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Understanding blood flukes infecting Southern Bluefin Tuna

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[*Understanding blood flukes infecting Southern Bluefin* Tuna]
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In submitting this report, the researcher has agreed to FRDC publishing this material in its edited form.

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Executive Summary

Background

Southern Bluefin Tuna (SBT), *Thunnus maccoyii*, is an important commercial species ranched off the coast of Port Lincoln, South Australia. A research team from RMIT University (RMIT), in conjunction with the Australian Southern Bluefin Tuna Industry Association (ASBTIA) and individual SBT ranching companies undertook this study into understanding blood flukes in SBT. The aporocotylids (blood flukes) *Cardicola forsteri* and *C. orientalis* are considered one of the most significant health concerns for SBT ranched off Port Lincoln, South Australia.

Objectives

The objectives of this study were to:

- Provide detailed SBT health data with regard to praziquantel treated and untreated cages to SBT industry.
- Understand differences (if any) between nearby praziquantel treated and untreated cages.
- Obtain a more detailed understanding of SBT pathogens in environmental samples.
- Provide baseline SBT health data for future longer-term projects.

Methods and Results

In this study we compared the effects of different treatment strategies on blood fluke infections in ranched SBT by sampling from one untreated and two praziquantel treated pontoons progressively throughout the 2018 season. Severity of infection was assessed relative to a number of criteria including adult fluke counts from hearts, egg counts from gill filaments and the use of specific quantitative polymerase chain reaction (qPCR) for detection of *C. forsteri* and *C. orientalis* ITS-2 DNA in SBT hearts and gills. *Cardicola forsteri* was the dominant species detected in this study, and intensity of *C. forsteri* infection in SBT was significantly higher in the untreated pontoon than the two treated pontoons at week 8 of ranching. *Cardicola orientalis* in SBT was rarely detected, and no significant differences were seen in mortalities or condition of SBT between pontoons. Comparing diagnostic methods for *C. forsteri* in SBT heart demonstrated qPCR as the gold standard. *Cardicola orientalis* was only detected via qPCR in the gills of SBT from companies F-H i.e. those with the lowest (company F ranged from 15-24 mg/kg), or no PZQ treatment. We were also able to detect DNA of blood flukes from biofouling samples collected from lease sites of 4 SBT companies, illustrating that environmental detection of SBT pathogens is possible, and that a well-developed environmental surveillance scheme may provide a non-destructive method for *Cardicola* spp. detection.

Key findings

- *Cardicola forsteri* was the dominant blood fluke species detected in 2018.
- Traditional methods (heart flush and gill microscopy) are limited to detecting adults or eggs, which means that they underestimate infections by classifying fish with other life stages as uninfected e.g. detecting adult *C. orientalis* in gills, or immature stages not yet producing eggs.
- Comparison of currently used diagnostic methods showed that molecular methods (i.e. qPCR) was most effective.
- Sensitivity between molecular and microscopy is similar, but microscopy is more labour intensive and time consuming.
- Quantitative PCR was more effective at detecting subtle changes in blood fluke infections.
- Praziquantel treatment does have a statistically significant effect on blood fluke prevalence and intensity, but increasing infection levels observed in this study did not decrease SBT condition or increase mortalities.

- *Cardicola orientalis* was only detected in SBT samples from companies where there was either no praziquantel treatment or treatment at 15 mg kg^{-1} or less
- Further investigation would be needed to identify whether dosage rate does influence *C. orientalis* presence in ranched SBT.
- Blood fluke DNA can be detected in biofouling collected from farming infrastructure (i.e. ropes and nets) even after the season has concluded.

Implications

This project has provided new insights into the understanding of blood flukes in SBT. An understanding of the effect of praziquantel on parasite loads has provided an important baseline for understanding the effects of different husbandry techniques. This will be further explored in FRDC 2018/170.

Recommendations

Findings from this project (2017/241) are being further explored in a new FRDC funded project (2018/170) where the aspects of different husbandry techniques will be assessed on a wider scale across the industry. Investigations into the levels of praziquantel treatment and the effect that has on parasite composition in SBT will be important for ongoing husbandry practices and management of the SBT health. The new project will also develop a new DNA diagnostic method for the detection of SBT pathogens which will enable DNA-based monitoring to occur in Port Lincoln.

Keywords

Blood flukes, Southern Bluefin Tuna, Praziquantel, *Cardicola fosteri, Cardicola orientalis*

Introduction

Southern Bluefin Tuna (SBT), *Thunnus maccoyii*, (Figure 1) is an important commercial species ranched off the coast of Port Lincoln, South Australia. Ranching was introduced in 1991 (Kirchhoff et al. 2011); whereby, wild juvenile SBT are caught in the Great Australian Bight and transferred to grow-out sites where they are fed daily with baitfish, enabling industry to maximise growth before harvest after for 2-6 months (Balli Garza et al. 2017). The primary health factor affecting ranched SBT over the last ten years has been the infection by blood flukes from the genus *Cardicola* (Trematoda: Aporocotylidae) (Neumann et al. 2018).

Cardicola is a species-rich genus that parasitise several teleost families, often species with high importance in fisheries and aquaculture (Palacios-Abella et al. 2015). *Cardicola forsteri* (Figure 2) was first described from the hearts of SBT ranched at Port Lincoln by Cribb et al. (2000), and *Cardicola orientalis* first reported from the branchial arteries of SBT by Shirakashi et al. (2013), all from SBT, and detected in SBT samples using molecular techniques (Polinski et al. 2013). Blood fluke infections in Port Lincoln ranched SBT have been monitored routinely during most ranching seasons (Aiken et al. 2008, 2009, 2015; Colquitt et al. 2001; Deveney et al. 2005; Hayward et al. 2010; Kirchhoff et al. 2011; Neumann et al. 2018; Nowak 2004; Polinski et al. 2013). Elevated mortalities were seen in 2008-09, and Dennis et al. (2011) showed that a large proportion of mortalities in ranched SBT were strongly associated with severe branchitis caused by blood flukes. Similarly, in Japan, *C. orientalis* is one of the main causes of mortality in farmed and ranched Pacific bluefin tuna (PBT), *Thunnus orientalis*, (Shirakashi et al. 2012b).

Since 2012, the anthelmintic praziquantel (PZQ) has been used as an effective treatment method of controlling blood fluke infection in farmed and ranched SBT and PBT (Hardy-Smith et al. 2012: Ishimaru et al. 2013; Shirakashi et al. 2012a). The introduction of praziquantel treatment has been attributed to a considerable decrease in ranched SBT mortalities in recent years (ASBTIA, pers. Comm.). However, associated treatment costs and a harvest withholding period have led some companies to leave SBT in pontoons untreated. This study analyses the effects of praziquantel treatment on prevalence and intensity of *C. forsteri* and *C. orientalis* infection in ranched SBT from pontoons with different treatment strategies.

Previous studies have shown that SBT pathogens, including *Cardicola* spp. can be detected from biofouling associated with SBT aquaculture infrastructure (e.g. Power et al., 2019a). The monitoring of environmental reservoirs of SBT pathogens may provide insights into parasite life cycles and be used to identify potential reservoirs of infection and inform parasite treatment/management programs for future ranching seasons. In this study we will explore whether parasite DNA can be detected from environmental samples collected form lease sites post-harvest.

Figure 1: Southern Bluefin Tuna ranched off Port Lincoln, Australia

Figure 2: Adult blood fluke, *Cardicola forsteri* (collected from the heart of SBT.

Objectives

The objectives of this study were to:

1. Provide detailed SBT health data with regard to praziquantel treated and untreated cages to SBT industry.
2. Understand differences (if any) between nearby praziquantel treated and untreated cages.
3. Obtain a more detailed understanding of SBT pathogens in environmental samples.
4. Provide baseline SBT health data for future longer-term projects.

Methods

SBT collection and processing

Wild SBT were purse-seined in the Great Australian Bight (33° 27' S, 132° 04' E) and towed to the Lincoln Aquaculture Zone in Lower Spencer Gulf near Port Lincoln, South Australia. At Port Lincoln fish were stocked into three 45m diameter pontoons managed by a single company (Company G) (Table 1).

