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SUMMARY REPORT

Optimising the use of praziquantel to manage blood fluke infections in commercially ranched Southern Bluefin Tuna - ASBTIA IPA

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In submitting this report, the researcher has agreed to FRDC publishing this material in its edited form.

Contents

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The South Australian Southern Bluefin Tuna ranching industry were integral to undertaking this work.

Executive Summary

This report provides a summary of work performed to develop methods to quantify *Cardicola forsteri* and *Cardicola orientalis* infections in Southern Bluefin Tuna (SBT) and an in vitro study to determine the efficacy of praziquantel as a treatment for *C. forsteri* infections.

The project was also intended to:

- determine the efficacy of praziquantel as a treatment for *C. orientalis* infections; determine minimum optimum dose to treat *Cardicola spp*.;
- determine safety margin / display target animal safety; determine ANZECC-compliant environmental trigger value and model environmental release;
- perform a dose optimisation field study; develop toxicology database; determine withholding period from residue studies; and collate occupational health and safety data.

However, due to changing industry practices and subsequent changes in research priorities, this project was ceased and did not address these additional outcomes. Despite this, the project delivered on its overarching aim of optimising the use of praziquantel to manage blood fluke infections, while developing data to underpin the requirements for an Australian Pesticides and Veterinary medicines Authority (APVMA) Minor Use Permit (MUP) application.

Given the outcomes of this project, the APVMA has indicated that they would accept a Minor Use Permit (MUP) application to be submitted for praziquantel. This application would make label claims against *C. forsteri* alone, with APVMA indicating that adequate supporting data for this application would comprise data on mortality and cardio-vascular health. This fulfils the primary reasons for undertaking this research project.

Keywords

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

Introduction

Ranching Southern Bluefin Tuna (SBT) is a successful Australian aquaculture sector. Since 1990, the industry has expanded to produce about 9000 tonnes of gilled and gutted SBT annually with an estimated value of between $AU100 - $AU300 million depending on factors such as SBT survival and performance, global tuna supply, availability of Pacific Bluefin Tuna, currency exchange rates and the status of economies in markets, principally in Japan. Direct and indirect employment in the industry is over 1500 FTE, resulting in around $50 million for households in the South Australian economy. From the mid 2000s, the SBT industry experienced increased mortality resulting in direct losses and changes in product quality that lowered the value of individual fish. Production issues cost the industry approximately $25m/season over this period.

To address this, FRDC invested in research that has identified a range of pathogens that affect the health of SBT. This has led to the development of diagnostic tools for the detection of known SBT pathogens and the establishment of husbandry and treatment strategies to mitigate against disease.

A range of SBT pathogens have been investigated, including protozoan (scuticociliate) parasites, bacteria, caligid copepods, viruses and blood flukes (FRDC Project 2001-253). Disease outcomes associated with these pathogens appear to be highly variable. While baseline mortality levels are generally low, with pathogens having no detectable impact on SBT condition index or performance between 2003 and 2006 (FRDC Project 2003-225), mortality rates of up to 12% have been reported. To address this, polymerase chain reaction (PCR) tests have been developed for the identification of several parasites and used to identify environmental reservoirs of key pathogens (FRDC Project 2004-085). It has also been demonstrated that SBT subjected to stress during the process of towing the fish to ranching sites are more likely to develop increased levels of parasitic pathogens, leading to increased morbidity and mortality (FRDC Project 2006-225).

Among the most important pathogens to the health of ranched SBT are the blood flukes *Cardicola forsteri* and *Cardicola orientalis*, which affect the heart and gills of fish and can cause significant levels of mortality (FRDC Project 2008-228). While these are only detected at very low levels in animals at the time of capture, the number of parasites can increase during the early months of ranching. This may, in part, be due to stress caused during towing, but may also be related to the presence of a terebellid polychaete that occurs in shallow waters and can attach to pontoons, nets and associated infrastructure. This species in an intermediate host of *C. forsteri* and may act as a vector to promote infection (FRDC Project 2008-228). The free living life stages of both species of blood flukes have also been detected in water samples collected from ranching operations.

Adult *C. orientalis* infect the branchial artery in the gills, while eggs lodge at the base of the primary lamellae (gill filaments). In contrast, adult *C. forsteri* occur primarily in the heart. In SBT, *C. forsteri* burdens are not correlated with mortality and it is likely that *C. orientalis* is a key driver of mortality, as it is in Pacific Bluefin Tuna.

