2.4.1. Identification of SBT gene sequences

Successful amplification of at least partial genetic coding sequence was obtained for all southern bluefin tuna mRNA targeted in this study. Full length bluefin tuna mRNA transcripts were identified for βactin, EF1α, TNF1, TNF2, IL-1β, and IL-8, and partial mRNA sequences were identified for Hsp70, Hsc70, and Cox2 (Suplemental Figure 2.1.). A list of GenBank accession numbers is available in Table 2.1. The two TNFα homologues previously identified for Pacific bluefin tuna (TNF1 and TNF2) by Kadowaki et al. [11] were similarly identified here for SBT. Greater than 98% sequence identity was observed for both homologues between SBT and PBT, resulting in identical protein translation. Indeed, the only distinctive inter-species difference was employment of an alternative polyadenylation signal in the newly identified southern bluefin TNF2 sequence which resulted in a shortening of the 3' untranslated region by approximately 60 bases relative to the Pacific bluefin TNF2 transcript (data not shown). As was previously observed for PBT, SBT TNF1 and TNF2 transcripts only possessed 43% sequence homology and appear to have distinct phylogenetic separation and physiological roles [11]. Nevertheless, protein translation of both homologues identified a common TNF signature domain (CDD: cd00184) following a conserved domain database (CDD) search [20], indicating both mature peptides a have structure adequate to initiate receptor-mediated apoptosis signalling [21].

Full-length mRNA sequence obtained for both IL-1β and IL-8 represent to the best of our knowledge the first report of these transcripts in the *Thunnus* genus. Protein translation of the IL-1β open reading frame (ORF) showed both conserved IL-1 pro-peptide as well as functional peptide signatures (CDD: pfam02394 and smart00125, respectively), and phylogenetic comparison of mature protein with other published teleost IL-1β revealed bluefin tuna to cluster with other perch-like fishes with closest similarity to IL-1β identified in Atlantic halibut *Hippoglossus hippoglossus* (GenBank: ACY54774) (Figure 2.1.A), although a distinct separation of Sparidae (seabream) family members was observed.

Translation of the IL-8 ORF identified an ELR^+ conserved CXC chemokine domain (CDD: cd00273); however, like many other teleosts fishes SBT have a variant ELH rather than ELR tri-peptide motif. Phylogenetic mature protein comparison with sequences from other available teleost IL-8 showed bluefin tuna to cluster with other perch-like fishes in similar fashion to that observed for IL-1β, except with similar sequence homology occurring between the percomorphs and gadiforms for mature peptide translation (Figure 2.1.B).

Attempts to discover Hsp70 mRNA also led to the partial identification of the constitutively expressed 70 kDa heat shock cognate protein mRNA transcript Hsc70. Although only partial mRNA transcripts were obtained for both Hsp70 and Hsc70 consisting of 1672 and 1929 bases, respectively, this was enough to identify a conserved Hsp70 domain signature (CDD: pfam00012) following putative protein translation of both transcripts indicating similar chaperone functional roles. A 1267 base partial mRNA transcript was also identified for Cox2, which consisted of 1244 bases from the ORF encoding 414 of a putative 608 amino acid peptide. A prostaglandin endoperoxide synthase domain (CDD: cd09816) was identified in the partial amino acid translation.

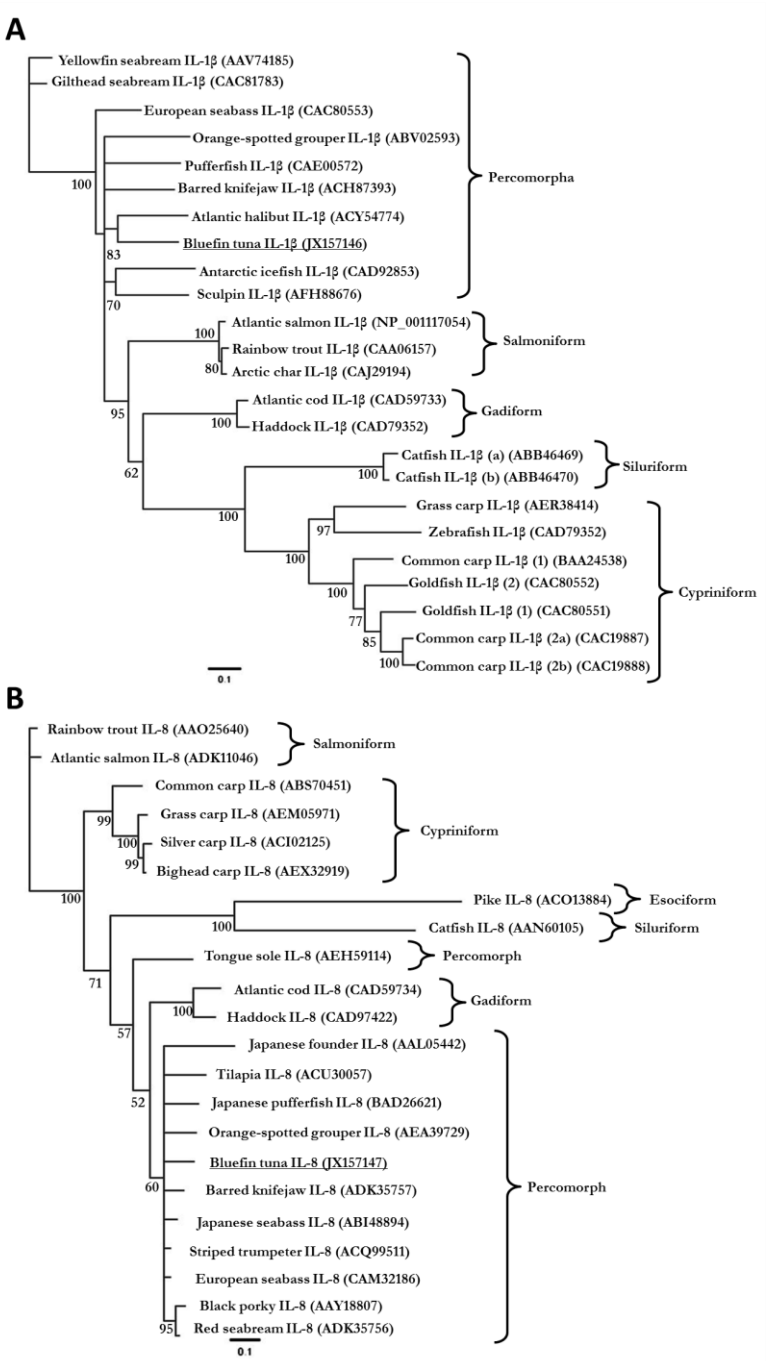


Figure 2.1. Phylogenetic trees of mature IL-1β (A) and IL-8 (B) proteins for bluefin tuna (underlined) with regard to other known fish species. The phylograms were constructed in the software program Geneious using the neighbour-joining method based on MUSCLE amino acid alignment. The numbers at the relevant branches refer to bootstrap values of 1000 replications. The accession numbers and common names of the IL-1β and IL-8 sequences retrieved in this study are provided. Brackets indicate similar taxonomic classification.

2.4.2. Transcriptional induction of inflammatory mediators following LPS stimulation and differential response to temperature

Mean transcriptional expression of TNF1 following LPS stimulation did not exceed more than a two-fold induction (the typical minimum threshold to indicate biological significance) in either PBL (Figure 2.2.) or HK (Figure 2.3.) cultures relative to PBS controls at any of the time points assessed during this trial. Additionally, NRQ values were statistically comparable between stimulated and non-stimulated populations for this gene transcript at each time point in either tissue, further highlighting the constitutive functional role of TNF1 previously identified in Pacific bluefin regarding pathogenic stimulation [11]. In contrast, there was an increased induction of mRNA transcription for inflammatory mediators TNF2, IL-1β, and IL-8 in both PBL and HK cell populations following stimulation with LPS, where an approximate 6-9 fold peak induction was observed at both temperatures for all four genes (Figures 2.2. and 2.3.). With regard to temperature, IL-1β, IL-8, and TNF2 were induced earlier at elevated (25°C) temperature. Significantly higher fold inductions were observed for all four inflammatory mediator transcripts at 3 h post stimulation at 25°C relative to 18°C cultures in both PBL (Figure 2.2) and HK (Figure 2.3.) cell cultures. Unfortunately the relationship of Cox2 with regard to temperature remains somewhat obscure, as general low levels of Cox2 transcript in the majority of assayed cell populations made analysing data across multiple cell populations impossible. Sufficient data were only available to give a complete Cox2 profile in response the LPS stimulation from two cell population sets (one HK, one PBL). In these cases, a 4-7 fold induction was observed in both PBL and HK cell populations indicating transcriptional induction of this transcript does occur in response to LPS in both cell population types (Supplemental Fig. 2.2.). There was no significant difference between unstimulated controls relative to temperature for Cox2 or any other gene transcript at any time point observed during this trial.

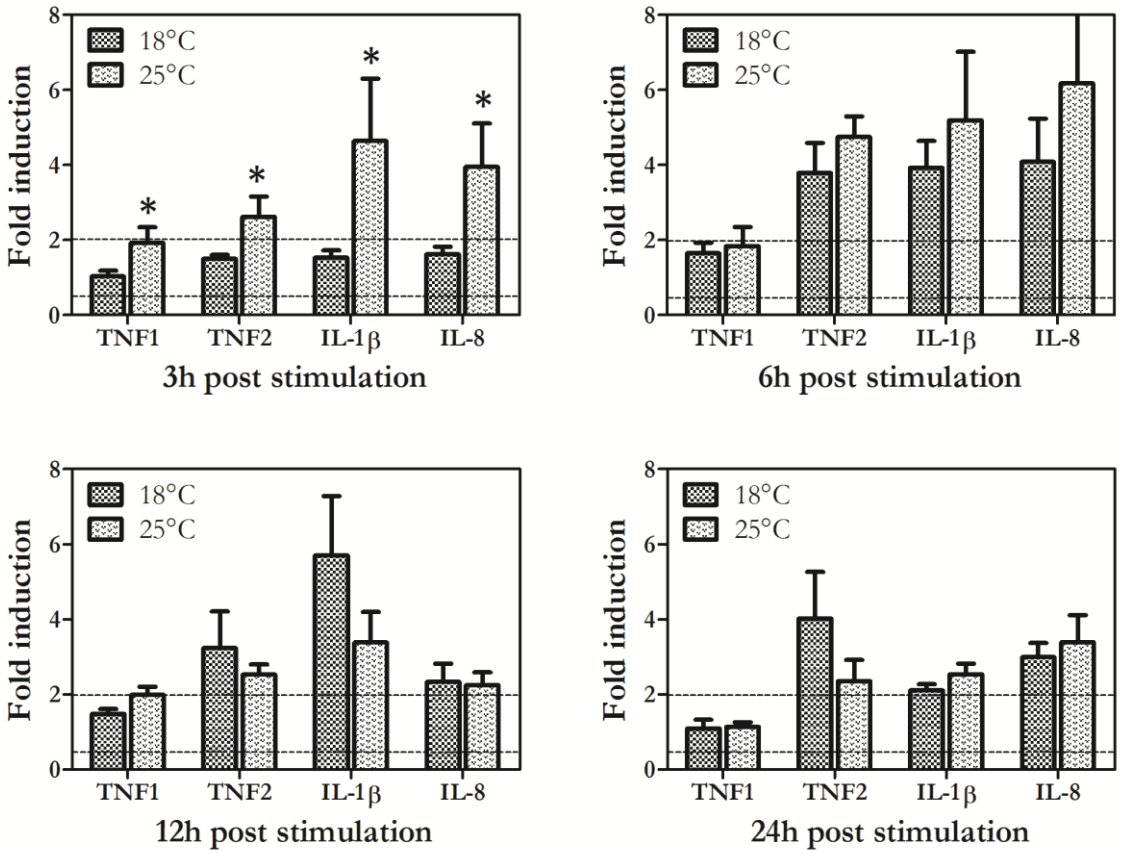


Figure 2.2. Kinetic profile of TNF1, TNF2, IL-1β and IL-8 in peripheral blood leukocytes. Cultures were stimulated with 10 µg mL^{-1} LPS. Data shown are the combined mean (± SE) of 6x biological replicates of PBL populations, assayed in duplicate by quantitative real-time PCR presented as fold induction relative to unstimulated PBS controls at 3, 6, 12, and 24 h post incubation. The dotted line presents the minimum fold change (2 fold) deemed biologically significant. * Identifies a significant ($P<0.05$) elevated induction relative to incubation temperature for the time point indicated.

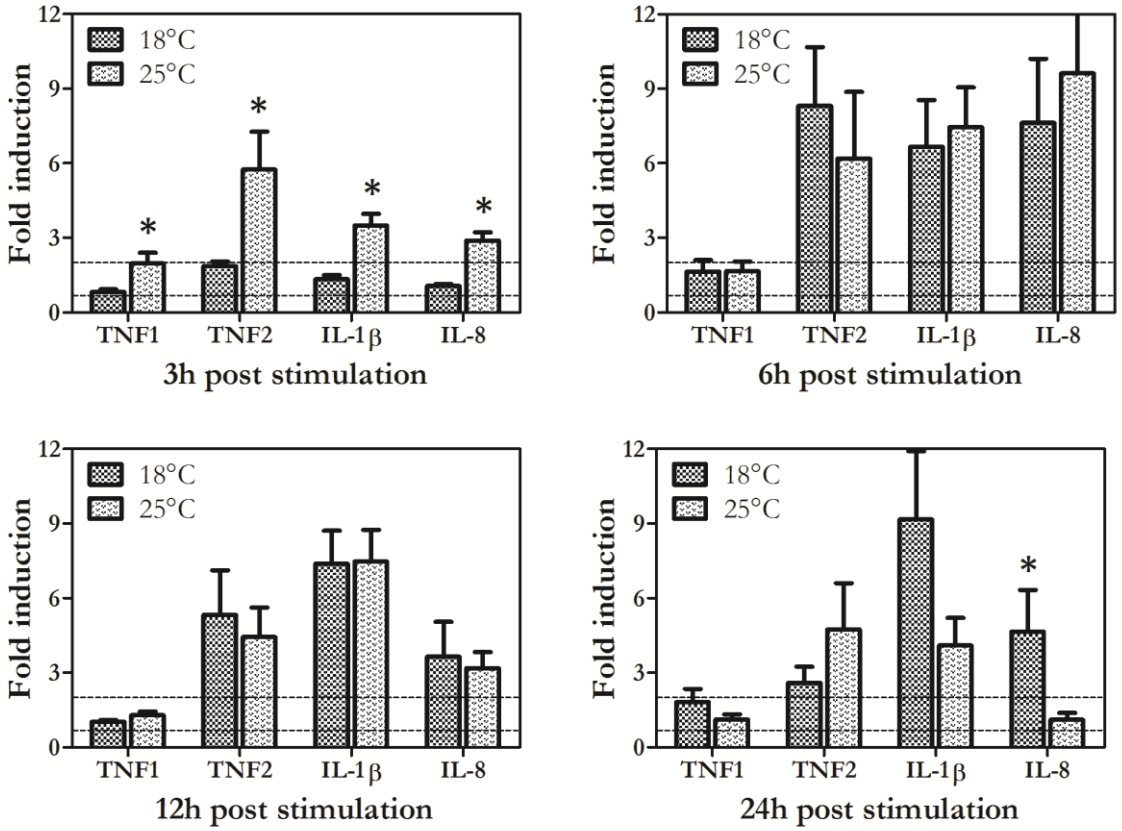


Figure 2.3. Kinetic profile of TNF1, TNF2, IL-1β and IL-8 in head kidney homogenate cultures. Cells were stimulated with 10 µg mL^{-1} LPS. Data shown are the combined mean (± SE) of 9x biological replicates of HK populations, assayed in duplicate by quantitative real-time PCR presented as fold induction relative to unstimulated PBS controls at 3, 6, 12, and 24 h post incubation. The dotted line presents the minimum fold change (2 fold) deemed biologically significant. * Identifies a significant ($P<0.05$) elevated induction relative to incubation temperature for the time point indicated.

2.4.3. Temperature induced induction of Hsp70 transcription and putative consequences on inflammatory mediator transcription.

Incubation for up to 24h at 18°C did not significantly induce expression of Hsc70 or Hsp70, and similarly the addition of 10 μg mL^{-1} LPS caused no change in overall expression of these transcripts at that temperature in either PBL or HK cell populations. However, incubation of PBLs at 25°C resulted in a 3-5 fold induction of Hsp70 relative to 18°C cultures following 24 h incubation, which was comparable regardless of LPS stimulation (Figure 2.4.). This was in contrast with HK cultures incubated at 25°C where no significant change in expression of Hsp70 was observed during the 24h period. Closer inspection of Hsp70 expression in PBL cultures also revealed a difference in timing between the two populations isolated from different cell sources used in this study. Specifically, one population set of PBLs appeared to be induced slightly earlier than the other. Faster responding population showed significant induction at 12h which continued through 24h incubation, whereas the other population set appeared to experience later induction where elevated expression was only observed following 24h (Supplemental Figure 2.2). As our experiment was only conducted through a 24h period, the subsequent effect of Hsp70 on immune signalling in this latter population was undetermined. Interestingly, in the cell population set with an earlier Hsp70 response, TNF2, IL-1β and IL-8 transcription remained significantly elevated at 24 h post stimulation in LPS stimulated cultures (Figure 2.5.). Additionally, It was observed that Hsp70 and IL-8 transcription in these 25°C LPS stimulated cultures showed strong linear correlation that was not observed in other cytokines such as TNF2 (Figure 2.6.B), and subsequent sequencing of the 5' flanking region of the IL-8 gene identified six putative heat shock response element binding sites (Figure 2.6.A).

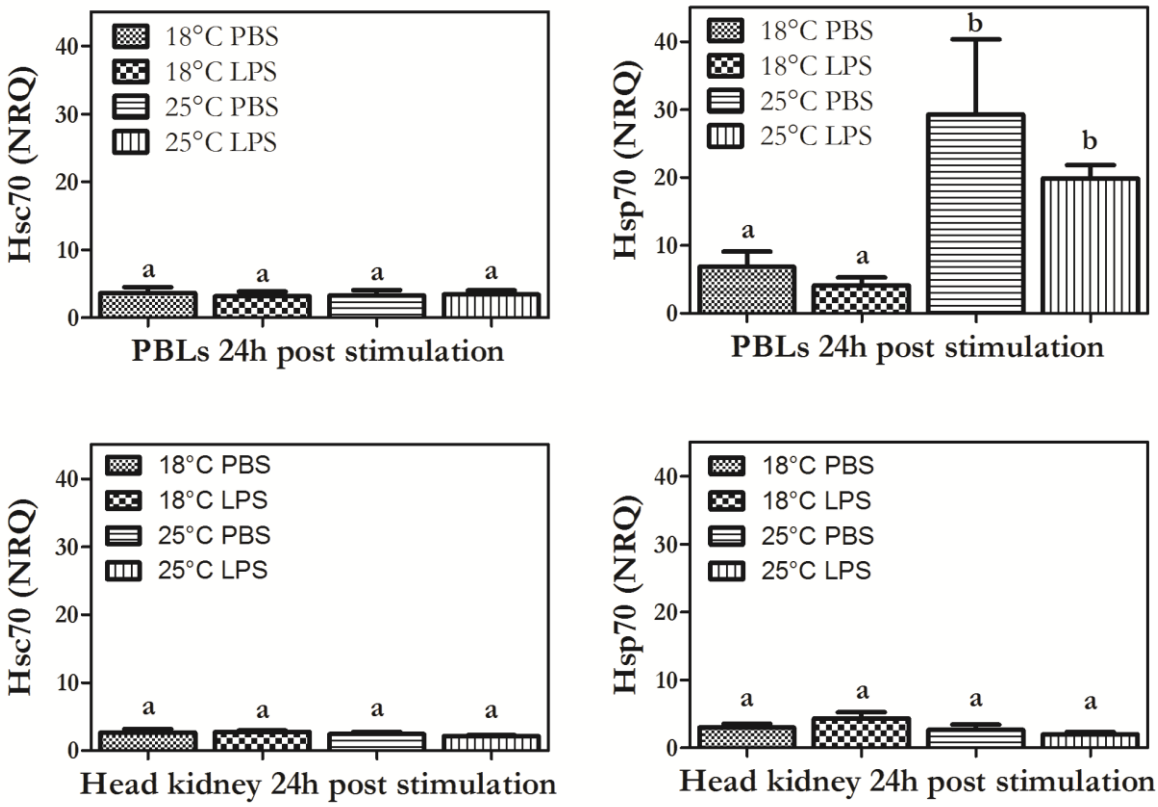


Figure 2.4. Hsp70 and Hsc70 mRNA transcription following LPS stimulation in PBL and HK cultures incubated at either 18 or 25°C. Data shown are the combined mean (± SE) of 9x biological replicates of HK populations and 6x biological replicates of PBL populations, assayed in duplicate by quantitative real-time PCR and presented as the relative quantity (NRQ) normalised to two stable housekeeping genes and scaled to the minimum observed value. Groups of significant ($P < 0.05$) similar transcriptional quantities are identified following 24 h incubation.

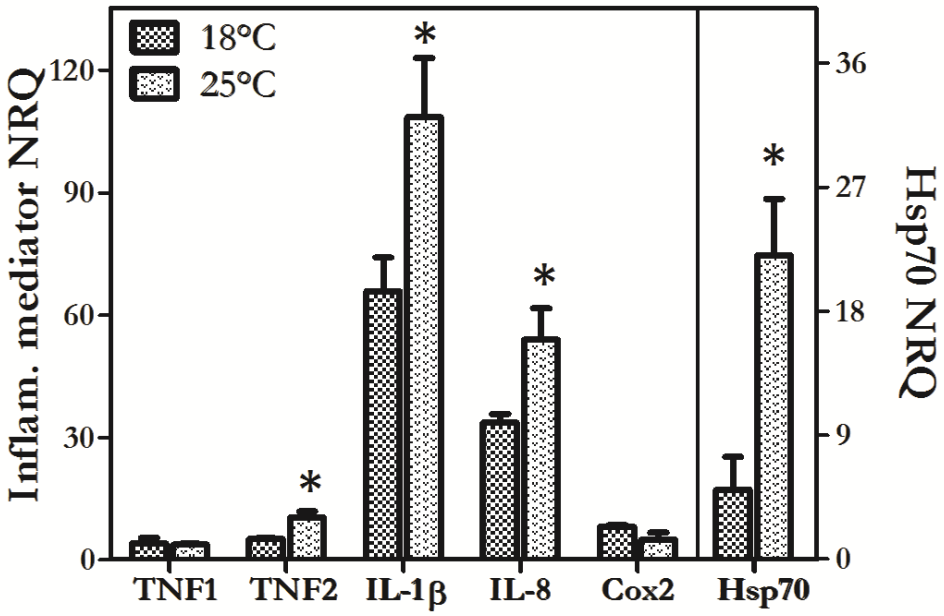


Figure 2.5. Relative transcription of inflammatory mediators with respect to Hsp70 in PBL cultures following heat shock and LPS stimulation. Data shown are the mean (± SE) of a triplicate set of biological replicates at 24h post LPS stimulation incubated at either 18 or 25°C. Quantitative real-time PCR was performed in duplicate and presents as the relative transcript quantity (NRQ) normalised to two stable housekeeping genes and scaled to the minimum observed value. * Identifies a significant ($P < 0.05$) elevated quantity relative to the alternative temperature at the time point indicated.

A

-840 *ggtaacaattttcacttgaaaaaaagtatgtgatggtgctgcaaaggctga* ***actttaacaacattaaacatggcagtac*** -761
-760 ***atgcagggcgtcatgtggaaattcttgttttaatagctgtccaacggttgttccagtatgtgagagacatgccaacaccaa*** -680
-679 ***agttaattatgtgaattaactcttggtacaacaaatttccctttttgagggttgatgacaaaacctgaagggctgcatttg*** -598
-597 ***tcacttgtccataaagaggattgcatgtaattgttcatttgattatatgccaacaatgaaatcacttgtcagttgatcaatgtc*** -516
-515 ***tattcataagcaattagtagcaattgtgcaatcagctcaaagcaataaggaaacaggaaattaattt gctaagaaaat*** -438
-437 ***ctctcaag****cgtccctgtcacaacattcacttcattaacaactttagtcacagtgttttccaaatgaaaatgctgtagtgtg* -355
-354 *acaaatagtgacccatgatccatcttgataaatcatttattgtagtc* ***aaatacttttcaagcacctc****actgtaaattcccact* -272
-271 *cttcagacaatccaagcttgcgcaaga****cttttaagg****aagtgactcattcaggag*AGGGCTATAAAAGGTGCCACT -197
-196 CTCCTCTTCAGCATTACAGTCAAAAAGCAAAGCGGAAGGGCAGCAAGAAAAGGAAAGCAGAGG -134
-133 AGAGAAGGAATTAGCAGAAGTAAAAGTTGCCTGCCTTTCTATAACATAGTGCTGCAGTCTTCATC -69
-68 TGAGAGCTTTCTGAAAGACGTTTTTATCTTTTAGTGTCTTAGTTGTTGCAGAATTTGTAAAAGGCA -3
-2 AAATG

B

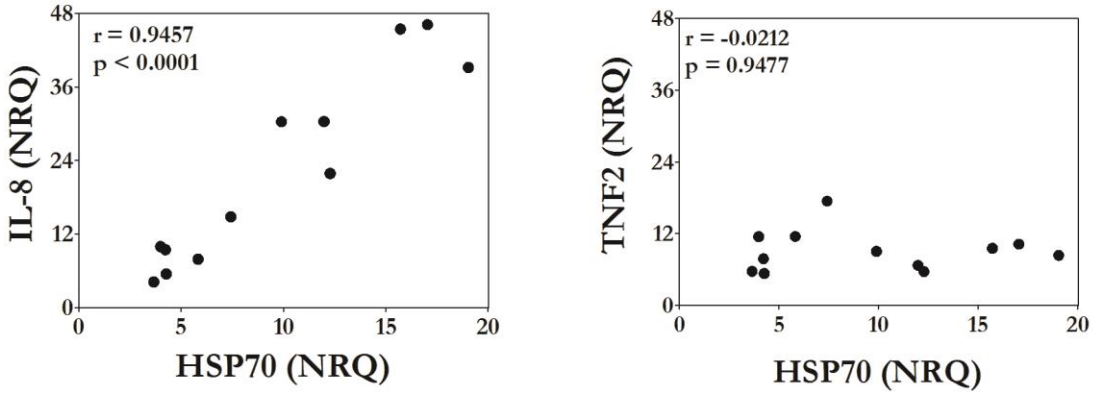


Figure 2.6. Putative evidence for co-activation of IL-8 by heat shock. (A) the 5'-upstream flanking region (lowercase, italised) of IL-8 relative to the mRNA sequence (capitalised). Putative recognition sites for HSF-1 binding are highlighted. Sites are categorised as either perfect or inverted dyads (nGAAnnTTCn or nCTTnnAAGn) or as a triad or longer provided there is only one mismatch per triplet (underlined) with at least one perfect triplet in the sequence (Bold text). Position is indicated in the negative relative to the start site of IL-8 protein translation (boxed). For correlation data (B), IL-8 transcription is presented in relation to Hsp70 transcription following LPS stimulation of PBL cultures at 25°C. TNF2 transcription is also provided for comparison.

2.5. Discussion

Ranching and investigations into hatchery-based aquaculture of SBT are currently being conducted in Australia, and given their growing aquaculture importance and potential concerns for natural populations, biological markers for identifying health and performance in these fish are becoming increasingly desirable. Here we identify five separate gene sequences associated with inflammatory and immune response signalling for SBT (TNF1, TNF2, IL-1β, IL-8 and Cox2) and demonstrate that four (TNF2, IL-1β, IL-8 and Cox2) are strongly induced using LPS, a well-known immune stimulant. Similar induction of TNF2 but not TNF1 has previously been reported in PBL leukocyte cultures from Pacific bluefin [11], and our present findings further support the distinct biological roles of these two paralogues in PBL populations and further expand these observations to HK homogenates. We also identify that the two forms of TNFα appear virtually identical between Pacific and southern bluefin species, and it may be presumed that the genetic sequences identified in this study will be useful for future work involving all three species of bluefin. This is the first report of complete genetic coding sequences of IL-1β and IL-8 in tuna. Although it is possible that additional homologues may be present for these two genes, such as the TNF1 and TNF2 homologues identified here in tuna or multiple IL-1β homologues previously identified in catfish [22] and common carp [23], our investigations have so far not identified any additional forms for either of these gene transcripts. Nevertheless, both IL-1β and IL-8 transcripts identified in this study demonstrate immunological responsiveness to pathogenic stimulation and thus present useful biological markers for accessing, at least in part, the immune and inflammatory responsiveness of bluefin tuna. Additionally, we identified coding sequence for the stress-induced protein chaperone Hsp70 as well as its constitutively expressed counterpart, Hsc70. Although a partial 574 base coding sequence for Hsp70 has

previously been identified for Pacific bluefin [12], our present identification of 1672 base Hsp70 and 1929 base Hsc70 partial transcripts for southern bluefin will allow greater specificity for singling out differential induction of these genetically similar homologues.

Bluefin are known to have a wide tolerance to environmental temperature (2-30°C) [24,25]. This is partially a result of their unique heterothermy, where heat generated in the muscle is conserved through counter-current vascularisation and allows for intraperitoneal temperature to be maintained in constant thermal excess of the ambient environment, typically in the range of 18-28°C in wild populations [26]. Nevertheless, captive Pacific bluefin held below 15°C and above 23°C for prolonged periods have been observed to exhibit acute stress behaviour [27]. A preferred environmental temperature is considered to be 18°C [28] which has also been shown to correspond to maintaining a minimum metabolic rate during swimming [25]. In this study, 18 and 25°C were chosen during *in vitro* cell culture to represent temperatures likely to be encountered by SBT PBLs within a 24h period *in vivo*, but also levels that might approach the lower and upper limits for long term unstressed physiological function in these cell populations.

Hsp70 is a well-known component of the cellular heat shock response enhanced during temperature related stress. Although typically associated with elevated temperature, Hsp70 expression is also known to occur in response to low temperature in fish [29] and has been observed to be enhanced in the spleen of Pacific bluefin held under chronic coldwater (15°C) conditions [12]. Both PBL and HK cultures held at 18°C in this study maintained relatively stable levels of Hsp70 throughout the 24 h test period, suggesting this temperature permissive for unstressed biological function of these cell populations in this time frame. In contrast, PBL cultures held for 24 h at 25°C showed significant induction of Hsp70 (Figure 2.4) indicating that this temperature surpasses the short-term homeostatic threshold for these cells. In HK cell cultures incubation at 25°C failed to induce Hsp70 expression, and we have

also observed Hsp70 non-responsiveness to that temperature in similarly cultured spleen homogenates (unpublished data). This tissue-specific variability was not entirely unexpected, as intraperitoneal temperatures have often been observed in excess of 25°C in wild fish [26], and further supports the differential induction of Hsp70 for organs in fish previously described by Dyer and colleagues for flathead minnow *Pimephales promelas* [30]. It also suggests that as a biomarker of elevated thermal stress, Hsp70 expression in blood (or specifically PBLs) may provide a closer parallel to that of the entire organism for heterothermic species such as tuna relative to internal organs typically assessed in fish and should be considered in future biological monitoring of this species.

In addition to temperature shock, Hsp70 expression is known to be induced by a variety of proteotoxic stressors such as toxin exposure, traumatic damage, radiation or nutritional deficiency. Induction of Hsp70, and the heat shock response in general, is primarily accomplished through activation of the transcriptional factor heat shock factor 1 (HSF-1) which under conditions of cellular stress binds to heat shock response elements in genomic DNA promoting transcription of heat shock proteins such as Hsp70. In a recent study by Singh *et al*. [31], heat shock response elements were identified on the promoter region of the human CXC chemokine IL-8. In that study, heat shock, via HSF-1 binding, was able to co-activate and enhance IL-8 transcription in conjunction with TNFα stimulation. This led the authors to hypothesise that ELR^+ CXC chemokines have co-opted elements of the heat shock response to facilitate neutrophil delivery at sites of infection or injury during febrile illness. In the present study, we identify six putative HREs in the IL-8 promoter of SBT, and although HSF-1 binding was not directly identified, a strong linear correlation between Hsp70 and IL-8 expression was observed following LPS stimulation and temperature shock (25°C incubation) that was not observed in other cytokines (Figure 2.6). This would suggest that co-activation of this chemokine occurs in SBT, and thus the co-opted

heat shock responsiveness in CXC chemokines is not only present in homoeothermic mammals but also in poikilothermic fish. Further work will be necessary to substantiate these findings; however this initial discovery opens the potential for interesting avenues for investigation concerning febrile temperature regulation in fish (particularly with regard to heterothermic tuna) and the role of inflammation during toxin or other stress exposure.