Heart and gills were collected from 12 SBT sampled from each pontoon at different time points during the 2018 ranching season (Table 1). SBT (n= 120) were sampled using a baited hook and line. Fish in Pontoon 1 were PZQ treated during week 2 of ranching, fish in Pontoon 2 were PZQ treated during week 6 of ranching, and fish in Pontoon 3 were not treated with PZQ. Treated SBT were orally administered PZQ at a dose of 15 mg kg^{-1} via industry standard PZQ injected baitfish (Table 1).

Table 1. Sampling of Southern Bluefin Tuna collected from company G in 2018.

Pontoon	Total stocked fish	Stocking date	Praziquantel treatment	Sampling size week post-stocking (n)			
				Week 0	Week 3	Week 8	Week 12
1	3128	28 February	Week 2	-	12	12	12
2	3205	28 Feb 2018	Week 6	-	12	12	11
3	2545	28 Feb 2018	Untreated	12	12	12	12

Hearts and gills from ranched SBT were collected from eight companies (A – H) during the 2018 harvest (Table 2). SBT were sampled using a baited hook and line. A 5cm x 5cm section of filaments from the middle region of the second left gill arch from each fish was placed in an individual sealed bag and stored on ice (Shirakashi et al. 2013). A 0.125cm^3 piece of heart was taken by removing tip of apex and targeting cardiac endothelium, and additional filaments from the second left gill arch were preserved in 1.5ml RNA*later*® and stored on ice for subsequent DNA extraction. The heart of each fish was then placed in an individual labelled plastic container and stored on ice.

In the laboratory, hearts were dissected 2 – 4 hours after removal and flushed with water to dislodge adult flukes as per Aiken et al. (2006). Adult flukes dislodged from each heart were counted. Egg counts were completed on gill samples 12 – 24 hours after sample collection, or the gills were stored at -20˚C and thawed at a later date. Four gill filaments were dissected and eggs examined under a compound microscope using a 40 × objective (as per Shirakashi et al., 2012b). Eggs were quantified as egg number per mm filament length.

Gilled and gutted body weights (kg) and total lengths (m) for each SBT sampled were also obtained, and a Fulton's K condition index (condition index) was calculated using the formula: whole body weight /length^3. As SBT were sampled during commercial processing operations that removes gills and gut from fish, whole weights were estimated using the industry formula, total weight = gilled and gutted weight / 0.87.

Table 2. Collection of SBT hearts and gill samples from companies A – G at harvest.

Company	Stocking date	Treatment date	Praziquantel dose (mg kg^{-1})	Sampling date	n
A	08 Mar 2018	12 Apr 2018	42	11 Jul 2018	15
B	13 Mar 2018	17 Apr 2018	30	13 Jul 2018	15
C	27 Feb 2018	28 Mar 2018	30	16 Jul 2018	15
D	10 Mar 2018	15 Apr 2018	24	12 Jul 2018	15
E	05 Feb 2018	13 Mar 2018	20	10 Jul 2018	15
F	28 Mar 2018	26 Apr 2018	15-24	20 Jul 2018	15
G	28 Feb 2018	03 Apr 2018	15	18 Jul 2018	15
Ha	26 Feb 2018	No treatment	0	15 Jul 2018	15
Hb	24 Feb 2018	No treatment	0	20 Jul 2018	15

Primer and probe design

The species-specific primers and probes used to detect *C. forsteri* and *C. orientalis* were designed in previous studies, which confirmed their specificity (Polinski et al. 2013). These were targeted against heterogeneous areas of the internal transcribed spacer-2 (ITS-2) region of rDNA specific to each species available on GenBank https://www.ncbi.nlm.nih.gov/genbank/.

DNA extraction

DNA was extracted from 25mg of gill and heart samples preserved in RNA*later*® using the Isolate II Genomic DNA Kit (Bioline, UK) following the manufacturer's instructions. Using the NanoDrop™ Lite Spectrophotometer, concentration (ng μl^{-1}) was measured to determine DNA quantity. All samples had concentration levels sufficient for PCR, ranging between 80 – 200 ng μl^{-1}. Spectrophotometry was also used to determine A_{260}:A_{280} ratios as a measure of DNA purity, with ~1.8 considered 'pure'. All samples had acceptable A_{260}:A_{280} ratios ranging between 1.6 – 2.0.

Statistics

The effects of time, praziquantel treatment and condition index of SBT infected with *C. forsteri* and *C. orientalis* were analysed using GraphPad Prism 8 (GraphPad software, CA, USA). Severity of infection for *C. forsteri* and *C. orientalis* was described by prevalence and intensity as per Bush et al. (1997). Prevalence was calculated using a Sterne's exact method at N=1000±95% confidence interval.

Infection data did not meet the assumptions of normality so non-parametric tests were used. The effect of time and treatment on infection prevalence was determined using Chi square analysis or Fisher's exact test. Differences in infection intensity of SBT between pontoons at each time point,

condition index of SBT and mortalities of SBT between pontoons over time were compared using Kruskal-Wallis followed by Dunn's or Mann-Whitney test for pairwise comparisons. Spearman's rank correlation coefficients were used to determine the relationship between condition index and adult *C. forsteri* intensity. The sensitivity of traditional vs molecular diagnostic methods was compared using a two-tailed McNemar χ^2 test (Cleophas & Zwinderman 2011). Significance for all statistical analysis was assumed at $p \leq 0.05$.

Biofouling

Biofouling samples were collected from four SBT ranching company lease sites following the SBT harvest in October 2018. Samples were frozen upon collection. Each company collected at least four samples, east and west net samples, and east and west rope samples. Company D collected 2 different depths (1m and 4m) and three replicates for each rope and net sample. Additionally, Company D collected three replicates of east and west benthic samples and three replicates of east and west 'navigation cross' samples at 4m (Table 13).

Sample preparation and sorting

Each biofouling sample was defrosted for up to two hours to allow for taxonomic sorting of invertebrates. Using gloves and a sterile spatula, biofouling was removed from 6sterilised bags and placed in a sterilised Petri dish. Samples were identified based on morpho-groupings into broad taxonomic levels using an M60 stereomicroscope (Leica, Germany). A time limit of two hours was placed on taxonomic sorting to 6minimise DNA degradation, and subsequently the entire sample could not be processed. The unidentified material was treated as a mixed biofouling sample. After sorting, taxonomic groups were placed in separate sterile tubes and the mixed biofouling placed in its own tube and stored at -20ºC. Samples were 6lyophilised (freeze dried) for a period of 24-48 h using the Alpha 1-2 Ldplus Freeze Dryer (CHRIST, Germany). After freeze drying, samples were ground to a fine powder with a mortar and pestle and stored at 4ºC until further analysis. Samples were weighed both prior and post freeze drying to the nearest 0.1 g using a XS105 Analytical Balance (Mettler-Toledo, Port Melbourne, VIC).

DNA extraction

Sub-samples taken from taxonomic groups (e.g. bivalves) were approximately 5g so DNA was extracted using the PowerSoil® DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) was used following the manufacturer's instructions. The quantity and quality of samples were estimated using spectrophotometry as described above. All samples had concentration levels sufficient for PCR.

Quantitative PCR

All samples for molecular analysis were analysed using quantitative polymerase chain reaction (qPCR). Quantitative PCR assays were performed on a Rotor-Gene™ Q (Qiagen, Germany) in a final volume of 20µL containing 10µL of SensiFAST™ Probe 2x No-ROX Mix (Bioline, UK), 0.8µL of each 10µM primer (Integrated DNA Technologies, USA), 0.2µL of 10µM probe (Thermo Fisher Scientific, AUS), 6.2µL of Rnase/Dnase free water and 2µL of template DNA. Reaction cycling conditions were: 95ºC for 5 min, followed by 40 cycles of 95ºC for 10 s (denaturing) and 60ºC for 25 s (annealing), with relative fluorescence measured on the annealing cycle. All samples tested were analysed in duplicate, including a positive control and no template control for each run. For each assay, the limit of detection (LOD) and the limit of quantification (LOQ) were calculated using triplicates of four serial dilutions from a synthetic double stranded gBlock® standard (Integrated DNA Technologies, USA) and converted to a copy number per mass using a nucleotide copy number calculator

(http://www.endmemo.com/bio/dnacopynum.php) as previously described (Neumann et al. 2018). Samples were considered positive if they had a copy number / mg greater than the LOQ.

