Aquaculture 487 (2018) 1–6

Contents lists available at ScienceDirect

Aquaculture

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

Annual variability of infection with *Cardicola forsteri* and *Cardicola orientalis* in ranched and wild southern bluefin tuna (*Thunnus maccoyii*)☆

L. Neumann*, A. Bridle[1], M. Leef, B. Nowak*

IMAS, University of Tasmania, Australia

ARTICLE INFO

Keywords:
Cardicola
Blood fluke
Tuna
qPCR
Quantification
Wild
Ranched

ABSTRACT

Infections by blood flukes from the genus *Cardicola* are the primary health concern in ranched bluefin tuna. This study explores annual variability of infection by *C. forsteri* and *C. orientalis* in ranched southern bluefin tuna (SBT) from Port Lincoln, South Australia, as well as in wild SBT caught at Pedra Branca, Tasmania. A hydrolysis probe-based qPCR assay was developed and validated for the quantification of *C. orientalis*, *C. forsteri*, and *C. opisthorchis* ITS2 rDNA. Having identified and confirmed *C. forsteri* and *C. orientalis* as the two species known to infect SBT the assay was used to quantify their ITS2 rDNA in SBT heart and gill samples from 2013 to 2015. Significant annual variability of infection in ranched SBT hearts was identified and *C. orientalis* was only detected in the 2013 samples. This study also presents the first detection of *C. orientalis* in wild SBT.

Significance:

- Validation of a hydrolysis probe-based qPCR assay for the detection and quantification of *C. orientalis*, *C. forsteri*, and *C. opisthorchis* ITS2 rDNA
- First detection of *C. orientalis* in wild SBT
- Significant annual variability in detection of *Cardicola forsteri* and *Cardicola orientalis* in ranched SBT heart from 2013, 2014, and 2015 harvests

1. Introduction

Southern bluefin tuna (SBT) (*Thunnus maccoyii*) is a highly profitable and important species to the Australian seafood industry (ABARES, 2013). In recent decades the SBT fishery has moved toward ranching to augment total saleable yields while abiding by annual wild catch quotas (AFMA, 2007). Wild juvenile SBT are caught in the Great Australian Bight and are towed in cages to ranching sites near Port Lincoln (Kirchhoff et al., 2011; Kirchhoff et al., 2014; Nan et al., 2016). The SBT are transferred to grow-out sea cages where they are fed daily to maximize growth before harvest, this process generally increases individual fish weight by approximately 10–20 kg over 6 months (Volpe, 2005). The primary health factor affecting ranched SBT in Australia is infection by blood flukes from the genus *Cardicola* (Trematoda: Aporocotylidae) (Colquitt et al., 2001; Cribb et al., 2000; Munday et al., 2003). Seasonal mortalities of SBT have been attributed to infections with *Cardicola forsteri* and *Cardicola orientalis* (Cribb, 2000; Deveney et al., 2005; Dennis et al., 2011). Similarly in Japanese cultured PBT, infection with *C. orientalis* is one of the main causes of mortality in hatchery-reared juveniles (Shirakashi et al., 2012b; Sugihara et al., 2016). Infections with *C. forsteri*, *C. orientalis* and *C. opisthorchis* are the primary health concern in sea-cage reared Atlantic bluefin tuna (ABT), though no significant difference in the level of blood fluke infections has been documented between wild and ranched fish (Forte-Gil et al., 2016).

At Kinki University in Japan, > 50% of juvenile PBT mortality has been associated with blood fluke infection, with infection prevalence reaching 100% within 3 months of transfer to sea cages (Ishimaru et al., 2013). Since 2012 the anthelmintic praziquantel (PZQ) has been used as an effective treatment method of controlling blood fluke infection in farmed and ranched tuna (Ishimaru et al., 2013; Shirakashi et al., 2012a). A study at Kinki University investigating PZQ doses found that an administered 15 and 150 mg kg^{-1} bodyweight (BW) PZQ eradicated adult worms within 8 days post treatment (Shirakashi et al., 2012a). PZQ has been used in the Australian SBT industry since early 2013, since then annual mortality has been reported at < 1%, much lower than 10–15% in previous years (Polinski et al., 2014). This study examined SBT from the first three consecutive years after the introduction

☆ This research was supported by John Bicknell Scholarship.

* Corresponding authors at: IMAS, University of Tasmania, Locked Bag 1370, Launceston, TAS 7250, Australia.
E-mail addresses: lukas.neumann@utas.edu.au (L. Neumann), bnowak@utas.edu.au (B. Nowak).
[1] Contributed equally to this work: Lukas Neumann, Andrew Bridle.

https://doi.org/10.1016/j.aquaculture.2017.12.042
Received 30 September 2017; Received in revised form 17 December 2017; Accepted 26 December 2017
Available online 27 December 2017

Table 1
Oligonucleotide primers and probes used to amplify *Cardicola forsteri*, *Cardicola orientalis*, and *Cardicola opisthorchis* for real-time qPCR detection.