Although the heat shock response has definitive impacts on immunity and inflammation, it is not considered a direct part of the innate immune or inflammatory signalling cascade. Indeed, induction of Hsp70 during the heat shock response is known to be an inflammatory antagonist by decreasing expression of pro-inflammatory signalling through inhibition of the transcription factor NF-κβ (reviewed by [32]). Additionally, no evidence has been presented to suggest the activation of HSF-1 following pathogen recognition. Nevertheless, Hsp70 has been directly associated with LPS stimulation in carp immune cells [7,8], leading to some speculation for a potential role of Hsp70 in direct immune signalling in fish. In the present study, transcriptional regulation of Hsp70 in SBT PBL populations occurred independent of LPS stimulation. At 18°C, LPS induced large fold changes in cytokine (TNFα and IL-1β), chemokine (IL-8) and prostaglandin associated synthesis enzyme (Cox2) transcription (Figure 2.2; Figure 2.3), but failed to induce Hsp70 (Figure 2.4). At 25°C, Hsp70 transcription was induced to the same relative level with or without LPS stimulation. This would indicate that at least for SBT, Hsp70 is not directly associated with inflammatory or immune response signalling. This is not necessarily in contrast with the previous findings in carp phagocytes and PBLs, as in those studies high levels ($\geq$30 μg mL^{-1}) of LPS were needed to elicit an Hsp70 response and in fact 10 μg mL^{-1} LPS (the concentration used in this study) failed to significantly induce Hsp70 expression in grass carp *Ctenopharyngodon idella* PBL populations [8]. Although fish are known to be resistant to bacterial endotoxin, 10 μg mL^{-1} LPS has generally been observed to induce significant

responsiveness in immune cells of fish [33], exemplified here in PBL cultures from SBT. Additionally, 10 μg mL^{-1} LPS would conservatively correspond to > 10^8 *E. coli* ml^{-1} (more for other Gram-negative bacteria such as *Flavobacterium spp.*) [34,35], which is a bacteria/LPS load unlikely to be encountered during *in vivo* septicaemia [36,37]. We therefore conclude that Hsp70 is unlikely to be a direct component of immune or inflammatory response signalling following endoxtoxin recognition in fish under normal physiological conditions. This is not to say that components of the inflammatory/immune response do not have secondary effects on Hsp70 expression. On the contrary, we observed a possible limited increase in expression of Hsp70 at 24 h post stimulation in LPS stimulated cultures of PBLs following its temperature induced activation at 25°C, suggesting that inflammatory signalling may have a secondary ability to enhance Hsp70 expression in SBT PBLs. However, initial induction or at least co-activation by a proteotoxic stressor such as temperature appears to be required for transcriptional induction of Hsp70.

Lastly, we identify that inflammatory mediators are differentially expressed in response to temperature in SBT PBLs. Transcription of both TNF2 and IL-1β was induced earlier at elevated temperature (Figure 2.2; Figure 2.3). This differential transcription occurred prior to Hsp70 induction in PBL populations at 25°C and without Hsp70 induction entirely in HK populations, thus indicating independence from any heat shock response. Although the role of temperature for influencing inflammatory signal induction in fish has not been well articulated, a study by Zou and colleagues [5] has shown low temperature to suppress short-term (4 h) IL-1β transcription during *in vitro* culture of LPS stimulated rainbow trout leukocytes. We observed a similar initial response between the two temperatures used in this study, but additional sampling at later time points revealed that initial suppression at lower temperature was not maintained and in fact the influence of temperature was to delay rather than suppress overall transcription. Both TNF2 and IL-1β

expression were significantly delayed for 3-6 h with the 7°C reduction in incubation temperature, but relative peak transcript quantities were comparable at both temperatures for each cytokine (Figure 2.2; Figure 2.3). This is in contrast with mammalian leukocytes, where enhanced and suppressed cytokine expression have been observed in cases of hypothermia and hyperthermia, respectively [1]. The unaltered peak transcription observed here is not completely unexpected, as the poikilothermic nature of fish requires a physiology adapted to operate under variable temperature conditions, even for heterothermic species such as tuna. Nevertheless, fish immune functions can be differentially regulated in response to temperature, and a general shift from specific to nonspecific immunity has been observed during low temperature exposure (reviewed by [38]). Therefore, the potential for lowered temperature to delay inflammation may have significant implications for the effectiveness of an innate immune response in fish. Specifically with regard to heterothermic species such as SBT, findings in this study highlight the external gill relative to the internal gut as a potential 'weak point' for infection or injury repair due to a possible slower inflammatory immune response resulting from lowered environmental temperature.

2.6. Acknowledgments

The authors would like to thank the research staff of the Australian Southern Bluefin Tuna Industry Association and the ranching companies and crews for their cooperation and assistance in collecting blood samples. We would also extend our appreciation to the Lincoln Marine Science Centre for providing laboratory space and equipment used during preparation and maintenance of primary cell cultures. This research was supported by Fisheries Research and Development Corporation, Southern Bluefin Tuna industry, and the Australian

Department of Education, Employment, and Workspace Relations Endeavour International Postgraduate Award.

2.7 References

1. Fairchild KD, Viscardi RM, Hester L, Singh IS, Hasday JD (2000) Effects of hypothermia and hyperthermia on cytokine production by cultured human mononuclear phagocytes from adults and newborns. Journal of Interferon Cytokine Research 20:1049-1055.

2. Fairchild KD, Singh IS, Patel S, Drysdale BE, Viscardi RM, Hester L, et al. (2004) Hypothermia prolongs activation of NF-κB and augments generation of inflammatory cytokines. American Journal of Physiology - Cell Physiology 287:C422-C431.

3. Fairchild KD, Singh IS, Carter HC, Hester L, Hasday JD (2005) Hypothermia enhances phosphorylation of IkB kinase and prolongs nuclear localization of NF-kB in lipopolysaccharide-activated macrophages. American Journal of Physiology - Cell Physiology 289:C1114-C1121.

4. Chen Y, Currie RW (2006) Small interfering RNA knocks down heat shock factor-1 (HSF-1) and exacerbates pro-inflammatory activation of NF-κB and AP-1 in vascular smooth muscle cells. Cardiovascular Research 69:66-75.

5. Zou J, Holland J, Pleguezuelos O, Cunningham C, Secombes CJ (2000) Factors influencing the expression of interleukin-1β in cultured rainbow trout (*Oncorhynchus mykiss*) leucocytes. Developmental and Comparative Immunology 24:575-582.

6. Deane EE, Woo NYS (2005) Evidence for the disruption of Na^+ -K^+ -ATPase and HSP70 during vibriosis in sea bream, *Sparus* (=Rhabdosargus) *sarba* Forsskal. Journal of Fish Diseases 28:239-251.

7. Stolte EH, Chadzinska M, Przybylska D, Flik G, Salelkoul HFJ, Verburg-van Kemenade BML (2009) The immune response differentially regulates Hsp70 and glucocorticoid receptor expression *in vitro* and *in vivo* in common carp (*Cyprinus carpio* L.). Fish and Shellfish Immunology 27:9-16.

8. Zhang A, Zhou X, Wang X, Zhou H (2011) Characterization of two heat shock proteins (Hsp70/Hsc70) from grass carp (*Ctenopharyngodon idella*): Evidence for their differential gene expression, protein synthesis and secretion in LPS-challenged peripheral blood lymphocytes. Comparative Biochemistry and Physiology, Part B 159:109-114.

9. Graham JB, Dickson KA (2004) Tuna comparative physiology. Journal of Experimental Biology 207:4015-4024.

10. Kirchhoff NT, Rough KM, Nowak BF (2011) Moving Cages Further Offshore: Effects on Southern Bluefin Tuna, *T. maccoyii*, Parasites, Health and Performance. PLoS One 6:e23705.

11. Kadowaki T, Harada H, Sawada Y, Kohchi C, Soma G-I, Takahashi Y, et al (2009) Two types of tumor necrosis factor-α in bluefin tuna (*Thunnus orientalis*) genes: Molecular cloning and expression profile in response to several immunological stimulants. Fish and Shellfish Immunology 27:585-594.

12. Mladineo I, Block BA (2009) Expression of Hsp70, Na+/K+ ATP-ase, HIF-1α, IL-1β and TNF-α in captive Pacific bluefin tuna (*Thunnus orientalis*) after chronic warm and cold exposure. Journal of Experimental Marine Biology and Ecology 374:51-57.

13. Drummond A, Ashton B, Buxton S, Cheung M, Cooper A, Heled J, et al. (2011) Geneious V4.8, Available from http://www.geneious.com.

14. Altschul S, Gish W, Miller W, Myers E, Lipman D (1990) Basic local alignment search tool. Journal of Molecular Biology 215:403-410.

15. Nagarsekar A, Hasday JD, Singh IS (2005) CXC Chemokines: A New Family of Heat-Shock Proteins? Immunological Investigations 34:381-398.

16. Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J (2007) qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biology 8:R19.

17. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3:0034.1-11.

18. Hellemans J, Vandesompele J (2011) Quantitative PCR data analysis – unlocking the secret to successful results. In: Oswald N, Kennedy S, editors. PCR troubleshooting and optimization: the essential guide. Norwich, UK: Caister Academic Press.

19. Rieu I (2009) Real-Time Quantitative RT-PCR: Design, Calculations, and Statistics. The Plant Cell 21:1031-1033.

20. Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, et al. (2011) CDD: a Conserved Domain Database for the functional annotation of proteins. Nucleic Acids Research 39:D225-D229.

21. Lam J, Nelson C, Ross F, Teitelbaum S, Fremont D (2001) Crystal sructure of the TRANCE/RANKL cytokine reveals determinants of receptor-ligand specificity. Journal of Clinical Investigation 108:971-979.

22. Wang Y, Wang Q, Baoprasertkul P, Peatman E, Liu Z (2006) Genomic organization, gene duplication, and expression analysis of interleukin-1beta in channel catfish (*Ictalurus punctatus*). Molecular Immunology 43:1653-1664.

23. Engelsma M, Stet R, Saeij J, Verburg-van Kemenade BM (2003) Differential expression and haplotypic variation of two interleukin-1beta genes in the common carp (*Cyprinus carpio* L.). Cytokine 22:21-32.

24. Block BA, Dewar H, Blackwell SB, Williams TD, Prince ED, Farwell CJ, et al. (2001) Migratory movements, depth preferences, and thermal biology of Atlantic Bluefin tuna. Science 293:1310-1314.

25. Blank JM, Morissette JM, Farwell CJ, Price M, Schallert RJ, Block BA (2007) Temperature effects on metabolic rate of juvenile Pacific bluefin tuna *Thunnus orientalis*. Journal of Experimental Biology 201:4254-4261.

26. Lawson GL, Castleton MR, Block BA (2010) Movements and diving behavior of Atlantic bluefin tuna *Thunnus thynnus* in relation to water column structure in the northwestern Atlantic. Marine Ecology Progress Series 400:245-265.

27. Farwell CJ (2001) Tunas in captivity. In: Hoar WS, Randall D, Farrekk A, editors. Tunas: Physiology, Ecology and Evolution. San Diego, CA: Academic Press, 391-412.

28. Marcinek DJ, Blackwell SB, Dewar H, Freund EV, Farwell C, Dau D, et al. (2001) Depth and muscle temperature of Pacific bluefin tuna examined with acoustic and pop-up satellite archival tags. Marine Biology 138:869-885.

29. Ju ZJ, Dunham RD, Liu ZL (2002) Differential gene expression in the brain of channel catfish (*Ictalurus punctatus*) in response to cold acclimation. Molecular Genetics and Genomics 268:87-95.

30. Dyer SD, Dickson KL, Zimmerman EG, Sanders BM (1991) Tissue-specific patterns of synthesis of heat-shock proteins and thermal tolerance of the fathead minnow (*Pimephales promelas*). Canadian Journal of Zoology 69:2021-2027.

31. Singh IS, Gupta A, Nagarsekar A, Cooper Z, Manka C, Hester L, et al. (2008) Heat shock co-activates interleukin-8 transcription. American Journal of Respiratory Cell and Molecular Biology 39:235-242.

32. Chen Y, Voegeli TS, Liu PP, Noble EG, Currie RW (2007) Heat shock paradox and a new role of heat shock proteins and their receptors as anti-inflammation targets. Inflammation and Allergy - Drug Targets 6:91-100.

33. Swain P, Nayak SK, Nanda PK, Dash S (2008) Biological effects of bacterial lipopolysaccharide (endotoxin) in fish: A review. Fish and Shellfish Immunology 25:191-201.

34. Magalhaes PO, Lopes AM, Mazzola PG, Rangel-Yagui C, Penna TCV, Pessoa A (2007) Methods of endotoxin removal from biological preparations: a review. Journal of Pharmacy and Pharmaceutical Sciences 10:388-404.

35. Saghee MR, Sandle T, Tidswell EC, editors (2011) Microbiology and Sterility Assurance in Pharmaceuticals and Medical Devices. New Delhi: Business Horizons.

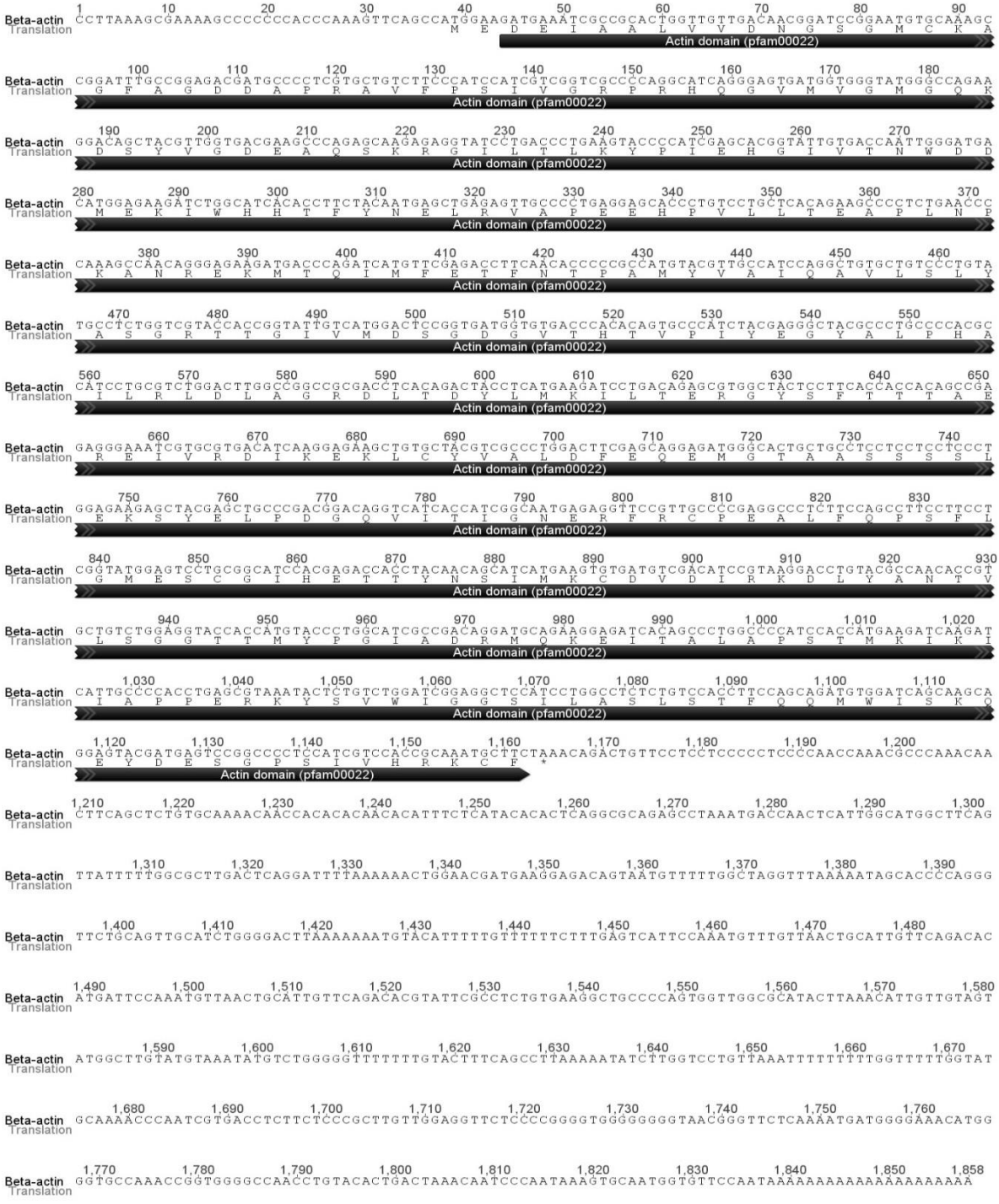
36. Kim KS, Cross AS, Zollinger W, Sadoff J (1985) Prevention and therapy of experimental *Escherichia coli* infection with monoclonal antibody. Infection and Immunity 50:734-737.

37. Fernandez RD, Yoshimizu M, Kimura T, Ezura Y (1993) Establishment and Characterization of Seven Continuous Cell Lines from Freshwater Fish. Journal of Aquatic Animal Health 5:137-147.

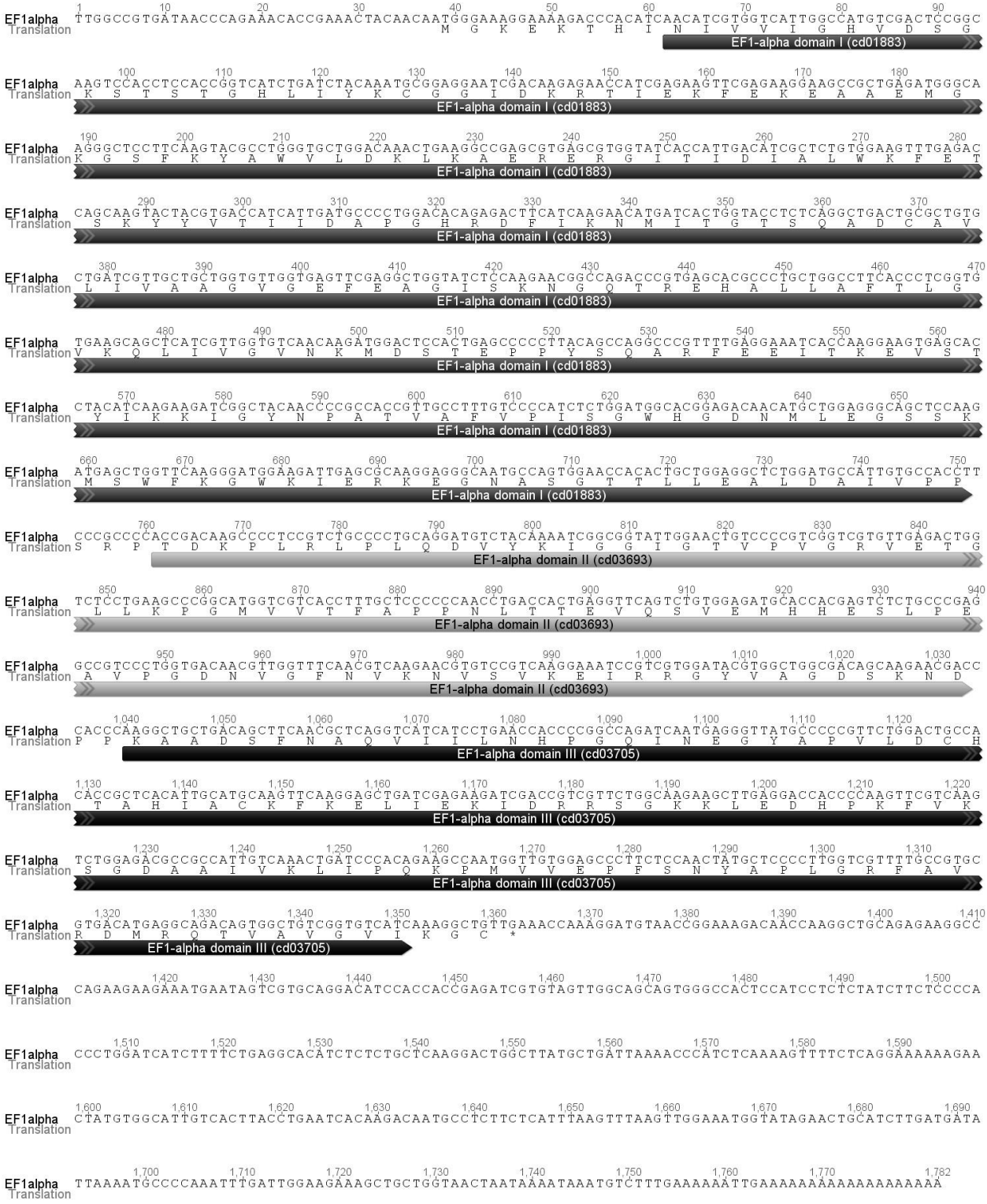
38. Le Morvan C, Troutaud D, Deschaux P (1998) Differential effects of temperature on specific and nonspecific immune defences in fish. Journal of Experimental Biology 201:165-168.

2.8. Supplemental material

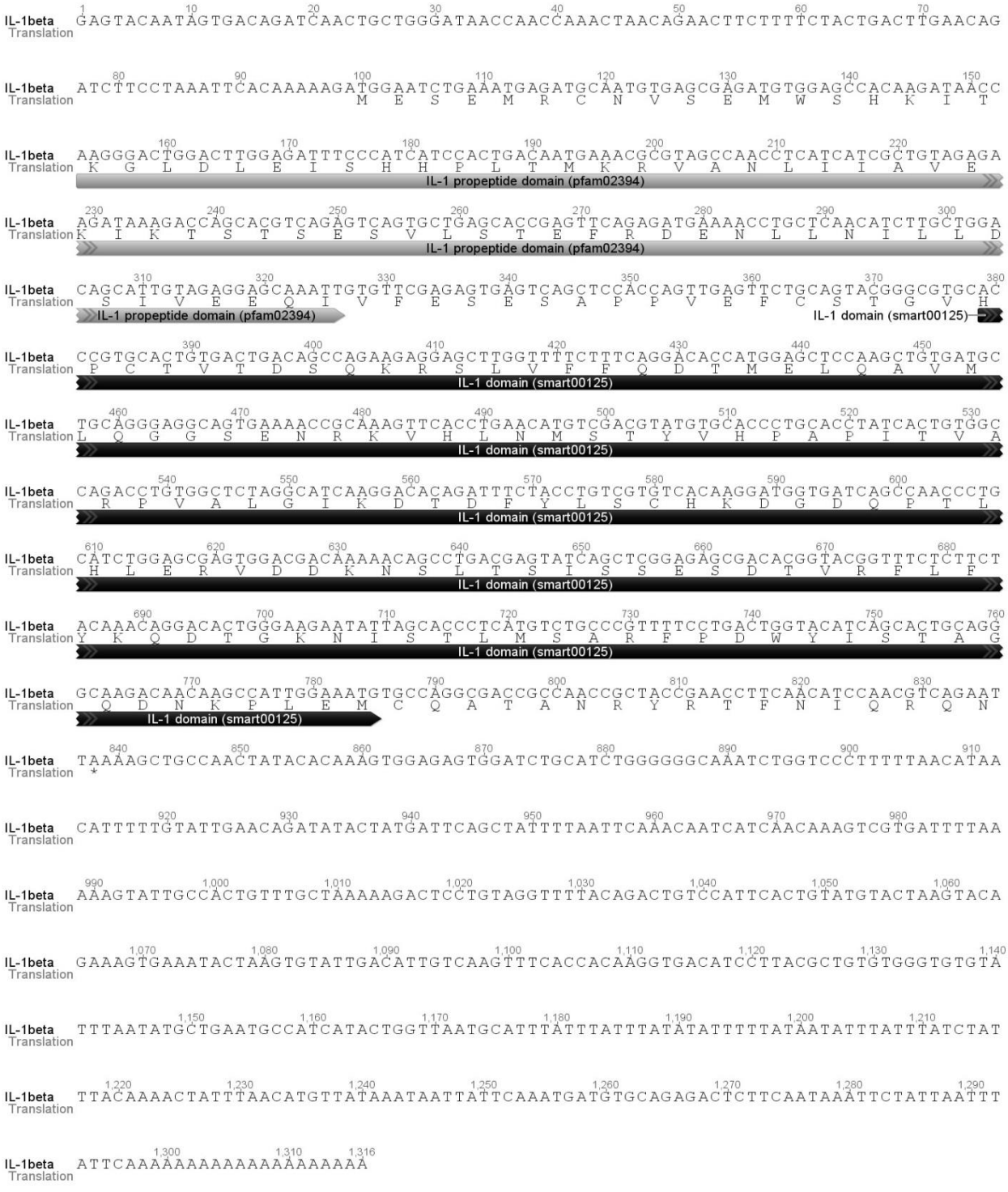
A



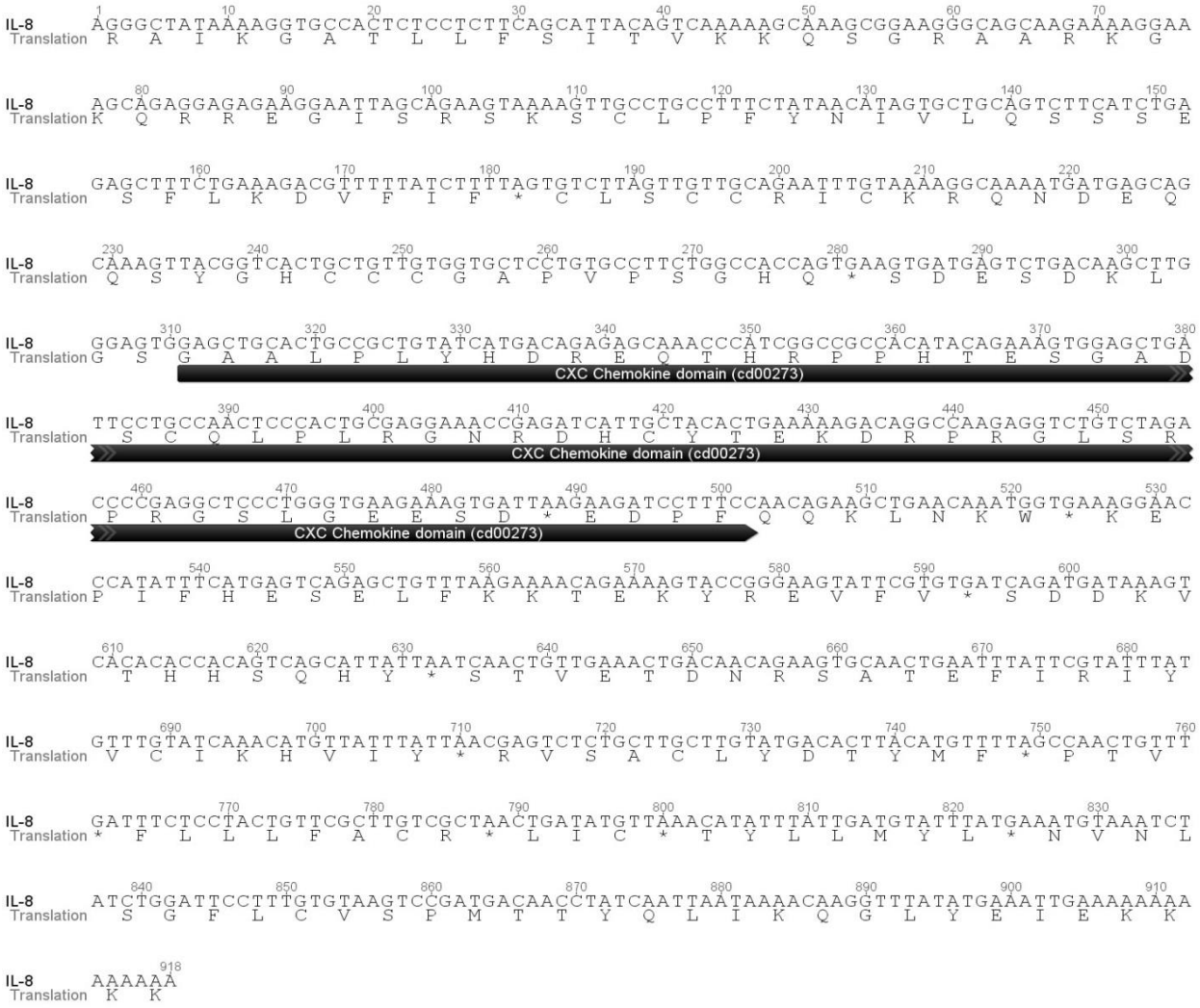
B


EF1alpha
Translation
EF1-alpha domain I (cd01883)
EF1-alpha domain II (cd03693)
EF1-alpha domain III (cd03705)

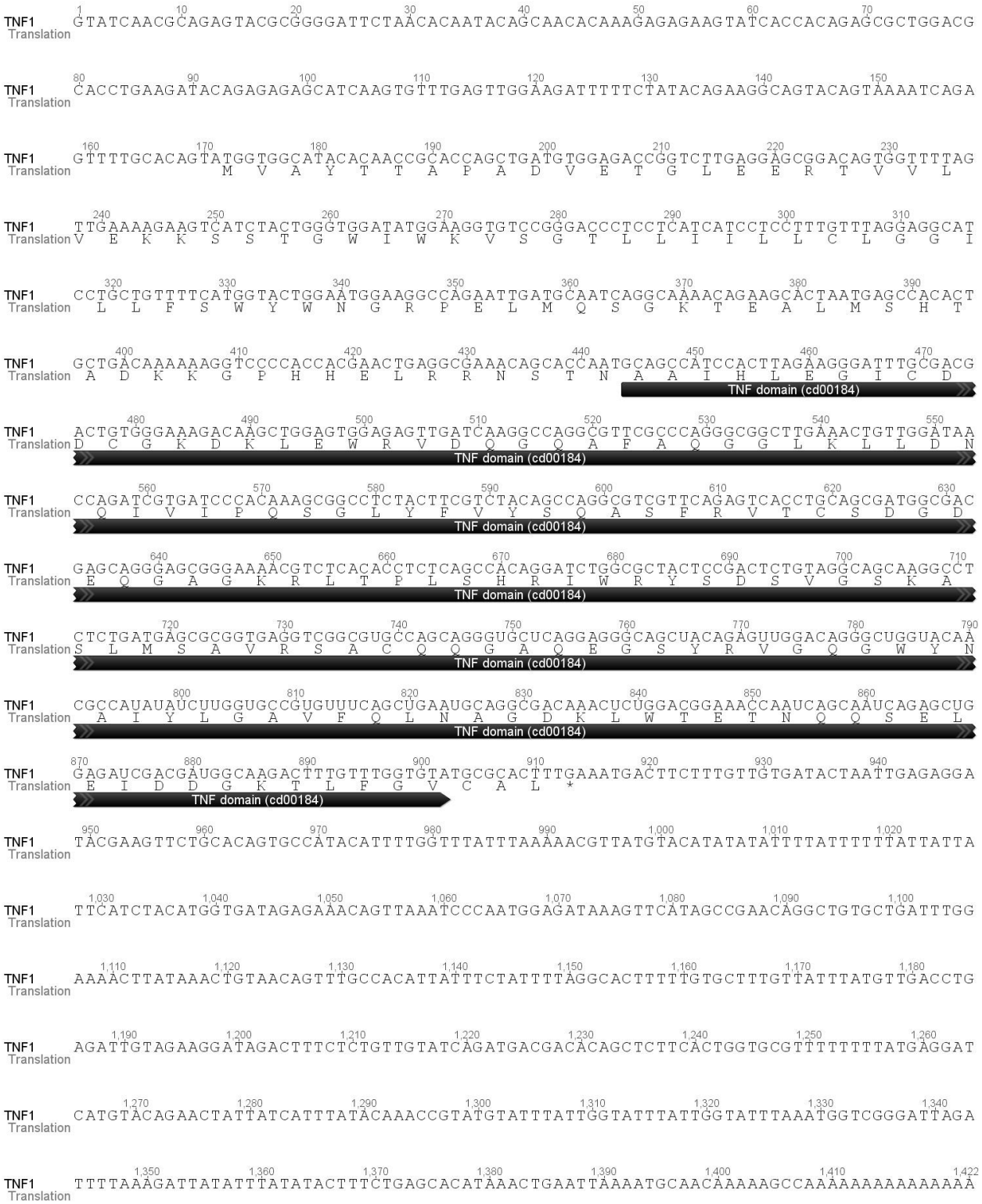
C


IL-1beta
Translation
IL-1 propeptide domain (pfam02394)
IL-1 domain (smart00125)