Results

Company G during ranching 2018

Survival and SBT size characteristics

In total, 119 SBT were sampled from Company G throughout the entire ranching season (Table 1). No significant differences in mortalities were seen between pontoons at any time point (Table 3). Mean condition index increased over time for Pontoon 1 ($p = 0.0049$), Pontoon 2 ($p < 0.0001$) and Pontoon 3 (untreated) ($p < 0.0001$) (Figure 3). There was also a significant difference in condition of SBT in different pontoons at all sampling periods (Fig 3).

Table 3. Mortalities of ranched Southern bluefin tuna from company G in 2018.

Pontoon	Praziquantel treatment	Week: Number of mortalities 1	4	8	12	16	Total mortalities	Cumulative mortality relative to total stocked fish (%)
1	Week 2	2	0	0	2	1	5	0.16
2	Week 6	0	2	4	3	1	10	0.31
3	Untreated	1	2	1	1	-	5	0.20

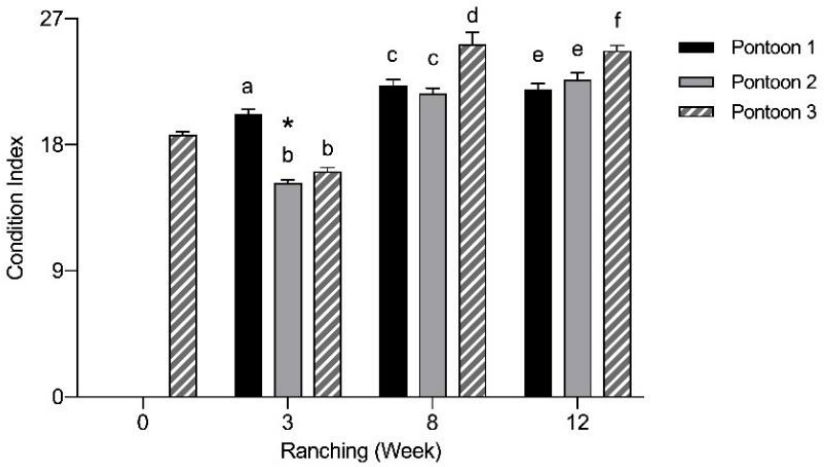

Figure 3. Mean (±SE, n = 12 fish per sampling) condition index of SBT treated with PZQ (pontoon 1 – treated week 2, pontoon 2 treated week 6) or not treated (pontoon 3) by company G. * indicates sampling prior to PZQ treatment. No samples were collected at week 0 for Pontoon 1 and 2. Different letters denote statistical differences at $p \leq 0.05$ among pontoons at each time point.

Treated v Untreated

Samples were collected over a 16 week period from SBT that were treated with praziquantel (n=74) as well as SBT that were left untreated (n=60). Whilst prevalence of adult blood flukes was not statistically significant between treated and untreated cages, abundance (Table 4) and intensity (Table 5) of adult flukes and eggs was significantly higher in untreated cages. Table 6 shows the prevalence of *C. forsteri and C. orientalis* detection from heart and gill samples using accepted methods of detection; heart flush and qPCR for hearts, and egg counts from gill filaments and qPCR for gills. For gill egg counts, no differentiation is made between *C. forsteri* and *C. orientalis*

Table 4. Prevalence (%), mean abundance (number of parasites per host) and mean intensity (number of parasites per infected host) of *Cardicola* spp. adults in SBT heart samples collected in 2018 from company G (treated n=74, untreated n=60).

	Treated	**Untreated**	**P value**
Prevalence	33.8%	45.0%	p = 0.188
Mean abundance (all fish)	1.1	4.4	p < 0.001
Mean intensity (infected fish)	3.4	9.7	p < 0.001

Table 5. Prevalence (%), mean abundance (number of eggs per host) and mean intensity (number of eggs per infected host) of *Cardicola* spp. eggs per mm of gill filament in SBT samples collected in 2018 from company G (treated n=74, untreated n=60).

	Treated	**Untreated**	**P value**
Prevalence	79.5%	85.0%	p = 0.412
Mean abundance (all fish)	0.60	1.75	p < 0.001
Mean intensity (infected fish)	0.79	2.02	P = 0.001

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Table 6. Prevalence (%) of *C. forsteri* and *C. orientalis* in SBT determined from counts of adults within heart samples and counts of eggs within samples of gill filaments compared to results from qPCR analysis of heart and gill samples collected from company G.

Sample	Method used for parasite determination	Target	Treated (n = 74)	Untreated (n = 60)
Heart	Heart flush	Adult flukes	33.8%	45.0%
	qPCR	*C. forsteri*	75.7%	68.3%
		C. orientalis	0%	0%
Gill	Gill filament - histological examination	Eggs	79.5%	85.0%
	qPCR	*C. forsteri*	87.7%	76.7%
		C. orientalis	5.5%	10.0%

Detection of Cardicola forsteri in SBT

Prevalence of infection with adult *C. forsteri* increased during ranching for Pontoon 1 (X^2 = 6.107, df = 2, p = 0.0472) and Pontoon 3 (untreated) (X^2 = 40.62, df = 2, p < 0.0001) (Figure 4a). No significant differences were seen for Pontoon 2 over time. Prevalence of adult *C. forsteri* in hearts of SBT sampled from Pontoon 3 (untreated) was significantly greater than fish in Pontoon 1 (p = 0.0003) or Pontoon 2 (p <0.0001) at week 8, and also significantly greater than fish in Pontoon 1 (p = 0.0046) or Pontoon 2 (p < 0.0001) at week 12. No significant differences in prevalence of adult *C. forsteri* in hearts of SBT sampled from were seen between fish in Pontoons at week 3, or Pontoon 1 and 2 at any time point. Mean intensity of infection with adult *C. forsteri* increased during ranching for Pontoon 1 (p = 0.0044) and Pontoon 3 (untreated) (p = 0.0242) (Figure 4c). No significant differences were seen for Pontoon 2 over time. Mean intensity for Pontoon 3 (untreated) was significantly higher than Pontoon 1 at week 8 (p = 0.0132) and week 12 (p = 0.0176), and higher than Pontoon 2 at week 12 (p = 0.0220). No significant differences were seen between Pontoons at week 3, Pontoon 2 and 3 at week 8, or Pontoon 1 and 2 at any time point.