It has been noted that in Pacific Bluefin Tuna, *C. orientalis* is susceptible to praziquantel treatments at the same doses used to treat *C. opisthorchis* (a heart blood fluke similar to *C. forsteri* that affects Pacific Bluefin Tuna). Praziquantel also appears to be an effective treatment for blood flukes in SBT, with the use of this medicine significantly reducing mortality in recent years (FRDC 2008-228). As such, praziquantel may be applied more widely in the SBT ranching industry to prevent mortality. However, the use of praziquantel in the SBT industry requires approval from Australian Pesticides and Veterinary medicines Authority (APVMA). This approval requires that overseas studies on other species of hosts and parasites be confirmed using Australian studies on local host and parasite species. In addition, the use of praziquantel in SBT requires optimisation to meet regulatory requirements for veterinary medicine use, minimised environmental impact, maximised efficacy and to prevent the emergence of widespread resistance to the product in SBT blood flukes.

A critical aspect of showing efficacy is being able to quantify parasite burdens. This is currently undertaken using a laborious manual method (flushing the heart with saline) for heart blood flukes, and no reliable

method exists for gill blood flukes. Difficulties in quantifying adult gill blood flukes in tuna have previously been reported and the technique is not considered to be efficacious. Instead, egg counts in gill tissue have been used to assess parasite intensity. However, these methods have only been applied to smaller, 200 g Pacific Bluefin Tuna, two orders of magnitude smaller than ranched SBT, and it may not be feasible to count eggs in the gills of ranched SBT. An alternative strategy to quantitate the intensity of *Cardicola spp*. infections in SBT is required.

Quantitative polymerase chain reaction (qPCR) assays, that have the potential to quantitate the intensity of infections, have been developed for *C. orientalis* and *C. forsteri* (FRDC Projects 2008-228 and 2004-085). As well as detecting the presence of either species, it is also possible to use these assays to differentiate between *C. forsteri* and *C. orientalis* DNA in SBT blood. It is proposed, therefore, that these qPCR assays should be validated and a study undertaken to determine if they can be used to accurately and reliably quantitate the intensity of *Cardicola spp*. infections by assessing SBT blood samples.

While it has been demonstrated that *C. orientalis* and *C. opisthorchis* occurring in Pacific Bluefin Tuna are susceptible to praziquantel treatments at the same dose rate, it is currently unknown if the same relationship exists for *C. orientalis* and *C. forsteri* in SBT. This is a key consideration as simultaneous treatment for both species of blood flukes would streamline treatment programs, while enabling regulatory approval for praziquantel as a treatment for both pathogens.

This project aims to optimise the use of praziquantel to manage blood fluke infections while developing research data to underpin the regulatory requirements for an APVMA Minor Use Permit (MUP) application. Ultimately, this will contribute to maintaining high health status of ranched SBT.

Objectives

Objectives of the project – as agreed in the contract:

1. Develop methods to quantify *C. forsteri* and *C. orientalis* infections
2. In vitro study to show equivalent efficacy of praziquantel as a treatment for *C. forsteri* and *C. orientalis* infections
3. Determine minimum optimum dose to treat *Cardicola* spp.
4. Determine safety margin / display target animal safety
5. Determine ANZECC-compliant environmental trigger value and model environmental release
6. Dose optimisation field study
7. Develop toxicology database
8. Determine withholding period from residue studies
9. Collate occupational health and safety data

Due to changing industry practices and subsequent changes in research priorities, this project was ceased at the end of 2013. As such, this summary final report only reports on activities and results aligned to parts of Objectives 1 and 2.

Method

Develop methods to quantify *Cardicola forsteri* and *Cardicola orientalis* infections

Preparation of fish

To assess blood fluke loads, SBT were fed a high fat diet for 30-40 days to maximise blood fluke infestation (ASBTIA unpublished data). Fish were then killed using standard commercial methods prior to a blood sample being taken for subsequent qPCR analysis. Hearts and gills were then removed from each fish to manually assess the abundance of *C. forsteri* and *C. orientalis*.

Manually Assessing the abundance of *C. forsteri* and *C. orientalis* in SBT heart and gills

Hearts of SBT were flushed with saline, with the resultant solution being examined using a dissecting microscope to count the number of adult *C. forsteri* present.

Individual gill arches were removed and observed under a dissecting microscope. The total number of *Cardicola spp.* eggs present were counted. Eggs were assessed visually and identified to species level where possible. An attempt was also made to assess the abundance of adult *C. orientalis* in gills. First, the gills were stripped of the tissue lateral to the branchial arteries using forceps, a vegetable peeler or a scalpel, and the lumen of the artery was flushed with saline using a squirt bottle. The afferent vessel was canulated to a piece of rubber tubing and the tubing was ligated to the vessel using surgical thread. A 50mL syringe was used to force saline through the gills. The saline was collected and examined using a dissecting microscope to assess the presence of adult *C. orientalis*.