Target	GenBank accession #	Name	Amplicon size	Sequence (5′- 3′)
C. forsteri (ITS2 rDNA)	EF661575	Cfor_F	287 bp	TGATTGCTTGCTTTTTCTCGAT
		Cfor_R		TATCAAAACATCAATCGACATC
		Cfor_probe		HEX – CCACGACCTGAGCACAAGCCG – BHQ1
C. orientalis(ITS2 rDNA)	HQ324226	Cori_F	191 bp	TGCTTGCTATTCCTAGATGTTTAC
		Cori_R		AACAACTATACTAAGCCACAA
		Cori_probe		HEX – CACAAGCCGCTACCACAATTCCACTC – BHQ1
C. opisthorchis (ITS2 rDNA)	HQ324228	Copis_F	272 bp	TTCCTAAATGTGTGTGCA
		Copis_R		TCAAAACATCAATCGACACT
		Copis_probe		HEX – CACGACCTGAGCACAAGCCG – BHQ1

of PZQ treatment.

Research concerning blood fluke detection and annual variation in prevalence has historically relied on a limited suite of methods including histology and detection of adult blood flukes in heart flushes (Bullard et al., 2004; Colquitt et al., 2001; Cribb, 2000; Dennis et al., 2011; Deveney et al., 2005; Kirchhoff et al., 2012; Kirchhoff et al., 2014; dos Santos et al., 2012; Shirakashi et al., 2013). Histological analysis permits the quantification of eggs and granulomas in hematoxylin and eosin (H&E) stained organ sections without the ability to identify which species is being detected. Heart flushes are used to quantify adult blood flukes in the whole heart (*C. forsteri* adults), though differentiation of *C. forsteri* and *C. orientalis* is possible, this requires time and experience (Aiken, 2009). *C. forsteri* and *C. orientalis* can be detected and differentiated to species using PCR analysis, even in samples containing trace amounts of the parasite's DNA thereby surpassing every other method with its detection sensitivity, specificity and speed (Polinski et al., 2013a; Polinski et al., 2013b).

This study documents annual variability of wild and ranched SBT infection with *C. forsteri and C. orientalis* infection and prevalence in wild and ranched SBT using a highly sensitive and specific quantitative PCR (qPCR) to analyze samples from three consecutive annual harvests following the introduction of PZQ in 2013.

2. Materials and methods

2.1. Ethics statement

All wild SBT sampling procedures were approved by the University of Tasmania Animal Ethics Committee (A0013175).

2.2. Sample collection and processing

Samples of organs from ranched SBT were collected at the same time of each year (early July), during commercial SBT harvests with the same company which used the same husbandry practices over those years, in Port Lincoln (Coordinates: 34°41′26.6″S 135°57′37.8″E), South Australia in 2013 (n = 50), 2014 (n = 60), and 2015 (n = 40). Wild SBT were caught between January and May 2014 (n = 30) and 2015 (n = 16) by trolling in waters surrounding Pedra Branca, Tasmania.

A 5 cm × 5 cm section of filaments from the center region of the second left gill arch was placed in an individual sealed bag and stored on ice (dos Santos et al., 2012; Shirakashi et al., 2012b). A 0.5 cm^3 piece of heart (taken from near the apex of the ventricle) and additional gill filaments from the center region of the second left gill arch were preserved in 1 mL RNA preservation reagent (4 M ammonium sulphate, 25 mM sodium citrate, 10 mM EDTA, pH 5.2) and placed on ice for subsequent DNA extraction. After taking the sample for molecular analysis, the heart was placed in a 450 mL plastic container and kept on ice until heart flush processing upon arrival at the laboratory 3–4 h later.

For histology, a 1 cm × 1 cm piece of the 5 cm × 5 cm piece of gill was placed in 10% neutral-buffered formalin (NBF). A thin section of heart 0.5 cm × 0.5 cm comprised of both compacta and spongy muscle from near the apex was placed in 10% NBF for histology. After 24 h fixation, the samples were transferred to 70% ethanol. The samples were dehydrated using a sequence of alcohols at increasing concentrations (80%, 95% and 100%), embedded in paraffin, sectioned at 5 μm and then stained with (H&E) using standard methods (Brown, 2002). Gill samples were decalcified in rapid decalcifying fluid (Australian Bio-stain, VIC, Australia) for 2 h prior to processing.

Heart flushes were conducted for quantification of adult *Cardicola* spp. in each heart (Aiken et al., 2006; Colquitt et al., 2001). A vertical incision was made, partially exposing the bulbous arteriosus, ventricle and atrium without cutting the heart in half. Vertical and horizontal cuts were made in the ventricle without cutting through the muscle. The two halves were then washed with a 50:50 mixture of sea water and fresh water, which was collected and poured into clean petri dishes (3–5 per heart) and left for 10 min to allow the red blood cells to settle. The dishes were then examined for adult flukes using a dissecting microscope and quantified.

2.3. Primer and probe design

The F and R primers used in this study (Table 1) were designed in a previous study, which confirmed their specificity (Polinski et al., 2013b). The probes were designed using Beacon Designer™ 8 (Premier Biosoft, CA, USA) and Geneious® 6 software. Probes were targeted against heterogeneous areas of the internal transcribed spacer-2 (ITS2) region of rDNA specific to *C. forsteri*, *C. orientalis*, and *C. opisthorchis* available on GenBank (Aiken et al., 2007; Cribb et al., 2011; Ogawa et al., 2010; Shirakashi et al., 2013).