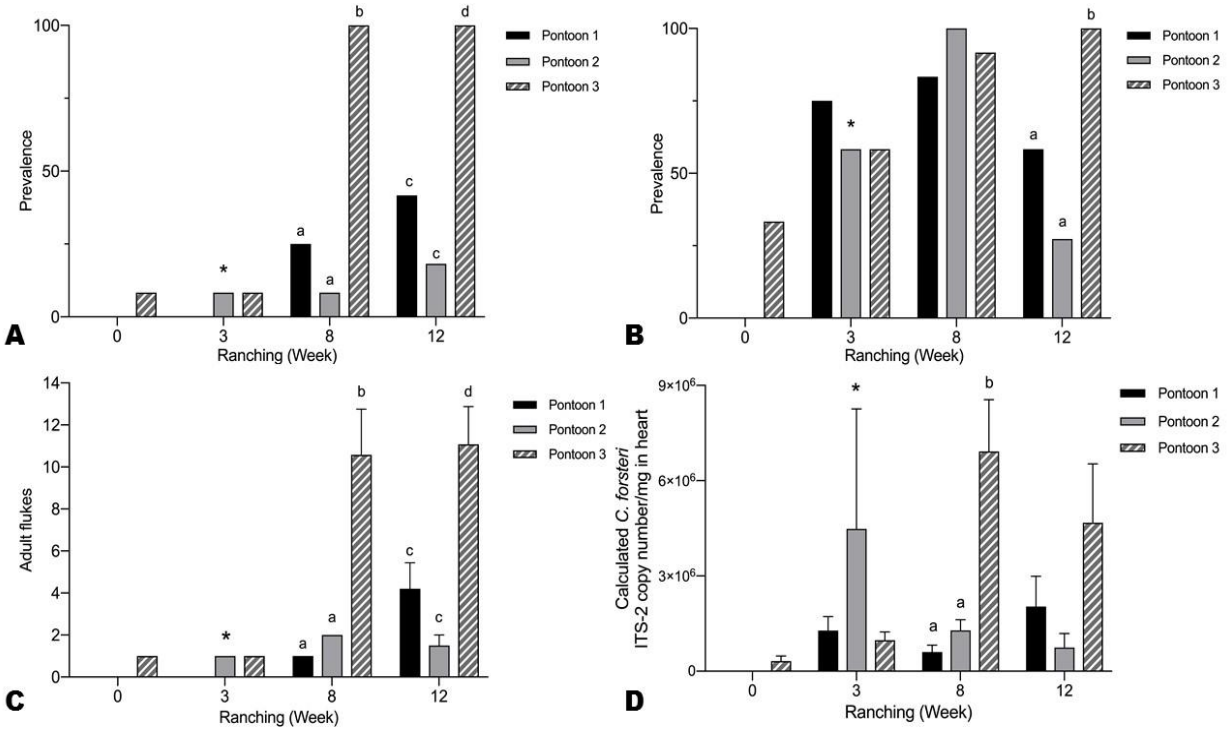

Figure 4. Prevalence and mean intensity of *Cardicola forsteri* infection in heart samples (n = 12 SBT sampled from each pontoon at each time point) from SBT treated with PZQ (pontoon 1 – treated week 2, pontoon 2 treated week 6) or not treated (pontoon 3) by company G. (a) Prevalence of adult *C. forsteri*; (b) prevalence of *C. forsteri* internal transcribed spacer-2 (ITS-2) in heart; (c) mean intensity (±SE) of adult *C. forsteri*; and (d) mean (±SE) calculated *C. forsteri* ITS-2 copy number mg^{-1} intensity in heart samples. * indicates sampling prior to PZQ treatment. No samples were collected at week 0 for Pontoon 1 and 2. Significant letters denote statistical differences at $p \leq 0.05$ between pontoons at each time point.

Prevalence of *C. forsteri* determined by ITS-2 copy number per mg of heart from fish sampled from Pontoons 1 and 2 increased between week 3 and week 8, then decreased at week 12 (Figure 4b). Difference in prevalence over time was significant for Pontoon 2 ($X^2 = 13.16$, df = 2, p = 0.0014) but not for Pontoon 1. Prevalence in Pontoon 3 (untreated) increased at every time point ($X^2 = 16.54$, df = 2, p = 0.0009). Significant differences were seen between Pontoon 1 (treated) and Pontoon 3 (untreated) at week 12 (p = 0.0373) and between Pontoon 2 (treated) and Pontoon 3 (untreated) at week 12 (p = 0.0003). No differences were seen between any Pontoons at week 3 or week 8, or Pontoon 1 and 2 at any time point. Mean intensity of infection based on calculated *C. forsteri* ITS-2 copy number per mg in Pontoon 3 (untreated) increased week 3 to week 8, then decreased week 12 (p = 0.0355) (Figure 4d). A significant difference was seen between Pontoon 3 (untreated) and Pontoon 1 at week 8 (p = 0.0065), and a marginal difference between Pontoon 3 (untreated) and Pontoon 2 at week 8 (p = 0.0578). No significant differences were seen between Pontoons week 3 and week 12, or within Pontoons 1 and 2 over time.

Prevalence of *C. forsteri* ITS-2 in gill samples from SBT in Pontoon 3 (untreated) decreased from week 0 to week 3, then increased to 100% at week 8 and week 12 ($X^2 = 16.89$, df = 2, p = 0.0007) (Figure 5a). No significant differences in prevalence of *C. forsteri* ITS-2 in gills were seen between fish sampled from any Pontoons at any time point, or within Pontoons 1 and 2 (treated) over time. Mean intensity of *C. forsteri* ITS-2 copy number in gills of fish from Pontoon 2 (treated) and Pontoon 3 (untreated) peaked at week 8 then decreased at week 12 (Pontoon 2, p = 0.0002; Pontoon 3, p = 0.0003) (Figure 5b). Mean intensity in Pontoon 3 (untreated) increased at week 8, significantly higher than Pontoon 1 ($p < 0.0001$) or Pontoon 2 (p = 0.0150). Mean intensity continued to be highest in Pontoon 3 (untreated) at week

12, significantly higher than Pontoon 2 (p = 0.0003) but not Pontoon 1 (p = 0.2688). No significant differences in intensity of *C. forsteri* ITS-2 copy number in gills of fish were seen between Pontoons at week 3, between Pontoon 1 and 2 at any time point, or within Pontoon 1 over time.

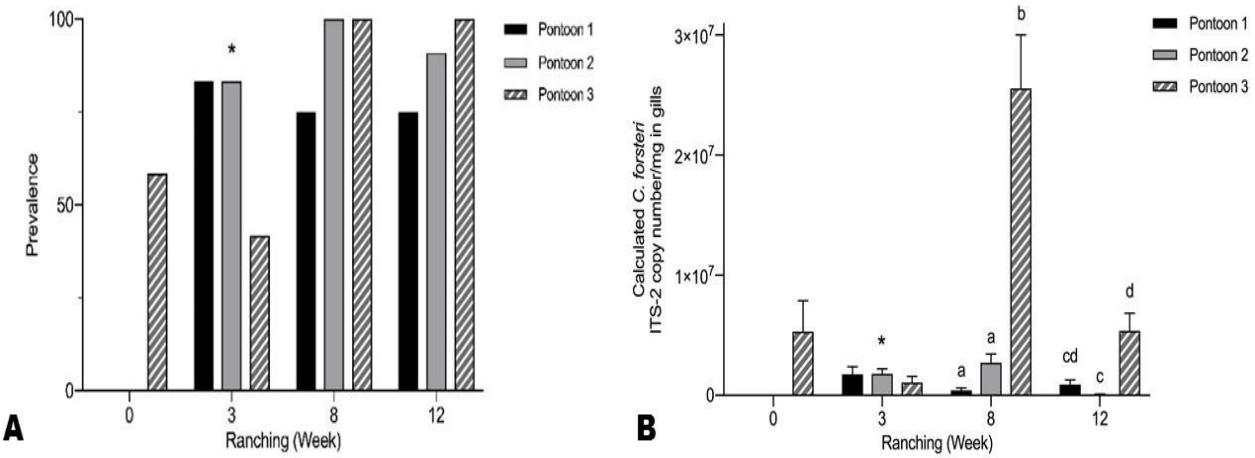

Figure 5. *Cardicola forsteri* ITS-2 in gill samples (n = 12 SBT sampled from each pontoon at each time point) from SBT treated with PZQ (pontoon 1 – treated week 2, pontoon 2 treated week 6) or not treated (pontoon 3) by company G. (a) Prevalence of *C.forsteri* ITS-2 in gills; and (b) mean (±SE) intensity of *C. forsteri* ITS-2 (copy number mg^{-1}) in gill samples. * indicates sampling prior to PZQ treatment. No samples were collected at week 0 for Pontoon 1 and 2. No statistical differences at $p \leq 0.05$ were seen between pontoons at each time point.

Detection of Cardicola orientalis in SBT

C. orientalis was detected in hearts of SBT sampled from Pontoon 2 (treated) and Pontoon 3 (untreated) (Figure 6). No significant difference in prevalence was seen between pontoons at any time point.

