

**Epidemiology of blood fluke, *Cardicola forsteri*, infection in  
southern bluefin tuna, *Thunnus maccoyii***

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## **Statement of Co-Authorship**

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

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## Abstract

*Cardicola forsteri* Cribb, Daintith and Munday, 2000 (Digenea: Sanguinicolidae), is a digenean parasite of southern bluefin tuna, *Thunnus maccoyii* (Castelnau) that can cause disease in an aquaculture environment. The aim of this research was to gain information about factors affecting the epidemiology of blood fluke, *C. forsteri*, infection in farmed southern bluefin tuna in South Australia. Comparative analysis of blood fluke of other bluefin tunas was undertaken to determine the host range of *C. forsteri*. We found, through comparisons of ITS2 and partial 28S rDNA, *C. forsteri* from multiple hosts and localities: southern bluefin tuna (*T. maccoyii*) from South Australia and northern bluefin tuna (*T. thynnus*) from two localities in the Mediterranean (Spain and Croatia). Host migration seems likely to be responsible for the widespread occurrence of *C. forsteri*, although it is also possible that *C. forsteri* was translocated recently by the spread of infected intermediate hosts in international shipping, either as biofouling attached to hulls, or in ballast waters.

*C. forsteri*, was examined in cultured southern bluefin tuna, *T. maccoyii*, over a six month growout season in Port Lincoln, South Australia. *C. forsteri* infections declined after an initial peak two months after transfer from the wild and no effect was observed on tuna condition index despite high intensities being recorded. It is concluded that *T. maccoyii* may be able to control blood fluke infection.

Stochastic models were developed to describe the infection pattern of *Cardicola forsteri* in farmed southern bluefin tuna, *T. maccoyii*. Observed field data on the lengths of flukes over a growout season were used as the basis for the models. An estimated time of infection was produced from the models and it was shown that infections mostly occurred after introduction to sea-cages from the wild indicating the presence of the intermediate host in the farming environment.

Factors influencing blood fluke intensity, abundance and prevalence were investigated by examining southern bluefin tuna collected from commercial harvests over a three-year period. Blood fluke prevalence was observed to be approximately 60% in tuna over a growout season. Annual means of intensity were fixed around six fluke per infected host and annual means of abundance between three and five fluke per host. A universal factor in explaining variation in *C. forsteri* intensity, abundance and prevalence was company. Year did not influence intensity or abundance although a decrease in prevalence in 2005 was evident. Tuna harvested in winter had a significantly greater abundance and prevalence of blood fluke than harvest in autumn. Interestingly, the period of time that tuna are in captivity does not significantly influence intensity of infection even though it does affect blood fluke abundance and prevalence. Intensity or abundance did not affect the condition of tuna.

An adaptive immune response was investigated by developing an ELISA to detect and quantify an antibody response against the blood fluke in southern bluefin tuna serum. Antibody titres and seroprevalence increased during the growout period.

Parasitological and serological values from were compared from a cohort introduced to the tuna farming zone in 2005 to a cohort introduced in 2006 to determine if prior infection in the 2005 cohort elicited any protection against infection in 2006.

Although significant differences were not observed in intensities between cohorts it was shown that the cohort that had a history of infection had significantly lower abundances and prevalences of blood fluke infection than the naïve cohort demonstrating the development of acquired resistance against *C. forsteri*.

The accuracies of a gross gill pathology test and a histopathological test for detecting *C. forsteri* were evaluated. The sensitivity of gross gill pathology was the only high estimate of diagnostic accuracy. Although the other estimates of diagnostic accuracy

were low, the high sensitivity of gross gill pathology suggests that this may be a useful tool for future epidemiology studies.

A Bayesian approach to the estimation of prevalence was carried out using two populations of tuna and two different diagnostic tests, an ELISA and parasitological examination. The prevalence of infection was shown to be higher than previously thought. ELISA was shown to have poor estimates of accuracy whereas a high sensitivity for parasitological examination was demonstrated. Parasitological examination is probably the best current method for detecting blood fluke infections.