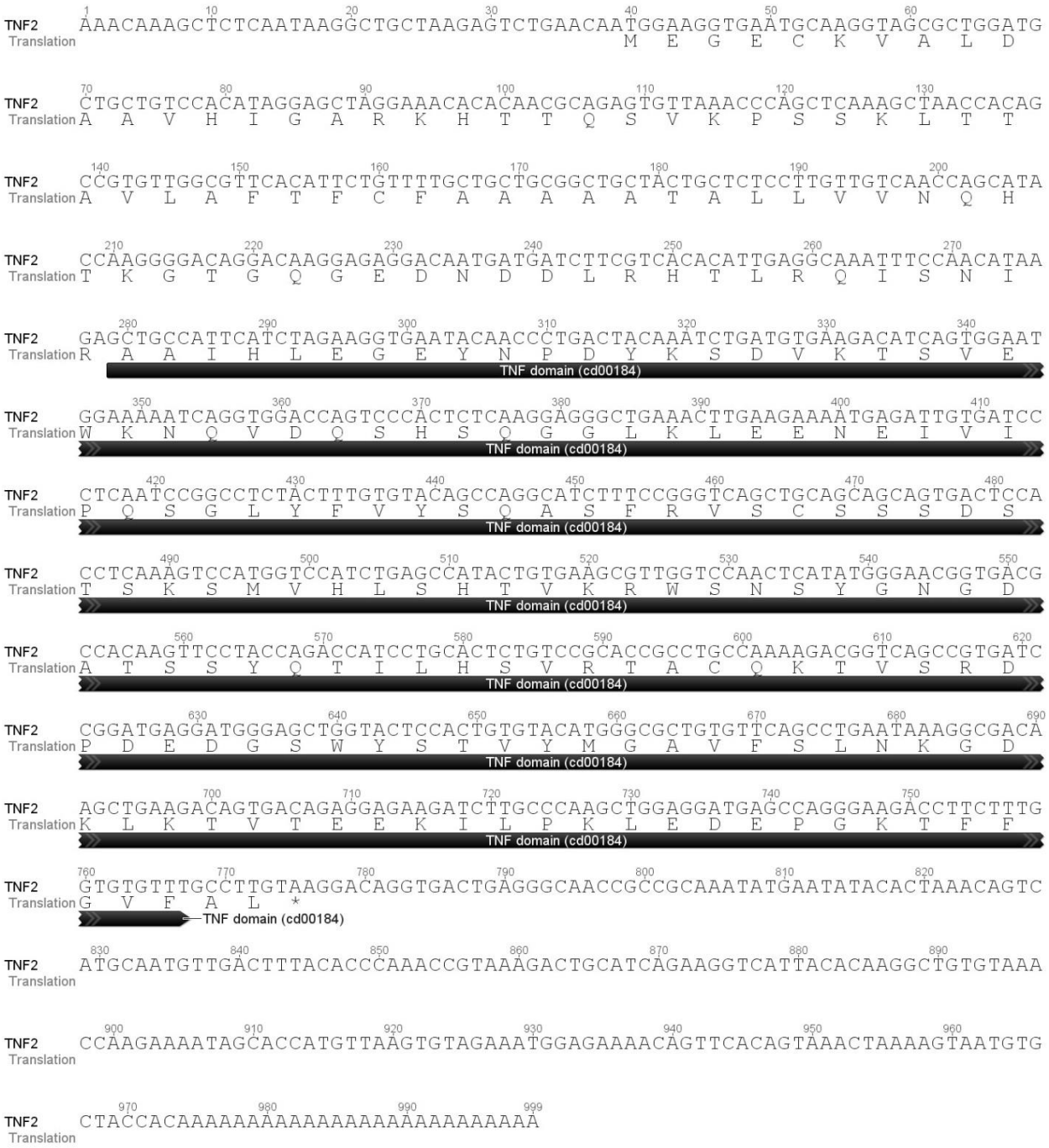
D


IL-8
Translation
CXC Chemokine domain (cd00273)
CXC Chemokine domain (cd00273)
CXC Chemokine domain (cd00273)

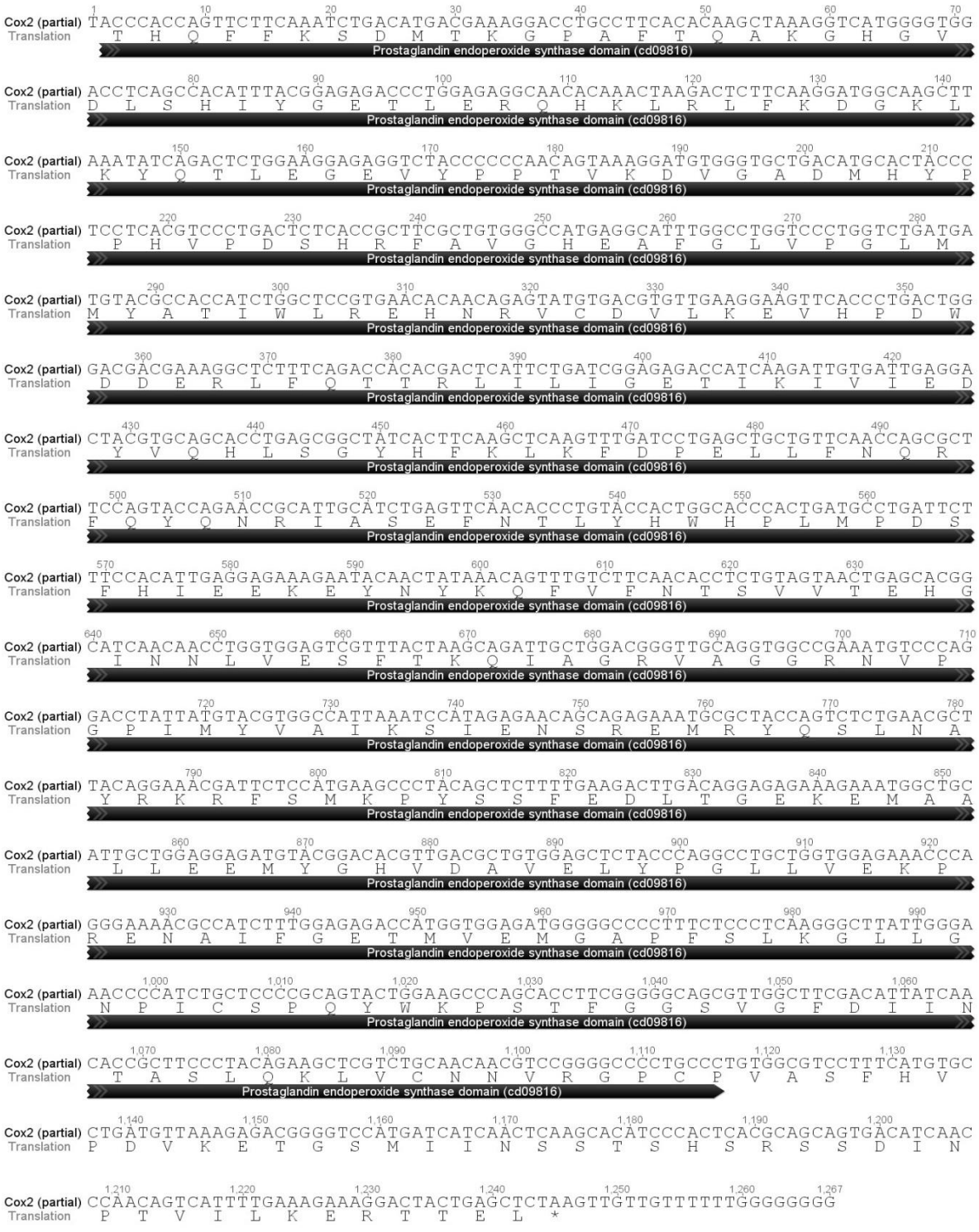
E



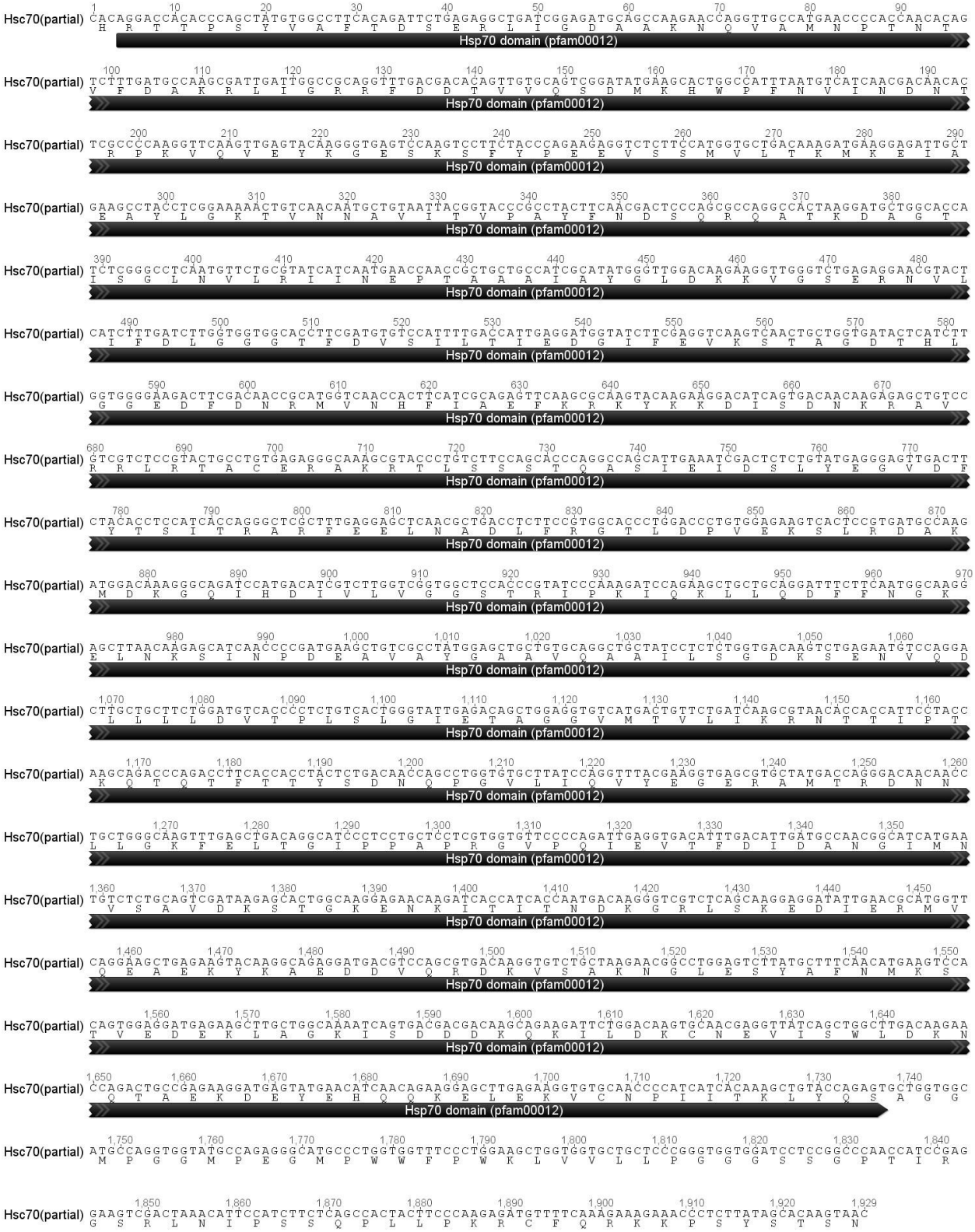
F



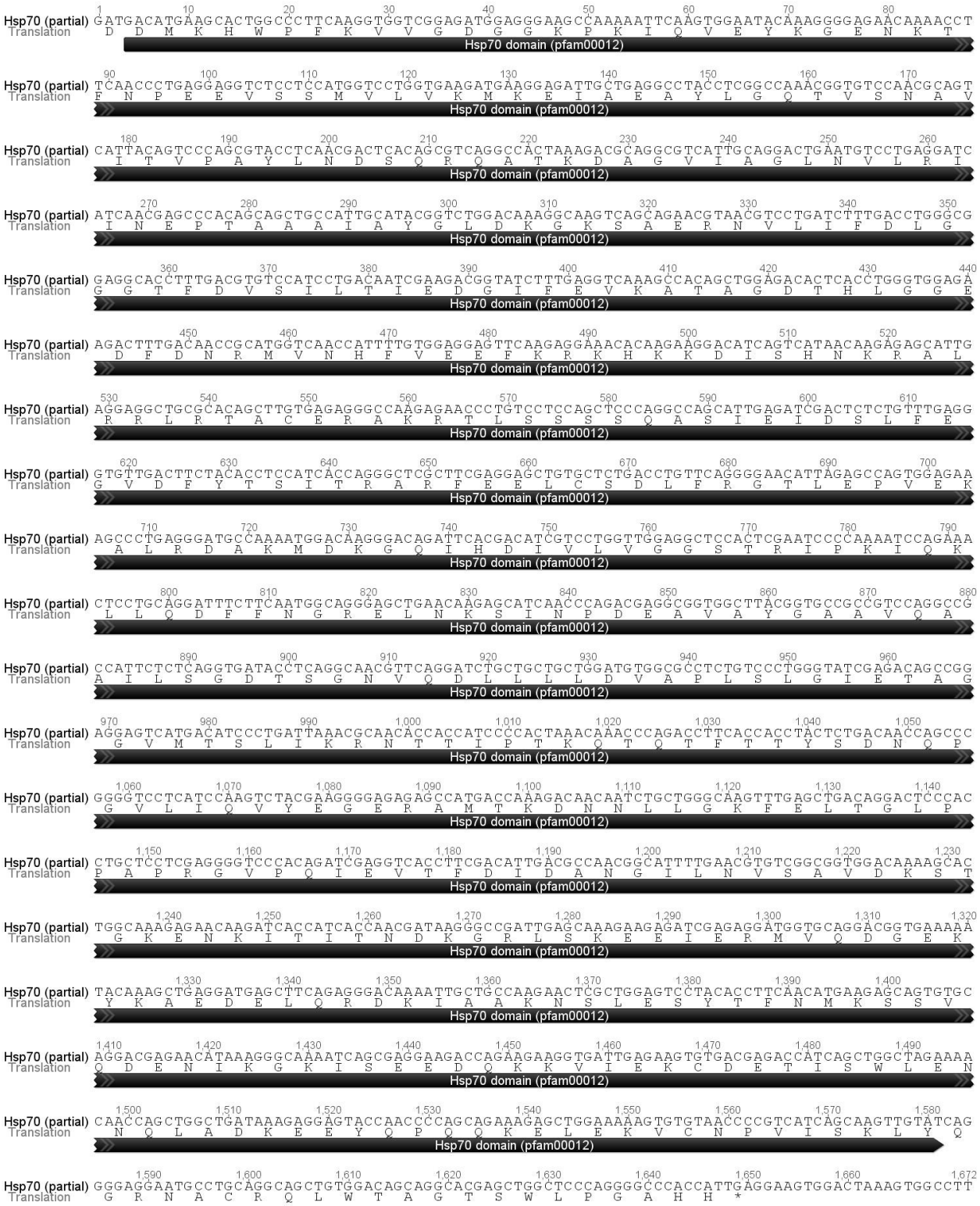
G


Cox2 (partial)
Translation
Prostaglandin endoperoxide synthase domain (cd09816)

H

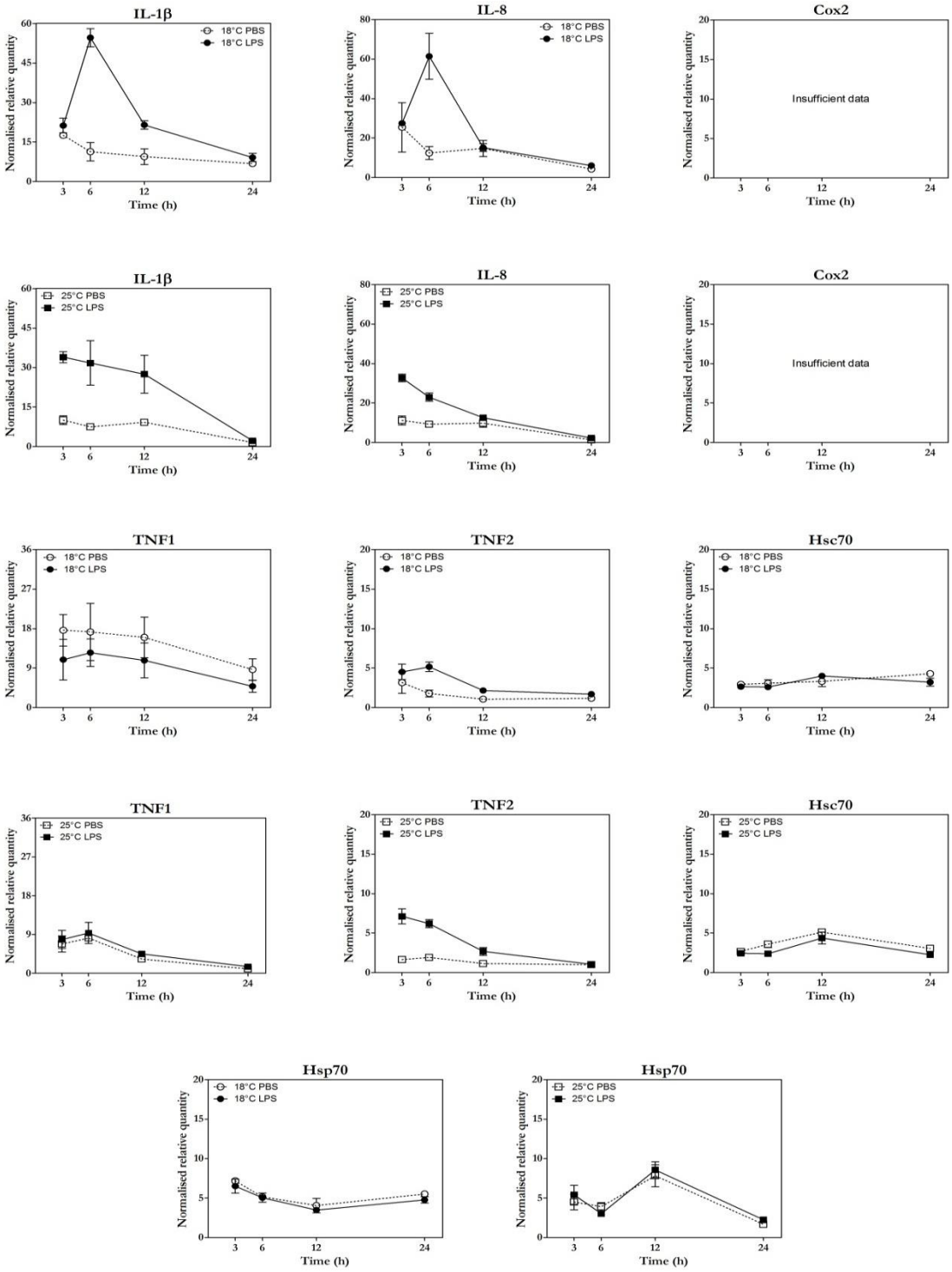


I

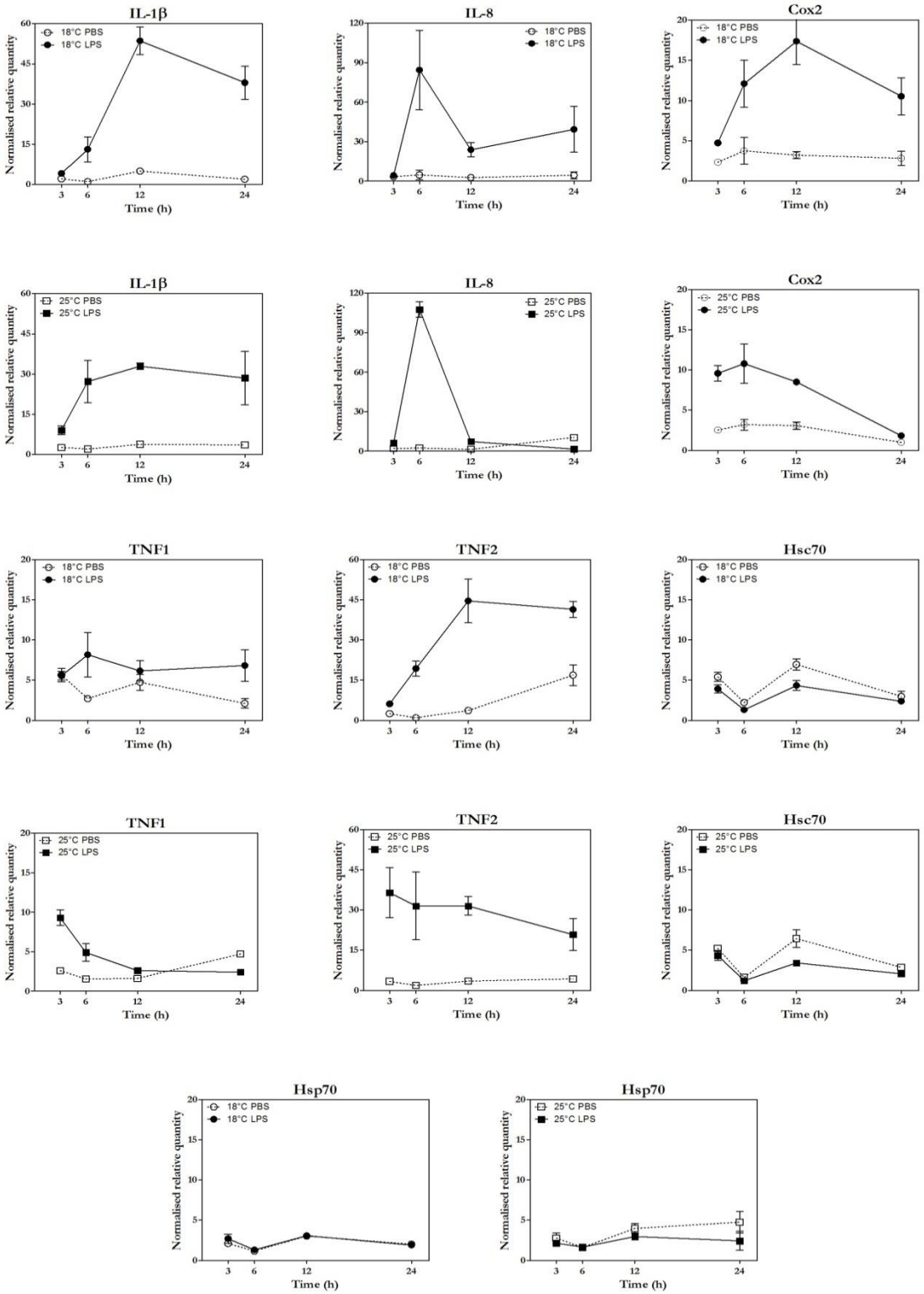


Supplemental Figure 2.1. Nucleotide cDNA sequence for βactin (A), EF1α (B), IL-1β (C), IL-8 (D), TNF1 (E), TNF2 (F), Cox2 (G), Hsc70 (H) and Hsp70 (I) identified in this study along with amino acid translation of the open reading frame. Conserved mature protein family signatures are annotated with conserved domain database (CDD) accession numbers provided.

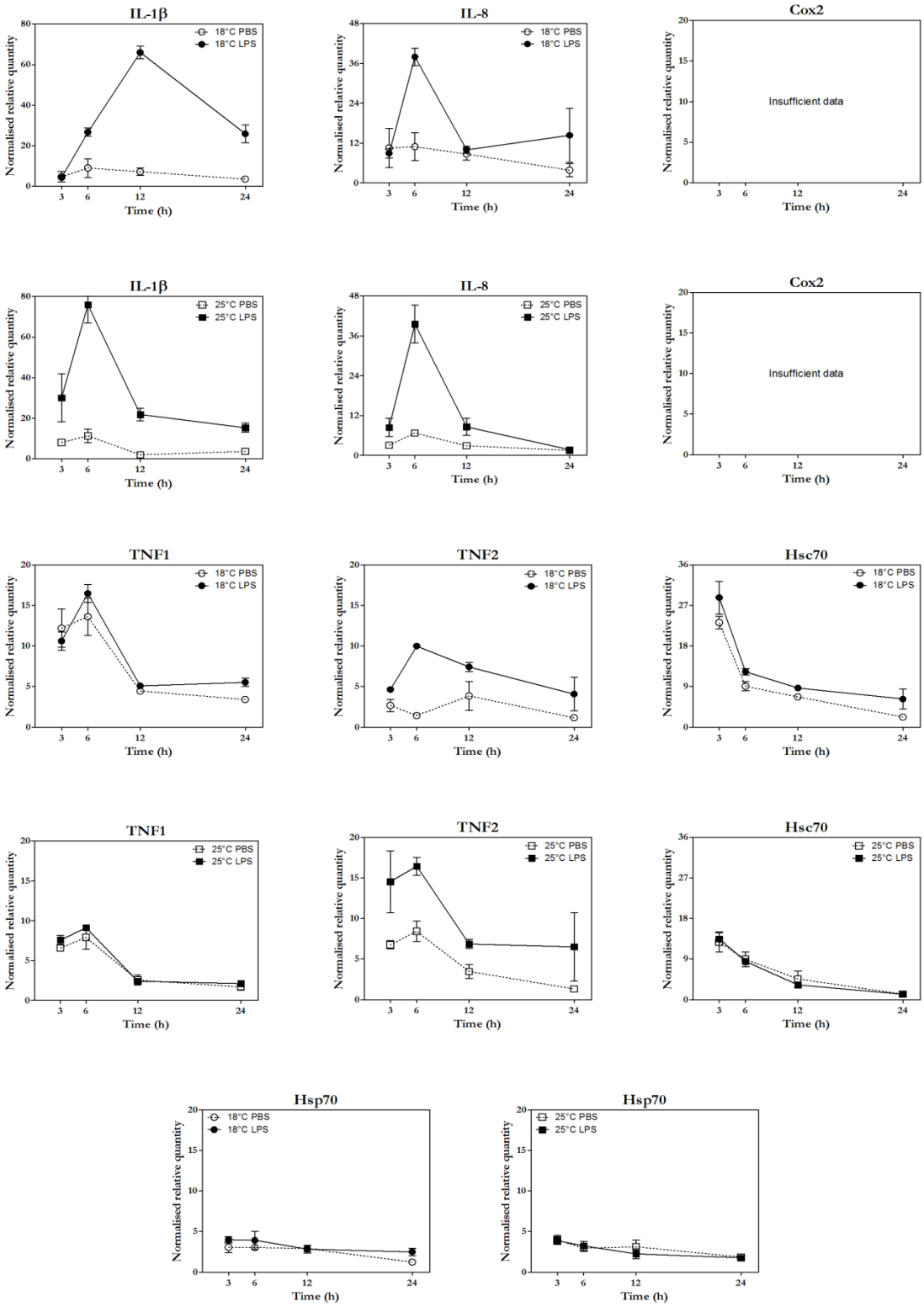
A


IL-1β
IL-8
Cox2
TNF1
TNF2
Hsc70
Hsp70
18°C PBS
18°C LPS
25°C PBS
25°C LPS
Insufficient data
Normalised relative quantity
Time (h)

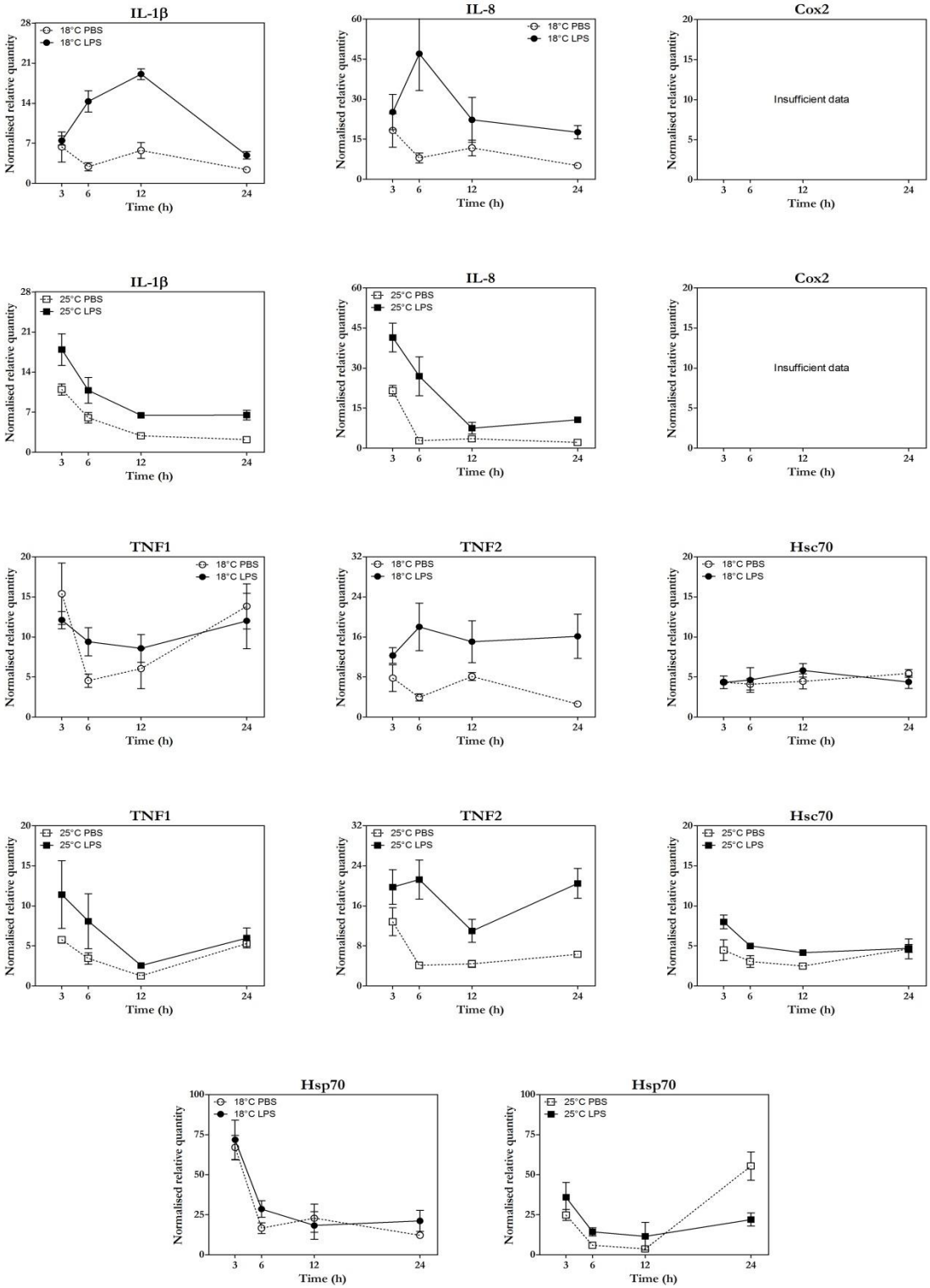
B


IL-1β
IL-8
Cox2
IL-1β
IL-8
Cox2
TNF1
TNF2
Hsc70
TNF1
TNF2
Hsc70
Hsp70
Hsp70
18°C PBS
18°C LPS
25°C PBS
25°C LPS
Normalised relative quantity
Time (h)

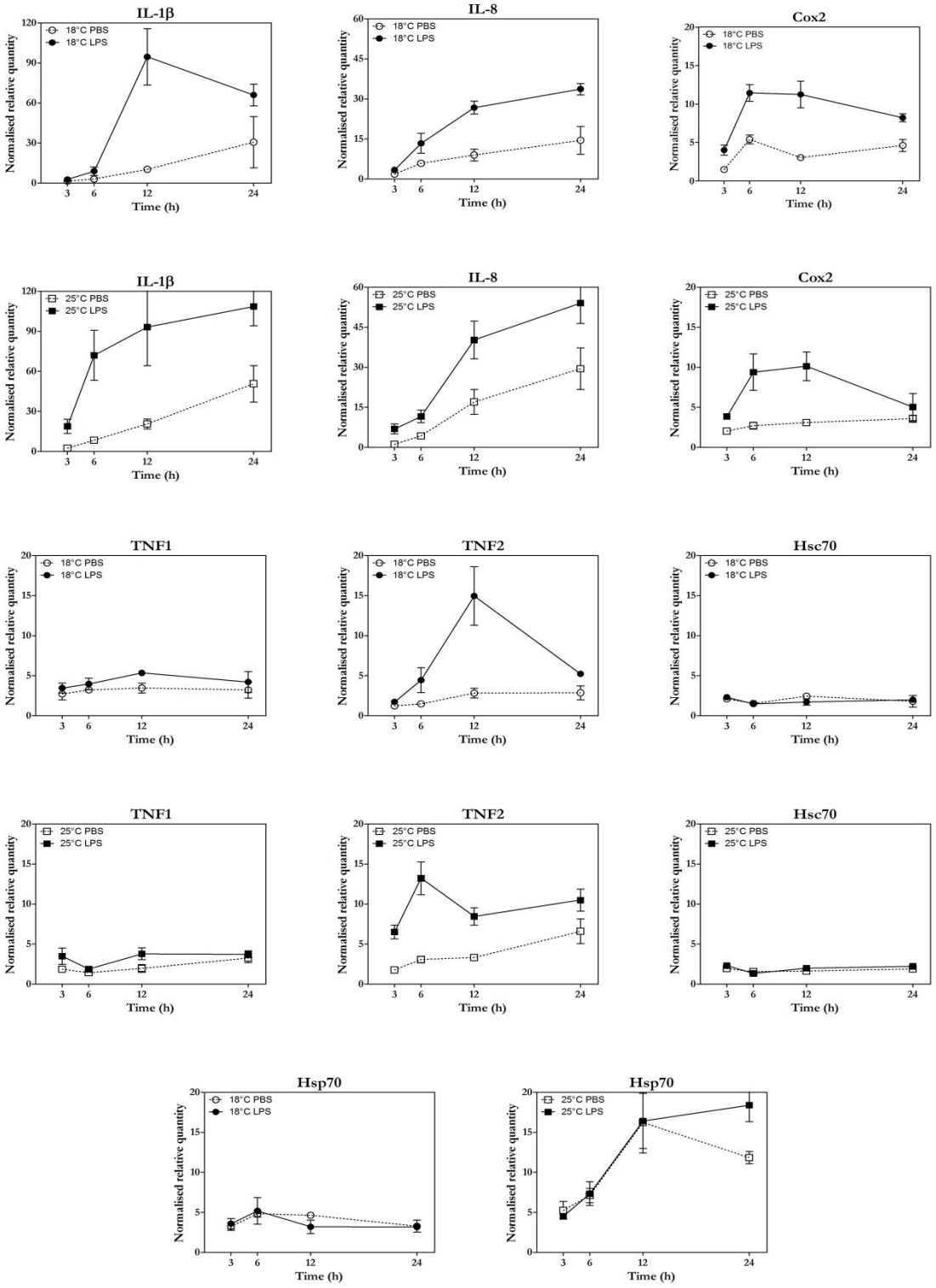
C


IL-1β
IL-8
Cox2
TNF1
TNF2
Hsc70
Hsp70
18°C PBS
18°C LPS
25°C PBS
25°C LPS
Insufficient data
Normalised relative quantity
Time (h)

D


IL-1β
18°C PBS
18°C LPS
Normalised relative quantity
Time (h)
IL-8
18°C PBS
18°C LPS
Normalised relative quantity
Time (h)
Cox2
Insufficient data
Normalised relative quantity
Time (h)
IL-1β
25°C PBS
25°C LPS
Normalised relative quantity
Time (h)
IL-8
25°C PBS
25°C LPS
Normalised relative quantity
Time (h)
Cox2
Insufficient data
Normalised relative quantity
Time (h)
TNF1
18°C PBS
18°C LPS
Normalised relative quantity
Time (h)
TNF2
18°C PBS
18°C LPS
Normalised relative quantity
Time (h)
Hsc70
18°C PBS
18°C LPS
Normalised relative quantity
Time (h)
TNF1
25°C PBS
25°C LPS
Normalised relative quantity
Time (h)
TNF2
25°C PBS
25°C LPS
Normalised relative quantity
Time (h)
Hsc70
25°C PBS
25°C LPS
Normalised relative quantity
Time (h)
Hsp70
18°C PBS
18°C LPS
Normalised relative quantity
Time (h)
Hsp70
25°C PBS
25°C LPS
Normalised relative quantity
Time (h)

E


IL-1β
IL-8
Cox2
IL-1β
IL-8
Cox2
TNF1
TNF2
Hsc70
TNF1
TNF2
Hsc70
Hsp70
Hsp70
18°C PBS
18°C LPS
25°C PBS
25°C LPS
Normalised relative quantity
Time (h)

Supplemental Figure 2.2. Relative transcriptional quantity of gene transcripts evaluated in this study following 10 µg mL^{-1} LPS (Filled; solid) or PBS (open; dashed) stimulation at either 18 or 25°C. Data are presented independently for each of three populations of head kidney homogenates (A, B, & C) and two populations of peripheral blood leukocytes (D & E). Data shown are mean (± SE) of triplicate samples assayed in duplicate by quantitative real-time PCR presented as the relative quantity at 3, 6, 12, and 24 h post incubation normalised to two stable housekeeping genes and scaled the minimum observed value.