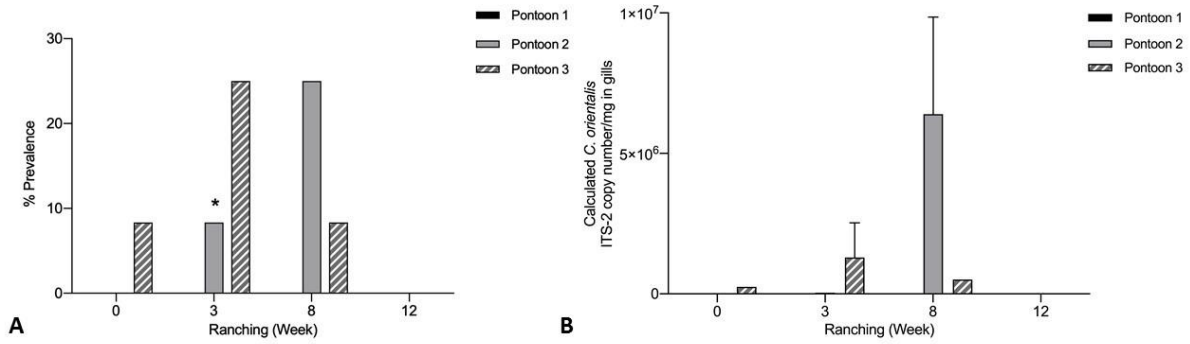

Figure 6. *Cardicola orientalis* ITS-2 in gill samples (n = 12 SBT sampled from each pontoon at each time point) from SBT treated with PZQ (pontoon 1 – treated week 2, pontoon 2 treated week 6) or not treated (pontoon 3) by company G. (a) Prevalence of *Cardicola orientalis* infection in heart samples (n = 12 SBT sampled from each pontoon at each time point) from SBT treated with PZQ (pontoon 1 – treated week 2, pontoon 2 treated week 6) or not treated (pontoon 3) by company G. (b) mean (±SE) intensity of *C. orientalis* ITS-2 (copy number mg^{-1}) in gill samples. * indicates sampling prior to PZQ treatment. No samples were collected at week 0 for Pontoon 1 and 2.

Mean intensity of calculated *C. orientalis* ITS-2 copy number per mg in gill increased in Pontoon 2 from 4.12×10^4 copy number/mg at week 3 to 6.40×10^6 copy number/mg week 8, but *C. orientalis* was not detected in week 12. Mean intensity in Pontoon 3 (untreated) increased from 8.48×10^4 copy number/mg week 0 to 1.30×10^6 copy number/mg week 3, then decreased to 5.08×10^5 copy number/mg week 8 and was not detected in week 12. No significant differences in overall mean intensity were seen between pontoons. Intensity could not be directly compared statistically between pontoons at different time points or within pontoons over time as the infection was absent or pontoons had only one infected individual. *Cardicola orientalis* ITS-2 was not detected in gills of SBT from Pontoon 1, and hearts of SBT from any pontoon at any time point.

Detection of Cardicola spp. *eggs in gill filaments of SBT*

Prevalence of *Cardicola* spp. eggs in gill filaments in Pontoon 3 (untreated) increased during ranching ($X^2 = 14.40$, df = 3, p = 0.0024) and decreased during ranching for Pontoon 2 ($X^2 = 10.08$, df = 2, p = 0.0065) (Figure 7a). Prevalence for Pontoon 3 (untreated) was significantly higher than Pontoon 2 at week 12 (p = 0.0046). No other significant differences were seen. Mean intensity of *Cardicola* spp. egg/mm of gill filament for Pontoon 3 (untreated) increased at every time point (p < 0.0001). Mean intensity in Pontoon 3 (untreated) was significantly higher than Pontoon 1 at week 8 (p = 0.0060) and higher than Pontoon 1 (p = 0.0009) and Pontoon 2 (p = 0.0024) at week 12 (Figure 7b). No significant differences were seen between Pontoons at week 3, Pontoon 2 and 3 at week 8, Pontoon 1 and 2 at any time point or within Pontoons over time.

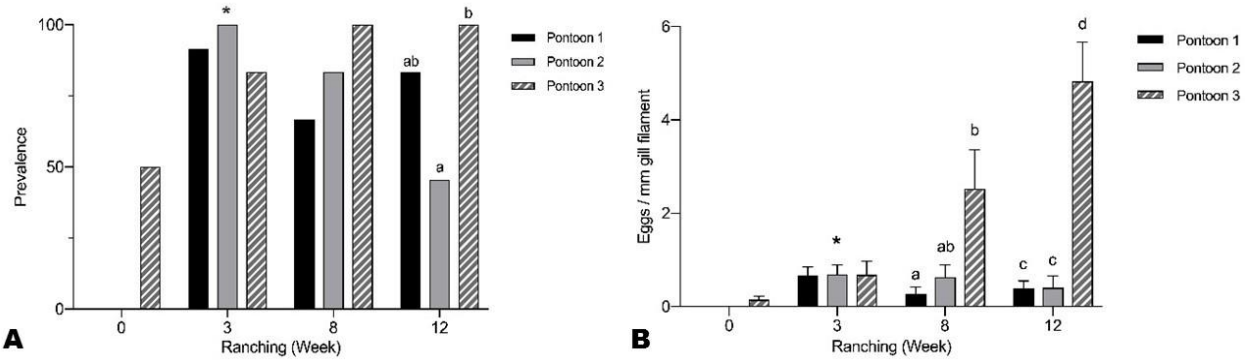

Figure 7. (a) Prevalence (%) of *Cardicola* spp. eggs in gill filament; and (b) mean (±SE) intensity of *Cardicola* spp. eggs per mm of gill filament from SBT (n = 12 SBT sampled from each pontoon at each time point) treated with PZQ (pontoon 1 – treated week 2, pontoon 2 treated week 6) or not treated (pontoon 3) by company G. * indicates sampling prior to PZQ treatment. No samples were collected at week 0 for Pontoon 1 and 2. Significant letters denote statistical differences at $p \leq 0.05$ between pontoons at each time point.

Comparisons between Cardicola spp. *diagnostic methods*

When comparing diagnostic methods for presence of *C. forsteri* in SBT hearts, detection of *C. forsteri* using qPCR (ITS-2) had a higher sensitivity (n = 82 positive) than detection of *C. forsteri* using heart flush microscopy (n = 38 positive) (McNemar's test $\chi 2$ test, $p < 0.0001$) (Table 7). When comparing diagnostic methods for presence of *Cardicola* spp. in SBT gills, detection of *C. forsteri* (ITS-2) and *C. orientalis* (ITS-2) using qPCR (n = 97 positive) showed similar sensitivity to detection of *Cardicola* spp. eggs using gill microscopy (n = 95 positive) (McNemar's test $\chi 2$ test, $p = 0.83$) (Table 8).

Table 7. Comparison of methods for detecting *C. forsteri* in heart samples from the same fish (**+** = *C. forsteri* detected by method/s, **-** = *C. forsteri* not detected by method/s)

METHOD		**qPCR (ITS-2)**		
		+	**-**	Total
Heart flush microscopy	**+**	34	4	38
	-	48	33	81
	Total	82	37	119

Table 8. Comparison of methods for detecting *Cardicola* spp. in gills samples from the same fish. **+** =*Cardicola* detected by method/s, **-** = *Cardicola* not detected by method/s

METHOD		**qPCR (ITS-2)**		
		+	**-**	Total
Gill filament microscopy	**+**	85	10	95
	-	12	12	24
	Total	97	22	119

Companies A – H during harvest 2018

A total of 135 samples were collected from eight SBT ranching companies (A – H) during the July 2018 harvest period. Mean prevalence of *Cardicola* spp. determined from heart flush microscopy was 52.6% (Table 9), however prevalence varied considerably, with ranges from 26.7% to 93.3% (Table 10, 11). Using qPCR analysis *C. forsteri* was shown to be the dominant species, with an overall prevalence of 76.7% and 76.3% detected in heart (Figure 8(and gill samples (Figure 9) respectively, while *C. orientalis* was detected at a prevalence of 0% and 3% in heart and gill samples respectively (Table 9, not shown in graphs). Prevalence (%), mean abundance and mean intensity of *Cardicola* spp. adults identified via heart flush microscopy is shown in Table 11. Mean abundance (Figure 10) and mean intensity of *Cardicola* spp. adults identified via heart flush microscopy varied from 0.47 to 3.73 and 1.17 to 4.00 respectively (Table 11). Prevalence of *Cardicola* spp. eggs/mm of gill based on microscopy varied across the companies, varying from 80-100% (Table 12). Mean abundance (Figure 11) and mean intensity of *Cardicola* spp. eggs/mm of gill, based microscopy varied from 0.1 to 1.47 and 0.13 to 1.47 respectively (Table 12).

Table 9. Prevalence (%) and 95% confidence interval of *C. forsteri* and *C. orientalis* in heart flush microscopy, gill filament microscopy and qPCR analysis of heart and gill samples collected during harvest in 2018 (n= 135).