2.4. Nucleic acid extraction

Total nucleic acid (TNA) was extracted from 10 mg of RNA preservation reagent preserved gill and heart samples. Each sample was suspended in 500 μL extraction buffer (4 M Urea, 1% SDS, 0.2 M NaCl, 1 mM Na Citrate) containing 5 μL Proteinase K (Bioline, NSW, Australia) and incubated at 37 °C for 1 h with a 5 s vortex every 10 min. Samples were then cooled on ice for 5 min; protein was precipitated by adding 350 μL 7.5 M ammonium acetate, 20 s vortex and centrifugation at 16,000 × *g* for 3 min at 18 °C. TNA was subsequently precipitated from the supernatant by adding an equal volume of isopropanol containing co-precipitant pink (Bioline) followed by centrifugation at 16,000 x g for 30 min. The resulting TNA pellet was rinsed twice with 1 mL 75% ethanol and re-suspended in 35 μL nuclease-free buffered water (0.05% Triton X-100, 10 mM Tris, pH 7.5). A 1:10 dilution of the original re-suspension was used for detection and quantification.

2.5. Real-time PCR

A CFX Connect Real-Time PCR Detection System (Bio-Rad, NSW, Australia) was used for real-time qPCR analyses. For *Cardicola* spp. detection, 2 × MyTaq™ HS mix (Bioline) was used in combination with

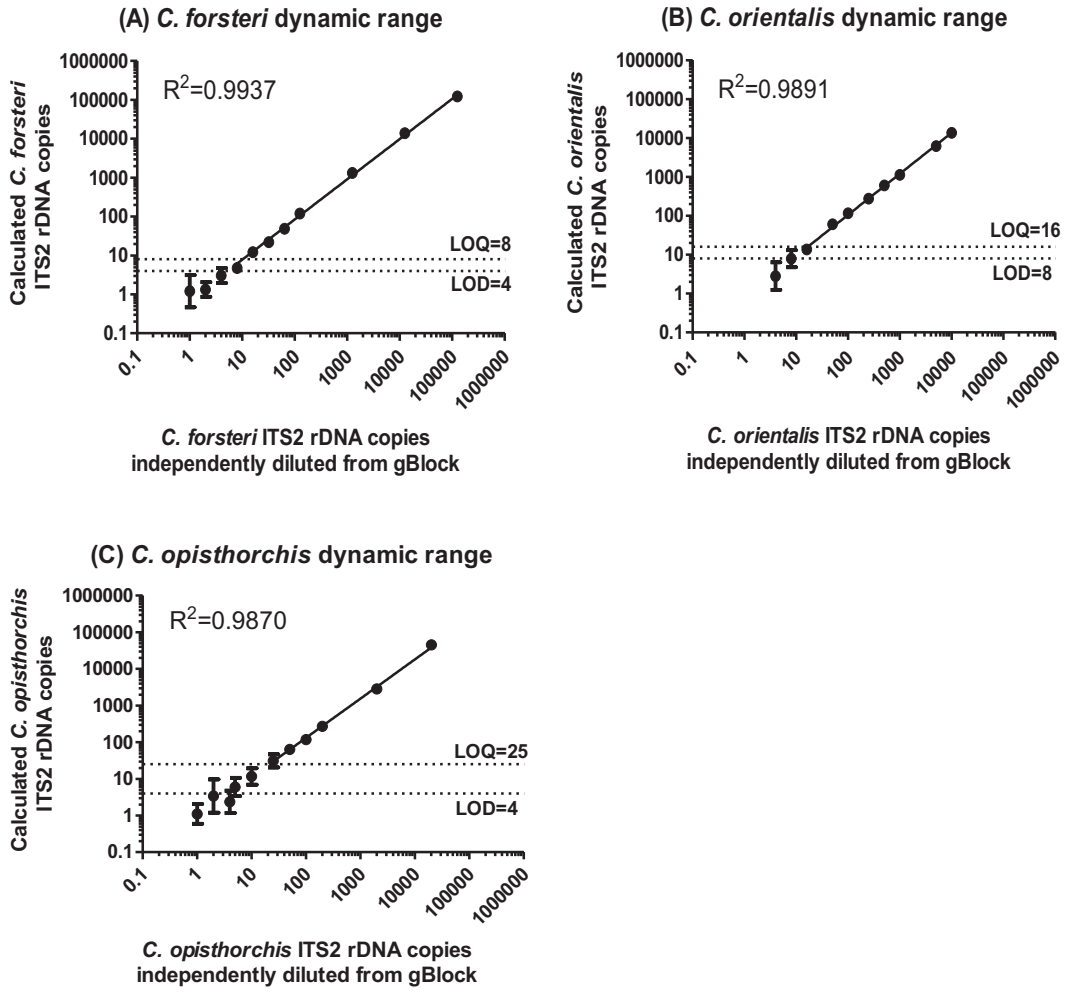

Fig. 1. The dynamic range, limit of quantification (LOQ) and limit of detection (LOD) for the three *Cardicola* species-specific hydrolysis probed-based quantitative real-time PCR assays. Data were obtained using 8 independently replicated dilutions of at least 10 concentrations of a synthetic double-stranded gBlock DNA standard. Data shown are the mean (± 95% CI)