Adult *C. forsteri* and *C. orientalis* collected using these methods were subsequently used to redesign and validate molecular detection techniques (qPCR) and to assess in vitro sensitivity to praziquantel (see below).

Molecular detection of *C. orientalis* and *C. forsteri*

Existing molecular detection methods for both *C. orientalis* and *C. forsteri* were redesigned for this study. The assays of Polinski et al. were considered, but are not in a suitable format for the high-throughput environment of SARDI's molecular diagnostics laboratory. Environmental sequencing also suggests they may not be specific for assessment of blood fluke eggs and larval stages from environmental samples.

Assessing the in vitro sensitivity of *C. forsteri* and *C. orientalis* to praziquantel

To assess in vitro sensitivity to praziquantel, 125 adult *C. forsteri* were collected from SBT hearts as described above. Insufficient adult *C. orientalis* were collected to assess the sensitivity of this species to praziquantel.

In vitro testing was performed according to the methods of Hardy-Smith et al. (2012). Briefly, *C. forsteri* collected from SBT hearts were washed three times in 2 mL Dulbecco's Modified Eagle's Medium (DMEM) adjusted to mimic tuna serum and randomly allocated into 12-well plates. The plates were then incubated at 20 °C for 1 h. After this time, any nonresponsive individuals were removed. The surviving flukes were then redistributed such that there was a minimum of five responsive flukes in each well.

The sensitivity of *C. forsteri* to praziquantel was assessed by exposing them to praziquantel concentrations of 0 (control), 0.1, 0.15, 0.5, 1, 5 and 6.6 mg/L. Praziquantel concentrations were randomly assigned to each well. Plates were incubated at 20 °C for 24 hours. The flukes were observed continually for the first 15 minutes and then at regular intervals for 24 hours and mortalities recorded. Based on these observations,

the praziquantel concentration at which 99% of *C. forsteri* were killed (LC99) was determined at various time points.

Results and Discussion

Quantification of *Cardicola forsteri* and *Cardicola orientalis* infections

Assessing the abundance of *C. forsteri* and *C. orientalis* in SBT heart and gills

Using saline flush, 140 adult *C. forsteri* were recovered from SBT hearts confirming that this technique is an effective means of collecting adult *C. forsteri*. Of these, 125 adult blood flukes were stored in cell culture medium adjusted to mimic tuna serum, and subsequently used to assess the sensitivity of *C. forsteri* to praziquantel. The remaining 15 adult *C. forsteri* were preserved for DNA sequencing.

Assessment of gills for the abundance of *Cardicola spp.* eggs was attempted. Gills of ranching size SBT are large and can harbour tens of thousands of *Cardicola spp.* eggs. Counting eggs in individual gill arches took over 90 minutes. While this is an effective means of detecting the presence of *Cardicola spp.* eggs, the process is extremely time consuming. As such, it is not feasible to use this approach to compare egg abundance in large numbers of fish. SBT gill tissue is also so thick as to prohibit the identification of *Cardicola spp.* eggs to the species level. Assessment of egg numbers in gills was rejected as a viable method to assess the abundance of *C. orientalis* in the gills of large numbers of fish.

The gill flush technique used in this report was not an efficient technique to collect adult *C. orientalis.* The project team was unable to reliably dislodge *C. orientalis* from the gill, and in many cases gills that had numerous *C. orientalis* eggs yielded no adult flukes. Assessment of numbers of adult *C. orientalis* in the branchial arteries proved logistically infeasible. Despite these difficulties, 12 adult *C. orientalis* were recovered. These were preserved for subsequent DNA sequencing.

Molecular detection of *C. orientalis* and *C. forsteri*

New qPCR assays for ITS-2 rDNA sequences were developed. The qPCR assay for *C. orientalis* was redesigned with the aim of achieving greater sensitivity, while the assay for *C. forsteri* was redesigned to prevent late cross-reactivity with *C. orientalis* DNA. The new assays are: *C. orientalis* Forward: 5'-TGCTTGCTATTCCTAGATGTTTAC-3' Reverse: 5'-AACAAGCCACAAAACTATACT-3' TaqMan MGB probe: 5'-6FAM-CATCCGCTCCGACGACACGA-MGB-3'; *C. forsteri* Forward: TGATTGCTTGCTTTTTCTCGAT Reverse: TATCAAAACATCAATCGACATC TaqMan MGB probe: 5'-6FAM-CCACACGGTCTCGCACTGGC-MGBNFQ-3'. These assays require further laboratory and field validation.

In Vitro Sensitivity of *C. forsteri* to Praziquantel

Adult *C. forsteri* succumbed rapidly to praziquantel exposure at concentrations ≥0.5 mg/L, with all individuals unresponsive after 60 minutes of exposure. At lower doses, mortality was reduced. When adult *C. forsteri* were exposed to 0.15 mg/L, only 60% of flukes were dead after 60 minutes of exposure, while flukes exposed to 0.1 mg/L did not succumb to praziquantel during the study period. No *C. fosteri* died in the control group (Fig. 1).