	Method	**Target**	**Prevalence**	**95% Confidence Interval**
Heart	Heart flush	Adult flukes	52.6%	44.0% - 61.2%
	qPCR	*C. forsteri*	76.7%	69.4% - 84.0%
Gill	Gill filament	Eggs	92.4%	87.8% - 97.0%
	qPCR	*C. forsteri*	76.3%	69.0% - 83.7%
		C. orientalis	3.1%	0% - 6.0%

Table 10. Prevalence (%) (95% confidence interval) of *C. forsteri* and *C. orientalis* determined from heart flush microscopy, gill filament microscopy and qPCR analysis of heart and gill harvest samples (n=15, except company H where n=30) from SBT collected during harvest from companies A – H.

	Method	**Target**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Heart	Heart flush	Adult flukes	50.0 (20.0-79.9)	46.7 (18.1-75.3)	40.0 (11.9-68.1)	42.9 (13.2-72.5)	53.3 (24.7-81.9)	26.7 (1.3-52.0)	93.3 (79.0-100)	60.0 (41.4-78.6)
	qPCR	*C. forsteri*	85.7 (64.8-100)	60.0 (31.9-88.1)	66.7 (39.6-93.7)	64.3 (35.6-93.0)	86.7 (67.2-100)	33.3 (6.3-60.4)	100 (79.6-100)	96.7 (90.0-100)
Gill	Gill filament	Eggs	100 (79.6-100)	80.0 (57.1-100)	84.6 (61.9-100)	86.7 (67.2-100)	100 (79.6-100)	80.0 (57.1-100)	100 (79.6-100)	100 (88.7-100)

qPCR	*C. forsteri*	80.0 (57.1-100)	40.0 (11.9-68.1)	61.5 (30.9-92.1)	53.3 (24.7-81.9)	93.3 (79.0-100)	73.3 (48.0-98.7)	100 (79.6-100)	93.1 (83.3-100)
	C. orientalis	0 (0-20.4)	0 (0-20.4)	0 (0-20.4)	0 (0-20.4)	0 (0-20.4)	6.7 (0-21.0)	7.1 (0-22.6)	6.9 (0-16.7)

Table 11. Prevalence (%) (95% confidence interval), mean abundance (±SE) (average number of parasites per host) and mean intensity (±SE) (average number of parasites per infected host) of *Cardicola* spp. adults in heart flush microscopy of harvest samples collected in 2018 from company A – H (n=15, except company H where n=30)

	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Prevalence	50.0 (20.0-79.9)	46.7 (18.1-75.3)	40.0 (11.9-68.1)	42.9 (13.2-72.5)	53.3 (24.7-81.9)	26.7 (1.3-52.0)	93.3 (79.0-100)	60.0 (41.4-78.6)
Mean abundance	1.50 (±0.55)	0.60 (±0.19)	0.60 (±0.24)	0.50 (±0.17)	0.87 (±0.29)	0.47 (±0.27)	3.73 (±0.79)	1.33 (±0.30)
Mean intensity	3.00 (±0.76)	1.29 (±0.18)	1.50 (±0.34)	1.17 (±0.17)	1.63 (±0.38)	1.75 (±0.75)	4.00 (±0.80)	2.22 (±0.37)

Table 12. Prevalence (%) (95% confidence interval), mean abundance (±SE) (average number of eggs per host) and mean intensity (±SE) (average number of eggs per infected host) of *Cardicola* spp. eggs/mm in gill filament microscopy of harvest samples collected in 2018 from company A – H (n=15, except company H where n=30)

	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Prevalence	100 (96.7-100)	80.0 (57.1-100)	84.6 (61.9-100)	86.7 (67.2-100)	100 (96.7-100)	80.0 (57.1-100)	100 (96.7-100)	100 (99.7-100)
Mean abundance	0.66 (±0.25)	0.23 (±0.16)	0.10 (±0.04)	0.19 (±0.07)	1.00 (±0.22)	0.35 (±0.16)	1.33 (±0.23)	1.47 (±0.18)
Mean intensity	0.66 (±0.25)	0.29 (±0.14)	0.13 (±0.04)	0.22 (±0.08)	1.00 (±0.22)	0.43 (±0.20)	1.33 (±0.23)	1.47 (±0.18)

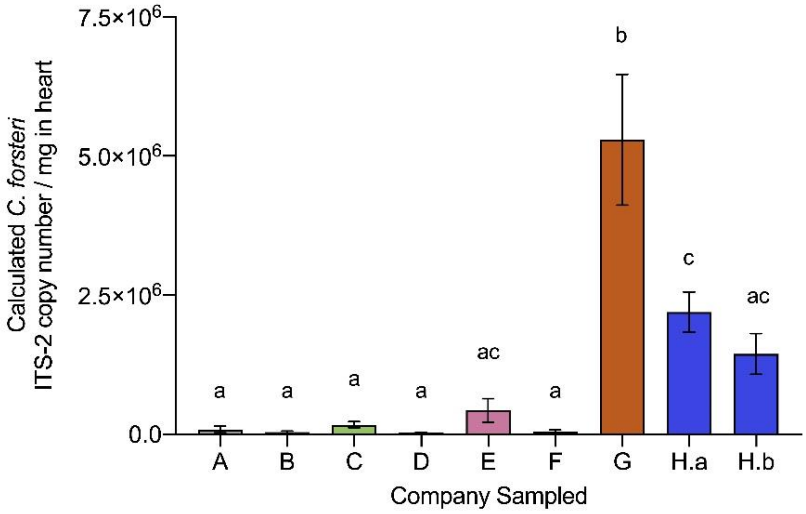

Figure 8. Mean (±SE) calculated *Cardicola forsteri* (ITS-2) copy number/mg in heart samples collected from ranched Southern Bluefin Tuna at harvest 2018. Significant letters denote statistical differences at $p \leq 0.05$ between companies.

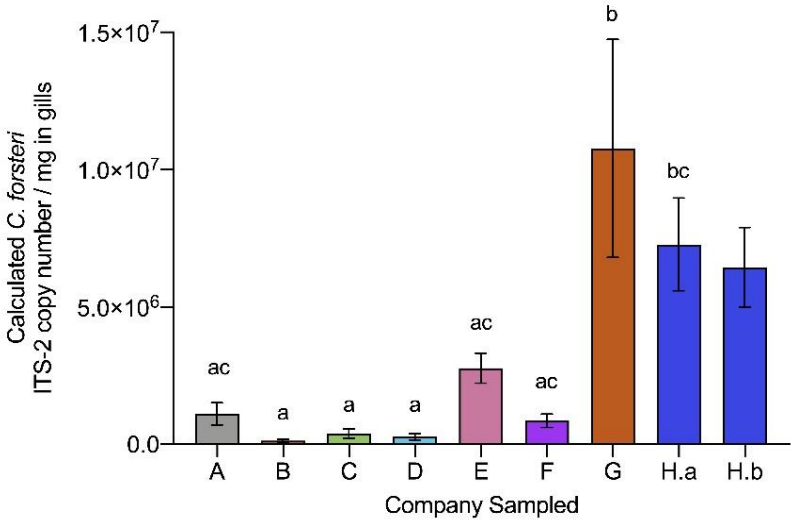

Figure 9. Mean (±SE) calculated *Cardicola forsteri* (ITS-2) copy number/mg in gill samples collected from ranched Southern Bluefin Tuna at harvest 2018. Significant letters denote statistical differences at $p \leq 0.05$ between companies.

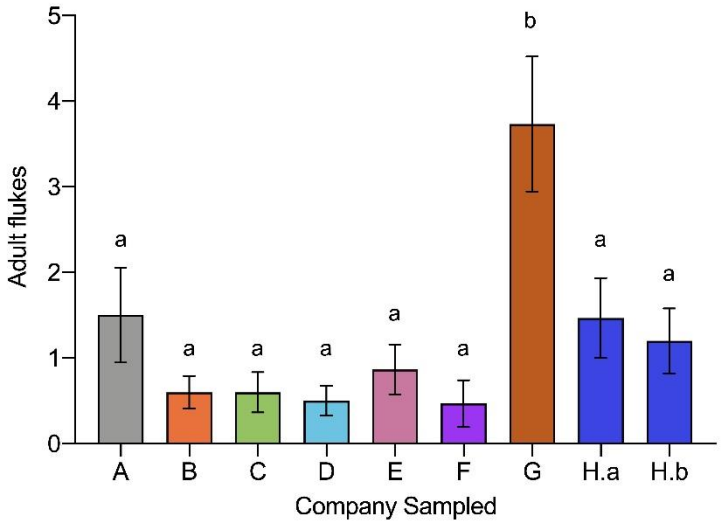

Figure 10. Mean abundance of *Cardicola forsteri* adults in SBT hearts collected at harvest from all participating companies. Different letters denote statistical differences at $p \leq 0.05$.