150 nM species-specific probe; 400 nM forward and reverse primer, and molecular water in each 10 μL reaction volume. Probes were labeled at the 5′ end with 6-carboxy-2,4,4,5,7,7-hexachlorofluorescein succinimidyl ester (HEX) together with a Black Hole Quencher® (BHQ1, Biosearch Technologies, CA, USA) added to the 3′ terminus. Cycling conditions comprised an activation of DNA polymerase at 95 °C for 3 min, followed by 45 cycles of 10 s at 95 °C and 30 s at 60 °C, with relative fluorescence measured at the end of each 60 °C combined annealing and extension step.

2.6. Validation of assay: limit of detection (LOD), limit of quantification (LOQ) and copy number calculation

The limit of detection (LOD) for each assay was defined as the lowest analyte concentration at which at least 95% of the independent biological replicates were positive. The limit of quantification (LOQ) refers to the lowest independent analyte dilution that can be reliably quantified with a mean coefficient of variance (CV) < 35% (Armbruster and Pry, 2008). Both the LOD and LOQ for each assay were determined using 8 replicates of at least 10 DNA ITS2 copy number concentrations each independently diluted from a synthetic double stranded gBlock DNA standard. The overall range of these concentrations was 1–1.25 × 10^5 copies. Target DNA used for the each of the dilutions consisted of a synthetic double-stranded DNA named a gBlock® that was manufactured by Integrated DNA Technologies (Iowa, USA). Each of the gBlock®s contained the respective *Cardicola* spp. specific 287 bp (*C. forsteri*), 191 bp (*C. orientalis*), and 272 bp (*C. opisthorchis*) ITS2 rDNA sequences flanked by 413 bp, 333 bp and 248 bp of non-specific DNA, respectively. Each gBlock sequence was verified by the manufacturer and accurately supplied as a 200 ng quantity that was converted to a copy number per mass using a nucleotide specific DNA copy number calculator (http://www.endmemo.com/bio/dnacopynum.php).

Calculated analyte concentrations were determined using a mechanistic model termed 'cm3' developed by Carr and Moore (2012) that is incorporated into the qpcR package (Ritz and Spiess, 2008) within R software v3.2.2 (R Core Team, 2013) that was manipulated using R Studio© v0.99.902 software (RStudio Team, 2015). The cm3 mechanistic model uses fluorescence signals of later cycles and considers variables such as the influence of baseline adjustment errors, reaction inefficiencies, signal loss per cycle and template abundance, thereby making it independent of qPCR reaction efficiencies that are a common source of error when using existing methods based on the crossing threshold (Cq or Ct). The D0 parameter derived from the cm3 model was calibrated for each assay using a minimum of 16 independently replicated gBlock dilutions each estimated to contain 1 copy. These gBlock dilutions allowed a more precise and accurate copy number to be established when an algorithm was used that models the number of unamplified nil qPCR reactions to the amplified positive qPCR reactions using a Poisson distribution analogous to digital PCR (Rutledge and Stewart, 2010).

2.7. Statistical analysis

GraphPad Prism 5 (GraphPad software, CA, USA) was used to perform one-way analysis of variance (ANOVA). Tukey's posthoc test was used to determine significant difference between *C. forsteri* ITS2 rDNA between years in gill and heart. Linear regression analysis was used to determine the relationship between independent gBlock dilutions and calculated DNA copies. Correlation analysis was used to compare detected *C. forsteri* ITS2 rDNA calculated in heart/gill and adult flukes counted using heart flush microscopy in the same fish. A $P < 0.05$ was considered significant. Shapiro-Wilk's W test was used to test assumption of normality. Levene's F test was used to confirm homogeneity of variances.

3. Results

3.1. Limit of detection (LOD) and limit of quantification (LOQ) for qPCR

The assay limit of quantification with a 95% confidence level was 8 copies when quantifying *C. forsteri*, 16 copies for *C. orientalis* and 25 copies for *C. opisthorchis* (Fig. 1.A–C). The limit of detection, where the assay reliably detected expected ITS2 rDNA numbers was 4 copies for *C. forsteri* and *C. opisthorchis*, and 8 copies for *C. orientalis* (Fig. 1).