Chapter Three

SYBR, TaqMan, or both: Highly sensitive, non-invasive detection of *Cardicola* blood fluke species in southern bluefin tuna (*Thunnus maccoyii*)

Published in:

Polinski M, Belworthy Hamilton D, Nowak B, Bridle A (2013) SYBR, TaqMan, or both: Highly sensitive, non-invasive detection of *Cardicola* blood fluke species in Southern bluefin tuna (*Thunnus maccoyii*). Molecular and Biochemical Parasitology 191: 7-15.

3.1. Abstract

Three species of blood fluke from the genus *Cardicola* are known to parasitise and cause disease in bluefin tunas – *C. forsteri*, *C. orientalis*, and *C. opisthorchis*. Although initially believed to be separated by geography and host specificity, recent identification of at least two *Cardicola* spp. concurrently present within all three bluefin species has raised questions concerning pathogenicity, relative abundance, and distribution of these parasites within bluefin populations. Here, we present sensitive and differential real-time qPCR nucleic acid detection of these *Cardicola* spp. by targeting the ITS2 region of the parasite rDNA for PCR amplification. A limit of sensitivity of 1-5 genome copy equivelents was achieved for each of the three *Cardicola* species tested without cross-species or host genomic amplification. Similar sensitivity was further achieved in the presence of up to 20 ng/µL non-target host gDNA using SYBR Green chemistry alone, or in the presence of up to 160 ng/µL host gDNA through the utilisation of a TaqMan probe common-reporter detection system. These methods were subsequently used to positively identify both *C. forsteri* and *C. orientalis* DNA in preserved samples of serum, gill, and heart from ranched Southern bluefin tuna *Thunnus maccoyii*. Both methods were more sensitive for positively and differentially identifying the presence of *Cardicola* spp. than either histological or heart-flush microscopy techniques previously employed, and also possess the ability to be applied in non-lethal blood sampling of these highly valued fish. This is the first report for rapid and differential molecular quantitative detection of *Cardicola*, and opens the potential for effective monitoring of infection in cultured bluefin populations. Further, it is anticipated that the use of SYBR Green for melt-curve analyses in conjunction with a common-reporter TaqMan assay will present a flexible, accurate, and cost-effective approach for differential detection of a variety of other pathogens in future.

3.2. Introduction

Bluefin tuna make up one of the most monetarily important food fisheries in the world. The combined harvest of Atlantic *Thunnus thynnus* (ABT), Pacific *T. orientalis* (PBT), and Southern bluefin tuna *T. maccoyii* (SBT), within the tight regulatory quotas set for each species, can conservatively be estimated to be worth over $600 million USD per annum [1]. Although the fishery continues to rely heavily on wild stocks, increased success has been achieved in recent years in the intensive culture of these highly specialised fish [2-8]. Indeed, aquaculture and ranching practices for bluefin tunas have grown to exceed more than 20% of the total annual harvest in recent years [1].

Blood flukes in the genus *Cardicola* have emerged as a serious pathogenic concern to the developing culture of bluefin. In Japan, *C. orientalis* has been shown to cause significant mortality in young PBT shortly following their transfer to sea cages [9,10], and in Australia *C. forsteri* has been linked with mortality events in ranched populations of juvenile SBT [11,12]. A third *Cardicola* spp. (*C. opisthorchis*) has also been identified to cause pathology in both PBT and ABT cultured stocks, and although not yet directly linked to mortality, is suspected to contribute significantly toward an unhealthy status [13,14]. Most digenean organisms possess high host specificity and it was initially believed that each *Cardicola* spp. was likely highly host-specific and present only in a single host species. However, continued research has revealed that each bluefin species can be parasitised by at least two species of *Cardicola*, sometimes in concurrent infections [14,15]. Pacific bluefin has now been shown to be susceptible to both *C. orientalis* and *C. opisthorchis* [9,14], ABT to *C. forsteri* and *C. opisthorchis* [13,16], and SBT to *C. forsteri* and *C. orientalis* [15,17,18].

Current methods for the detection of *Cardicola* have exclusively relied on microscopic observation for primary diagnosis. This technique has been applied in situ

[9,14], following saline heart flushes [19], or from formalin-fixed tissue specimens [20]. In many instances, PCR has been used as a confirmatory tool for species identification; but as yet has not been applied in the initial detection of *Cardicola* infection. Nevertheless, given the potentially severity of infection caused by blood fluke parasites in conjunction with the difficult and time consuming methodology for detecting and identifying species by microscopy, a rapid method for sensitively detecting, differentiating, and potentially quantifying *Cardicola* infections in bluefin tuna would be highly advantageous. Further, the ability for non-lethal sampling is also highly desirable due to the high market value of each individual fish.

Real-time quantitative PCR (qPCR) has emerged as a powerful and rapid tool for detection of pathogens [21] and presents a logical choice for development with regard to *Cardicola* detection in tuna. Although a variety of other nucleic acid amplification methods have also been developed, none as yet have been implemented to such a pervasive extent in molecular research as the qPCR, which appears primarily due to its highly sensitive and rapid 'real-time' quantitative detection ability [22]. Currently, there are two main methods for nucleic acid detection upon which the qPCR operates; one based on fluorescent dyes which bind indiscriminately to double stranded DNA, such as SYBR® Green 1 [23], the other on a target-specific hybridization of a fluorescent-tagged sequence to single-stranded DNA, such as a TaqMan probe [24]. Each method has potential advantages depending on the desired application and outcome. For example, probe-based detection although relatively expensive can be multiplexed to detect several targets of interest in a single reaction, whereas binding dyes are cheaper and allow for melt curve specificity analysis but are precluded from quantitative multiplex analysis due to indiscriminate binding. Historically, these techniques have been implemented separately; however, recent data have suggested they may be combined to incorporate quantitative multiplexed detection of a target sequence while

ensuring specificity against non-specific amplification by melt curve analyses [25]. Further, the utilisation of a common-reporter (CR) on a tailed sequence-specific primer has also been shown to add flexibility and decrease costs involved in some hybridisation-based detection methods by allowing the relatively expensive TaqMan reporting probe to be designed against a common sequence for multiple use, rather than to a specific target [26]. As such, it is interesting to consider applying such a method in a combined SYBR/TaqMan system, which may further enhance the flexibility and decrease the costs associated with this technique.

Our aim was to develop a real-time PCR technique for sensitive and specific identification of *C. forsteri*, *C. orientalis*, and *C. opisthorchis*, which could be applied in various sample matrices, particularly those which could be non-lethally obtained such as blood or blood components, and investigate the effectiveness for possible multiplexed analyses. Further, we explored the possibility for combining the SYBR Green 1 nucleic acid dye with a TaqMan probe-based chemistry to ensure specific sensitivity by incorporation of melt curve analyses in the context of a CR real-time detection system.

3.3. Materials and methods

3.3.1 Ethics statement

All work with animals and methods for recovering samples were approved by the University of Tasmania Animal Ethics Committee, project number A0010593.

3.3.2. Field collection and processing of southern bluefin tissues

Peripheral blood and visceral organs were collected from 162 individual SBT weighing 35-40 kg (approximately 2-3 years of age) between 2008 and 2012 during

commercial harvest activities near Port Lincoln, South Australia, with capture and sea conditions as those previously described [5]. Blood was collected from the pectoral recess of the severed lateral artery of each fish into 10 mL Serum Z collection tubes (Sarstedt, SA, Australia). Visceral organs along with the entire branchial gill basket were removed as per industry practice, sealed in waterproof plastic bags, and immediately placed on ice. Hearts were separated from all other organs immediately following dissection and sealed in 500mL plastic containers. All samples were held on ice during transit to a laboratory setting (approximately 1-2 h) for further processing.

Following collection, blood was allowed to coagulate overnight at 4°C, and then spun at 800 × g for 10 min to separate serum from the cellular material. A portion of serum from each sample was subdivided into two 1 mL aliquots and frozen for subsequent DNA extraction and stored at -80°C. A piece of gill tissue from the second left gill arch and heart tissue from the apex of the ventricle (approximately 1 g) were collected from each specimen, minced with a scalpel, and placed in approximately five tissue volumes (5 mL) of nucleic acid preservation solution (4 M ammonium sulphate, 25 mM sodium citrate, 10 mM EDTA; pH 5.5) and stored at -20°C. Additionally, a similar portion of both gill and heart tissue was examined for the presence of *Cardicola* infection following histological processing as previously described [20]. Hearts were also dissected and flushed with physiological saline for microscopic identification and quantification of adult *Cardicola* [19].

3.3.3. Primer design and copy number estimation

All primes utilised in this study were designed with Beacon Designer™ 7.8 (Premier Biosoft, CA, USA) and Geneious® 6 software (http://www.geneious.com) and are presented in Table 3.1. *Cardicola* species-specific primers were targeted against heterogeneous areas of the internal transcribed spacer-2 (ITS2) region of rDNA specific to each species available on

GenBank [14,15,18]. The construction of primers and probes utilised in CR qPCR was carried out as previously described by Rickert *et al*. [26]. Briefly, sequence for each species-specific forward primer was tailed in a 5' direction with additional nucleotides to incorporate both a TaqMan probe and universal primer binding sight. This tailed sequence was structured in a manner to ensure exonuclease activation of bound TaqMan probe following polymerase extension initiated by the universal primer once a primary species-specific amplification had taken place.

In all subsequent qPCR analyses, copy number was estimated by serial dilutions of linearised plasmid DNA containing the ITS2 rDNA gene fragment specific to each *Cardicola* spp. tested. To this end, total DNA was extracted from individual ethanol-preserved specimens of *C. forsteri*, *C. orientalis*, and *C. opisthorchis* by methods described in section 3.2.4; for which the origin and initial identification of each specimen had previously been described [9,14,19]. A fragment (between 190-290 bp) of ITS2 rDNA from each of the three *Cardicola* spp. was amplified using specifically designed restriction-free (RF) bridging primers (Table 3.1.) and inserted into a Litmus 28i cloning vector using Phusion® polymerase (Thermo Fisher Scientific, VIC, Australia) as described by Bond and Naus [27]. The recombinant plasmid was grown in α-Select™ competent cells (Bioline, NSW, Australia), purified using a Qiaprep spin miniprep kit (Qiagen, VIC, Australia), and linearised by PvuII restriction enzymatic digestion (Thermo Fisher Scientific) at 37°C for 4 hours. Plasmid DNA concentration was determined using an Invitrogen Qubit fluorometer and Quant-iT dsDNA HS assay kit (Invitrogen, VIC, Australia) from quadruplicate subsamples. The number of construct copies in the plasmid solution was calculated using a DNA copy number calculator (http://www.endmemo.com/bio/dnacopynum.php) based on plasmid and recombinant insert nucleotide composition.

Table 3.1. Oligonucleotide primers and probes used to amplify *Cardicola* spp. for real-time qPCR detection and restriction free cloning.

Target	Accession #	Name	Product size	Sequence (5'→3')
C. forsteri **(ITS2 rDNA)**	EF661575	Cfor_F	287 bp	TGATTGCTTGCTTTTTCTCGAT
		L_Cfor_F		TGCACAATTCACGACTCACGATCCACACGGTCTCGCACTGGCACGGGTGATTGCTTGCT TTTTCTCGAT
		Cfor_R		TATCAAAACATCAATCGACATC
		RF_Cfor_F		CGACTCACTATAGGGCAGATCTTCGAATGATTGCTTGCTTTTTCTCGATATG
		RF_Cfor_R		GGCCTTGACTAGAGGGTACCAGATATCAAAACATCAATCGACATCTCA
C. orientalis **(ITS2 rDNA)**	HQ324226	Cori_F	191 bp	TGCTTGCTATTCCTAGATGTTTAC
		L_Cori_F		TGCACAATTCACGACTCACGATCATCCGCTCCGACGACACGAACGGGTGCTTGCTATTC CTAGATGTTTAC
		Cori_R		AACAACTATACTAAGCCACAA
		RF_Cori_F		CGACTCACTATAGGGCAGATCTTCGAATGCTTGCTATTCCTAGATGTTTACG
		RF_Cori_R		GGCCTTGACTAGAGGGTACCAGAAACAACTATACTAAGCCACAACCT
C. opisthorchis **(ITS2 rDNA)**	HQ324228	Copt_F	272 bp	TTCCTAAATGTGTGTGCA
		Copt_R		TCAAAACATCAATCGACACT
		RF_Copt_F		CGACTCACTATAGGGCAGATCTTCGAATTCCTAAATGTGTGTGCATTTGTG
		RF_Copt_R		GGCCTTGACTAGAGGGTACCAGATCAAAACATCAATCGACACTTCAC
All L-tailed primers	--	L_UP	334 bp (forsteri) 238 bp (orientalis)	GCACAATTCACGACTCACGA
L_Cfor_F	--	L_FAM_1	--	FAM-CCACACGGTCTCGCACTGGC-BHQ1
L_Cfor_F	--	L_HEX_1	--	HEX-CCACACGGTCTCGCACTGGC-BHQ1
L_Cori_F	--	L_HEX_2	--	HEX-CATCCGCTCCGACGACACGA-BHQ1

3.3.4. Nucleic acid extraction

Total nucleic acid (TNA) was extracted from 100 uL of serum, a 20 µm section of formalin-fixed paraffin-embedded (FFPE) heart tissue, approximately 10 mg of ammonium sulphate preserved gill and heart tissues, or ethanol-preserved adult *Cardicola* in 500 µL Extraction Buffer (4 M Urea, 0.5% SDS, 0.2M NaCl, 10% glycerol) supplemented with 20 U Proteinase K (Bioline) incubated at 55°C for 30 min with occasional vortexing. Protein was removed by precipitation with the addition of 300 µL 7.5 M ammonium acetate and centrifugation at 14,000 x g for 5 min. Nucleic acids were then precipitated from the supernatant with the addition of one volume isopropanol and centrifugation at 16,000 x g for 10 min. The nucleic acid pellet was rinsed with 75% ethanol and resuspended in 50 µL TE buffer (10 mM Tris, 1 mM EDTA, pH 8). For FFPE samples, paraffin was removed prior to DNA extraction by two 10 minute incubations in xylene followed by two 10 min washes in ethanol.

3.3.5. Real-time PCR Cardicola detection

All real-time qPCR analyses were conducted on a CFX Connect Real-Time PCR Detection System (Bio-Rad, NSW, Australia) with efficiency and stringency of standard curves held to between 90-105% and 0.99-1.00, respectively, for all assays. For assays exclusively using SYBR® Green 1 chemistry, each PCR reaction consisted of 2X SensiFast^{TM} +SYBR® mastermix (Bioline), forward and reverse primers (500 nM each), and 1 µL TNA template in molecular grade water to a final volume of 10 µL. Cycling conditions consisted of an initial activation of DNA polymerase at 95°C for 3 min, followed by 40 cycles of 5 s at 95°C, 20 s at 60°C, and 8 s at 72°C, with relative florescence measured at the end of each 72°C extension. Following the cycling protocol a melt curve analysis was run to ensure amplification specificity with florescent detection conducted at 0.5°C intervals.

For single-species detection utilizing a TaqMan CR system, 2X SensiFast™ +SYBR® mastermix was used in conjunction with 50 nM tailed species-specific forward primer, 500 nM universal L and species-specific reverse primers, and 250 nM TaqMan probe in molecular grade water. In this study, probes were labelled at the 5' end with either 6-carboxyfluorescein (FAM) or 6-carboxy-2 ,4,4 ,5 ,7,7 -hexachlorofluorescein succinimidyl ester (HEX) and used in conjunction with a Black Hole Quencher® (BHQ; Biosearch Technologies, CA, USA) added to the 3' terminus. In conditions of multiplexed detection, the above primer concentrations were preserved for each additional species added to the reaction. As some crossover in detection of SYBR can occur at many of the wavelengths used in TaqMan fluorescent monitoring (particularly FAM), cycling conditions were modified to assess relative fluorescence following DNA denaturing to minimise dsDNA SYBR fluorescence. This consisted of 3 min polymerase activation at 95°C, followed by 40 cycles of 25 s at 60°C, 5 s at 72°C, 5 sat 95°C, 10 s at 90°C, with the relative fluorescence measured at the end of each 90°C step. As with the purely SYBR-based detection described above, amplification specificity was confirmed by melt curve analysis.

3.3.6. Determination of real-time PCR specificity and sensitivity

Specificity was ensured for each *Cardicola* species-specific primer set, as well as for the TaqMan CR system, by comparing qPCR amplification in quadruplicate reactions spiked with approximately 0.1-1.0 ng of C. *forsteri*, *C. orientalis*, or *C. opisthorchis* TNA, 1.0 ng SBT genomic DNA (gDNA), or molecular grade water for 42 amplification cycles. Replicates of each treatment were then pooled and visualised on a 2% agarose gel stained with GelRed™ (Biotium, SA, Australia) run at 90 v for 1 h in TBE Buffer (Bioline).

Sensitivity of the qPCR assay without the presence of host gDNA was conducted for each of the three *Cardicola* spp. targeted in this study using fluorescence generated by SYBR

Green 1 chemistry or TaqMan chemistry in a CR system in conjunction with SYBR Green 1 for melt curve analysis. Calibration curves using linearised recombinant plasmid described in section 3.2.3 were generated for each *Cardicola* spp. by serial dilution in molecular grade water. Each dilution series was analysed in 10 PCR replications alongside no-template controls in a dynamic range of $1 – 1.00 \times 10^7$ genome equivalents (copies) of ITS2 rDNA per reaction. To determine the impact of varying amounts of host gDNA on qPCR detection, SBT gDNA was spiked into each of four PCR replicates to a final concentration of 0, 10, 20, 40, 80, or 160 ng/µL and analysed for each 10 serial dilution of linearised recombinant *C. forsteri* plasmid in a dynamic range of $10 – 1.00 \times 10^6$ copies.

3.3.7. Statistical analysis

The comparative inhibition of host gDNA in qPCR detection of *C. forsteri* was assessed for each quantity of host gDNA tested against no-host controls by a one-way analysis of variance followed by a Dunnett's post-test using GraphPad Prism 5 (GraphPad software, CA, USA) based on the relative difference in copy number estimation throughout the dynamic range of each treatment (N=24) following Log_{10} transformation of the data. An estimate of agreement between real-time qPCR and the non-reference standard gross detection methods of histopathology and heart-flush microscopy were evaluated by calculating positive and negative percent agreement between each detection method as described by Meier [28]. This was followed by a Cohen's Kappa calculation for a relative comparison of overall agreement.

3.4. Results

3.4.1. Specificity of real-time PCR Cardicola spp. detection

Both SYBR-based and TaqMan CR qPCR detection methods employed in this study specifically amplified *C. forsteri*, *C. orientalis*, or *C. opisthorchis* genomic DNA without cross-species or non-targeted host amplification (Figure 3.1.). Further, both TaqMan CR and SYBR-based detection systems were successful in duplexed detection of both separate and concurrent *C. forsteri* and *C. orientalis* DNA templates. For SYBR-based detection assays, species identification could be accomplished for *C. forsteri* and *C. orientalis* based on the different peak melt temperatures between products (83.5 and 82.0°C, respectively). This technique could also be employed for differentiating *C. opisthorchis* and *C. orientalis*, as a similar discrepancy in product melt temperatures was observed (83.5 and 82.0°C, respectively). However, SYBR-based melt curve differentiation of *C. forsteri* and *C. opisthorchis* was not possible under the current assay conditions as both targeted products melted at a similar temperature (83.5°C) and were also of a similar size (287 vs. 272 bp) so as to necessitate gel-based differentiation. Nevertheless, successful duplexed differential detection was achieved in all three combinations of *Cardicola* species utilizing FAM- and HEX-labelled TaqMan probes in a CR system. Assurance of specificity was further provided in this context with the incorporation of SYBR into the reaction for melt curve analysis, as it allowed for differentiation of any potential false-positive non-specific products. This was further highlighted in instances involving *C. forsteri* detection in this study, where primer-dimer was sometimes minimally amplified late (>35 cycles) in both TaqMan CR and SYBR-based detection assays in the absence of target template, but was easily differentiated from targeted product amplification by melt temperature (Figure 3.1.). Unfortunately, secondary species confirmation for *C. forsteri* / *C. orientalis* and *C.opisthorchis* / *C. orientalis* co-infections during TaqMan CR or SYBR duplexing was unaided by melt curve analysis as melt profiles were consistent with product of highest melt temperature even when both target

products were confirmed to be present by gel electrophoresis or during probe-specific amplification (Figure 3.2.).

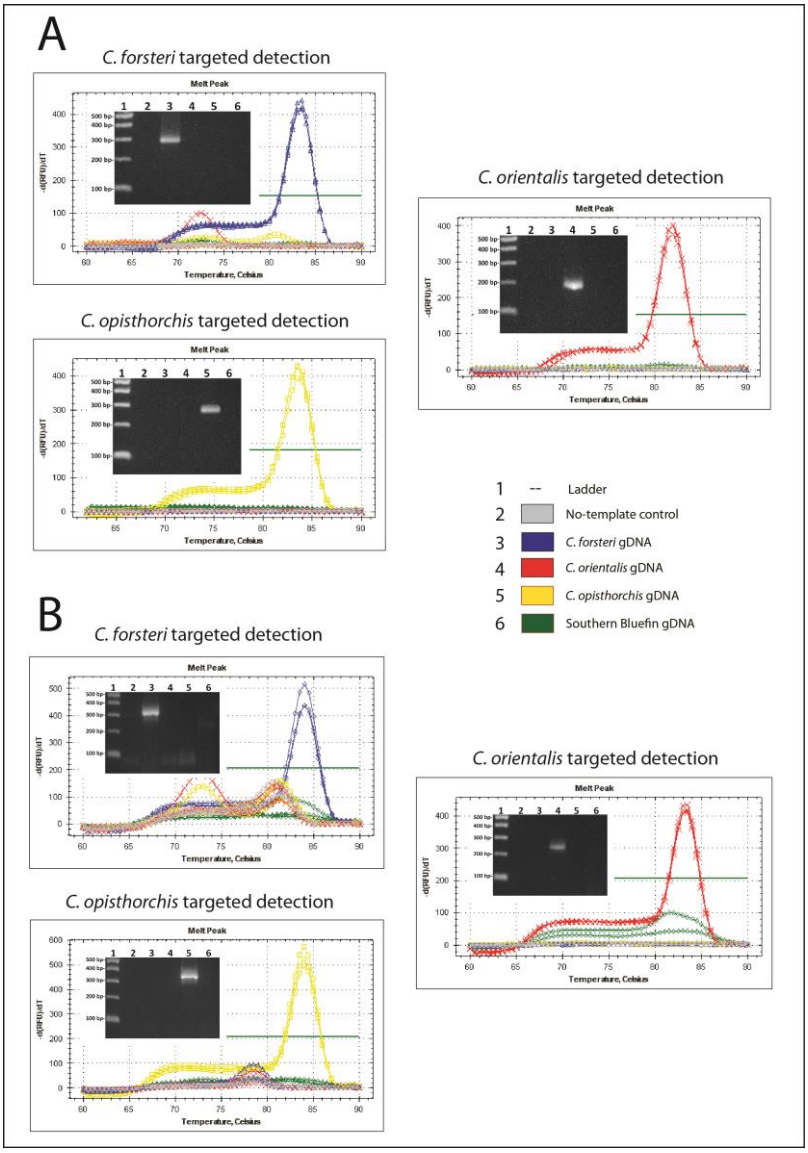


Figure 3.1. Specificity of real-time PCR detection of *C. forsteri*, *C. orientalis*, and *C. opisthorchis*. Images provide melt curve data after 42 cycles of real-time PCR amplification followed by product visualization on a 2% agarose gel for various genomic DNA templates. Real-time PCR detection was based either exclusively on SYBR Green 1 chemistry (A) or using a TaqMan common-reporter system followed by SYBR melt curve analysis (B). The horizontal green bar provides reference for the baseline threshold of florescence used during amplification.

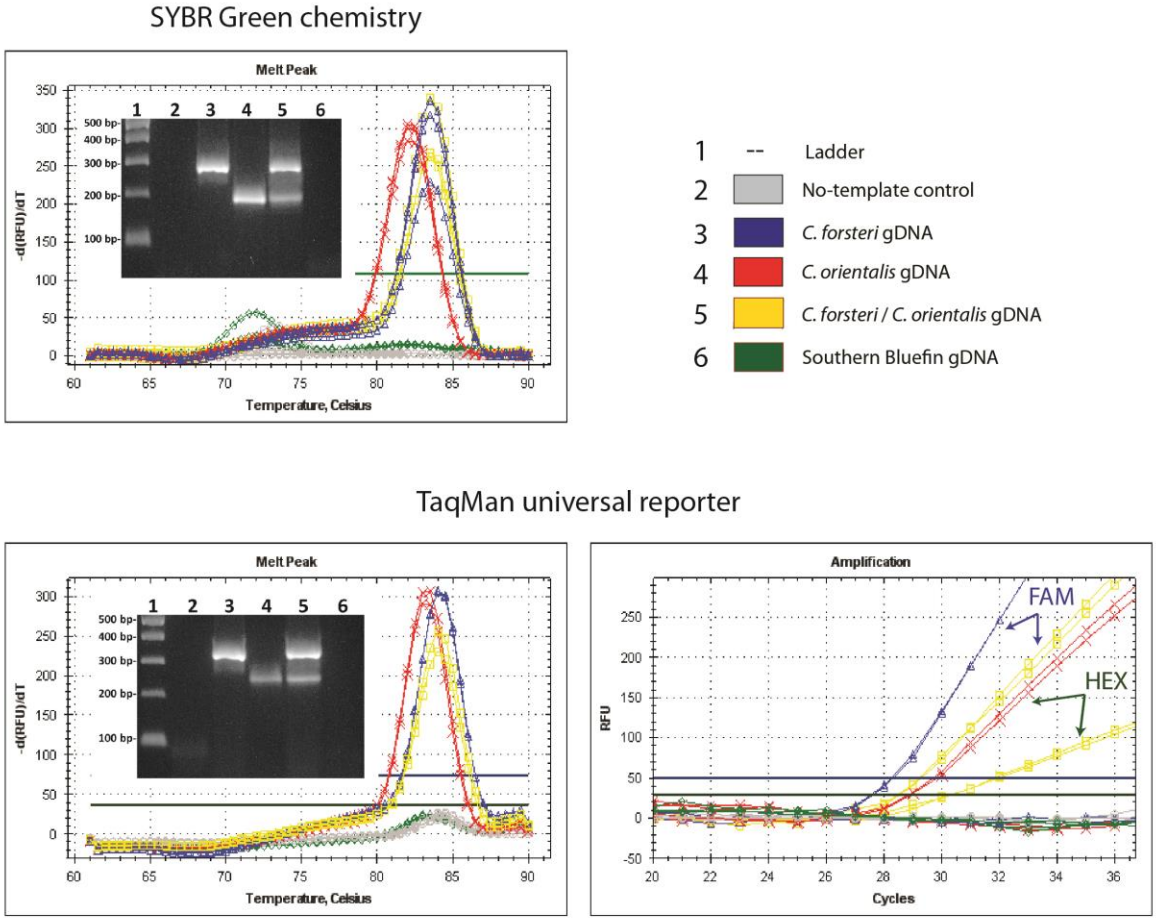


Figure 3.2. Specificity of duplexed real-time qPCR detection of *C. forsteri* and *C. orientalis* following SYBR or TaqMan common-reporter amplification and melt curve analysis. Products were visualised on a 2% agarose gel. During TaqMan common-reporter amplification, detection of *C. forsteri* and *C. orientalis* were assessed separately using FAM- and HEX-labelled reporters, respectively.

3.4.2. Sensitivity of real-time PCR Cardicola spp. detection

SYBR-based and TaqMan CR qPCR detection assays were similar in sensitively reporting the presence of *Cardicola*-specific DNA for each of the three *Cardicola* spp. targeted. The limit of detection, as defined by a greater than 95% chance of target detection following adjustment for dilution probability as described by Rodrigo et al. [29], was between 1 and 5 genomic copy equivalents for all three *Cardicola spp*. tested by either of these methods (Table 3.2.). Detection limits were comparable between SYBR-based and TaqMan CR qPCR assays for each target species; however, the limit of quantification, as defined by less than a 20% coefficient of variance in copy estimation between identical replicates (N=10), was consistently lower using the TaqMan CR technique (between 5 and 10 copies) relative to the SYBR-based method (between 10 and 100 copies) (Table 3.2.). Further, the presence of host genomic DNA significantly reduced the sensitivity and quantitative ability for SYBR-based real-time detection of *Cardicola* spp. in a concentration dependent manner; whereby copy estimation was comparable to non-genomic standards in reactions containing up to 20 ng/µL host DNA, but at 40 ng/µL a mean 0.3 $\log_{10}$ reduction was observed which increased to a 1.3 $\log_{10}$ reduction at 160 ng/µL (Figure 3.3.). In contrast, employment of a FAM-labelled TaqMan probe prolonged accuracy similar to no-host controls in up to more than 80 ng/µL host DNA, which was further improved to be comparable to no-host controls in up to 160 ng/µL with a HEX-labelled probe.

Table 3.2. Real-time qPCR sensitivity for *C. forsteri, C. orientalis, and C. opisthorchis* detection. Data present the experimental and theoretical limits of detection (LOD_E and LOD_T, respectively) based on the minimum number of ITS2 rDNA copies required for successful qPCR amplification with > 95% confidence following adjustment for dilution probability (N=10). The experimental limit of quantification (LOQ_E) identifies the lowest number of copies tested for which a coefficient of variance was < 20% between replicates (N=10). Two real-time detection methods were analysed; one based exclusively on SYBR Green 1 chemistry, the other on a HEX-labelled TaqMan CR system run in conjunction with SYBR for melt curve analysis.