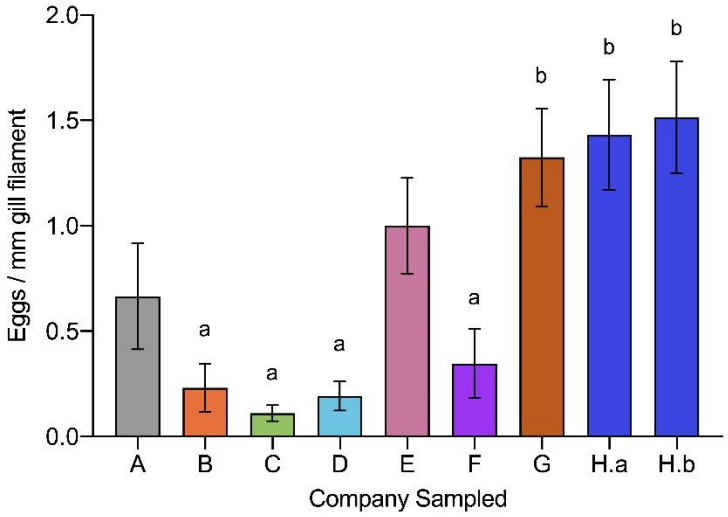

Figure 11. Mean abundance of *Cardicola* spp. eggs in gill filament collected from ranched Southern Bluefin Tuna at harvest 2018. Significant letters denote statistical differences at $p \leq 0.05$ between companies.

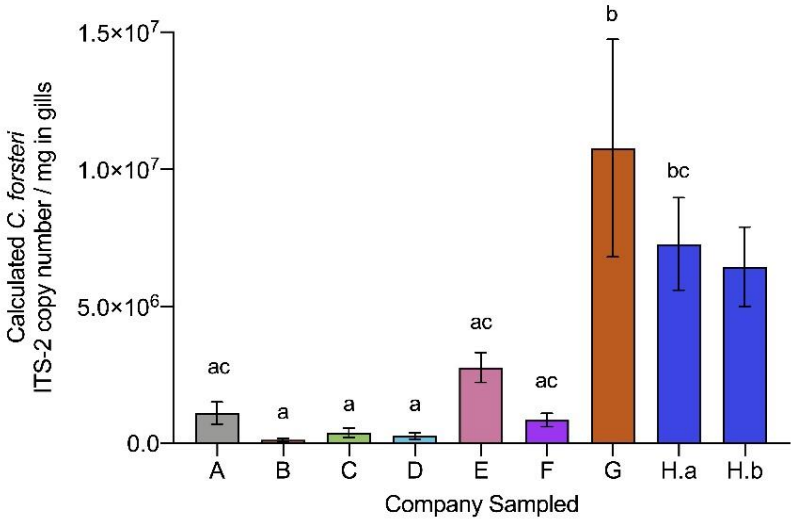

Figure 11. Mean (±SE) calculated *Cardicola forsteri* (ITS-2) copy number/mg in gill samples collected from ranched Southern Bluefin Tuna at harvest 2018. Significant letters denote statistical differences at $p \leq 0.05$ between companies.

Biofouling

Biofouling samples were collected from rope and nets at an east and west points of the lease from Companies A, B, E and G. Company E collected biofouling samples from 1m and 4m depths. Presence of *C. forsteri* and *C. orientalis* DNA was determined by qPCR analysis (Table 13). No *C. orientalis* was detected from any of the biofouling samples. *C. forsteri* was detected from Company A (Net sample, East), Company E (Net sample, West 4m), and Company G (Rope sample, East and West).

Table 13. Results of qPCR analyses of biofouling collected from net and rope samples from SBT ranching companies A, B, E and G.

Company	Substrate	Direction	Depth	*C. forsteri* ITS-2 rDNA Copy Number (per 5g of freeze-dried biofouling)	*C. orientalis* ITS-2 rDNA Copy Number (per 5g of freeze-dried biofouling)
G	Rope	East		2,429,276	-
G	Rope	West		417,238	-
G	Net	East		-	-
G	Net	West		-	-
B	Rope	East		-	-
B	Rope	West		-	-
B	Net	East		-	-
B	Net	West		-	-
A	Rope	East		-	-
A	Rope	West		-	-
A	Net	East		2,897,229	-
A	Net	West		-	-
E	Net	East	1m	-	-
E	Net	East	4m	-	-
E	Net	West	1m	-	-
E	Net	West	4m	10,007,847	-

Discussion

Company G during ranching 2018

Intensity of *C. forsteri* infection in SBT was significantly higher in the untreated pontoon compared to two PZQ treated pontoons at week 8 and week 12 of ranching. This was shown through prevalence of adult *C. forsteri* in heart, *C. forsteri* ITS-2 DNA in heart and gills, and *Cardicola* spp. eggs in gill filaments. These results demonstrate the continued efficacy of PZQ at controlling adult *C. forsteri* in ranched SBT. In treated pontoons, calculated *C. forsteri* ITS-2 DNA in gill samples was greater than calculated *C. forsteri* ITS-2 DNA in heart samples at each time point, and this is probably due to PZQ not being able to kill *Cardicola* spp. eggs or miracidia (Hardy-Smith et al. 2012; Shirakashi et al. 2012a). Although PZQ treatment correlated with a significant effect on intensity of blood fluke infection, mortalities were similar between the three pontoons sampled. This may be due to severity of infection not being strong enough to cause mortality. In 2008, before PZQ was used in the sector and SBT mortalities peaked, *C. forsteri* infection reached a maximum mean intensity of 268.3 adults in SBT hearts (Hayward et al. 2010), far greater than a maximum mean intensity of 11.1 adults (Figure 4c) seen in this study.

Prevalence and intensity of *C. forsteri* in SBT sampled from pontoons 2 and 3 at week 3 of ranching, prior to PZQ treatment, show similar results to samples taken at the time of stocking (week 0) from pontoon 3. There were also no significant differences in prevalence and intensity between the two treated pontoons (1 and 2) at week 8 and week 12, indicating that treatment later into ranching may be an effective method for controlling adult *C. forsteri*. This strategy could reduce the number of PZQ doses administered throughout the season or allow for a longer SBT ranching period in that particular pontoon.

Results from the mean condition index of sampled SBT were inconsistent. At week 3, SBT in Pontoon 1 (treated at week 2) had significantly higher mean condition index than fish in the other pontoons. There was no significant difference for mean condition index for fish in any pontoon at week 8 and week 12.

Comparisons across companies

Companies A-G were placed in order of PZQ treatment dosage, with dosage rates varying from company A at 42 mg kg^{-1} through to company G at 15 mg kg^{-1} (Table 2). Company H did not untreat fish with PZQ. Company G had the highest counts of adult *C. forsteri* in heart, as well as *C. forsteri* ITS-2 DNA in heart and gills (Figures 8-11). Company G had the highest number of *Cardicola* spp. eggs in gill filaments and significantly differed in infection rate from all other companies with the exception of company H. Previous studies found the universal factor in explaining variation in *C. forsteri* intensity, abundance and prevalence was different ranching companies (Aiken et al., 2015). They hypothesised that differences in infection levels between different companies may be related to differences in husbandry measures employed on each farm, or due to different average sizes of tuna farmed by each of the companies, or due to the location of the operations (Aiken et al., 2015).

Effect of PZQ treatment on *Cardicola*

Of particular note is the observation that companies F-H i.e. those with the lowest PZQ treatment dosages (company F ranged from 15-24 mg kg^{-1}), or no PZQ treatment (Company H) were the only companies for which we were able to detect *C. orientalis* DNA from the gills using qPCR. This finding further supports the observation by Neumann et al. (2018) that detection of *C. orientalis* in SBT has dropped significantly since the introduction of PZQ treatment in the SBT industry. This may indicate that PZQ has a higher efficacy on *C. orientalis* than it does on *C. forsteri.* Further investigation of this

from past and future SBT seasons would be advantageous in order to understand this more completely. Developing a deeper understanding of the different effects of PZQ on the two different blood fluke species may have future implications for PZQ treatment strategies to be employed by the Australian SBT industry.