3.2. Detection of C. forsteri and C. orientalis in SBT

In heart of ranched SBT, *C. forsteri* was detected in 97.1% of samples in 2013, 98.3% in 2014 and 60% in 2015 using qPCR (Table 2). Being limited to visual identification of *Cardicola* spp. eggs, histological examination of heart sections of the same fish presented an egg prevalence of 10% in 2013, 73% in 2014 and 17.5% in 2015. No *Cardicola* eggs were seen in 2013 gill histology, whereas qPCR analysis of respective gills showed a 100% prevalence of *C. forsteri* and 15.7% *C. orientalis*. Histological examination of 2014 and 2015 gills, presented an egg prevalence of 68% and 5%, respectively. qPCR analysis showed a *C. forsteri* prevalence of 18.3% in 2014 and 95% in 2015 gill, and no *C. orientalis* was detected in ranched SBT heart and gill in 2014 and 2015. Further, qPCR analysis of 2013 heart and gill samples showed a mean ITS2 rDNA copy number/mg of 27.26 (SD 8.69) in heart and 72.34 (SD 41.39) in gill when present (intensity of infection). Of the 2014 heart samples, 20 were examined for *C. opisthorchis* using qPCR, and all were negative. No further harvest samples were examined for *C. opisthorchis* ITS2 rDNA.

Analysis of variance followed by Tukey's posthoc analysis showed a significant difference between *C. forsteri* ITS2 rDNA copy number/mg in ranched SBT 2014 heart samples compared to 2013 and 2015 while there was no significant effect of year on gill *C. forsteri* ITS2 rDNA copy number/mg (Fig. 2). *C. forsteri* ITS2 rDNA copy numbers were significantly higher in 2013 ranched SBT heart and gill compared to *C. orientalis* of the same year.

No *Cardicola* eggs were detected via histology (heart) and qPCR ITS2 rDNA analysis (heart and gill) in wild SBT (n = 30) in 2014. Real-time qPCR analysis of 2015 wild SBT (n = 16) heart and gill, showed the presence of *C. forsteri* in 3 fish (18.8%) and *C. orientalis* in 2 fish (12.5%). To the best of our knowledge this is the first detection of *C. forsteri* in wild SBT caught along the Tasmanian coast, and the first case of *C. orientalis* in wild SBT.

There was no association between calculated *C. forsteri* ITS2 rDNA copy number/mg of heart and adult fluke numbers in heart of the same individual SBT ($R^2 = 0.0407$, $P = 0.1254$).

Table 2
Prevalence (%) of *C. forsteri* and *C. orientalis* in heart flush microscopy, histological examination and qPCR analysis of heart and gill of samples collected in 2013, 2014, and 2015 ranched SBT harvest.

	Method	Target	2013 (n = 50)	2014 (n = 60)	2015 (n = 40)
Heart	Heart flush	Adult flukes	48%	76.6%	40%
	Histology	Eggs	10%	73.3%	17.5%
	qPCR	*C. forsteri*	97.1%	98.3%	60%
		C. orientalis	11.4%	0%	0%
Gill	Histology	Eggs	0%	68.3%	5%
	qPCR	*C. forsteri*	100%	18.3%	95%
		C. orientalis	15.7%	0%	0%

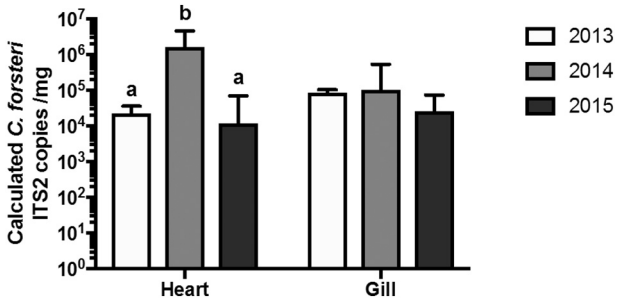

Fig. 2. Calculated *Cardicola forsteri* ITS2 rDNA quantity per mg in heart and gill samples from 2013, 2014 and 2015 ranched SBT. Data shown are the mean (± 95% Cl) of all SBT heart and gill samples examined in 2013 (n = 50), 2014 (n = 60), and 2015 (n = 40). Different letters denote significant differences at $P < 0.05$.

4. Discussion

The qPCR assay and species specific primers presented here provide unambiguous and rapid absolute quantification of all three species of *Cardicola* known to infect bluefin tuna, while remaining both exquisitely sensitive and specific at low target analyte concentrations. This improved qPCR assay was used to identify and quantify *C. forsteri* and *C. orientalis* ITS2 rDNA in wild and ranched SBT heart and gill samples. This molecular method was superior in terms of speed, sensitivity and specificity compared to traditional methods of *Cardicola* detection and diagnosis such as heart flushes and histology that are both time consuming and labor intensive (Aiken et al., 2006; Kirchhoff et al., 2011; Shirakashi et al., 2013). In contrast the qPCR assay described here can be performed in as little as 3 h starting from gill or heart samples providing rapid detection, species identification and quantification of pathogen load measured in terms of DNA quantity. Furthermore, the simplicity of the qPCR provides an opportunity for this assay to be adapted to a portable Real time PCR platform that could be used by SBT industry.