Species	SYBR			TaqMan common-reporter		
	LOD_E (copies)	LOD_T (copies)	LOQ_E (copies)	LOD_E (copies)	LOD_T (copies)	LOQ_E (copies)
C. forsteri	2	1	>10	5	4	5
C. orientalis	5	4	>10	2	1	10
C. opisthorchis	5	2	>10	5	3	10

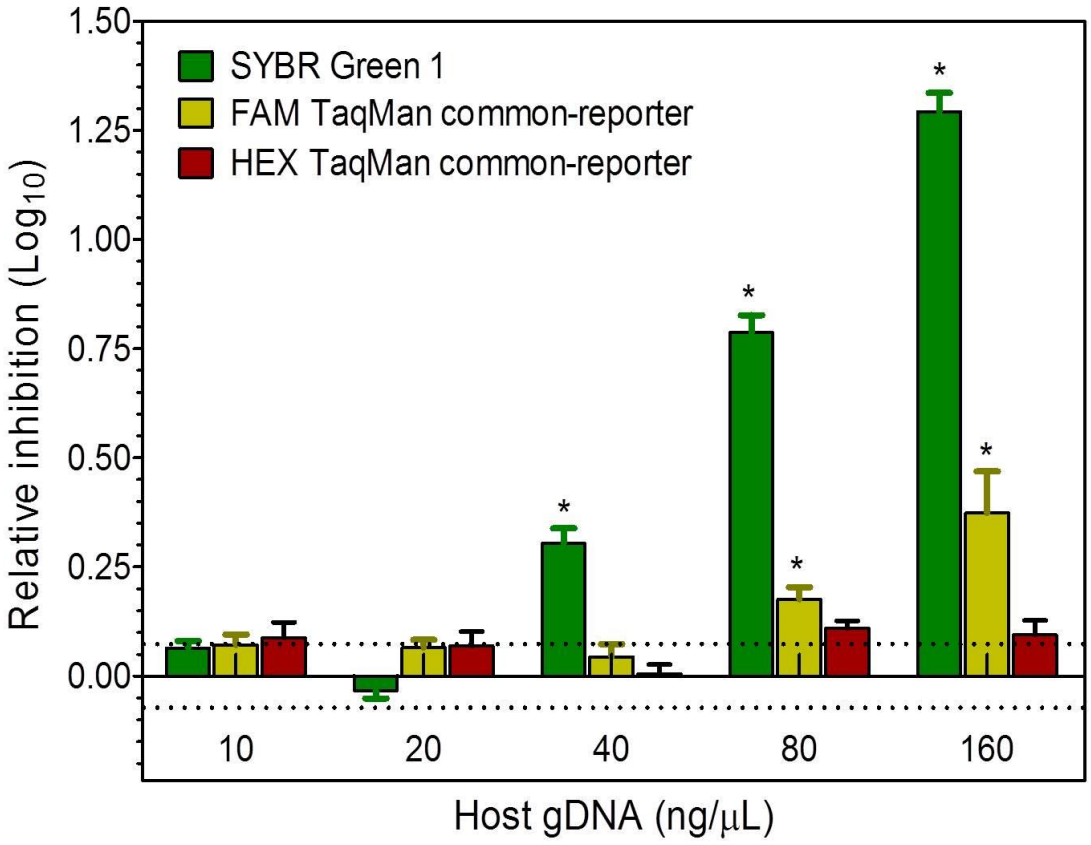


Figure 3.3. Inhibition of *C. forsteri* real-time qPCR detection by host genomic DNA. Data present the Log_{10} reduction in *C. forsteri* copy number estimation by real-time qPCR in the presence of varying amounts of Southern bluefin tuna gDNA relative to no-host controls utilizing SYBR Green 1 dye or FAM- and HEX-labelled TaqMan probe common-reporter systems. Significant (*) reduction relative to no-host controls is indicated at $p < 0.01$. The dotted lines present the upper and lower 95% confidence interval for variation within no-host controls (N=24).

3.4.3. Identification of Cardicola spp. in ranched SBT

SYBR-based qPCR detection chemistry was successful in identifying both *C. forsteri* and *C. orientalis* DNA from the serum, heart, and gill of multiple ranched SBT specimens incorporated in this study. Indeed, 92% of the individuals screened using this technique were observed to possess DNA from at least one of these two *Cardicola* spp.; 86% being positive for *C. orientalis* and 36% positive for *C. forsteri* DNA by qPCR (Figure 3.4.). Detection of parasitic DNA in serum was generally pattered similar to that of the multi-tissue assessment with the most notable discrepancy being a 32% lower prevalence of *C. orientalis* in serum relative to the other tissues. Both heart and gill tissues were observed to be highly infected (95% or greater for both tissue types) from the portion of the population examined.

Comparison of real-time qPCR to non-reference detection methods of *Cardicola* spp. in SBT revealed qPCR to be more sensitive in detecting the presence of the parasite relative to either saline heart-flush or heart histological microscopy techniques previously employed (Table 3.3). The only instance where both non-reference techniques proved more sensitive than qPCR was when qPCR was applied to previously prepared FFPE histology sectioning, which was likely a result of nucleic acid degradation during fixation/paraffin removal or possible carryover of PCR inhibitors during TNA extraction as a result of these processes.

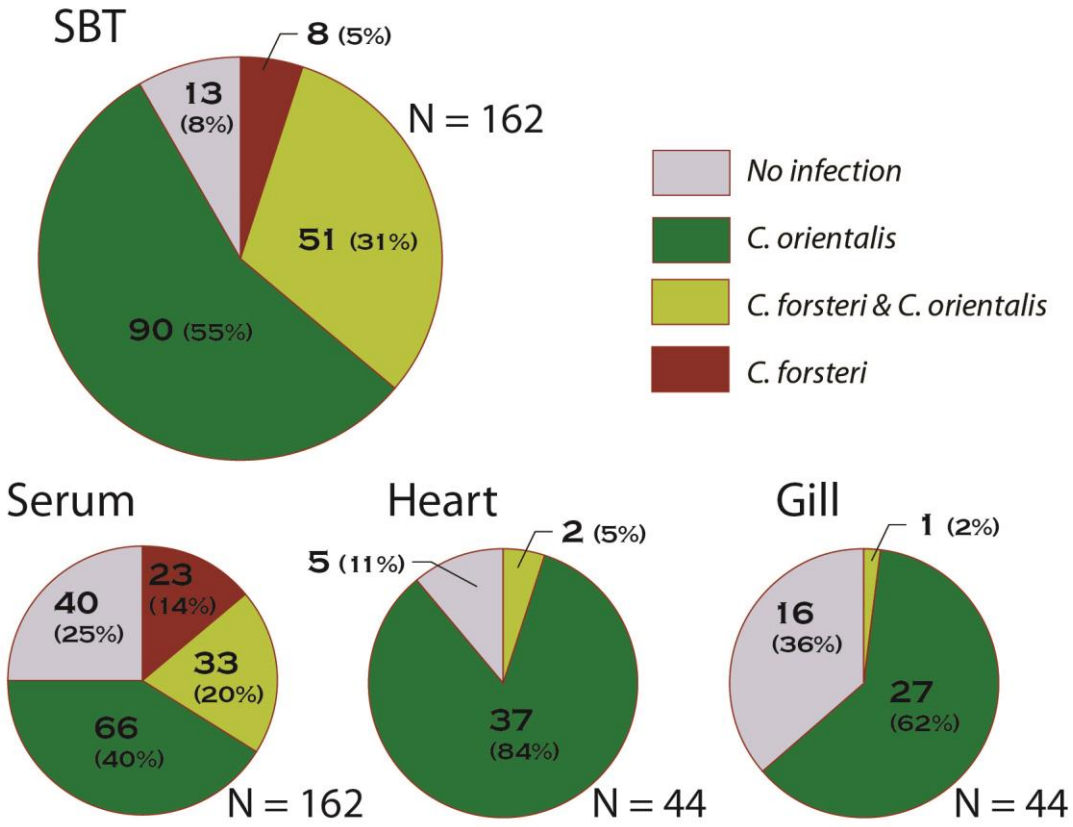


Figure 3.4. Prevalence of *C. forsteri* and *C. orientalis* in ranched Southern bluefin tuna (SBT). Data present the number of infected samples and relative percentages within the sampled population as accessed by real-time PCR (serum and gill), or real-time PCR in conjunction with heart-flush and histological microscopy (heart and whole organism). The total number of samples (N) is also provided in each instance.

Table 3.3. Comparison of real-time PCR to non-reference detection methods of *Cardicola* spp. in Southern bluefin tuna. In each instance, presence (+) or absence (-) of *Cardicola* spp. identified by real-time PCR from either serum, heart, gill, or formalin-fixed paraffin-embedded histological sections (FFPE) is compared separately to the non-reference standard detection methods involving either saline heart-flushes or heart histological sectioning followed by microscopy. The positive percent agreement (PPA), negative percent agreement (NPA), along with the total sample number is provided.

Agreement	PCR:	Serum		Heart		FFPE heart		Gill	
	non-ref:	Heart-flush	Heart-histo	Heart-flush	Heart-histo	Heart-flush	Heart-histo	Heart-flush	Heart-histo
+/+		78	2	15	4	19	19	13	5
+/-		44	21	24	35	7	7	15	23
-/+		22	4	4	1	13	14	6	0
-/-		18	30	1	4	7	6	10	16
Total		162	57	44	44	46	46	44	44
PPA		78	33	78	80	59	58	68	100
NPA		29	58	4	10	50	46	40	41

3.5. Discussion

The ITS2 region of rDNA is considered to be an excellent candidate for the DNA barcoding of plants and animals [30], and has previously been incorporated into the identification of all three *Cardicola* spp. utilised in this study [9,14,18]. However, some of the desirable characteristics for barcoding, such as regions of high conservation useful in universal primers design, can prove a challenge in designing species-specific primer sets. Indeed, the pairwise identity between the published ITS2 sequences of the three *Cardicola* spp. incorporated into this study is greater than 85% (>94% similarity between *C. forsteri* and *C. opisthorchis*) resulting in a fairly limited latitude for effective species-specific real-time primer design. Nevertheless, primer sets developed in this study were successful in both SYBR-based and TaqMan CR qPCR detection assays and specifically and sensitively amplified *C. forsteri*, *C. orientalis*, or *C. opisthorchis* genomic DNA without cross-species or non-targeted host amplification (Figure 3.1.). Additionally, we successfully used the CR technique previously described by Rickert et al. [26] for incorporating TaqMan probes into species-specific screening without making the probes themselves species specific; thus minimizing cost and also circumventing the difficulty for finding additional sufficient heterogeneously distinct gene regions in these highly similar species.

As at least two *Cardicola* spp. are known to infect each species of bluefin, duplexed or multiplexed screening of *Cardicola* spp. may be required for effectively and rapidly identifying co-infections. Although either SYBR-based or TaqMan CR qPCR techniques could successfully differentiate and quantify co-infections in separate analyses, only TaqMan probes reporting fluorescence on discrete wavelengths could independently identify and quantify co-infection during duplexing. In addition, the TaqMan CR method yielded a lower limit for effective target quantification relative to the exclusively SYBR based method (Table

3.2.), which will likely prove valuable during quantitative assessment of low-level infections or highly diluted samples in future. In this context, specificity and quantitative accuracy was further ensured in this study by incorporating SYBR melt curve analysis following amplification, as it readily differentiated target versus non-specific products which sometimes occurred in late cycle amplification or when analysis conditions were suboptimal. In an absolute sense, quantification may be irrelevant with regard to *Cardicola* as DNA quantities of adult flukes, eggs, miracidium, and cercaria are unknown and likely highly variable. Nevertheless, the relative quantification of DNA will undoubtedly prove useful in future work elucidating questions for assessing pathological responsibility or regarding the effectiveness of an anti-parasitic treatment.

In addition to providing a means for duplexed quantitative detection, we also observed targeted probes to be more resilient to inhibition by high concentrations of non-target host genomic DNA than SYBR-based analyses (Figure 3.3.). This was to be somewhat expected, as the binding of SYBR to any dsDNA including non-targeted host can result in an initial saturation of florescence that masks the reporting of targeted amplification at low concentrations. During assays utilising TaqMan chemistry, this is typically avoided as reporting fluorescence is only generated following targeted amplification; however, in this study we observed some inhibition when utilizing FAM-labelled TaqMan chemistry. This was likely a result of the SYBR, which was incorporated into all TaqMan assays for melt curve analysis and reports fluorescence on a similar wavelength (520nm) to that of FAM (517 nm). We were able to minimise this respective crossover by measuring fluorescence at 90°C during PCR cycling – as the majority of dsDNA would denature at this temperature and thus minimise SYBR fluorescence while still preserving much of the FAM generated fluorescence as suggested by previous work [25]. Nevertheless, this technique was clearly insufficient in completely denaturing host gDNA and/or preventing SYBR-based fluorescence because

monitoring the fluorescence of HEX at a substantially higher wavelength (556 nm) virtually eliminated this crossover in detection. Thus, although SYBR and FAM-labelled TaqMan probes could be combined for the effective screening of *Cardicola* in most circumstances, it is advisable to utilise alternative wavelength reporters in conjunction with SYBR melt curves when high DNA concentrations may be present, such as during extraction from whole blood or large amounts of tissue, to effectively preserve quantitative ability and accuracy by this method.

Until now, primary diagnostics for identifying bluefin infected with *Cardicola* have revolved around the visualization of a parasite by microscopy, typically after either 'panning' for adult organism or eggs [10,19], or *in situ* by histological sectioning [20]. While such methods have been used successfully, their scope is limited to detecting either adult parasites or eggs within a targeted organ (usually heart or gill) and thus overlooks migrating stages such as infecting cercaria, emerging miracidia, or eggs and adults in most circumstances during circulatory transit. Microscopy has further limitations: it is time consuming, experience of the examiner is required for accurate screening, and species differentiation of parasites can sometimes be difficult. For the detection of many other parasitic diseases such as malaria, PCR has become accepted as a 'gold standard' for accurate and sensitive identification [31] for which real-time qPCR specifically has shown to be highly effective with the potential for rapid and automated processing [32]. In this study, real-time qPCR was shown to be more sensitive for detecting *Cardicola* infection than either heart-flush or histological microscopy methods previously employed; and as such, presents a new relative standard for the detection of these parasites in bluefin tuna. The high positive agreements in accompaniment with low negative agreements between qPCR and these previously employed detection methods further supports qPCR as a new reference standard for *Cardicola* detection, as it suggests expansion of detectible parasitic life stages or reservoirs without

compromising previously achieved sensitivity. Previously similar comparisons of qPCR and other quantitative detection methods have evolved similar conclusions for identifying *Plasmodium* spp. in humans [33]. Further, we successfully applied qPCR for identifying parasitic DNA in serum in addition to the previously assessed heart and gill tissues which presents the first method for potentially detecting migrating life stages of *Cardicola* within the host circulatory system. It also presents the first screening tool which may be applied to non-lethally procured samples and thus a potential means for monitoring presence of *Cardicola* in tuna prior to terminal harvest; of particular importance for this species where individual organisms hold such high economic value. Indeed, such a monitoring tool may provide specific applicability for assessing net-pen culture locations given the recent identification of a benthic intermediate terrebelid polychaete host (*Longicarpus modestus*) for *C. forsteri* [34] and the potential for decreasing disease by physical separation of these two host organisms [5].

Prior to this study, only one report confirmed the presence of *C. orientalis* in the gill of an SBT [15]. It was therefore somewhat surprising to discover that more than 80% of the individual SBT screened by qPCR in this study showed signs of infection with *C. orientalis*, which was prevalent in serum, heart, as well as gill components (Figure 3.4). It was also unexpected to discover that *C. forsteri*, previously the species targeted for detection in SBT, was present in only a comparatively low (36%) portion of the same SBT population. Previous screening of 24 SBT specimens prior to 2007 failed to identify *C. orientalis* from similar ranched populations [18], suggesting that perhaps *C. orientalis* infections of ranched SBT is a relatively new occurrence. However, we identified *C. orientalis* from many of the 2008 samples with continued detection through 2012 demonstrating *C. orientalis* has been present for at least the past 5 years in ranched SBT populations. One explanation for why *C. orientalis* may have been overlooked could be due to a potential bias resulting from

previously used isolation techniques. For example, during the PCR conformational screening of more than 20 *Cardicola* specimens isolated by saline heart-flush methods during the course of this study, all but one was observed to be *C. forsteri* (data not shown), even when *C. orientalis* DNA was show by PCR to have high prevalence in heart tissues in some specimens (Figure 3.4). Interestingly, previous screening of PBT has suggested that adult *C. orientalis* have a tropism for the gill arteries and that eggs are also highly prevalent in those tissues rather than what has been observed in the heart [10], which appears to be juxtaposed to what we observed here by qPCR screening of SBT for *C. orientalis* DNA. It may be speculated that this discrepancy is a result of the qPCR detection of free DNA from broken down adult flukes and eggs, which has been released into circulation and accumulates in blood-rich organs such as the heart. This hypothesis may be supported by the relatively high levels of *C. orientalis* DNA that was detected in serum which appears likely to be in a free circulating form due to its relatively low concentration and location in the serum rather than cellular-associated blood component following centrifugation. Alternatively, the high levels of *C. orientalis* DNA observed in heart tissues during this study may have resulted from alternative life stages such as miracidium or cercaria that have previously been overlooked in assessing infection status. Further work will need to be employed to definitively answer the complete distribution profile of these parasites within the host, but whatever the case, we have demonstrated here that *C. orientalis* can and does infect SBT in addition to *C. forsteri* and will need to be considered with regard to management of this species in future.

In conclusion, this study outlines two sensitive, quantitative, and discriminating molecular techniques for identifying *Cardicola* blood fluke infections in bluefin tuna which has the potential for application in non-lethally procured blood components. It is anticipated that such methods may be easily adapted for screening of other internal parasites or alternative pathogens in a variety of sample matrices in future. These methods were also

successfully utilised to identify the continued concurrent presence of both *C. forsteri* and *C. orientalis* in ranched SBT populations throughout the past five years in Australia, and have indicated that *C. orientalis* rather than *C. forsteri* exhibits higher prevalence and distribution within these culture organisms and will need to be considered in future management decisions of these highly important fish.

3.6. Acknowledgments

The authors would like to extend our sincerest appreciation to Dr Sho Shirakashi and Dr Kazuo Ogawa of Kinki and Tokyo Universities, respectively, for providing *C. orientalis* and *C. opisthorchis* specimens for use in this study and to Karine Cadoret, Melanie Leef, Nicole Kirchhoff, Victoria Valdenegro, and all other University of Tasmania personnel involved with field collection of samples utilised in this study. We would also thank the research staff of the Australian Southern Bluefin Tuna Industry Association and the ranching companies and crews for their cooperation and assistance in collecting blood and organ samples, as well as the Lincoln Marine Science Centre for providing laboratory space to conduct parasite isolations. This research was supported by the Fisheries Research and Development Corporation (project 2008/228), the Southern Bluefin Tuna industry, as well as the Australian Department of Education, Employment, and Workplace Relations via the Endeavour International Postgraduate Award and University of Tasmania Australian Maritime College John Bicknell Scholarship.

3.7. References

1. Food and Agriculture Organization of the United Nations (2010) Yearbook of Fishery Statistics 2010. Rome: FAO. 107 p.

2. Biswas BK, Ji S-C, Biswas AK, Seoka M, Kim Y-S, et al. (2009) Dietary protein and lipid requirements for the Pacific bluefin tuna *Thunnus orientalis* juvenile. Aquaculture 288: 114-119.

3. Gwo H-H, Weng T-S, Fan L-S, Lee Y-H (2005) Development of cryopreservation procedures for semen of Pacific bluefin tuna *Thunnus orientalis*. Aquaculture 249: 205-211.

4. Honryo T, Okada T, Kurata M, Tamura T, Ishibashi Y (2012) Optimal periods of night-time lighting in the sea cage culture of Pacific bluefin tuna, *Thunnus orientalis*, juvenile (Temminck and Schlegel). Aquaculture Research: doi:10.1111/are.12059.

5. Kirchhoff NT, Rough KM, Nowak BF (2011) Moving Cages Further Offshore: Effects on Southern Bluefin Tuna, *T. maccoyii*, Parasites, Health and Performance. PLoS One 6: e23705.

6. Mourente G, Tocher DR (2009) Tuna Nutrition and Feeds: Current Status and Future Perspectives. Reviews in Fisheries Science 17: 373-390.

7. Sawada Y, Okada T, Miyashita S, Murata O, Kumai H (2005) Completion of the Pacific bluefin tuna *Thunnus orientalis* (Temminck et Schlegel) life cycle. Aquaculture Research 36: 413-421.

8. Tsuda Y, Sakamoto W, Yamamoto S, Murata O (2012) Effect of environmental fluctuations on mortality of juvenile Pacific bluefin tuna, *Thunnus orientalis*, in closed life-cycle aquaculture. Aquaculture 330–333: 142-147.

9. Ogawa K, Tanaka S, Sugihara Y, Takami I (2010) A new blood fluke of the genus *Cardicola* (Trematoda: Sanguinicolidae) from Pacific bluefin tuna *Thunnus orientalis*

(Temminck and Schlegel, 1844) cultured in Japan. Parasitology International 59: 44-48.

10. Shirakashi S, Kishimoto Y, Kinami R, Katano H, Ishimaru K, et al. (2012) Morphology and distribution of blood fluke eggs and associated pathology in the gills of cultured Pacific bluefin tuna, *Thunnus orientalis*. Parasitology International 61: 242-249.

11. Colquitt SE, Munday BL, Daintith M (2001) Pathological findings in southern bluefin tuna, *Thunnus maccoyii* (Castelnau), infected with *Cardicola forsteri* (Cribb, Daintith & Munday, 2000) (Digenea: Sanguinicolidae), a blood fluke. Journal of Fish Diseases 24: 225-229.

12. Dennis MM, Landos M, D'Antignana T (2011) Case–Control Study of Epidemic Mortality and Cardicola forsteri–Associated Disease in Farmed Southern Bluefin Tuna (*Thunnus maccoyii*) of South Australia. Veterinary Pathology Online 48: 846-855.

13. de Ybañez RR, Peñalver J, Martínez-Carrasco C, del Río L, Dolores EM, et al. (2011) Blood Fluke Infection of Cage Reared Atlantic Bluefin Tuna *Thunnus thynnus* in West Mediterranean. Fish Pathology 46: 87-90.

14. Ogawa K, Ishimaru K, Shirakashi S, Takami I, Grabner D (2011) *Cardicola opisthorchis* n. sp. (Trematoda: Aporocotylidae) from the Pacific bluefin tuna, *Thunnus orientalis* (Temminck & Schlegel, 1844), cultured in Japan. Parasitology International 60: 307-312.

15. Shirakashi S, Tsunemoto K, Webber C, Rough K, Ellis D, et al. (2013) Two species of Cardicola (Trematoda: Aporocotylidae) found in Southern Bluefin Tuna *Thunnus maccoyii* ranched in South Australia. Fish Pathology 48: 1-4.

16. Bullard SA, Goldstein RJ, Goodwin RH, Overstreet RM (2004) *Cardicola forsteri* (Digenea: Sanguinicolidae) from the Heart of a Northern Bluefin Tuna, *Thunnus thynnus* (Scombridae), in the Northwest Atlantic Ocean. 71: 245-246.

17. Cribb TH, Daintith M, Munday BL (2000) A new blood-fluke, *Cardicola forsteri*, (Digenea : Sanguinicolidae) of southern blue-fin tuna (*Thunnus maccoyii*) in aquaculture. Transactions of the Royal Society of South Australia 124: 117-120.

18. Aiken HM, Bott NJ, Mladineo I, Montero FE, Nowak BF, et al. (2007) Molecular evidence for cosmopolitan distribution of platyhelminth parasites of tunas (Thunnus spp.). Fish and Fisheries 8: 167-180.

19. Aiken HM, Hayward CJ, Nowak BF (2006) An epizootic and its decline of a blood fluke, *Cardicola fosteri*, in farmed souther bluefin tuna, *Thunnus maccoyii*. Aquaculture 254: 40-45.

20. Norte dos Santos C, Leef M, Jones B, Bott N, Giblot-Ducray D, et al. (2012) Distribution of Cardicola forsteri eggs in the gills of southern bluefin tuna (*Thunnus maccoyii*) (Castelnau, 1872). Aquaculture 344–349: 54-57.

21. Johnson M, Brzoska P, Petrauskene O, Melancon C (2005) Using Real-Time PCR for Pathogen Detection. Genetic Engineering News 25: www.genengnews.com.

22. VanGuilder HD, Vrana KE, Freeman WM (2008) Twenty-five years of quantitative PCR for gene expression analysis. BioTechniques 44: 619-626.

23. Morrison TB, Weis JJ, Wittwer CT (1998) Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. Biotechniques 24: 954-962.

24. Livak KJ, Flood SJ, Marmaro J, Giusti W, Deetz K (1995) Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for

detecting PCR product and nucleic acid hybridization. PCR Methods and Applications 4: 357-362.

25. Van Poucke M, Van Zeveren A, Peelman LJ (2012) Combined FAM-labeled TaqMan probe detection and SYBR green 1 melting curve analysis in multiprobe qPCR genotyping assays. BioTechniques 52: 81-86.

26. Rickert AM, Lehrach H, Sperling S (2004) Multiplexed Real-Time PCR Using Universal Reporters. Clinical Chemistry 50: 1680-1683.

27. Bond SR, Naus CC (2012) RF-Cloning.org: an online tool for the design of restriction-free cloning projects. Nucleic Acids Research: 10.1093/nar/gks396.

28. Meier K (2007) Statistical guidance on reporting results from studies evaluating diagnostic tests. Silvers Springs, MD: United States Department of Health and Human Services. 19 p.

29. Rodrigo AG, Goracke PC, Rowhanian K, Mullins JI (1997) Quantitation of target molecules from PCR-based limiting dilution assays. AIDS Research and Human Retroviruses 13: 737-742.

30. Yao H, Song J, Liu C, Luo K, Han J, et al. (2010) Use of ITS2 region as the universal DNA barcode for plants and animals. PLoS One 5: e0013102.

31. Ndao M, Bandyayera E, Kokoskin E, Gyorkos TW, MacLean JD, et al. (2004) Comparison of blood smear, antigen detection, and nested-PCR methods for screening refugees from regions where malaria is endemic after a malaria outbreak in Quebec, Canada. Journal of Clinical Microbiology 42: 2694-2700.

32. Perandin F, Manca N, Calderaro A, Piccolo G, Galati L, et al. (2004) Development of a real-time PCR assay for detection of *Plasmodium falciparum*, *Plasmodium vivax*, and *Plasmodium ovale* for routine clinical diagnosis. Journal of Clinical Microbiology 42: 1214-1219.

33. Rosanas-Urgell A, Mueller D, Betuela I, Barnadas C, Iga J, et al. (2010) Comparison of diagnostic methods for the detection and quantification of the four sympatric *Plasmodium* species in field samples from Papua New Guinea. Malaria Journal 9: 361.

34. Cribb TH, Adlard RD, Hayward CJ, Bott NJ, Ellis D, et al. (2011) The life cycle of *Cardicola forsteri* (Trematoda: Aporocotylidae), a pathogen of ranched southern bluefin tuna, *Thunnus maccoyi*. International Journal of Parasitology 41: 861-870.

Chapter Four

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

Published in:

Polinski M, Shirakashi S, Bridle A, Nowak B (*In press*) Transcriptional immune response of cage-cultured Pacific bluefin tuna during infection by two *Cardicola* blood fluke species. Fish and Shellfish Immunology, doi: j.fsi.2013.10.008.

4.1 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.

4.2. Introduction

Pacific bluefin tuna (PBT) *Thunnus orientalis*, along with its two sibling species (Southern bluefin tuna *T. maccoyii* and Atlantic bluefin tuna *T. 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 maximise 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 – *C. orientalis* and *C. 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 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 *C. forsteri* in Southern bluefin tuna heart, gill, and serum [12] (Chapter 3). These analyses demonstrated a surprising prevalence and distribution of *C. orientalis* which had only recently been identified in ranched SBT [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] (Chapter 2), 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 SBT [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.

4.3. Materials and Methods

4.3.1. 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 (6,000 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 (Figure 4.1.). 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].

4.3.2. 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 5,000 × 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, 0.2M 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 homogenised 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 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] (Chapter 2). 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.

4.3.3. 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 efficiency and stringency of standard curves held to between 85-105% and 0.98-1.00, respectively, for all assays. All primer sets used in targeted qPCR amplification are presented in Table 1. Targeted quantitative PCR amplification of the internal transcribed spacer-2 (ITS2) region of rDNA specific to *C. orientalis* or *C. opisthorchis* was carried out separately for each organ and blood sample utilizing methods previously described [12] (Chapter 3). Briefly, PCR reactions utilizing gill and heart-derived templates were prepared in 2X SensiFast^{TM} +SYBR® mastermix (Bioline), forward and reverse primers (500 nM each), and 1 µL TNA template (2% of total eluted sample) in molecular grade water to a final volume of 10 µL per reaction. For blood-derived samples, large amounts of host DNA (> 0.5 µg/µL) necessitated target-specific fluorometric detection for accurate quantification [12] (Chapter 3). Thus, reactions were modified to consist of 2X SensiFast^{TM} +SYBR® mastermix, 50 nM tailed species-specific forward primer, 500 nM universal L and species-specific reverse primers, 250 nM TaqMan HEX-labelled probe, and 1 µL TNA template in molecular grade water. Cycling conditions for both SYBR and TaqMan detection consisted of an initial activation of DNA polymerase at 95°C for 3 min, followed by 40 cycles of 5 s at 95°C, 20 s at 60°C, and 8 s at 72°C, with relative fluorescence measured at the end of each 72°C extension. Following the cycling protocol a melt curve analysis was run to ensure amplification specificity with fluorescent detection conducted at 0.5°C intervals. Samples were assayed in duplicate alongside a six-step ten-fold

dilution series of previously prepared *C. orientalis* or *C. opisthorchis* linearised recombinant plasmid [12] (Chapter 3) assayed in triplicate representing a dynamic range of 10-10^6 genome equivalents (copies) of ITS2 rDNA per reaction for both parasite species targeted.

For gene expression analysis, extracted and reversed transcribed cDNA described above was used to measure the expressions of IL-1β, TNFα (TNF2), and IL-8 inflammatory associated genes in reference to two stable housekeeping genes – β actin and elongation factor 1α (EF1α) – using previously designed primers [14] (Chapter 2). For IgM heavy chain (IgM), T-cell receptor β subunit (TCRβ) and major histocompatibility complex-2 (MHC2) gene expression, primers were newly designed using Beacon Designer 7 software based on transcripts assembled from Northern bluefin tuna expressed sequence tag (EST) GenBank libraries. Transcripts were identified by the Basic Local Alignment Search Tool (BLAST) [18] to previously identified transcripts in other Perciform species (Table 4.1.). Each PCR reaction consisted of 2X SensiFast™ +SYBR® mastermix, forward and reverse primers (400 nM each), and 1 µL cDNA template in molecular grade water to a final volume of 10 µL. Samples were assayed in duplicate with a five-step, four-fold dilution series of pooled cDNA included in each run to calculate amplification efficiency and linearity. Cycling conditions consisted of an initial activation of DNA polymerase at 95°C for 3 min, followed by 40 cycles of 5 sec at 95°C, 20 sec at 60°C, and 10 sec at 72°C. At the end of the cycling protocol melt curve analysis was run to ensure amplification specificity.

Table 4.1. Oligonucleotide primers and probes used in real-time qPCR *Cardicola* detection and host gene expression analysis.

Name	Target	Accession	Product size	Sequence 5' → 3'
tuna_q_actin_F tuna_q_actin_R	βactin	JX157141	89 bp	TATCCTGACCCTGAAGTA CATTGTAGAAGGTGTGATG
tuna_q_EF1a_F tuna_q_EF1a_R	EF1α	JX157143	86 bp	TTGGTGTCAACAAGATGG GATGTAGGTGCTCACTTC
tuna_q_IL1_F tuna_q_IL1_R	IL-1β	JX157146	92 bp	AGCCACAAGATAACCAAG TTCTCTACAGCGATGATG
tuna_q_IL8_F tuna_q_IL8_R	IL-8	JX157147	108 bp	CTACTGTTCGCTTGTCGCTAA TTGATAGGTTGTCATCGGACTTAC
tuna_q_IgM_F tuna_q_IgM_R	$IgM_{(H)}$	EC917943	113bp	GTTCCACTTGACATCACAT AGTCCTACAGTCCTCCTT
tuna_q_MHC2_F tuna_q_MHC2_R	MHC2	EH667387	344bp	CAAGTTGGAGCACATCAG ATCTCATCGGTGGAAGTG
tuna_q_TCR_F tuna_q_TCR_R	TCRβ	EC092872	101 bp	AAGAGCAGCGTCTATGGA CTTGAGAGTTCACTTGTTCAG
tuna_q_TNF2_F tuna_q_TNF2_R	TNFα(2)	JX157149	76 bp	ATCTGAGCCATACTGTGAA AGGATGGTCTGGTAGGAA
tailed_C_ori_F C_ori_R	*C. orientalis* ITS2	HQ324226	191 bp	TGCACAATTCACGACTCACGATCATCCGCTCCGACGACACGAACGGGTGCTTGCTATTCCTAGATGTTTAC AACAACTATACTAAGCCACAA
tailed_C_opt_F C_opt_R	*C. opisthorchis* ITS2	HQ324228	272 bp	TGCACAATTCACGACTCACGATCATCCGCTCCGACGACACGAACGGGTTCCTAAATGTGTGTGCA TCAAAACATCAATCGACACT
Uni_F Uni_HEX	Universal tail Universal tail	[12]	subjective	GCACAATTCACGACTCACGA HEX-CATCCGCTCCGACGACACGA-BHQ1

4.3.4. Statistical analyses

Genomic copies of *C. orientalis* and *C. opisthorchis* were estimated using the Bioline CFX interface software. Gene expression was analysed with qBase software as described by Hellmans et al. [19] where mRNA expression was normalised using the mean expressions of the two reference genes [20]. The corrected normalised relative quantity (CNRQ) was calculated for each gene and scaled to the minimum value. Data from grouped biological replicates were then combined following a $\log_{10}$ transformation as suggested by Hellmans and Vandesompele [21], and used for statistical comparisons. The effects of *Cardicola* infection on fold induction of immune response genes was compared for each gene by a Mann-Whitney one-tailed U-test. For these comparisons, data were rescaled relative to mean control values at the time point specified and therefore standard errors were propagated as described by Rieu [22] prior to comparison. Additionally, Pearson's r bivariate correlation coefficient was generated using Graphpad Prism 5.01 (Graphpad Software, CA, USA) to compare the relative quantities of selected genes to relative quantities of *C. orientalis* and/or *C. opisthorchis* for infected tissues. Significance was assessed for both the Mann-Whitney U and Pearson's r test at a p-value < 0.05.