After 15 minutes, the LC99 value was 1.728 mg/L (95% CI 0.859 - 4.759), while the 30 min LC99 was 1.641 mg/L (95% CI 0.812 - 4.603). These LC99 values will be used with pharmacokinetic information to determine starting points for dose optimisation and be submitted as supporting information for the APVMA Minor Use Permit (MUP).

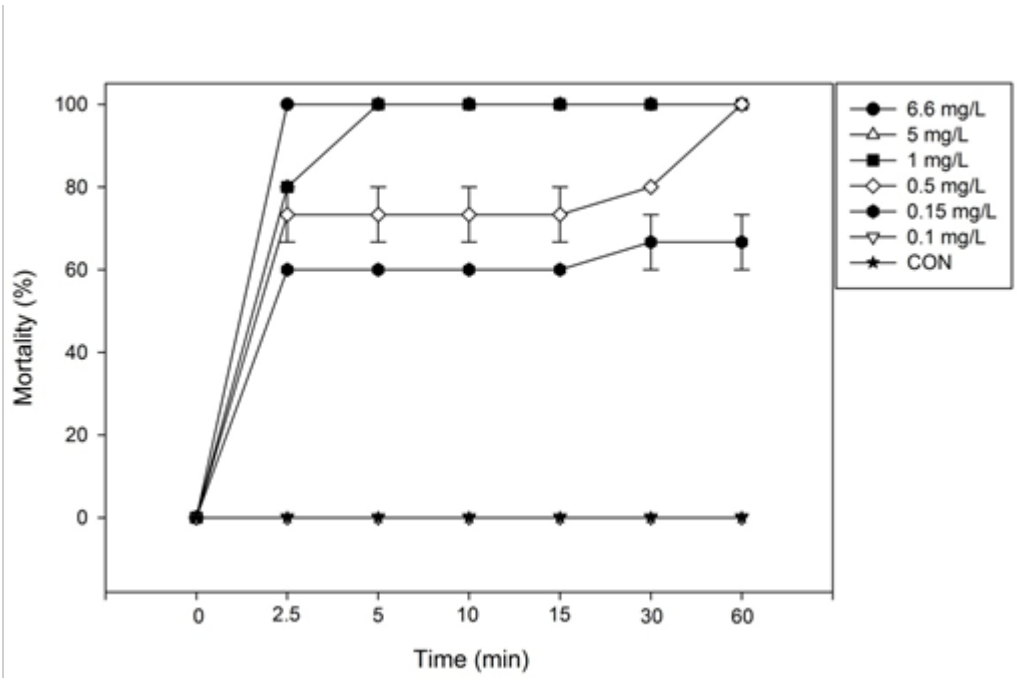

Fig. 1. Cumulative mortality curves for in vitro analysis of *C. forsteri* exposed to praziquantel.

Conclusion and Implications

Changes to research priorities during this project led to a realigning of its scope and desired outcomes. Despite this, the project has delivered on its overarching aim of maintaining the high health status of ranched SBT by optimising the use of praziquantel to manage blood fluke infections, while developing data to underpin the requirements for an APVMA Minor Use Permit (MUP) application.

It has been demonstrated that *C. orientalis* adults and eggs cannot be feasibly counted in ranching sized SBT using existing methods. However, adult *C. forsteri* can be readily collected by flushing the heart of infected fish with saline water and examining the flushed saline under dissecting microscope. Adult *C. forsteri* collected in this way were used to demonstrate the sensitivity of this pathogenic blood fluke to praziquantel at concentrations exceeding 0.15 mg/L. In Vitro LC99 values of 1.73 mg/L and 1.64 mg/L were determined after 15 and 30 minutes of exposure respectively.

This project has also delivered the key data required to develop quantitative molecular diagnostic methods for assessment of *C. forsteri and C. orientalis* in SBT blood, culture water and environmental sediments. These were developed in a format suitable for the diagnostics laboratory at SARDI and will underpin the development of rapid and reliable diagnostic tools that can quantify the abundance of these important SBT pathogens. Such tests will enable a refinement of strategies used by SBT ranching operations to mitigate against blood flukes into the future.

Discussions with the APVMA have indicated that, given the outcome of this project, they would accept a Minor Use Permit (MUP) application to be submitted for praziquantel. This application would make label claims against *C. forsteri* alone, with APVMA indicating that adequate supporting data for this application would comprise data on mortality and cardio-vascular health. This fulfils one of the primary reasons for undertaking this research project.