Though previous studies have developed qPCR-based quantification of *Cardicola* in SBT, these methods utilized DNA intercalating SYBR green chemistry which, to ensure maximum sensitivity and specificity, requires expert interpretation of melt curve analysis at target analyte concentrations nearing the assay LOQ and LOD (Polinski et al., 2013a). Moreover, in this study we decided to adopt a traditional hydrolysis probe design rather than use either the SYBR green chemistry or the TaqMan CR approach also designed in our lab and described by Polinski et al. (2013a). While both the TaqMan CR assay and the more recent hydrolysis probe assay have almost identical sensitivities and specificities to the currently known *Cardicola* we believe the extra specificity of the traditional fluorophore labeled probe as opposed to the common reporter probe was an advantage given more recent identification of another species of *Cardicola* infecting bluefin tuna (Palacios-Abella et al., 2015) and the likely presence of other similar and yet to be identified *Cardicola*. The ease of use, accuracy and sensitivity of the assay described and validated in this paper facilitates early *C. forsteri* and *C. orientalis* detection in ranched SBT.

Prior to 2012, *C. forsteri* was believed to be the only species of *Cardicola* infecting SBT and detection was based on counting adult flukes in the heart so all the data until then are only for this species. In

2004–2006 the mean prevalence of infection of ranched SBT with *C. forsteri* was 62.64% based on results of heart flushes (Aiken et al., 2015). Methods of detection and species differentiation have since become more sensitive and precise, where microscopy methods have led to the description of *C. orientalis* in PBT in 2010 (Ogawa et al., 2010) and qPCR methods resulted in the confirmation of the species in SBT (Polinski et al., 2013b; Shirakashi et al., 2013). *C. orientalis* has since been identified as the predominant species of *Cardicola* having been found in 86% of SBT samples from 2008 to 2012 while *C. forsteri* was present in 36% of SBT, that were examined using qPCR (Polinski et al., 2013b). *C. forsteri* loads were more prevalent and greater than *C. orientalis* in 2013 and *C. orientalis* was absent in 2014 and 2015. This was unexpected, as this species had been documented as the main blood fluke in ranched SBT populations in the previous five years (Polinski et al., 2013b).

Using qPCR methods, future studies should determine the dynamics of prevalence of different species of *Cardicola* throughout the season, particularly before and after PZQ treatments. In 2013 PZQ was introduced as a method of mitigating infections with blood fluke and has been applied since by the industry. The change of the most prevalent species of *Cardicola* may demonstrate differences in anthelmintic efficacy against the two blood fluke species.

Traditional methods including heart flush data from previous studies reported a 5–10% prevalence of *C. forsteri* in wild juveniles caught in the Great Australian Bight in 2001 (Aiken et al., 2006; Colquitt et al., 2001; Kirchhoff et al., 2012; Kirchhoff et al., 2014). Comparatively low prevalence of *Cardicola* spp. in wild SBT (compared to ranched) indicates that levels of infection seen in ranched systems could result from increased prevalence of the infected intermediate host in the ranching areas, thereby facilitating completion of the parasite's life-cycle (Cribb et al., 2000; Cribb et al., 2011).

The proximity of ranched SBT to *C. forsteri* and *C. orientalis* intermediate hosts could propagate the infection of ranched SBT (Bullard and Overstreet, 2002; Montero et al., 1999; Munday et al., 2003). Confined SBT probably come in contact with larger numbers of cercariae than wild, free-swimming SBT, thereby increasing the likelihood of infection (Bullard and Overstreet, 2002). In a previous study, offshore relocation of sea cages resulted in no *C. forsteri* and *C. orientalis* infection of ranched SBT and maximized fish growth (Kirchhoff et al., 2011).

The high variability of wild SBT infection with blood flukes is in agreement with reports about these parasites in wild PBT and ABT. In wild PBT juveniles annual prevalence of *C. orientalis* and *C. opisthorchis* was highly variable (Sugihara et al., 2016). Quantitative PCR showed that *C. orientalis* prevalence was 20.9% in 2011 and dropped below 5% the following years. *C. opisthorchis* prevalence in wild PBT ranged between 74.6% in 2011, 4.1% in 2013, and 32.1% in 2014 (Sugihara et al., 2016). In ABT no statistically significant difference in prevalence was detected among the three detected blood fluke species in either wild or ranched specimens (Forte-Gil et al., 2016). Prevalence of blood flukes in ABT was 6.5% and 37.5% when only a single blood fluke species was detected, whereas it was 60.9% and 12.5% for prevalence of all three species in wild and cultured respectively (Forte-Gil et al., 2016).

Sampling location within the organ, particularly the gill, may affect the documented prevalence of each species of *Cardicola*. Adult *C. forsteri* reside primarily in the heart, while adult *C. orientalis* have been found in the afferent gill artery, branchial arteries as well as in the heart (Ogawa et al., 2010; Ogawa et al., 2011; Shirakashi et al., 2012b). In the present study, the central filaments of the left second gill arch were analyzed to assess *Cardicola* infection. This area has been shown to give most accurate indication of species composition and prevalence for eggs (dos Santos et al., 2012; Shirakashi et al., 2012b). Therefore, when investigating *C. forsteri* and *C. orientalis* prevalence in gill the inclusion of the afferent artery in qPCR analyses may contribute to a clearer exposition of species prevalence.

4.1. Conclusion

This hydrolysis probe-based qPCR assay allows accurate quantification of blood flukes from genus *Cardicola* and as a result their prevalence in ranched and wild SBT. The first detection of *C. orientalis* in wild SBT and *C. forsteri* in wild SBT caught along the Tasmanian coast (Pedra Branca) is a highlight of this study.