4.4. Results

4.4.1. Relative prevalence of C. orientalis and C. opisthorchis in cultured PBT

Both *C. opisthorchis* and *C. orientalis* DNA were first identified by qPCR in tissues collected 22 days post-transfer (dpt) to a sea-cage environments. Comparatively, gill microscopy first detected the presence of *Cardicola* eggs at 66 dpt (Figure 4.1.). Gill samples developed the highest relative abundance for both *C. opisthorchis* and *C. orientalis* DNA,

which reached approximately 2.2 and 6.9×10^6 copies/mg, respectively, by 85 dpt. *Cardicola opisthorchis* DNA also became highly prevalent in heart samples (1.4×10^6 copies/mg); however, *C. orientalis* culminated in substantially lower (3.9×10^4 copies/mg) levels of DNA in that organ and it was generally observed that heart became infected more rapidly and to a greater extent with *C. opisthorchis*, while gills more quickly and severely infected with *C. orientalis* (Figure 4.2. A & B). Blood had comparatively low levels of parasite DNA, for which the total mean concentration did not reach higher than 69 copies/µL and consisted primarily of *C. opisthorchis* rather than *C. orientalis* DNA.

Administration of the anthelmintic praziquantel treatment did not appear to have an immediate effect on the relative quantity of either *C. opisthorchis* or *C. orientalis* DNA, as quantities measured 48 hours after PZQ treatment were comparable to those from non-treated fish. However, the relative quantities of DNA for both parasite species were significantly reduced in both heart and gill tissues by 30 days post treatment relative to maximum pre-treatment levels (Figure 2B).

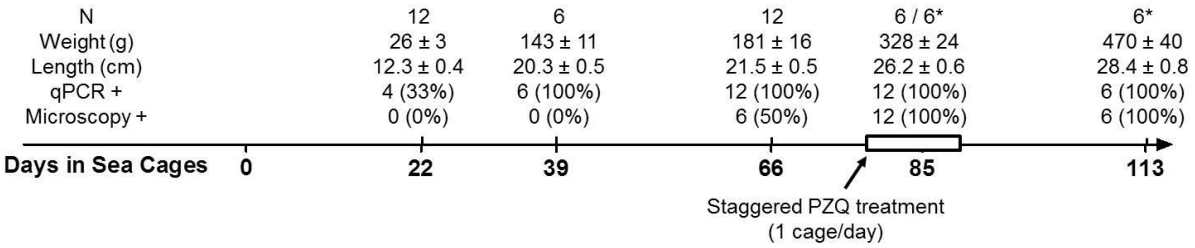


Figure 4.1. Linear presentation for the time course and sampling events. Arrow indicates the time course for early cage culture of juvenile Pacific bluefin tuna with marked sampling events, for which sample number (N), mean weight, and fork length (± SEM) of fish are provided. The number of individuals 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.

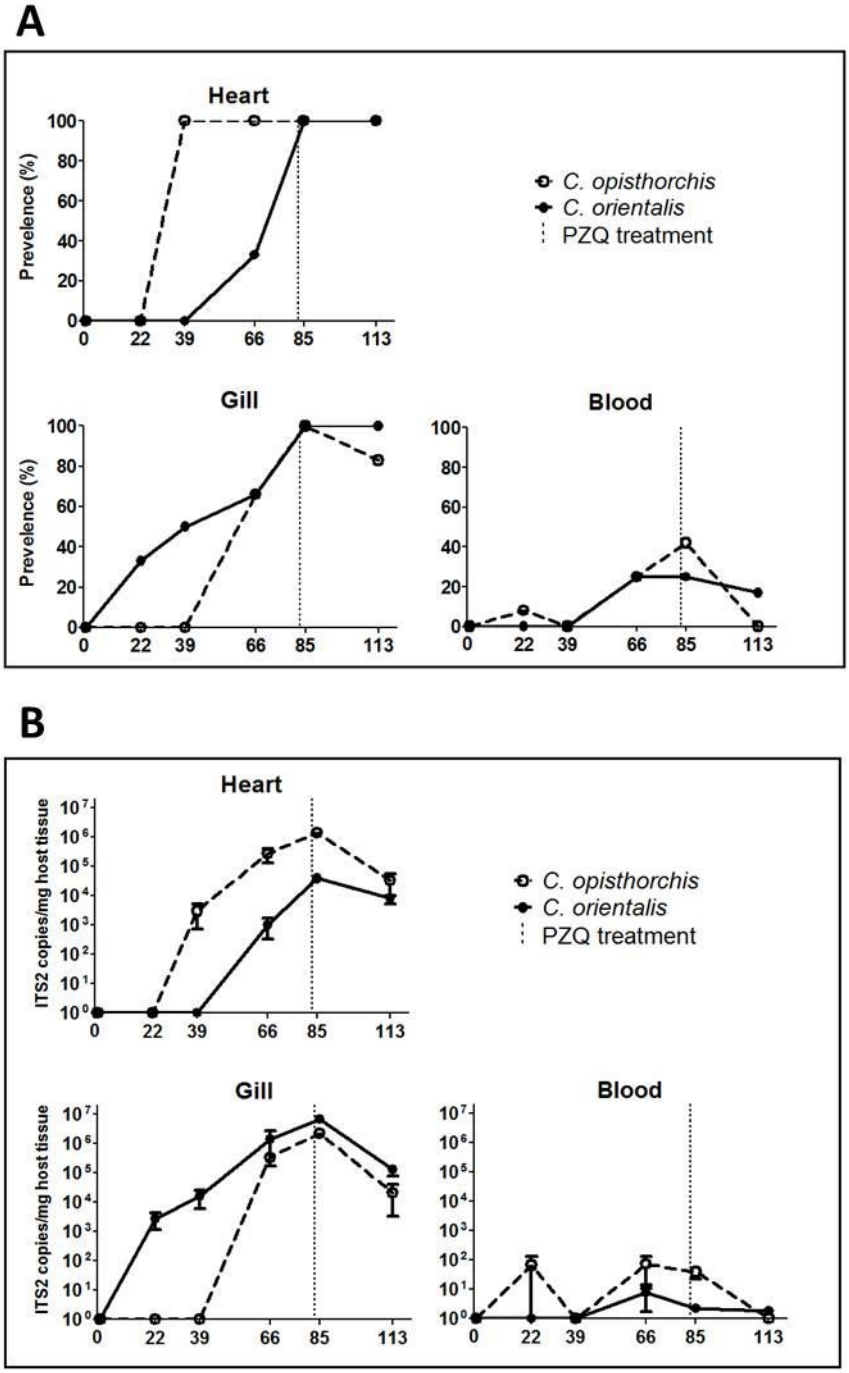


Figure 4.2. Relative prevalence of *Cardicola* spp. in Pacific bluefin during cage-culture. (A) Data indicate the proportion of infected individuals in the sample population at the time point indicated. B) Data present the mean (± SEM) genomic copies of parasite DNA detected in host tissues by qPCR during the first 113 days of cage culture. In both instances, the dotted line designates timing of an oral anthelmintic praziquantel (PZQ) treatment (150 mg/kg).

4.4.2. Transcriptional immune responsive in PBT caused by Cardicola infection

Of the six immune response genes evaluated (IL-1β, TNF2, IL-8, IgM, MHC2, and TCRβ), transcription of four (IL-8, IgM, MHC2, and TCRβ) were significantly up regulated in fish parasitised by *Cardicola* relative to non-infected fish for samples collected throughout this 113 day study period; for which expression was induced differentially for each sample type analysed. In heart samples, a 3-6 fold induction of IL-8, IgM, MHC2, and TCRβ was observed during infection. In gills, IgM was induced approximately 11 fold in infected individuals; however, induction of all other genes including IL-8, MHC2, and TCRβ was not significant in that organ. In blood, IgM and TCRβ transcriptions were modestly but significantly (approximately 2 fold) induced in infected individuals (Figure 4.3.). Neither of the inflammatory cytokines IL-1β or TNF2 was significantly induced by the presence of *Cardicola* in any of the three types of samples examined. Reference genes maintained stable and comparable expression within each sample type analysed in this study ($M < 0.55$; $CV < 0.25$).

For immune genes significantly induced during *Cardicola* infection (IgM, MHC2, TCRβ, and IL-8 in heart; IgM in gill; IgM and TCRβ in blood), relative mean transcription trended in a similar fashion to that of *Cardicola* DNA detected in gill and heart samples. This resulted in low levels of transcription in naïve fish immediately following the transfer of fish to sea-cages, which steadily increased in a similar pattern to infection prevalence and abundance until 85 dpt when an anthelmintic praziquantel treatment was administered. Following treatment, elevated transcriptional quantities of immune genes were observed to modestly subside (Figure 4.4.). This trend was observed for IgM, TCRβ, and MHC2 expression; however, gene transcription of IL-8 appeared to peak slightly earlier (66 dpt) relative to infection levels or other gene transcripts analysed.

As co-infections with both *Cardicola* spp. were common, it was difficult to determine if host immune gene transcription was preferentially induced by a single species. Indeed, correlations between parasitic DNA quantity and relative transcriptional quantity were not significant for any of the genes induced by infection in either heart or blood samples. However, IgM transcription in gills was highly correlated to the relative quantity of *C. orientalis* DNA but not that of *C. opisthorchis* (Figure 4.5.).

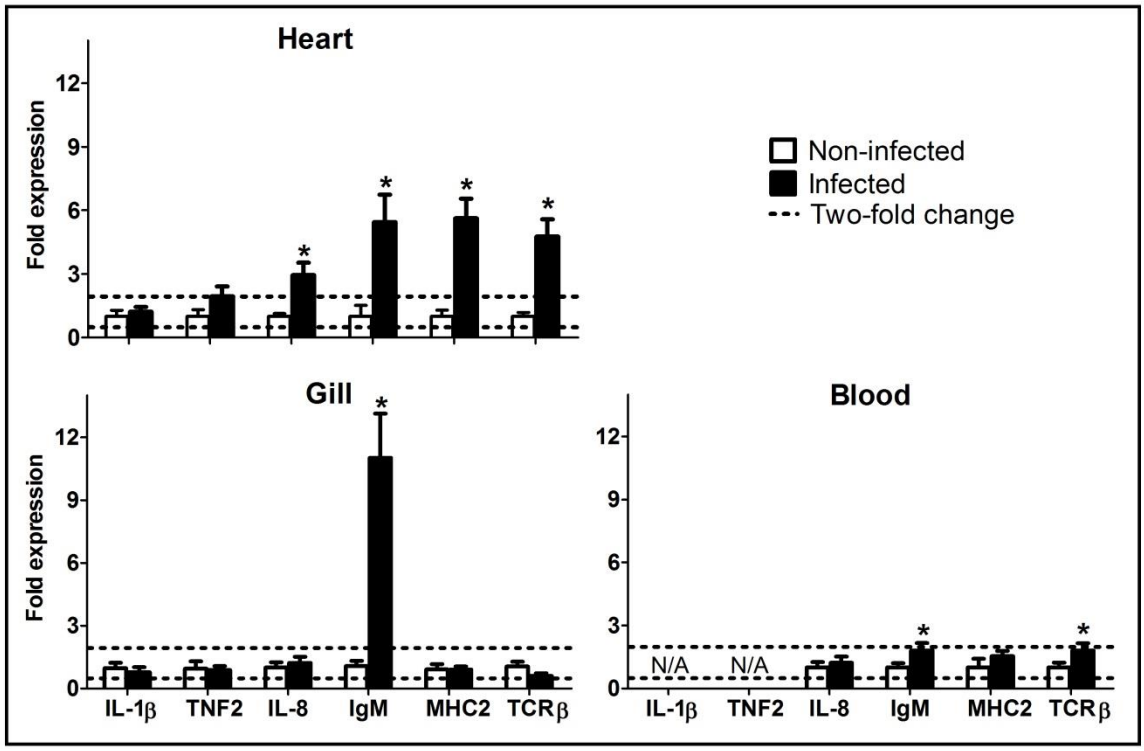


Figure 4.3. Induction of immune response genes during *Cardicola* blood fluke infection of Pacific bluefin tuna. Data identify mean (± SEM) fold induction of six selected immune genes from a population of infected fish (N=24) relative to non-infected (N=14) individuals collected over a period of 113 days assayed in duplicate by qPCR. The dotted lines present the minimum fold change (2 fold) typically deemed biologically significant. (*) Identifies a significant ($P<0.05$) elevated induction relative to non-infected individuals.

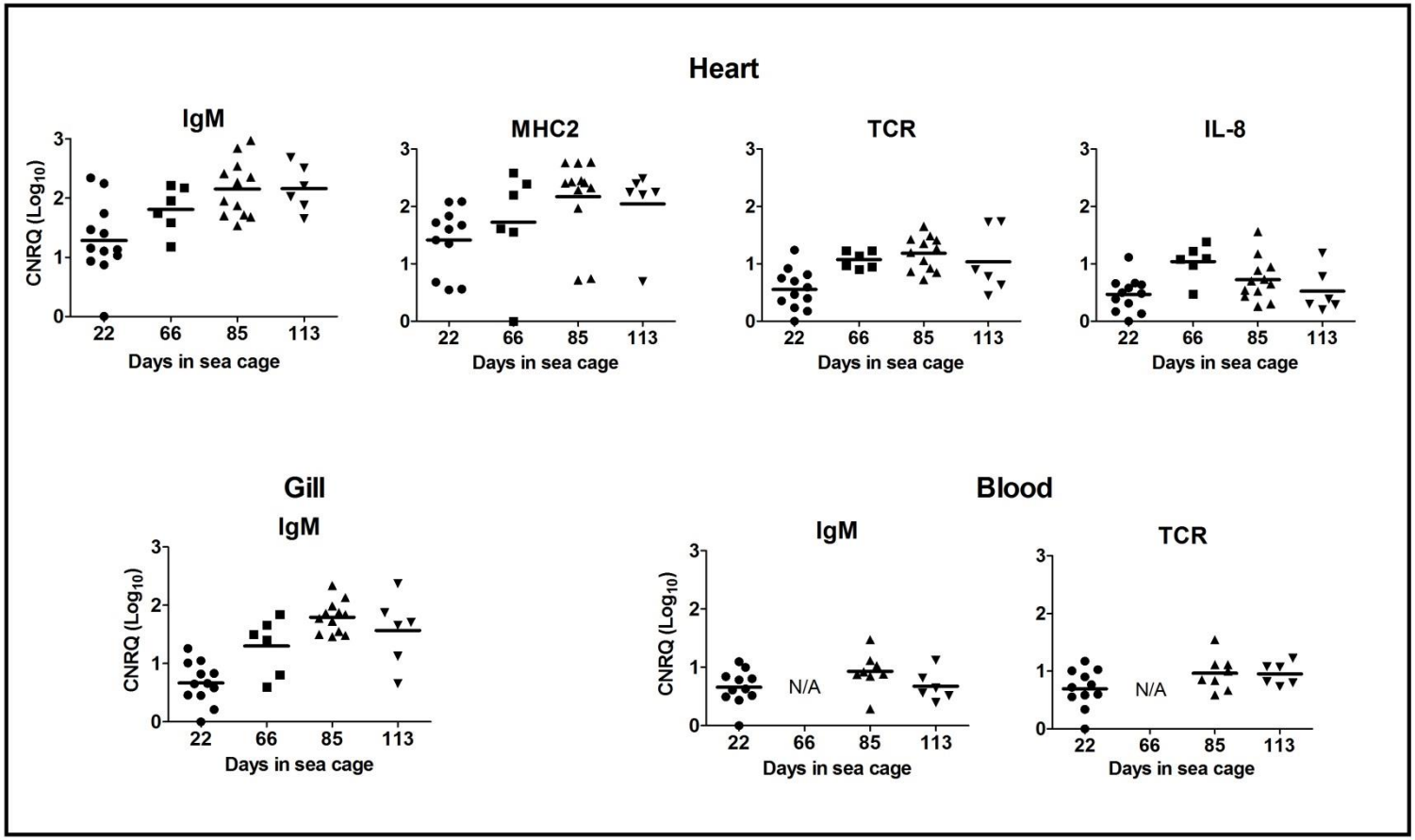


Figure 4.4. Relative transcription of immune genes responsive to *Cardicola* infection during early cage-culture of Pacific bluefin tuna. Data show the corrected normalised relative quantity (CNRQ) of genes identified to be induced during infection in heart, gill, and blood components. Samples were assayed in duplicate by qPCR and are presented in relation to duration of sea-cage culture. Horizontal bars identify the mean transcriptional quantity at each time point.

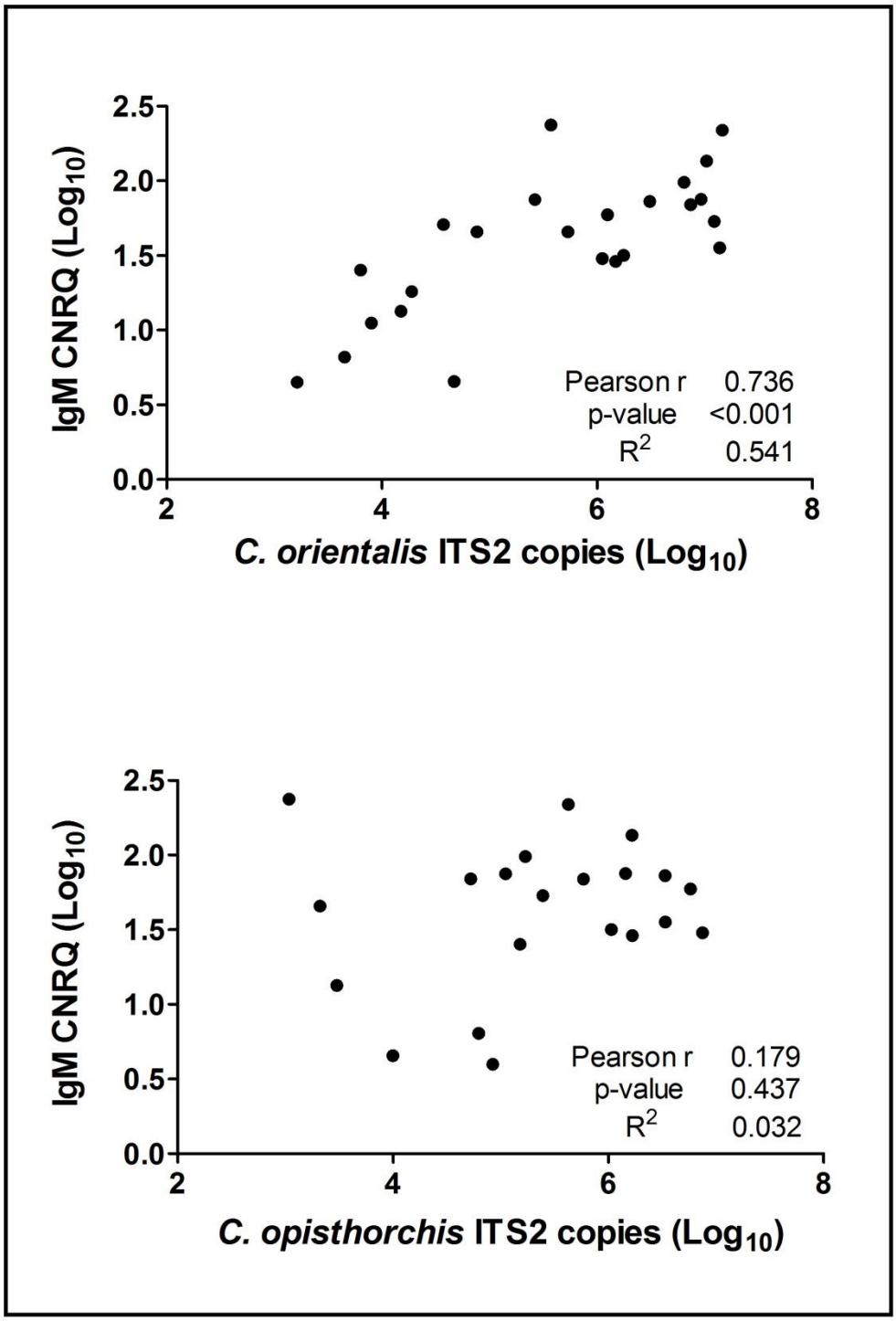


Figure 4.5. Relationship for relative prevalence of *Cardicola* to IgM transcription in the gill of cultured Pacific bluefin tuna. The corrected normalised relative quantity (CNRQ) for IgM transcription is presented in relation to *C. orientalis or C. opisthorchis* ITS2 genomic copy estimation following Log_{10} transformation of each variable.

4.5. Discussion

Cardicola infection currently represents the most important disease affecting the cage-culture production of PBT, and as such, early and rapid detection of these parasites will be critical for furthering success of PBT culture. We have recently reported sensitive and differential detection of *C. forsteri* and *C. orientalis* from serum, heart, and gill samples of SBT using qPCR, which provided strong evidence for its adoption as a reference standard for the diagnosis of *Cardicola* infection [12] (Chapter 3). In this study, we confirm the effectiveness of qPCR for differentially detecting *C. orientalis* and *C. opisthorchis* in PBT, as well as expand its utility to the detection of parasitic DNA from within whole blood.

Further, and for the first time, we take advantage of the relative quantitative ability of qPCR analysis to aid in mapping *Cardicola* prevalence within host organs during natural infection. Somewhat surprisingly, we observed relatively high quantities of *C. orientalis* DNA in sampled heart ($> 10^4$ copies/mg), even though this species have generally been observed (both adult flukes and eggs) in blood vessels of the gill by microscopy [8,9]. We have recently also observed *C. orientalis* DNA in hearts of ranched SBT, but as the detection was not assessed quantitatively in that instance, we attributed this occurrence to either the accumulation of free parasite DNA to a blood-rich organ, or to the presence of intermediate parasitic life stages such as eggs or immature worms which have thus far proven undetectable by microscopy screening [12] (Chapter 3). During the quantitative analysis conducted in this study, it appears unlikely that the high levels of *C. orientalis* DNA observed in the heart can be attributed to free-floating DNA alone, as similar volumes of blood contained comparatively little or no DNA from *C. orientalis* (Figure 4.2.). Additionally, as *C. orientalis* DNA was detected more than 40 days prior to our first observation of mature eggs or adults in the gill, it appears likely that much of the *C. orientalis* DNA identified during early

infection or within heart samples is associated with an intermediate parasitic life stage. The current methods for DNA-based detection cannot differentially identify parasitic life stage and the number of copies associated with each life stage are unknown and likely highly variable. Furthermore the inability for live/dead parasite distinction by qPCR must also be acknowledged when considering the DNA distribution and relative prevalence of this parasite *in situ*. Nevertheless, these findings confirm that the distribution of *C. orientalis* within the host is more extensive than previously identified in cultured PBT.

In general, helminths are often known to modify host immunity in the interest of their own longevity, reproduction and persistence [23]. In particular, digeneans such as *Fasciola hepatica* and *Schistosoma* spp. appear to actively suppress inflammation and T-helper cell responses by inducing host IL-10 secretion, a potent anti-inflammatory [24,25]. As blood flukes from genus *Cardicola* have only recently been identified in tuna, much of the host-pathogen relationship remains unclear; however, large quantities of both *C. orientalis* and *C. opisthorchis* in gill and heart samples in this study failed to elicit the production of either of the classic inflammatory signalling cytokines TNFα or IL-1β (Figure 4.3.). Further, the non-induction for MHC2 and TCRβ transcription in heavily infected gills also indicates a minimal T-cell and antigen presenting cell involvement in that organ. Unfortunately the genetic coding sequence for IL-10 has yet to be identified in bluefin tuna thus precluding its use in the current study. However, these current findings suggest that similar to blood flukes from genus *Schistosoma* and liver flukes from genus *Fasciola, Cardicola* species may have evolved an evolutionary strategy against the innate host response by supressing inflammation. This may be further supported by the fact that in the heart samples which even exhibited elevated IL-8, TCRβ, and MHC2 transcription caused by parasitic infection, elevated transcription of TNFα and IL-1β was not observed, and the overall immune response failed to inhibit exponential proliferation of the parasites (Figures 4.2. & 4.3.). The induction of IL-10

will need to be confirmed; but nevertheless, the ability of *Cardicola* spp. to suppress inflammatory cytokines and remain relatively unimpeded by a host primary cellular immune response has significant implications for the management and continued research concerning bluefin tuna aquaculture. Specifically, this indicates that unless enhanced immune signalling can be obtained and shown effective against parasitic infection, continued anthelmintic treatment will be necessary for combating at least primary *Cardicola* infections.

It has previously been indicated that SBT can produce antibody specific to *C. forsteri*, and that *C. forsteri*-specific antibody serum levels increased in fish experiencing moderate heart parasitism [16]. Additionally, parasite-induced mortality and severe infection occur following primary exposure in PBT during the first year of sea-cage culture, but surviving fish are not known to experience mortality in subsequent years of culture and lowered prevalence/clearance of infection is generally observed during continued culture [6]. In this study, we identify PBT to generate an elevated IgM transcription response to *C. orientalis/opisthorchis* co-infections in heart, gill, and blood samples of infected fish. Interestingly however, IgM transcription was only found to be significantly correlated to *C. orientalis* DNA prevalence in the gill (Figure 4.5.), even though *C. opisthorchis* DNA also became highly prevalent in that organ. As previous microscopy has revealed that adult *C. opisthorchis* are primarily restricted to the heart [8], whereas eggs are known to accumulate in the gill [10], our observations therefore suggest that the transcriptional induction of IgM is targeted at *C. orientalis* (both adults and eggs are gill associated [9,10]) but not the eggs of *C. opisthorchis*. This would also suggest that it is likely the adult flukes rather than eggs of *C. orientalis* which induce IgM expression; given that *C. opisthorchis* eggs appear not to induce a specific response. As the currently used molecular detection methods for *Cardicola* do not differentiate between life stages it is impossible to determine if only adults are inducing changes in IgM gene expression. It is also possible that if we could differentiate between all

life stages we could find significant relationships with other immune gene expression and particular parasite life stage. Further investigation will be needed to establish a more complete understanding for the induction and potential specificity of IgM to *Cardicola* infection; however, these initial findings warrant further examination into the antibody response in PBT and introduce exiting prospects for immunity enhancement, pathogen recognition, or for cross-species antibody protection against *Cardicola* infection.

In conclusion, this study supports qPCR as a new diagnostic standard for identifying the presence of *Cardicola* in tuna, and demonstrates the usefulness for quantitative detection in assessing the consequences of blood fluke infection on host tissues. Here, young PBT were shown to have at least some immunological responsiveness to *C. orientalis* and *C. opisthorchis* infection; however, this response appeared primarily focused toward increased transcription of IgM which seemed ultimately unable to effectively clear or even mitigate a primary infection. Thus, given current culture practices, anthelmintic treatment will continue to be necessary for combating primary *Cardicola* infections in PBT. However, continued investigation into the adaptive immunity of PBT will likely prove valuable for identifying and enhancing primary resistance or life-long protection against these blood fluke parasites during the future aquaculture production of bluefin tunas.

4.6. Acknowledgments

The authors would like to extend our sincerest appreciation to Hiroe Ishitani for aiding in the collection of samples utilised in this study. This research was supported in part by the SATREPS program of the Japan Science and Technology Agency (JST), the Australian Department of Education, Employment, and Workplace Relations via the

Endeavour International Postgraduate Award, and the University of Tasmania Australian Maritime College John Bicknell Scholarship.

4.7. References

1. Food and Agriculture Organization of the United Nations (2010) Yearbook of Fishery Statistics 2010. Rome; FAO. 107 p.

2. Ishibashi Y, Izumi T, Kurata M, Okada T (2013) Effects of tank wall pattern on survival, bone injury rate, and stress response of juvenile Pacific bluefin tuna, Thunnus orientalis. Aquacultural Engineering 56: 13-17.

3. Sawada Y, Okada T, Miyashita S, Murata O, Kumai H (2005) Completion of the Pacific bluefin tuna *Thunnus orientalis* (Temminck et Schlegel) life cycle. Aquaculture Research 36: 413-421.

4. Tsuda Y, Sakamoto W, Yamamoto S, Murata O (2012) Effect of environmental fluctuations on mortality of juvenile Pacific bluefin tuna, *Thunnus orientalis*, in closed life-cycle aquaculture. Aquaculture 330–333: 142-147.

5. Tsutsumi Y, Matsumoto T, Honryo T, Agawa Y, Sawada Y, et al. (2014) Effects of light wavelength on growth and survival rate in juvenile Pacific bluefin tuna, *Thunnus orientalis*. Environmental Biology of Fishes 97: 53-58.

6. Ishimaru K, Mine R, Shirakashi S, Kaneko E, Kubono K, et al. (2013) Praziquantel treatment against *Cardicola* blood flukes: Determination of the minimal effective dose and pharmacokinetics in juvenile Pacific bluefin tuna. Aquaculture 402–403: 24-27.

7. Aiken HM, Hayward CJ, Nowak BF (2006) An epizootic and its decline of a blood fluke, *Cardicola fosteri*, in farmed souther bluefin tuna, *Thunnus maccoyii*. Aquaculture 254: 40-45.

8. Ogawa K, Ishimaru K, Shirakashi S, Takami I, Grabner D (2011) *Cardicola opisthorchis* n. sp. (Trematoda: Aporocotylidae) from the Pacific bluefin tuna, *Thunnus orientalis* (Temminck & Schlegel, 1844), cultured in Japan. Parasitology International 60: 307-312.

9. Ogawa K, Tanaka S, Sugihara Y, Takami I (2010) A new blood fluke of the genus *Cardicola* (Trematoda: Sanguinicolidae) from Pacific bluefin tuna *Thunnus orientalis* (Temminck & Schlegel, 1844) cultured in Japan. Parasitology International 59: 44-48.

10. Shirakashi S, Kishimoto Y, Kinami R, Katano H, Ishimaru K, et al. (2012) Morphology and distribution of blood fluke eggs and associated pathology in the gills of cultured Pacific bluefin tuna, *Thunnus orientalis*. Parasitology International 61: 242-249.

11. Shirakashi S, Andrews M, Kishimoto Y, Ishimaru K, Okada T, et al. (2012) Oral treatment of praziquantel as an effective control measure against blood fluke infection in Pacific bluefin tuna (*Thunnus orientalis*). Aquaculture 326–329: 15-19.

12. Polinski MP, Belworthy Hamilton D, Nowak BF, Bridle A (2013) SYBR, TaqMan, or both: Highly sensitive, non-invasive detection of Cardicola blood fluke species in Southern Bluefin Tuna (*Thunnus maccoyii*). Molecular and Biochemical Parasitology 191: 7-15.

13. Shirakashi S, Tsunemoto K, Webber C, Rough K, Ellis D, et al. (2013) Two species of Cardicola (Trematoda: Aporocotylidae) found in Southern Bluefin Tuna Thunnus maccoyii ranched in South Australia. Fish Pathology 48: 1-4.

14. Polinski M, Bridle A, Nowak B (2013) Temperature-induced transcription of inflammatory mediators and the influence of Hsp70 following LPS stimulation of southern bluefin tuna peripheral blood leukocytes and kidney homogenates. Fish and Shellfish Immunology 34: 1147-1157.

15. Mladineo I, Block BA (2010) Expression of cytokines IL-1β and TNF-α in tissues and cysts surrounding *Didymocystis wedli* (Digenea, Didymozoidae) in the Pacific bluefin tuna (*Thunnus orientalis*). Fish and Shellfish Immunology 29: 487-493.

16. Aiken HM, Hayward CJ, Crosbie P, Watts M, Nowak BF (2008) Serological evidence of an antibody response in farmed southern bluefin tuna naturally infected with the blood fluke *Cardicola forsteri*. Fish and Shellfish Immunology 25: 66-75.

17. Aranda PS, LaJoie DM, Jorcyk CL (2012) Bleach gel: a simple agarose gel for analyzing RNA quality. Electrophoresis 33: 366-369.

18. Altschul S, Gish W, Miller W, Myers E, Lipman D (1990) Basic local alignment search tool. Journal of Molecular Biology 215: 403-410.

19. Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J (2007) qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biology 8: R19.

20. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, et al. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3: 0034.0031-0034.0011.

21. Hellemans J, Vandesompele J (2011) Quantitative PCR data analysis – unlocking the secret to successful results. In: Oswald N, Kennedy S, editors. PCR troubleshooting and optimization: the essential guide. Norwich, UK: Caister Academic Press.

22. Rieu I (2009) Real-Time Quantitative RT-PCR: Design, Calculations, and Statistics. The Plant Cell 21: 1031-1033.

23. Hewitson J, Grainger J, Maizels R (2009) Helminth immunoregulation: The role of parasite secreted proteins in modulating host immunity. Molecular and Biochemical Parasitology 167: 1-11.

24. Walsh KP, Brady MT, Finlay CM, Boon L, Mills KHG (2009) Infection with a Helminth Parasite Attenuates Autoimmunity through TGF-β-Mediated Suppression of Th17 and Th1 Responses. The Journal of Immunology 183: 1577-1586.

25. van den Biggelaar AHJ, van Ree R, Rodrigues LC, Lell B, Deelder AM, et al. (2000) Decreased atopy in children infected with *Schistosoma haematobium*: a role for parasite-induced interleukin-10. The Lancet 356: 1723-1727.

Chapter Five

Preliminary Evidence of Transcriptional Immunomodulation by Praziquantel in Bluefin Tuna and Atlantic Salmon *In Vitro* Cultures

Submitted as:

Polinski M, Bridle A, Nuemann L, Nowak B (2013) Preliminary Evidence of Transcriptional Immunomodulation by Praziquantel in Bluefin Tuna and Atlantic Salmon *In Vitro* Cultures. Fish and Shellfish Immunology.

5.1. Abstract

Praziquantel (PZQ), long-used in veterinary and human medicine for the treatment of helminth parasites, is known to enhance humoral and cellular immune responsiveness in mammals but has unknown direct immunomodulatory capabilities in fish. In the present study, we examined the ability of PZQ to induce gene transcriptional changes in immune-competent primary tissue/organ cultures of two highly important yet evolutionarily discrete fish species – Southern bluefin tuna *Thunnus maccoyii* and Atlantic salmon *Salmo salar*. These cultures consisted of mixed blood cell population for both species, as well as intestinal explants from bluefin. Although expression profiles varied between species and tissue/organ type, PZQ was observed to significantly induce both T-cell receptor (more than twofold) and IL-8 transcriptional expression (more than fourfold). Additionally, increased expression of other inflammatory cytokines including IL-1β was detected in blood cell cultures from both species, and a general pattern of heightened antiviral signalling was observed. Specifically, this included elevated transcription of Type I (IFNα) and Type II (IFNγ) interferon in Atlantic salmon blood cultures along with elevated expression of MHC class I in blood cultures of both species. These findings provide preliminary evidence for direct immunomodulation by PZQ in fish and insight into its potential capacity as an immune stimulant/adjuvant in the rapidly expanding aquaculture industry.

5.2. Introduction

Since its discovery in the mid-1970s, praziquantel (PZQ) has gained prominence in the anthelmintic treatment of flatworms [1-3] and is listed as an Essential Medicine by the World Health Organization [4]. It is also a commonly used therapeutic in veterinary

medicine for the treatment of a variety of Platyhelminth-associated diseases in both mammals and fish [5,6]. Specifically, and relative to the current study, PZQ has recently been applied to the treatment of *Cardicola* spp. infections in cultured Southern (*Thunnus maccoyii*) and Pacific (*Thunnus orientalis*) bluefin tuna [7,8].

Early investigations into the anti-parasitic properties of PZQ identified the drug to cause paralysis of the worm musculature [9], presumably due to a rapid influx of calcium ions [10], and to induce morphological alterations of the worm tegument including vacuolization and blebbing [11]. The drug was also observed to increase antigen exposure of the parasite [12], promoting a heightened immune response of the host, which was hypothesised to contribute to the increased effectiveness observed for PZQ *in vivo* [13,14]. More recently however, PZQ has been identified to directly induce $CD8^+$ T-cell responsiveness [15] and act as an adjuvant during administration of influenza and hepatitis DNA vaccines to mice [16,17]. At least part of the adjuvant capabilities of PZQ appear to stem from creating a suppressed state of immune regulation through the down-regulation of TGF-β/Smad2,3 signalling and decrease in T-regulatory cell function [17,18]. Nevertheless, a comprehensive understanding of the influences PZQ has on host immune-signalling is far from complete. This is particularly evident with regard to administration of PZQ to fish, as there has been limited investigations into the immunomodulatory ability of PZQ in teleost fish despite its long-use in an aquaculture context [5]. Recently, PZQ treatment of the gill fluke *Dactylogyrus intermedius* in goldfish *Carassius auratus* showed an up-regulation of the inflammatory mediators IL-8, IL-1β, and TNFα and a down-regulation of TGF-β following administration [19]. However, it remains unclear if this change in expression was a direct result of the drug or a consequence of reducing the parasite load.

In the present study, our aim was to assess the direct immunomodulatory ability of PZQ in fish using immune-competent tissue/organ cultures from two highly valued food fish

species – the Southern bluefin tuna (SBT) and the Atlantic salmon (*Salmo salar*). Here, we applied qPCR gene expression analysis to identify both potential systemic (blood cells) and localised (mucosa/sub mucosa of intestinal explants) responsiveness to PZQ to better understand the anthelminthic capabilities of this drug as applied to bluefin tuna and also give a preliminary indication for the possible use of PZQ as an adjuvant and/or immunostimulatory compound in fish.

5.3. Materials and Methods

5.3.1. Ethics statement

All work with animals and methods for recovering samples were performed in strict accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes [20] and approved by the University of Tasmania Animal Ethics Committee (UTAS AEC project numbers A0010593 and A0012145).

5.3.2. Field collection

Peripheral blood and visceral organs were collected from three individual SBT weighing 35–40 kg (approximately 2–3 years of age) during commercial harvest of ranched populations held in off-shore sea cages near Port Lincoln, South Australia, similar to methods previously described [21] (Chapter 3). Blood was collected from the pectoral recess of the severed lateral artery of each fish into heparinised blood collection tubes (Sarstedt) and visceral organs were removed as per industry practice, sealed in waterproof plastic bags, and placed on ice for transit to a laboratory setting (approximately 1–2 h). Separately, blood of Atlantic salmon was collected from three 150-200 g fish held at the University of Tasmania

Aquaculture Centre, Launceston, Tasmania, following anesthesia using a heparinised syringe and a 22 gauge needle [22].

5.3.3. In vitro cultures and stimulation

Blood cell cultures were prepared by spinning heparinised whole blood at 800 × *g* for 10 min at 4°C. Plasma was removed, cells washed twice with phosphate-buffered saline (PBS) and suspended to a final concentration of 10^8 cells/mL in L-15 culture media supplemented with GlutaMax® and 10% fetal bovine serum (Invitrogen). Aliquots of 100 µL were seeded to 1.6 mL centrifuge tubes and stimulated in triplicate to a final concentration of 50 µg/mL PZQ (Sigma) in PBS alongside non-stimulation PBS controls. Cultures were incubated for 6 h at 20°C and then diluted 1:7 in a nucleic acid preservation solution (NAPS; 4 M ammonium sulphate, 25 mM sodium citrate, 10 mM EDTA; pH 5.5). Preserved suspensions were held overnight at 4°C and then stored at -20°C until RNA extraction.

Intestinal explant cultures (SBT only) were prepared in triplicate 1 cm² portions from a 10 cm piece of anterior intestine cut lengthwise using a scalpel, cleared of mucus and digestive remnants by gentle massage and repeated PBS washings, and sterilised in a 0.1% chlorohexidine-PBS solution for 5 min. Explants were washed twice in PBS, divided separately into each of a 12-well tissue culture plate containing 3 mL of L-15 supplemented growth media per well, and stimulated with PZQ to a final concentration of 10 µg/mL alongside PBS controls. Cultures were incubated for 3 h at 25°C and preserved in 5 mL NAPS at 4°C overnight and stored at -20°C until processing.

5.3.4. RNA extraction and cDNA synthesis

Both RNA extraction and cDNA synthesis were performed similar to methods previously described [23] (Chapter 2). Briefly, blood cells were recovered from the preservation solution by centrifugation at 5000 × *g* for 5 min. Intestinal explants were placed on a clean glass plate and approximately 10 mg of mucosa and submucosa were separated from the underlying *muscularis propria* and *serosa* layers using a scalpel. Cells were then lysed in an Extraction Buffer (4 M Urea, 1 % SDS, 0.2 M NaCl, 10% glycerol) supplemented with 20 U Proteinase K (Bioline). Protein, cellular debris, and detergent were removed by centrifugation in 3 M ammonium acetate at 14,000 × *g* for 5 min, and nucleic acids were recovered by isopropanol precipitation at 16,000 × *g* for 10 min followed by an ethanol wash of the nucleic acid pellet. Nucleic acids were then eluted in 100 µL water and RNA phase separated from DNA by addition of 700 mL RNAzol® and 50 mL BAN® phase separation reagent (Molecular Research Center) as outlined by the manufacturer. Complete removal of DNA was further ensured by treatment with 4 U Baseline-Zero DNAse (Epicentre) for 30 min at 37°C. RNA was quantified using a Qubit fluorometer (Invitrogen), and quality was visualised on a 1% agarose bleach denaturing gel [24]. A portion of RNA (100 ng) was reverse transcribed for each sample replicate using a cDNA Synthesis Kit (Bioline) with Oligo $(dT)_{18}$ primer mix as outlined by the manufacturer. A portion of remaining RNA from each sample was pooled, and 100 ng was reverse transcribed in triplicate for use in preparing qPCR standards. Three additional reactions of pooled RNA without reverse transcriptase provided no-reverse transcription controls.

5.3.5. Real-time qPCR analysis

All real-time qPCR analyses were conducted on a CFX Connect Real-Time PCR detection system (Bio-Rad). All primer sets used in targeting SBT and Atlantic salmon gene transcripts are presented in Table 5.1. Each PCR reaction consisted of 2X SensiFast™

+SYBR® mastermix (Bioline), forward and reverse primers (400 nM each), and 1 µL cDNA template in molecular grade water to a final volume of 10 µL. Samples were assayed in duplicate with a five-step, four-fold dilution series of pooled cDNA included in each run to calculate amplification efficiency and linearity. Cycling conditions consisted of an initial activation of DNA polymerase at 95°C for 3 min, followed by 40 cycles of 5 sec at 95°C, 25 sec at 60°C, and 10 sec at 72°C. At the end of the cycling protocol melt curve analysis was run to ensure amplification specificity.

5.3.6. Statistical comparisons

Gene expression was analysed using qBase software as described by Hellemans et al. [25] where mRNA expression was normalised using the mean expressions of two stable reference genes (β-actin and EF1-α in tuna, β-actin and RPS-2 in salmon). Reference gene stability was assessed using methods previously described ($M < 0.5$, $CV < 0.25$) [26]. The corrected normalised relative quantity (CNRQ) was calculated for each gene and scaled to the minimum value, and data from grouped biological replicates were combined following a $\log_{10}$ transformation as suggested by Hellemans and Vandesompele [27]. Fold induction or repression relative to PBS controls was assessed for each gene using a Mann-Whitney two-tailed U-test.

Table 5.1. Oligonucleotide primers used in real-time qPCR analyses.

Target species	Target gene	Name	Accession #	5' → 3' sequence
Southern bluefin	Beta actin	SBT-Actin-F	JX157141	TATCCTGACCCTGAAGTA
		SBT-Actin-R		CATTGTAGAAGGTGTGATG
	Elongation factor 1 alpha	SBT-EF1a-F	JX157143	TTGGTGTCAACAAGATGG
		SBT-EF1a-R		GATGTAGGTGCTCACTTC
	Interleukin 1 beta	SBT-IL1-F	JX157146	AGCCACAAGATAACCAAG
		SBT-IL1-R		TTCTCTACAGCGATGATG
	Interleukin 8	SBT-IL8-F	JX157147	CTACTGTTCGCTTGTCGCTAA
		SBT-IL8-R		TTGATAGGTTGTCATCGGACTTAC
	Tumor necrosis factor 2 alpha	SBT-TNF2-F	JX157149	ATCTGAGCCATACTGTGAA
		SBT-TNF2-R		AGGATGGTCTGGTAGGAA
	Immunoglobulin M heavy chain	SBT-IgM-F	EC917943	GTTCCACTTGACATCACAT
		SBT-IgM-R		AGTCCTACAGTCCTCCTT
	T cell receptor beta chain	SBT-TCR-F	EC092872	AAGAGCAGCGTCTATGGA
		SBT-TCR-R		CTTGAGAGTTCACTTGTTCAG
	Major histocompatibility complex 1	SBT-MHC1-F	EG667956	TGATGTACGGCTGTGAAT
		SBT-MHC1-R		TGTCTGACCTCTGTTATGAT
	Major histocompatibility complex 2	SBT-MHC2-F	EH667387	CAAGTTGGAGCACATCAG
		SBT-MHC2-R		ATCTCATCGGTGGAAGTG
Atlantic salmon	Beta actin	AS-Actin-F	BT047241	TTGCGGTATCCACGAGAC
		AS-Actin-R		TAGAGGGAGCCAGAGAGG
	ribosomal polymerase subunit 2	AS-RPL2-F	BT049591	TAACGCCTGCCTCTTCACGTTGA
		AS-RPL2-R		ATGAGGGACCTTGTAGCCAGCAA
	Interleukin 1 beta	AS-IL1-F	NM_001123582	AAGACACTGTTACCTACCTA
		AS-IL1-R		ATACCTCCAGATCCAGAC
	Interleukin 8	AS-IL8-F	HM162835	ACCAGCGAGATAACAA
		AS-IL8-R		CCAGGAGCACAATGACAA
	Immunoglobulin M heavy chain	AS-IgM-F	Y12457	TGAGGAGAACTGTGGGCTACACT
		AS-IgM-R		TGTTAATGACCACTGAATGTGCAT
	T cell receptor alpha chain	AS-TCR-F	EF467027	GTCTGACTCTGCTGTGTA
		AS-TCR-R		GTGTAGTAGGATGGCTCAT
	Major histocompatibility complex 1	AS-MHC1-F	L07605	ATTGTCCTCATCATTGTAG
		AS-MHC1-R		TTCTTGCTCTTCTTCTTC
	Major histocompatibility complex 2	AS-MHC2-F	BT150000	GAACATTCTTCCTCATCA
		AS-MHC2-R		TGCCATCTACACTCTATA
	Cluster of differentiation 8	AS-CD8-F	AY693393	AAGACAACGCTGGAATGG
		AS-CD8-R		TATCTGCTCCTCGCTGAA
	Cluster of differentiation 4	AS-CD4-F	EU153044	TGTTTCTGGATTCCTTTCAAT
		AS-CD4-R		CCATACACAAGCACATCTC
	Type 1 interferon (alpha)	AS-IFNa-F	NM_001123570	GCGAAGTTATTAGCAGTTG
		AS-IFNa-R		CCACAAGATAAGATTATTCCATT
	Type 2 interferon (gamma)	AS-IFNg-F	AY795563	CTTGGATAGTGTCAGATAC
		AS-IFNg-R		CTCAATAGTCCTCAATCG

5.4. Results

Transcriptional expression in response to PZQ was assessed for seven immune-associated mRNA transcripts in both intestinal explant and blood cell cultures from SBT. This suite of genes currently represents the full extent of immune-specific gene sequences identified for bluefin tunas [23,28] (Chapter 2, Chapter 4). This includes the transcripts for the two inflammatory cytokines TNFα (TNF2) and IL-1β, the IL-8 chemokine, both T-cell receptor (TCR) beta chain and immunoglobulin-M (IgM) heavy chain representing T-cell and B-cell specific markers, respectively, as well as both MHC class I (MHC1) and MHC class II (MHC2) receptors involved in antigen presentation. Additionally, temperature is known to have differential stress-associated and timing effects with regard to tissue/organ type in this uniquely heterothermic organism [23] (Chapter 2). As a result incubation temperature and duration of stimulant exposure of *in vitro* cultures in the present study were discretely applied to each tissue/organ type to maximise identification of gene expression changes without inducing temperature associated stress. Therefore intestinal explants were cultured at 25°C for 3 h, while blood cell cultures were held at 20°C for 6 h. In both instances, stimulation with PZQ resulted in significant (between two and six fold) increased expression of IL-8, TCR, and IgM transcripts (Table 5.2). Expression of both inflammatory cytokines TNF2 and IL-1β, as well as MHC class I was also up-regulated (three to seven fold) in blood cultures, whereas MHC class II was significantly down regulated (six fold) in intestinal explants.

Blood cell cultures were also prepared and screened for PZQ responsiveness from a second fish species – Atlantic salmon – representing a second order of fish with more than 25 million years of discrete evolution to that of tuna [29]. Like SBT derived cultures, blood cultures from Atlantic salmon also showed increased inflammatory (IL-1β and IL-8), TCR, and MHC1 transcription in response to PZQ (Table 5.3). Further, as the suite of known

immune-associated genes for salmon is more extensive than that available for tuna, four additional immune-gene targets were analysed to identify antiviral and T-cell specific signalling pathways previously implicated to be responsive to PZQ in mammals [15,16]. These included transcripts for both Type I (IFNα) and Type II (IFNγ) interferon as well as transcripts identifying clusters of differentiation 4 (CD4) and 8 (CD8) utilised in assessing T-cell functions. Here, we identified significantly increased expression of both IFNα (16 fold) and IFNγ (threefold) in conjunction with a significant down-regulation (threefold) of CD4 in response to PZQ stimulation. As these gene transcripts are currently unavailable in SBT, no interspecies comparisons could be made; however, all other gene analysed showed a common pattern of expression in blood cultures of both species in response to PZQ with the notable exception of IgM. This gene target appeared down-regulated approximately threefold in Atlantic salmon blood cultures whereas a twofold increase in expression was observed in similar cultures from SBT.

Table 5.2. Effects of praziquantel on immunological related gene expression in Southern bluefin tuna *in vitro* cultures. Data identify mean (± SE) fold expression of seven selected immune genes relative to un-stimulated PBS controls assayed in duplicate by real-time qPCR for both the intestinal explant (n=3) and blood cell (n=9) cultures. Significant induction or repression is indicated at $P < 0.05$ (*) and $P < 0.01$ (**).

Gene target	SBT intestinal explant (10 µg/mL PZQ for 3 h at 25°C)	SBT blood cell culture (50 µg/mL PZQ for 6 h at 20°C)
Tumor necrosis factor 2 (alpha)	1.2 (±0.3)	6.9 (±2.2)**
Interleukin-1 (beta)	-1.6 (±0.5)	3.4 (±0.6)*
Interleukin-8	5.4 (±1.1)*	4.3 (±0.7)**
Immunoglobulin M (heavy chain)	4.0 (±0.6)*	2.0 (±0.4)*
T-cell receptor (beta chain)	4.2 (±0.5)*	2.4 (±0.3)*
Major histocompatibility complex 1	-1.6 (±0.3)	3.5 (±0.6)**
Major histocompatibility complex 2	-5.9 (±0.9)*	1.4 (±0.1)

Table 5.3. Effects of praziquantel on immunological related gene expression in Atlantic salmon *in vitro* blood cell culture. Data identify mean (± SE) fold expression of ten selected immune genes relative to un-stimulated PBS controls assayed in duplicate by real-time qPCR (n=3). Significant induction or repression is indicated at $P < 0.05$ (*) and $P < 0.01$ (**).

Gene target	Salmon blood cell culture (50 µg/mL PZQ for 6 h at 20°C)
Interleukin-1 beta	2.9 (±0.3)**
Interleukin-8	7.2 (±1.1)**
Immunoglobulin M (heavy chain)	-3.5 (±0.7)*
T-cell receptor (alpha chain)	3.3 (±0.5)*
Major histocompatibility complex 1	2.7 (±0.4)*
Major histocompatibility complex 2	-4.3 (±0.6)**
Cluster of differentiation 8	1.1 (±0.3)
Cluster of differentiation 4	-3.1 (±1.0)*
Type 1 interferon (alpha)	16.5 (±4.1)**
Type 2 interferon (gamma)	3.2 (±0.5)*

5.6. Discussion

Early work with PZQ identified a synergy between the drug and a competent mammalian immune system with regard to the clearance and killing of helminth parasites. Specifically, this was attributed to a T- and B-cell mediated host response to worm antigen which exhibited increased presentation following treatment with PZQ [12,13,30]. A similar synergy for *in vivo* effectiveness has not been confirmed with regard to the PZQ treatment of helminths in fish; although such a relationship is possible. Low concentrations of PZQ (7.5 mg/kg/day) have been shown to be sufficient to completely eradicate adult *Cardicola opisthorchis* from the hearts of juvenile Pacific bluefin tuna [8], whereas a biologically equivalent dose *in vitro* appeared to be less effective in completely killing adult flukes under a similar time frame of exposure [31]. In this study, we found that PZQ induced a heightened immune signalling in SBT tissue/organ cultures independent of parasitic antigen. Elevation of the IL-8 chemokine as well as both T and B cell-specific transcriptional signalling was observed in representative systemic and localised tissue/organ-specific cultures of SBT in response to PZQ. This suggests a second mechanism potentially responsible for improvement of the *in vivo* effectiveness of PZQ treatment of helminth parasites in fish in comparison to *in vitro* results. For internal helminth parasites such as blood flukes from the genus *Cardicola*, a combination of both antigen-associated and immunostimulatory PZQ-induced pathways may be required for effective parasite control given the known ability of helminths to enhance host IL-10 and TGF-β mediated immunosuppression in mammals [32,33] and the ineffectiveness for antibody in clearing *Cardicola* infection from tuna without the addition of PZQ during primary exposure [28] (Chapter 4). This may be further supported by the recent findings by Zhang and colleagues [19] where TGF-β was observed to be suppressed while IL-8, IL-1β and TNFα inflammatory mediator expression was enhanced following PZQ administration in

goldfish experiencing *D. intermedius* gill fluke infections. Further work is necessary to fully identify the extent and proportional significance of either the immunostimulatory or the antigen-associated synergistic pathways regarding the anthelmithic effectiveness of PZQ in these lower vertebrates

In the present study, PZQ induced IL-8 and TCR transcription in all *in vitro* cultures of SBT and Atlantic salmon, suggesting heightened T-cell responses and immune-cell trafficking in fish as a direct effect of PZQ. Additionally, the generalised increased proportion of MHC1 relative to MHC2 transcription in all fish cultures and the heightened interferon production along with an elevated CD8 to CD4 ratio induced by PZQ in Atlantic salmon blood cultures suggested a shift toward an antiviral and cytolytic immune state similar to previous observations in mice [16]. While these results are preliminary, they provide encouraging evidence for further investigations into the potential application of PZQ as adjuvant or as an immunostimulant for fish, particularly during viral infections. Current adjuvants are often limited in enhancing cell-mediated immunity [34,35], and the enhanced cytotoxic T-cell function and efficacy of this drug as an adjuvant for H5N1 vaccination in mice warrants further investigation in the rapidly expanding and economically important aquaculture industry.

In the course of this study, PZQ stimulations *in vitro* were designed to emulate potentially attainable concentrations *in vivo*. Recent oral administration of PZQ at a low dose (15 mg/kg) to juvenile Pacific bluefin tuna identified PZQ to reach concentrations of between 2-10 µg/g in serum and internal organs that lasted up to 1.5 hours [8], and it is reasonable to assume that oral administration of higher doses (150 mg/kg) which have been applied to tuna without apparent side effect could reach *in vivo* concentrations of 10-50 µg/g for periods of up to a few hours [7,8,28,36]. Nevertheless, caution is needed in the application of this chemical compound at high concentrations or during prolonged administration.

In conclusion, this study provides preliminary evidence for direct immunomodulatory abilities by PZQ in fish cell cultures, with indications for enhancing T-cell, B-cell, and inflammatory associated signalling responses. This warrants further investigation of PZQ as an immunostimulatory compound and potential vaccine adjuvant for these organisms. Additionally, the parasite-independent immune stimulation by PZQ in fish likely increases the anthelmintic property of this drug during *in vivo* application and provides a possible explanation for its high efficacy in the treatment of *Cardicola* spp. infections in bluefin tuna.

5.6. Acknowledgments

The authors would like to thank the staff and crews of Marnikol Fisheries for their cooperation and assistance in collecting blood and organ samples of Southern bluefin tuna, as well as the Lincoln Marine Science Centre for providing laboratory space to conduct SBT-related experiments. We would also like to thank Dr Philip Crosbie and Victoria Valdenegro for providing and aiding in the collection of Atlantic salmon blood used in this study.

5.7. References

1. Cioli D, Pica-Mattoccia L (2003) Praziquantel. Parasitology Research 90: S3-S9.
2. Doenhoff MJ, Cioli D, Utzinger J (2008) Praziquantel: mechanisms of action, resistance and new derivatives for schistosomiasis. Current Opinion in Infectious Diseases 21: 659-667.

3. Doenhoff M, Hagan P, Cioli D, Southgate V, Pica-Mattoccia L, et al. (2009) Praziquantel: its use in control of schistosomiasis in sub-Saharan Africa and current research needs. Parasitology 136: 1825.

4. World Health Organization (2013) WHO Model List of Essential Medicines: 18th Edition. Geneva: WHO. 45 p.

5. Schmahl G, Taraschewski H (1987) Treatment of fish parasites. 2. Effects of praziquantel, niclosamide, levamisole-HCl, and metrifonate on monogenea (*Gyrodactylus aculeati*, *Diplozoon paradoxum*). Parasitology Research 73: 341-351.

6. Reich MR, Govindaraj R, Dumbaugh K, Yang B-m, Brinkmann A, et al. (1998) International Strategies for Tropical Disease Treatments - Experiences with Praziquantel - EDM Research Series No. 026. In: Reich MR, editor. Action Programme of Essential Drugs Division of Control of Tropical Diseases. Cambridge: Harvard University.

7. Hardy-Smith P, Ellis D, Humphrey J, Evans M, Evans D, et al. (2012) In vitro and in vivo efficacy of anthelmintic compounds against blood fluke (*Cardicola forsteri*). Aquaculture 334–337: 39-44.

8. Ishimaru K, Mine R, Shirakashi S, Kaneko E, Kubono K, et al. (2013) Praziquantel treatment against *Cardicola* blood flukes: Determination of the minimal effective dose and pharmacokinetics in juvenile Pacific bluefin tuna. Aquaculture 402–403: 24-27.

9. Gönnert R, Andrews P (1977) Praziquantel, a new broad-spectrum antischistosomal agent. Zeitschrift für Parasitenkunde 52: 129-150.

10. Pax R, Bennett JL, Fetterer R (1978) A benzodiazepine derivative and praziquantel: effects on musculature of *Schistosoma mansoni* and *Schistosoma japonicum*. Naunyn Schmiedebergs Arch Pharmacology 304: 309-315.

11. Becker B, Mehlhorn H, Andrews P, Thomas H, Eckert J (1980) Light and electron microscopic studies on the effect of praziquantel on *Schistosoma mansoni*, *Dicrocoelium dendriticum*, and *Fasciola hepatica* (Trematoda) *in vitro*. Z Parasitenkd 63: 113-128.

12. Harnett W, Kusel JR (1986) Increased exposure of parasite antigens at the surface of adult male *Schistosoma mansoni* exposed to praziquantel *in vitro*. Parasitology 93: 401-405.

13. Brindley PJ, Sher A (1987) The chemotherapeutic effect of praziquantel against *Schistosoma mansoni* is dependent on host antibody response. The Journal of Immunology 139: 215-220.

14. Joseph S, Jones FM, Walter K, Fulford AJ, Kimani G, et al. (2004) Increases in human T helper 2 cytokine responses to Schistosoma mansoni worm and worm-tegument antigens are induced by treatment with praziquantel. Jounal of Infectious Diseases 190: 835-842.

15. Zou Q, Yao X, Feng J, Yin Z, Flavell R, et al. (2011) Praziquantel Facilitates IFN-γ-Producing CD8+ T Cells (Tc1) and IL-17-Producing CD8+ T Cells (Tc17) Responses to DNA Vaccination in Mice. PLoS One e25525.

16. Zou Q, Hu Y, Xue J, Fan X, Jin Y, et al. (2012) Use of Praziquantel as an Adjuvant Enhances Protection and Tc-17 Responses to Killed H5N1 Virus Vaccine in Mice. PLoS One 7: e34865.

17. Zou Q, Zhong Y, Su H, Kang Y, Jin J, et al. (2010) Enhancement of humoral and cellular responses to HBsAg DNA vaccination by immunization with praziquantel through inhibition TGF-β/Smad2,3 signaling. Vaccine 28: 2032-2038.

18. Watanabe K, Mwinzi PN, Black CL, Muok EM, Karanja DM, et al. (2007) T regulatory cell levels decrease in people infected with Schistosoma mansoni on effective treatment. American Journal of Tropical Medicine and Hygiene 77: 676-682.

19. Zhang C, Ling F, Chi C, Wang G-X (2013) Effects of praziquantel and sanguinarine on expression of immune genes and susceptibility to *Aeromonas hydrophila* in goldfish (*Carassius auratus*) infected with *Dactylogyrus intermedius*. Fish and Shellfish Immunology 35: 1301-1308.

20. National Health and Medical Research Council (2013) Australian code for the care and use of animals for scientific purposes, 8th edition. Canberra: National Health and Medical Research Council.

21. Polinski MP, Belworthy Hamilton D, Nowak BF, Bridle A (2013) SYBR, TaqMan, or both: Highly sensitive, non-invasive detection of *Cardicola* blood fluke species in Southern Bluefin Tuna (*Thunnus maccoyii*). Molecular and Biochemical Parasitology 191: 7-15.

22. Valdenegro-Vega VA, Crosbie P, Vincent B, Cain KD, Nowak BF (2013) Effect of immunization route on mucosal and systemic immune response in Atlantic salmon (*Salmo salar*). Vet Immunology and Immunopathology 151: 113-123.

23. Polinski M, Bridle A, Nowak B (2013) Temperature-induced transcription of inflammatory mediators and the influence of Hsp70 following LPS stimulation of southern bluefin tuna peripheral blood leukocytes and kidney homogenates. Fish and Shellfish Immunology 34: 1147-1157.

24. Aranda PS, LaJoie DM, Jorcyk CL (2012) Bleach gel: a simple agarose gel for analyzing RNA quality. Electrophoresis 33: 366-369.

25. Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J (2007) qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biology 8: R19.

26. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, et al. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3: 0034.0031-0034.0011.

27. Hellemans J, Vandesompele J (2011) Quantitative PCR data analysis – unlocking the secret to successful results. In: Oswald N, Kennedy S, editors. PCR troubleshooting and optimization: the essential guide. Norwich, UK: Caister Academic Press.

28. Polinski M, Shirakashi S, Bridle A, Nowak B (*In press*) Transcriptional immune response of cage-cultured Pacific bluefin tuna during infection by two Cardicola blood fluke species. Fish and Shellfish Immunology doi: j.fsi.2013.10.008

29. Volff JN (2005) Genome evolution and biodiversity in teleost fish. Heredity 94: 280-294.

30. Sabah AA, Fletcher C, Webbe G, Doenhoff MJ (1985) Schistosoma mansoni: reduced efficacy of chemotherapy in infected T-cell-deprived mice. Experimental Parasitology 60: 348-354.

31. Ogawa K. (2013) Blood fluke infections of farmed Pacific Bluefin tuna. Australian Society of Parasitology Parasites in Mariculture Workshop; Launceston, TAS.

32. van den Biggelaar AHJ, van Ree R, Rodrigues LC, Lell B, Deelder AM, et al. (2000) Decreased atopy in children infected with Schistosoma haematobium: a role for parasite-induced interleukin-10. The Lancet 356: 1723-1727.

33. Walsh KP, Brady MT, Finlay CM, Boon L, Mills KHG (2009) Infection with a Helminth Parasite Attenuates Autoimmunity through TGF-β-Mediated Suppression of Th17 and Th1 Responses. The Journal of Immunology 183: 1577-1586.

34. Tafalla C, Bøgwald J, Dalmo RA (2013) Adjuvants and immunostimulants in fish vaccines: Current knowledge and future perspectives. Fish and Shellfish Immunology 35: 1740-1750.

35. Wack A, Baudner BC, Hilbert AK, Manini I, Nuti S, et al. (2008) Combination adjuvants for the induction of potent, long-lasting antibody and T-cell responses to influenza vaccine in mice. Vaccine 26: 552-561.

36. Shirakashi S, Andrews M, Kishimoto Y, Ishimaru K, Okada T, et al. (2012) Oral treatment of praziquantel as an effective control measure against blood fluke infection in Pacific bluefin tuna (*Thunnus orientalis*). Aquaculture 326–329: 15-19.