Cardicola forsteri is the dominant species detected from farmed SBT in this study

Cardicola forsteri was the dominant parasitic species detected in this study. After confirmation of *C. orientalis* in SBT (Polinski et al. 2013; Shirakashi et al. 2013), it was identified as the main species in ranched SBT samples from 2008 – 2012 (Polinski et al. 2013). Since 2013, *C. forsteri* has been the dominant species documented in ranched SBT populations (Neumann et al. 2018), coinciding with the introduction of PZQ treatment as a control measure against blood fluke infections. Treatment methods for PZQ delivery though injected baitfish have been commercialised and adopted by most industry participants in the SBT ranching sector. Results from this study further demonstrate the change in dominant *Cardicola* species since the introduction of PZQ. The change in species dynamic also corresponds to a lower rate of mortalities seen in ranched SBT. In 2008, mortalities reached 15% for the worst affected pontoons, a big jump from 2 – 3% seen in previous years (Dennis et al. 2011). This onset of increased mortalities coincided with a peak in infection loads of blood flukes (Hayward et al. 2010), of which the dominant species detected was *C. orientalis* (Polinski et al. 2013). Since the introduction of PZQ and a reduction in detection of *C. orientalis*, SBT mortalities have decreased significantly (ASBTIA, pers. Comm.). In PBT, *C. orientalis* is believed to have higher pathogenicity than *C. opisthorchis* as it produces a significantly greater number of eggs (Shirakashi et al. 2012b). Adult *C. orientalis* usually infect the afferent gill artery, whilst *C. forsteri* and *C. opisthorchis* infect the heart ventricle (Shirakashi et al. 2013), so it is possible that adult *C. orientalis* could contribute to pathogenicity in tunas by clogging the gill arteries.

The reasons for the decline of *C. orientalis* in SBT since 2013 are unknown. In the Mediterranean, *C. orientalis* infections might be associated with long-distance migration and overlapping of *Thunnus* spp. distribution, as opposed to other *Cardicola* spp. infections which appear to originate from nearby tuna pontoons (Forte-Gil et al. 2016). This may be the same for SBT, and *C. orientalis* infections could be a reflection of differences in the wild populations of SBT targeted each season. In this study *C. orientalis* was not detected in any pontoon at week 12 of ranching, but prevalence of *C. orientalis* may be low enough not to be detectable at this sample size. Aporocotylids have a two-host life cycle with a definitive fish host and an invertebrate intermediate host (Power et al. 2019). Near Japanese tuna farms, the intermediate host for *C. orientalis* is *Nicolea gracilibranchis*, a terebellid polychaete, and terebellids have also been identified as intermediate hosts for *C. forsteri* and *C. opisthorchis* (Shirakashi et al. 2016). Similarly, larval stages of *C. forsteri* were found in terebellid polychaete *Longicarpus modestus* near SBT pontoons in Port Lincoln (Cribb et al. 2011). Terebellids appear to have strong microhabitat preferences and are typically associated with certain biofouling organisms such as poriferans and tunicates, which are more abundant in shallower areas (Shirakashi et al. 2017).

The importance of molecular detection methods in characterising blood fluke infections is evident. After confirmation of *C. orientalis* in SBT (Shirakashi et al. 2013), use of qPCR led to the recognition of *C. orientalis* as the more prevalent species seen in SBT from 2008 – 2012 (Polinski et al. 2013). Traditional methods, including heart flushes and microscopy, are limited to detecting either adults or eggs, and cannot detect migrating stages such as infecting cercaria or emerging miracidia. It can also be difficult to detect adult *C. orientalis* using traditional methods (because their primary habitat is in the gills). In this study, comparing diagnostic methods for *C. forsteri* in SBT hearts showed qPCR as the gold standard. Whilst similar sensitivity was seen when comparing diagnostic methods for *Cardicola* spp. in gills, microscopy was more laborious, taking several weeks to complete, whereas DNA extraction and qPCR for this sample size could be achieved in one day. It is interesting to note that

prevalence and intensity of adult *C. forsteri*, as well as prevalence of *C. forsteri* (ITS-2) in heart samples, increased from week 3 to week 8 in Pontoon 2, even after PZQ treatment at week 6. However we see a decrease in calculated *C. forsteri* (ITS-2) copy number/mg heart samples in Pontoon 2 from week 3 to week 8, suggesting that qPCR is more effective at detecting subtle changes in blood fluke infections, particularly in smaller sample sizes.

The introduction of untreated pontoons has allowed industry to harvest SBT earlier in the season, opening up a market for fresh fish and reduce feed and maintenance costs (Kirchhoff et al. 2011). Whilst the absence of PZQ treatment does appear to have an effect on blood fluke prevalence and intensity, infection levels observed in this study were evidently too low to cause any significant decrease in the condition of SBT or increase in mortalities. Further studies should continue to monitor blood fluke infections in SBT as the industry looks to develop new treatment strategies in the future.

Biofouling

The results of the biofouling analysis at four SBT aquaculture leases (Table 13) illustrates that DNA from *Cardicola forsteri* can be detected on in situ equipment (i.e. ropes and nets) on lease sites up to 3 months after the conclusion of the SBT harvest. This is consistent with other findings, notably from Cribb et al. (2011) when the terebellid polychaete found to be infected with *C. forsteri* was collected from an area where no SBT had been stocked for over 6 months. Determining the composition of biofouling attached to SBT pontoons and their variations, both spatial and temporal, may help us to discover if there is an intermediate host for *C. orientalis* near SBT pontoons, and further our understanding of infection dynamics of blood flukes between intermediate and definitive host. A systematic and well-developed monitoring of biofouling, beyond the scope of this project, may be an important tool for non-destructive monitoring of pathogens.

Conclusions

Cardicola forsteri is the dominant blood fluke species detected in farmed SBT in 2018. Our results have shown that traditional methods (heart flush and gill microscopy) are limited to detecting adults or eggs. Comparisons of currently used diagnostic methods showed that molecular methods (i.e. qPCR) was most effective. Findings show that sensitivity between molecular and microscopy is similar, but microscopy is more labour intensive and time consuming and qPCR is more effective and detecting subtle changes in blood fluke infections.

A lack of PZQ treatment does have a statistically significant effect on blood fluke prevalence and intensity, but infection levels observed in this study did not decrease SBT condition or increase mortalities. In this study, *C. orientalis* DNA was only detected from companies F-H where there was either no PZQ treatment, or low dosage PZQ treatment at ~15 mg/kg. Further investigation is required to identify whether dosage rate influences *C. orientalis* presence in ranched SBT.

Blood fluke DNA can be detected in biofouling collected from infrastructure even after the season has concluded.

Implications

This project has provided new insights into the understanding of blood flukes in SBT. An understanding of the effect of PZQ on parasite loads has provided an important baseline for understanding the effects of different husbandry techniques. This will be further explored in FRDC 2018/170.

Recommendations

Findings from this project (2017/241) are being further explored in a new FRDC funded project (2018/170) where the aspects of different husbandry techniques will be assessed on a wider scale across the industry. Investigations into the levels of PZQ treatment and the effect that has on parasite composition in SBT will be important for ongoing husbandry practices and management of the SBT health. The new project will also develop a new DNA diagnostic method for the detection of SBT pathogens which will enable DNA-based monitoring to occur in Port Lincoln.

Extension and Adoption

Project results have been communicated to the SBT industry via regular written updates (milestones), as well as via presentations at annual SBT research updates.

Project materials developed

Publications

Power C, Webber C, Rough C, Staunton R, Nowak BF, Bott NJ (2019) The effect of different treatment strategies on *Cardicola* spp. (Trematoda: Aporocotylidae) infection in ranched Southern Bluefin Tuna (*Thunnus maccoyii*) from Port Lincoln, South Australia. Aquaculture 513: 73401 DOI: https://doi.org/10.1016/j.aquaculture.2019.734401

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