Acknowledgements

The authors would like to thank the IMAS research staff, particularly Dr. Sean Tracey, Dr. Phil Crosbie, and Jimena Balli Garza. We want to thank Dr. Daryl Evans and the Australian Southern bluefin tuna industry for their cooperation giving us the opportunity to collect samples aboard their boat. We want to thank the Lincoln Marine Science Center for allowing us to use their laboratory and equipment during sample processing. This research was supported by John Bicknell Scholarship.

References

ABARES, 2013. Agricultural commodity statistics 2013. In: D., o. (Ed.), Agriculture.
AFMA, 2007. Southern Bluefin Tuna Fishery. Australian Fisheries Management Authority.
Aiken, H., 2009. Epidemiology of the blood fluke, *Cardicola forsteri*, in Southern bluefin tuna and Northern bluefin tuna. In: Fish Health. University of Tasmania, pp. 241.
Aiken, H., Hayward, C., Nowak, B., 2006. An epizootic and its decline of a blood fluke, *Cardicola forsteri*, in farmed Southern Bluefin tuna (*Thunnus maccoyii*). Aquaculture 254, 40–45.
Aiken, H.M., Bott, N., Mladineo, I., Montero, F., Nowak, B., Hayward, C., 2007. Molecular evidence for cosmopolitan distribution of platyhelminth parasites of tunas (*Thunnus* spp.). Fish Fish. 8, 167–180.
Aiken, H.M., Hayward, C.J., Nowak, B., 2015. Factors affecting abundance and prevalence of blood fluke, *Cardicola forsteri*, infection in commercially ranched Southern bluefin tuna, *Thunnus maccoyii*, in Australia. Vet. Parasitol. 210, 106–113.
Armbruster, D.A., Pry, T., 2008. Limit of blank, limit of detection and limit of quantitation. Clin. Biochem. Rev. 29, S49–52.
Brown, S.H., 2002. Hematoxylin & Eosin. The Routine Stain. Sigma-Aldrich.
Bullard, S.A., Overstreet, R.M., 2002. Potential pathological effects of blood flukes (Digenea: Sanguinicolidae) on pen-reared marine fishes. In: 53rd Gulf and Caribbean Fisheries Institute, pp. 10–25.
Bullard, S.A., Goldstein, R.J., Goodwin, R.H., Overstreet, R.M., 2004. *Cardicola forsteri* (Digenea: Sanguinicolidae) from the heart of a Northern Bluefin tuna, *Thunnus thynnus* (Scombridae), in the northwest Atlantic Ocean. Comp. Parasitol. 71, 245–246.
Carr, A.C., Moore, S.D., 2012. Robust quantification of polymerase chain reactions using global fitting. PLoS One 7, e37640.
Colquitt, S.E., Munday, B.L., Daintith, M., 2001. Pathological findings in southern bluefin tuna, *Thunnus maccoyii* (Castelnau), infected with *Cardicola forsteri* (Cribb, Daintith & Munday) (Digenea: Sanguinicolidae), a blood fluke. J. Fish Dis. 24, 225–229.
Cribb, T.H., 2000. A new blood-fluke, *Cardicola forsteri* (Digenea: Sanguinicolidae) of Southern Bluefin tuna (*Thunnus maccoyii*) in aquaculture. In: Transactions of the Royal Society of South Australia, Incorporated. vol. 124. pp. 117.
Cribb, T.H., Daintith, M., Munday, B., 2000. A new blood-fluke, *Cardicola forsteri*, (Digenea: Sanguinicolidae) of Southern bluefin tuna (*Thunnus Maccoyii*) in aquaculture. Trans. R. Soc. S. Aust. 124, 117–120.
Cribb, T.H., Adlard, R.D., Hayward, C.J., Bott, N.J., Ellis, D., Evans, D., Nowak, B.F., 2011. The life cycle of *Cardicola forsteri* (Trematoda: Aporocotylidae), a pathogen of ranched Southern Bluefin tuna, *Thunnus maccoyi*. Int. J. Parasitol. 41, 861–870.
Dennis, M.M., 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. Vet. Pathol. 48, 846–855.
Deveney, M.R., Bayly, T.J., Johnston, C.J., Nowak, B.F., 2005. A parasite survey of farmed southern bluefin tuna, *Thunnus maccoyii* (Castelnau). J. Fish Dis. 28, 279–284.
Forte-Gil, D., Holzer, A.S., Pecková, H., Bartošová-Sojková, P., Peñalver, J., Dolores, E.M., Muñoz, P., 2016. Molecular and morphological identification of *Cardicola* (Trematoda: Aporocotylidae) eggs in hatchery-reared and migratory Atlantic bluefin tuna (*Thunnus thynnus* L.). Aquaculture 450, 58–66.
Ishimaru, K., Mine, R., Shirakashi, S., Kaneko, E., Kubono, K., Okada, T., Sawada, Y., Ogawa, K., 2013. Praziquantel treatment against *Cardicola* blood flukes: determination of the minimal effective dose and pharmacokinetics in juvenile Pacific bluefin tuna. Aquaculture 402, 24–27.
Kirchhoff, N.T., Rough, K.M., Nowak, B.F., 2011. Moving cages further offshore: effects on southern bluefin tuna, *T. maccoyii*, parasites, health and performance. PLoS One 6, e23705.
Kirchhoff, N.T., Leef, M.J., Valdenegro, V., Hayward, C.J., Nowak, B.F., 2012. Correlation of humoral immune response in southern bluefin tuna, *T. maccoyii*, with infection stage of the blood fluke, *Cardicola forsteri*. PLoS One 7, e45742.
Kirchhoff, N.T., Nelligan, J., Ellis, D., Cadoret, K., Leef, M., Nowak, B., 2014. Inter-annual and intra-annual variability in blood variables and parasitic loads of wild *Thunnus*