Chapter Six

General Discussion

6.1. mRNA identification

One of the primary objectives of this PhD project was to identify bluefin-specific mRNA transcripts useful for gene expression analysis. To this end, a variety of approaches were implemented in the novel identification of bluefin mRNA. This included BLAST search tool alignment comparisons of known gene targets from evolutionarily similar teleost fishes with expressed sequence tag (EST) libraries available for ABT [16] (as described in Chapters 4 and 5). If not identifiable within available EST libraries, methods involving degenerate and non-degenerate primer design targeting consensus sequence of previously identified transcripts from other fish were used (Chapter 2). Although somewhat crude given the current sequencing technologies available, these techniques were cost effective and nonetheless successful in at least partially identifying a total of 18 targeted mRNA sequences of bluefin tuna (Table 6.1); for which 13 have been utilised for gene expression analysis involving immune, cellular stress, and housekeeping roles in the proceeding chapters. This provides a substantial addition to the previous two full length and six partial mRNA transcripts which had been identified for bluefin prior to this project which had severely limited investigations into immune response in this group of fish.

The five protein transcripts which did not directly appropriate into the scope of the previous chapters (PolyUb, Wap65, StAR, P450scc and IGF-1) have thus far been precluded from discussion; however, it is worth mentioning them here as they are likely to prove useful in future work regarding stress and growth related processes in bluefins. For example, the additional monitoring of Wap65-2 may incorporate another aspect for linking temperature related effects to immune functioning in bluefin, as this protein has previously been identified to have both temperature acclimation and immune associated roles in the liver of catfish *Ictalurus punctatus* and appears structurally similar to hemopexins in mammals [24]. Given

the current implications of temperature stress on immune signalling in bluefins as outlined in Chapter 2, this may prove invaluable in assessing thermal tolerance, fever, and immune function during the continued culture of these physiologically unique fish. Further, StAR and P450scc are proteins specifically involved in cortisol production and are considered to be the rate limiting steps of this pathway [25]. The transcriptional expression of these proteins may provide an expanded understanding in the physiological and cellular stress responses in buefins which may be of particular importance as previously increased plasma cortisol levels have been observed in SBT during wild capture [26] and likely during other intensive culture related practices. Lastly, the identification of IGF-1 in SBT brings the first means for monitoring the expression of a growth-related hormone in this group of fishes and thus potential insight into the anabolic pathways within these organisms. This may provide a second avenue for monitoring the impacts of diseases (i.e. loss in growth) such as *Cardicola* spp. infection in bluefin in addition to the direct loss via mortality and inferences from immune responses.

During the early stages of this project interspecies sequence heterogeneity in targeted immune genes was unknown between the three species of bluefin. As a consequence, it was also unclear if primer sets and PCR thermocycling conditions designed for transcriptional profiling from one species would also be effective when using and assessing genetic material from the other two. Moderate sequence divergence in immune genes have been observed in salmonids [27] and can be further complicated by genome duplication events in fish which have resulted in multiple forms of a gene with discrete functional roles [28], such as TNF1 and TNF2 previously identified in PBT [29]. In Chapter 2, interspecies comparisons between SBT and PBT TNFs identified that these transcripts were more than 95% homologous, and sequences of TNF paralogous in ABT have subsequently been identified to also be highly homologous to those of SBT/PBT [30]. Additionally, sequence identified from EST libraries

available for ABT proved sufficient templates for interspecies primer design targeting more than 8 genes which was successful in mapping expression in both PBT (Chapter 4) and SBT (Chapter 5). Indeed, in the course of this research, only primers designed to identify Hsp70 in SBT failed in interspecies application when attempted in gene expression analysis in PBT (data not shown) which appeared due to non-target amplification of Hsc70 (~90bp product) in addition to Hsp70 (~120 bp product) . This was not necessarily surprising, as the sequence homology of Hsc70 and Hsp70 in SBT alone is greater than 70%, leaving limited possibilities for areas of interspecies conservation without also compromising homologue specificity. Nevertheless, taken together this work suggests that high sequence homogeneity is present within the immunological and housekeeping genes within bluefin tunas, and once a gene target has been identified in one species it can likely be used as a template for designing molecular screening analyses for all three species. This will prove highly advantageous in the continued annotation of the bluefin tuna genome, particularly in light of the recent publication for the complete but (as yet mostly un-annotated) genome of PBT [31]. Interspecies utility for assays developed as part of this thesis is also important with regard to the constraints inherent in working with such a rare, highly prized and coveted organism as bluefin tuna. For example, although initial work in this project was done on identifying immune associated mRNA transcripts in SBT, the influence of *Cardicola* infection on host immune responses was conducted in PBT due to their accessibility in a closed life cycle aquaculture environment and the lower relative cost associated with each individual organism. It is likely that continued investigations into the health of bluefin will prove similarly sporadic due to limits in accessibility and therefore the interspecies genetic similarity and ability for encompassing and standardised assay techniques identified here will have valuable timesaving effects on continued research with these organisms.

Table 6.1. Summary of bluefin tuna-specific mRNA sequences identified in the course of this PhD project. Species of origin, GenBank accession numbers, and indication of the complete (C) or partial (P) length mRNA transcript are provided in each instance.

Target	Abrev.	Accession #	Species	Length
Elongation factor-1alpha	EF1α	JX157143	SBT	1,782 bp (C)
Beta actin	βactin	JX157141	SBT	1,858 bp (C)
Ubiquitin	PolyUb	EG999975	ABT	500 bp (P)
Interleukin-1beta	IL-1β	JX157146	SBT	1,316 bp (C)
Interleukin-8	IL-8	JX157147	SBT	918 bp (C)
Tumor necrosis factor 1 alpha	TNF1	JX157148	SBT	1,422 bp (C)
Tumor necrosis factor 2 alpha	TNF2	JX157149	SBT	999 bp (C)
Cyclooxygenase-2	Cox-2	JX157142	SBT	1,267 bp (P)
Immunoglobulin M heavy chain	IgM	EC917943	ABT	591 bp (P)
T cell receptor beta chain	TCR	EC092872	ABT	498 bp (P)
Major histocompatibility complex 1	MHC-1	EH667956	ABT	551 bp (P)
Major histocompatibility complex 2	MHC-2	EH667387	ABT	653 bp (P)
Insulin-like growth factor-1	IGF-1	KF891352	SBT	700 bp (P)
Heat-shock cognate protein (70 kD)	Hsc70	JX157144	SBT	1,929 bp (P)
Heat-shock protein (70 kD)	Hsp70	JX157145	SBT	1,672 bp (P)
Warm-temperature acclimation-related protein 2 (65 kD)	Wap65-2	KF891353	SBT	690 bp (P)
Steroidogenic acute regulatory protein	StAR	KF891354	SBT	492 bp (P)
Cholesterol side-chain cleavage enzyme	P450scc	KF891355	SBT	506 bp (P)

6.2. *Cardicola* spp. detection

The molecular methods developed in this thesis provide a new standard for *Cardicola* spp. detection in bluefin tuna. These qPCR techniques have proved more sensitive and expedient in differentially identifying *Cardicola* spp. within a greater diversity of medium than microscopy techniques previously employed. Nevertheless, the most significant advantage for these molecular detection methods is almost certainly the ability to be applied during non-lethal sampling of bluefin; specifically from blood and blood components. In many aspects, research regarding distribution, timing of infection or clearance versus carrier status for these parasites has been severely limited due to the high economic value given to each cultured individual bluefin and the understandable unwillingness of farmers to submit apparently healthy fish to lethal scientific investigations. However, non-lethal methods for blood sampling present the first potential bridge for monitoring parasitic infection and providing critical information to farmers without sacrificing fish and production goals. These techniques have already proven useful in identifying the high prevalence of *C. orientalis* in ranched SBT previously thought to only be infected with *C. forsteri* (Chapter 3), and the rapid infection of PBT with *C. orientalis* and *C. opisthorchis* following transfer to sea-cage environments (Chapter 4). As wild fish have previously been observed to be infected by *Cardicola* [15], detection of *Cardicola* by qPCR may prove useful in monitoring wild bluefin caught for ranching. These techniques also provide a new instrument for monitoring infection dynamics and a means for monitoring treatment efficacy such as preliminarily implemented in Chapter 4 with regard to Praziquantel.

Whole blood provides the most straightforward medium for identifying parasite DNA by qPCR. However, large amounts of host DNA from nucleated erythrocytes along with

other PCR inhibitors such as haem and Ig [32] present potential pitfalls for utilising or condensing large quantities of blood during pathogen screening. Ammonium acetate protein precipitation coupled with selective alcohol precipitation of nucleic acids was effective in minimizing carryover of heam and protein inhibitors into PCR analysis during the present research project. Experiments incorporating dilution series of extracted *Cardicola* positive samples could be linearly estimated by qPCR without signs of concentration dependent inhibition. Further, the utilisation of TaqMan probes also aided in minimising inhibition caused by the presence of host bluefin DNA (Figure 3.3.); however, during preliminary screening of whole blood samples in Chapter 4 it became quickly evident that elution of DNA at concentrations greater than 2.5 µg/µL was virtually impossible due to viscosity. Concentrations greater than 250 ng/µL were also observed to become inhibitory during TaqMan qPCR, presumably due to polymerase or primer annealing inhabition. Thus, concentrating whole blood (> 5:1) for qPCR in hope of identifying whole parasites is impossible using the current techniques without severely sacrificing sensitivity.

In contrast, the relatively minimal amount of host DNA in the serum component of blood samples allowed for large volumes (1 mL) to be highly concentrated (>100:1) for qPCR analysis and was successful in sensitively identifying *C. forsteri* and *C. orientalis* from ranched SBT (Chapter 3). Although whole organism detection would be precluded by this method as all parasite life stages are of a density similar or greater to host cells and thus would be pelleted with the cellular component, free parasite DNA was easily identified in serum during the screening of SBT and general sensitivity was still greater than previous microscopy methods (Chapter 3; Table 3.3.). Further increased concentration of nucleic acids from serum (possibly > 1,000:1) may also be possible if TaqMan rather than SYBR chemistry is used during analysis given the typical DNA yields obtained in this research which was generally < 1µg/mL in serum. In any case, targeting the non-cellular components

of blood such as serum or plasma currently appears more suited for sensitive detection of *Cardicola* spp. during non-lethal sampling relative to the whole blood component.

In addition to identifying non-lethal sampling methods, the qPCR *Cardicola* detection techniques developed during this PhD project has shed new insight into the distribution of these parasites. This has been evident in identifying multiple *Cardicola* spp. within ranched SBT populations previously considered to be parasitised by only one species (Chapter 3), as well as confirming *Cardicola* spp. organ tropisms in PBT (Chapter 4). Evidence for high prevalence of *C. orientalis* in heart samples was also newly identified even though previous microscopy observations have linked both adult and egg life stages of this species mainly to the gill in both SBT and PBT (Chapter 3 and 4). In concert with the observed systemic prevalence of *Cardicola* DNA, these findings suggest that expanding qPCR *Cardicola* detection to additional immune sensitive organs in future such as kidney, spleen, or lymphoid specific tissues may provide a greater understanding for the distribution of these parasites within the host organism and also the associated immune responses. This may be further supported by the previous historical findings identifying eggs of *C. forsteri* in liver, kidney and spleen of SBT [33]. Lastly, although each bluefin species has demonstrated natural infection with only two of the three *Cardicola* spp., overlaps in susceptibility suggest that all three species of bluefin may be parasitised by all three *Cardicola* spp. given the right circumstances (i.e. environmental parameters and availability of an appropriate intermediate host). Therefore, continued occasional screening for all three *Cardicola* spp. in each species of bluefin will likely prove beneficial for future culture efforts.

6.3. Bluefin gene expression

As part of this project, 13 immune associated mRNA transcripts of bluefin tuna were utilised in gene expression analyses to identify and map various aspects of immune signalling. Of these, 12 were observed to be responsive to immune stimulation, either *in vitro*, *in vivo*, or both (Chapters 2, 4 & 5). The remaining transcript, that of TNF1 identifed in SBT, was constitutively expressed and easily detectable in multiple organ and tissue samples (gill, kidney, spleen, heart, blood) but did not become differentially regulated in response to known immune stimulants such as LPS (Chapter 2), PolyI:C (data not shown) or *Cardicola* infection (Chapter 4). These results are in agreement with previous *in vitro* work conducted with this transcript in leukocytes of PBT which also found expression of this gene unresponsive to LPS and four other well-known mitogenic compounds [29]. Interestingly, both recombinant TNF1 and TNF2 of PBT were demonstrated to produce similar functional proteins and significantly increased phagocytosis of PBLs [29], suggesting similar biological roles between the two paralogues. The physiological importance for the discrete expression profiles for TNF1 and TNF2 in response to mitogenic stimulation remains enigmatic; however it is evident that of these paralogues TNF2 appears to be most useful as a biomarker for monitoring innate immune and inflammatory responses. Additionally, as the presence of these two TNF paralogues likely originated from the tetraploidation event(s) in bony fish [29,34,35], it is also likely that similar paralogues exist (or have existed) for many if not all of the other immune associated genes now identified in bluefin, similar to the multiple paralogues of IL-1β, IL-10 and TGF-β1 that have been identified in rainbow trout [36-38]. Although these paralogues and their possible roles are as yet undiscovered, all 12 of the current immune associated transcripts identified in this thesis for bluefin have nevertheless shown differential responsiveness to stimulation and thus utility as biological markers for investigating immune function in this group of fishes.

In Chapter 2, temperature was shown to influence the timing of an inflammatory response in SBT, and suggested the external gill relative to the internal gut as a potential 'weak point' for infection or injury repair due to a possibly slower inflammatory response resulting from lower environmental temperature. In Chapter 4, natural infection of PBT by *C. orientalis* and *C. opisthorchis* induced a greater number of immune signalling responses including elevated transcription of IL-8, TCR, MHC2, and IgM in the heart, whereas only IgM transcription was induced by similar infection in the gill. This is consistent with much stronger granulomatous response (as observed by histology) in the heart, but it appears unlikely that this discrete response was entirely due to morphology or quantity of pathogen, as high pathogen loads were observed in both organs. Additionally, it is known that both egg and adult fluke life stages infect each of these organs [33,39]. Although at least part of the differential organ response is almost certainly due to differences in cell and tissue composition, it is possible that temperature may also be a contributing factor for retarding a potential inflammatory response in gills of *Cardicola* infected fish. Specifically, a slower induction of IL-8, known for its chemotactic and permeablising properties which aid in immune cell trafficking, could in turn cause a slower or lower response of phagocytic neutrophils, B-cells and T-cells. Further research will be needed in this unique context of heterothermic tuna; however, insight into the effects of temperature in relation to host responses during *Cardicola* infection will prove valuable in understanding the mechanisms involved in this disease.

During *Cardicola* infection of cage-cultured PBT a significant induction of IL-8 was observed in heart of infected fish, but there was a notable lack for up-regulation of other classic inflammation associated signalling molecules including IL-1β and TNFα despite severe pathology which is known to occur in this organ during periods of robust infection [43]. A further lack of inflammatory signalling was observed in similarly infected gill which

showed no significant induction of any of the inflammatory associated cytokines analysed (Figrue 4.3.). In well studied helminth parasites of mammals such as *Schistosoma* spp., host immune signalling can be altered by the parasite to promote induction of host IL-10 [5] and TGF-β [4] – two potent inflammatory cytokine, Th1 immune response, and delayed type sensitivity (DTH) inhibitors. This change in host signalling coupled with active parasite suppression of tegument antigen [13] causes an overall shift in host responses to favour a 'modifed' Th2 versus a Th1 response with suppressed inflammation which appears to benefit parasite subsistence [7-9] (Figure 6.1. A). Although the ability of *Cardicola* spp. to suppress tegument antigen or increase host IL-10 and TGF-β in bluefin is unknown, the ineffectiveness of a bluefin antibody response to clear a primary infection [15,23] despite increased IgM transcription and the apparent lack of inflammatory cytokine and Th1 specific responses in infected organs (Chapter 4) suggest that *Cardicola* spp. may implement similar evolutionary strategies to their mammalian counterparts (Figure 6.1. B).

Treatment of cage-cultured PBT with PZQ caused significant reductions in *C. orientalis* and *C. opisthorchis* DNA within host organs as measured 28 days post treatment (Figure 4.2.). This trend was mirrored to some degree by host immune gene expression which was generally slightly lower 28 days post treatment than recorded immediately prior to PZQ treatment (Figure 4.4.), suggesting that the PBT immune response was targeted at adult and not eggs of *C. orientalis* and *C. opisthorchis*. This was based on previous observations identifying PZQ to have minimal toxicity to the egg life stage of *Cardicola* [23]. Although PZQ appeared ultimately effective at decreasing *Cardicola* induced mortality and recovery of SBT and PBT [21,23], the mechanisms of action for this drug remain somewhat unclear. Previous findings have identified a direct killing ability of PZQ on helminths [20] and specifically *C. forsteri* [21]; however, an increased efficacy of this drug has been observed *in vivo* indicating increased (or at least more effective) host immune functions [18,40]. In the

present research, expression of immune associated genes including IgM, TCR, and inflammatory mediators were not significantly elevated in PBT three days post PZQ treatment relative to similar cohorts which had yet to be treated (Chapter 4). This suggested that, at least at three days post treatment, PZQ did not noticeably elevate host immune signalling. This was not entirely unsurprising, as PZQ is known to be cleared quickly (within 24h) from within blood and internal organs of tuna [41] and it is possible that PZQ induced immune responses against *Cardicola* infection would have also become reduced to previous levels over a three day period. However, PZQ was shown to significantly induce a variety of immune signalling transcription in both SBT and in Atlantic salmon cell cultures following 6 h incubation, identifying a direct short term immunomodulatory ability of PZQ in these early vertebrate cells (Chapter 5). Additionally, work involving the application of PZQ to treat *Dactylogyrus intermedius* infection of goldfish has also been shown to enhance host immune functions that likely aids in parasite clearance [42]. Taken together, these finding suggest that PZQ increases host immune responses, both parasite specific and independent, which ultimately enhances clearance of parasitic organism as seen in the treatment of *Schistosoma* infection of mammals (Figure 6.1.). It would also appear that such benefits may be short lived, and further research will be needed to elucidate the benefits (if any) of prolonged application of PZQ, either as a treatment for helmith parasites such as *Cardicola* infection of bluefin tuna, or as a potential immunestimulant / adjuvant in a general context of aquaculture.

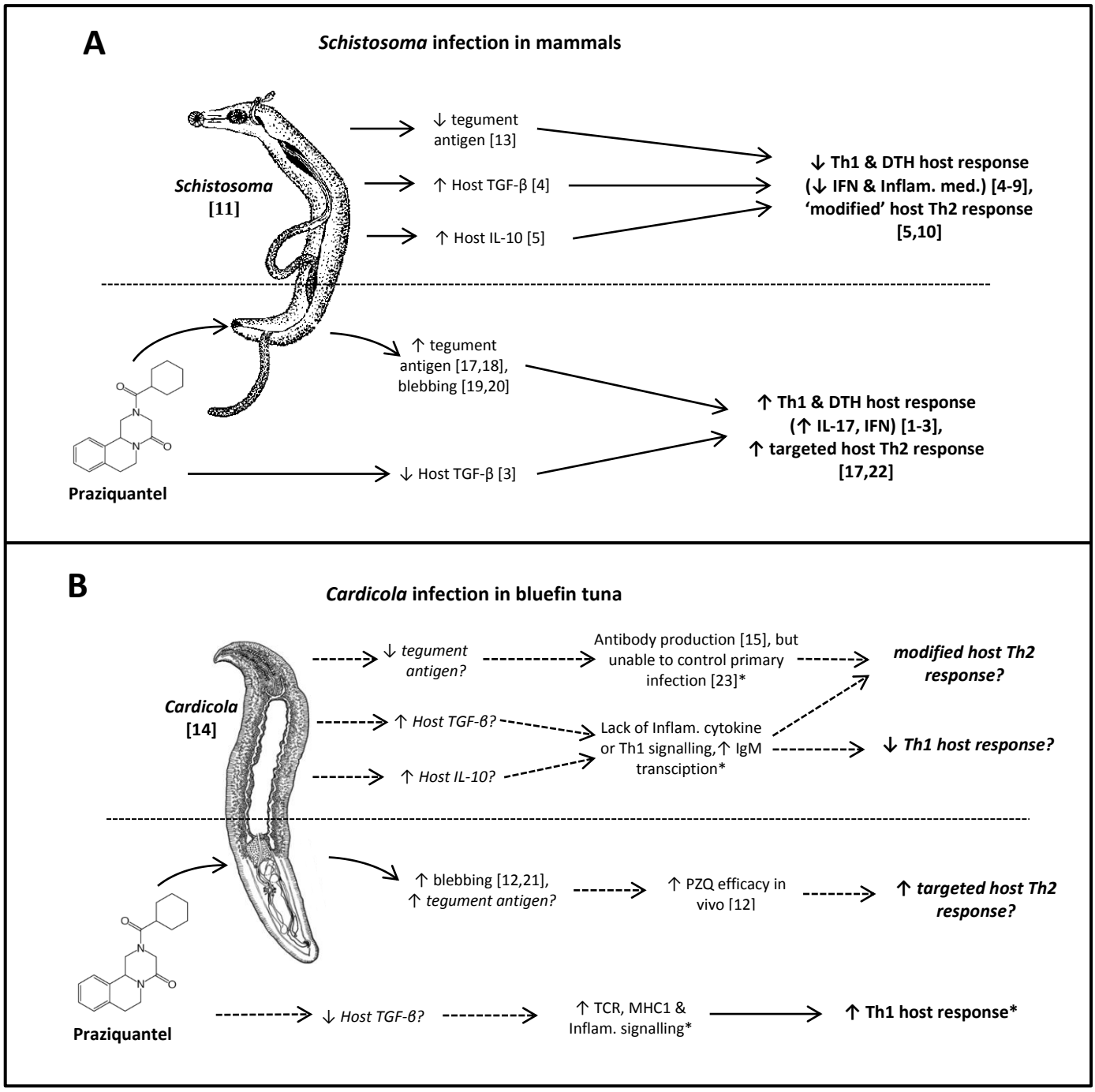


Figure 6.1 Changes in mammalian immune responses to *Schistosoma* (A) and bluefin tuna responses to *Cardicola* (B) infection as well as those induced by Praziquantel. Confirmed signalling pathways (solid arrow, normal text) are distinquished from unconfirmed pathways (dotted arrows, italisised text). * indicates findings first reported in this thesis.

6.4. Concluding statement

Until now molecular methods have not been fully implemented in research of bluefin tuna diseases. This thesis provides a strong initial step for introducing qPCR techniques to disease diagnosis and studies of these economically important organisms as well and new and important insight regarding disease and immune functions of these fish. As culture efforts continue to expand, it is expected that the gene targets, *Cardicola* detection methods, and the initial gene expression analyses conducted in this thesis will contribute significantly in continued development for sustainable wild and cultured bluefin tuna populations in future.

6.5. References

1. Zou Q, Hu Y, Xue J, Fan X, Jin Y, et al. (2012) Use of praziquantel as an adjuvant enhances protection and Tc-17 responses to killed H5N1 virus vaccine in mice. PLoS One 7: e34865.

2. Zou Q, Yao X, Feng J, Yin Z, Flavell R, et al. (2011) Praziquantel facilitates IFN-γ-producing CD8+ T Cells (Tc1) and IL-17-producing CD8+ T Cells (Tc17) responses to DNA vaccination in mice. PLoS One e25525.

3. Zou Q, Zhong Y, Su H, Kang Y, Jin J, et al. (2010) Enhancement of humoral and cellular responses to HBsAg DNA vaccination by immunization with praziquantel through inhibition TGF-β/Smad2,3 signaling. Vaccine 28: 2032-2038.

4. Graham BB, Chabon J, Gebreab L, Poole J, Debella E, et al. (2013) Transforming growth factor-beta signaling promotes pulmonary hypertension caused by *Schistosoma mansoni*. Circulation 128: 1354-1364.

5. van den Biggelaar AHJ, van Ree R, Rodrigues LC, Lell B, Deelder AM, et al. (2000) Decreased atopy in children infected with *Schistosoma haematobium*: a role for parasite-induced interleukin-10. The Lancet 356: 1723-1727.

6. Watanabe K, Mwinzi PN, Black CL, Muok EM, Karanja DM, et al. (2007) T regulatory cell levels decrease in people infected with *Schistosoma mansoni* on effective treatment. American Journal of Topical Medicine and Hygiene 77: 676-682.

7. Hewitson J, Grainger J, Maizels R (2009) Helminth immunoregulation: The role of parasite secreted proteins in modulating host immunity. Molecular and Biochemical Parasitology 167: 1-11.

8. Jackson JA, Friberg IM, Little S, Bradley JE (2009) Review series on helminths, immune modulation and the hygiene hypothesis: immunity against helminths and immunological phenomena in modern human populations: coevolutionary legacies? Immunology 126: 18-27.

9. Walsh KP, Brady MT, Finlay CM, Boon L, Mills KHG (2009) Infection with a helminth parasite attenuates autoimmunity through TGF-β-mediated suppression of Th17 and Th1 responses. The Journal of Immunology 183: 1577-1586.

10. Harrison RA, Doenhoff MJ (1983) Retarded development of *Schistosoma mansoni* in immunosuppressed mice. Parasitology 86: 429-438.

11. International Labour Organization (1979) Guide to health and hygiene in agricultural work. Geneva: International Labour Office. 328 p.

12. Ogawa K. (2013) Blood fluke infections of farmed Pacific bluefin tuna. Australian Society of Parasitoloyg Parasitic Diseases in Fish Mariculture Workshop. Launceston, TAS.

13. Dean DA (1977) Decreased binding of cytotoxic antibody by developing *Schistosoma mansoni*. Evidence for a surface change independent of host antigen adsorption and membrane turnover. The Journal of parasitology: 418-426.

14. Ogawa K, Tanaka S, Sugihara Y, Takami I (2010) A new blood fluke of the genus *Cardicola* (Trematoda: Sanguinicolidae) from Pacific bluefin tuna *Thunnus orientalis* (Temminck and Schlegel, 1844) cultured in Japan. Parasitology International 59: 44-48.

15. Aiken HM, Hayward CJ, Crosbie P, Watts M, Nowak BF (2008) Serological evidence of an antibody response in farmed southern bluefin tuna naturally infected with the blood fluke *Cardicola forsteri*. Fish and Shellfish Immunology 25: 66-75.

16. Chini V, Cattaneo AG, Rossi F, Bernardini G, Terova G, et al. (2008) Genes expressed in Blue Fin Tuna (*Thunnus thynnus*) liver and gonads. Gene 410: 207-213.

17. Brindley PJ, Sher A (1987) The chemotherapeutic effect of praziquantel against *Schistosoma mansoni* is dependent on host antibody response. The Journal of Immunology 139: 215-220.

18. Harnett W, Kusel JR (1986) Increased exposure of parasite antigens at the surface of adult male *Schistosoma mansoni* exposed to praziquantel *in vitro*. Parasitology 93: 401-405.

19. Becker B, Mehlhorn H, Andrews P, Thomas H, Eckert J (1980) Light and electron microscopic studies on the effect of praziquantel on *Schistosoma mansoni*, *Dicrocoelium dendriticum*, and *Fasciola hepatica* (Trematoda) *in vitro*. Z Parasitenkd 63: 113-128.

20. Gönnert R, Andrews P (1977) Praziquantel, a new broad-spectrum antischistosomal agent. Zeitschrift für Parasitenkunde 52: 129-150.

21. Hardy-Smith P, Ellis D, Humphrey J, Evans M, Evans D, et al. (2012) *In vitro* and *in vivo* efficacy of anthelmintic compounds against blood fluke (*Cardicola forsteri*). Aquaculture 334–337: 39-44.

22. Joseph S, Jones FM, Walter K, Fulford AJ, Kimani G, et al. (2004) Increases in human T helper 2 cytokine responses to *Schistosoma mansoni* worm and worm-tegument antigens are induced by treatment with praziquantel. Journal of Infectious Diseases 190: 835-842.

23. Shirakashi S, Andrews M, Kishimoto Y, Ishimaru K, Okada T, et al. (2012) Oral treatment of praziquantel as an effective control measure against blood fluke infection in Pacific bluefin tuna (*Thunnus orientalis*). Aquaculture 326–329: 15-19.

24. Sha Z, Xu P, Takano T, Liu H, Terhune J, et al. (2008) The warm temperature acclimation protein Wap65 as an immune response gene: Its duplicates are differentially regulated by temperature and bacterial infections. Molecular Immunology 45: 1458-1469.

25. Aluru N, Vijayan MM (2006) Aryl hydrocarbon receptor activation impairs cortisol response to stress in rainbow trout by disrupting the rate-limiting steps in steroidogenesis. Endocrinology 147: 1895-1903.

26. Kirchhoff NT, D'Antignana T, Leef MJ, Hayward CJ, Wilkinson RJ, et al. (2011) Effects of immunostimulants on ranched southern bluefin tuna *Thunnus maccoyii*: immune response, health and performance. J Fish Biol 79: 331-355.

27. Husain M, Bird S, Zwieten Rv, Secombes CJ, Wang T (2012) Cloning of the IL-1β3 gene and IL-1β4 pseudogene in salmonids uncovers a second type of IL-1β gene in teleost fish. Developmental and Comparative Immunology 38: 431-446.

28. Peatman E, Liu Z (2007) Evolution of CC chemokines in teleost fish: a case study in gene duplication and implications for immune diversity. Immunogenetics 59: 613-623.

29. Kadowaki T, Harada H, Sawada Y, Kohchi C, Soma G-I, et al. (2009) Two types of tumor necrosis factor-α in bluefin tuna (*Thunnus orientalis*) genes: Molecular cloning and expression profile in response to several immunological stimulants. Fish and Shellfish Immunology 27: 585-594.

30. Lepen Pleić I, Secombes CJ, Bird S, Mladineo I Characterization of three pro-inflammatory cytokines, TNFα1, TNFα2 and IL-1β, in cage-reared Atlantic bluefin tuna *Thunnus thynnus*. Fish and Shellfish Immunology.

31. Nakamura Y, Mori K, Saitoh K, Oshima K, Mekuchi M, et al. (2013) Evolutionary changes of multiple visual pigment genes in the complete genome of Pacific bluefin tuna. Proceedings of the National Academy of Sciences 110: 11061-11066.

32. Al-Soud WA, Rådström P (2001) Purification and characterization of PCR-inhibitory components in blood cells. Journal of Clinical Microbiology 39: 485-493.

33. Colquitt SE, Munday BL, Daintith M (2001) Pathological findings in southern bluefin tuna, *Thunnus maccoyii* (Castelnau), infected with *Cardicola forsteri* (Cribb, Daintith & Munday, 2000) (Digenea: Sanguinicolidae), a blood fluke. Journal of Fish Diseases 24: 225-229.

34. Haugland Ø, Mercy IS, Romøren K, Torgersen J, Evensen Ø (2007) Differential expression profiles and gene structure of two tumor necrosis factor-α variants in Atlantic salmon (*Salmo salar* L.). Molecular Immunology 44: 1652-1663.

35. Zou J, Wang T, Hirono I, Aoki T, Inagawa H, et al. (2002) Differential expression of two tumor necrosis factor genes in rainbow trout, *Oncorhynchus mykiss*. Developmental and Comparative Immunology 26: 161-172.

36. Pleguezuelos O, Zou J, Cunningham C, Secombes CJ (2000) Cloning, sequencing, and analysis of expression of a second IL-1 β gene in rainbow trout (*Oncorhynchus mykiss*). Immunogenetics 51: 1002-1011.

37. Harun NO, Costa MM, Secombes CJ, Wang T (2011) Sequencing of a second interleukin-10 gene in rainbow trout *Oncorhynchus mykiss* and comparative investigation of the expression and modulation of the paralogues *in vitro* and *in vivo*. Fish and Shellfish Immunology 31: 107-117.

38. Maehr T, Costa MM, Vecino JL, Wadsworth S, Martin SA, et al. (2013) Transforming growth factor-beta1b: a second TGF-beta1 paralogue in the rainbow trout (*Oncorhynchus mykiss*) that has a lower constitutive expression but is more responsive to immune stimulation. Fish and Shellfish Immunology 34: 420-432.

39. Shirakashi S, Kishimoto Y, Kinami R, Katano H, Ishimaru K, et al. (2012) Morphology and distribution of blood fluke eggs and associated pathology in the gills of cultured Pacific bluefin tuna, *Thunnus orientalis*. Parasitology International 61: 242-249.

40. Doenhoff MJ, Cioli D, Utzinger J (2008) Praziquantel: mechanisms of action, resistance and new derivatives for schistosomiasis. Current Opinion in Infectious Diseases 21: 659-667.

41. Ishimaru K, Mine R, Shirakashi S, Kaneko E, Kubono K, et al. (2013) Praziquantel treatment against *Cardicola* blood flukes: Determination of the minimal effective dose and pharmacokinetics in juvenile Pacific bluefin tuna. Aquaculture 402–403: 24-27.

42. Zhang C, Ling F, Chi C, Wang G-X (2013) Effects of praziquantel and sanguinarine on expression of immune genes and susceptibility to *Aeromonas hydrophila* in goldfish (*Carassius auratus*) infected with *Dactylogyrus intermedius*. Fish and Shellfish Immunology 35: 1301-1308.

43. Shirakashi S, Ishimaru K, Okada T, Sawada Y, Ogawa K (2012) Control of blood fluke infection in cultured Pacific bluefin tuna, *Thunnus orientalis*, in Japan. Australian Society of Parasitology annual Conference, Launceston, Australia.