maccoyii. Can. J. Fish. Aquat. Sci. 71, 1572–1578.
Montero, D., Izquierdo, M.S., Tort, L., Robaina, L., Vergara, J.M., 1999. High stocking density produces crowding stress altering some physiological and biochemical parameters in gilthead seabream, *Sparus aurata*, juveniles. Fish Physiol. Biochem. 20, 53–60.
Munday, B.L., Sawada, Y., Cribb, T., Hayward, C.J., 2003. Diseases of tunas, *Thunnus* spp. J. Fish Dis. 26, 187–206.
Nan, C.B., Hutchinson, W., Foster, C., 2016. Southern Bluefin Tuna Captive Breeding in Australia, Advances in Tuna Aquaculture: From Hatchery to Market. Academic Press.
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. Parasitol. Int. 59, 44–48.
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. Parasitol. Int. 60, 307–312.
Palacios-Abella, J.F., Rodriguez-Llanos, J., Mele, S., Montero, F.S., 2015. Morphological characterization and identification of four species of *Cardicola* Short 1953 (Trematoda: Aporocotylidae) infecting the Atlantic bluefin tuna *Thunnus thynnus* (L.) in the Mediterranean Sea. Syst. Parasitol. 91, 101–117.
Polinski, M., Bridle, A., Nowak, B., 2013a. Real-time pcr detection and differentiation of *Cardicola* blood fluke species from tissue and blood of southern bluefin tuna. Fish Shellfish Immunol. 34, 1730.
Polinski, M., Belworthy, H., Nowak, B., Bridle, A., 2013b. SYBR, TaqMan, or both: highly sensitive, non-invasive detection of *Cardicola* blood fluke species in southern bluefin tuna (*Thunnus maccoyii*). Mol. Biochem. Parasitol. 191, 7–15.
Polinski, M., Shirakashi, S., Bridle, A., Nowak, B., 2014. Transcriptional immune response of cage-cultured Pacific Bluefin tuna during infection by two *Cardicola* blood fluke species. Fish Shellfish Immunol. 36, 61–67.
R Core Team, 2013. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
Ritz, C., Spiess, A., 2008. qpcR: an R package for sigmoidal model selection in quantitative real-time polymerase chain reaction analysis. Bioinformatics 24, 1549–1551.
RStudio Team, 2015. RStudio: Integrated Development Environment for R. RStudio Inc., Boston, Massachusetts.
Rutledge, R.G., Stewart, D., 2010. Assessing the performance capabilities of LRE-based assays for absolute quantitative real-time PCR. PLoS One 5, e9731.
dos Santos, C.N., Leef, M., Jones, B., Bott, N., Giblot-Ducray, D., Nowak, B., 2012. Distribution of *Cardicola forsteri* eggs in the gills of southern bluefin tuna (*Thunnus maccoyii*) (Castelnau, 1872). Aquaculture 344, 54–57.
Shirakashi, S., Andrews, M., Kishimoto, Y., Ishimaru, K., Okada, T., Sawada, Y., Ogawa, K., 2012a. Oral treatment of praziquantel as an effective control measure against blood fluke infection in Pacific bluefin tuna (*Thunnus orientalis*). Aquaculture 326, 15–19.
Shirakashi, S., Kishimoto, Y., Kinami, R., Katano, H., Ishimaru, K., Murata, O., Itoh, N., Ogawa, K., 2012b. Morphology and distribution of blood fluke eggs and associated pathology in the gills of cultured Pacific bluefin tuna, *Thunnus orientalis*. Parasitol. Int. 61, 242–249.
Shirakashi, S., Tsunemoto, K., Webber, C., Rough, K., Ellis, D., Ogawa, K., 2013. Two species of *Cardicola* (Trematoda: Aporocotylidae) found in southern bluefin tuna (*Thunnus maccoyii*) ranched in South Australia. Fish Pathol. 48, 1–4.
Sugihara, Y., Yamada, T., Ichimaru, T., Matsukura, K., Kanai, K., 2016. Detection of bluefin tuna blood flukes (*Cardicola* spp.) from wild juvenile Pacific bluefin tuna (*Thunnus orientalis*) caught for aquaculture. Aquaculture 452, 9–11.
Volpe, J.P., 2005. Dollars without sense: the bait for big-money tuna ranching around the world. Bioscience 55, 301–302.