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## Publications

- Aiken, H. M., Hayward, C. J., Nowak, B. F. 2006. An epizootic and its decline of a blood fluke, *Cardicola forsteri*, in farmed southern bluefin tuna, *Thunnus maccoyii*. Aquaculture 254(1-4), 40-45.
- Aiken, H. M., Bott, N. J., Mladineo, I., Montero, F. E., Nowak, B. F., Hayward, C. J. 2007. Molecular evidence for cosmopolitan distribution of platyhelminth parasites of tunas (*Thunnus* spp.) Fish and Fisheries 8(3), 167-180.
- Aiken, H. M., Hayward, C. J., Crosbie, P., Watts, M., Nowak, B. F. 2008. Serological evidence of an antibody response in farmed southern bluefin tuna naturally infected with the blood fluke *Cardicola forsteri*. Fish and Shellfish Immunology 25(1-2), 66-75.
- Aiken, H. M., Hayward, C. J., Cameron, A., Nowak, B. F. 2009. Simulating blood fluke, *Cardicola forsteri*, infection in farmed southern bluefin tuna, *Thunnus maccoyii* using stochastic models. Aquaculture 293(3-4), 204-210.

**Note to Readers: This thesis is based on published papers,  
therefore some repetition between chapters occurs.**

## Chapter 1. General Introduction

The southern bluefin tuna, *Thunnus maccoyii*, is a long-lived and highly migratory fish that is widely distributed across the southern temperate oceans from the western Atlantic across the Indian to the western Pacific Ocean (Caton 1991; Farley and Davis 1998; Clear et al. 2000). Mitochondrial DNA analysis supports the existence of one population (Grewe et al. 1997) with adults migrating to a single spawning area in the north-east Indian Ocean south of Indonesia between September and April (Caton 1991; Grewe et al. 1997; Farley and Davis 1998). *T. maccoyii* are slow to mature reaching maturity at around eight years, but live for as long as forty years and grow to about 200 kilograms in weight and 200 cm in length (Davis et al. 2001). Juveniles tend to move south from the spawning ground to waters off the south west of Australia. Some juveniles then move westward to southern Africa, but most move east across the southern coastline of Australia inhabiting the coastal and continental shelf waters. These tuna continue to live off southern and south eastern Australia until six to nine years of age. At maturity, most *T. maccoyii* disperse into the deeper waters of the south Atlantic, Indian and south west Pacific Oceans (Love and Langenkamp 2003). Adult *T. maccoyii* are assumed to forage throughout the temperate waters of the Southern Hemisphere oceans during the austral winter, migrating to the spawning grounds of the north-western Indian Ocean from spring to autumn (Shingu 1978; Caton 1991) before returning to the foraging grounds in the following autumn/winter. Individuals do not remain on the spawning grounds over the whole season; instead there is a turnover of fish with the numbers of mature fish peaking in October and February (Farley and Davis 1998).

Commercial fishing for southern bluefin tuna by Australia and Japan began in the early 1950s. Overfishing has resulted in the species being listed as endangered

(Safina 1995) and a quota system was introduced in 1989-1990. Mature stock population numbers reached a critically low level, below 6-11% of the 1960 size, in the early 90s (Ward 1995). Australia's catch of southern bluefin tuna was restricted to 5265 tonnes annually. The high commercial value, reduced quotas and the endangered status led to the development of a commercial aquaculture industry for this species as the best way of utilising a limited resource (Lee 1998). At present capture and on-growing in sea-cages is being used successfully in Port Lincoln, South Australia (Watts et al. 2001). Juvenile fish (2-3 years old) are caught from December to March off the South Australian coast, corresponding with the annual migration of southern bluefin tuna through the Great Australian Bight fishery, using netting techniques. Tuna are transferred to special purpose built towing cages in which they are towed slowly back to growout farms near Port Lincoln at an average speed of 1 knot. During the next three to eight months the tuna are held in 40–50 metre diameter polar circle type farm pontoons. Tuna are fattened on a diet of frozen and fresh baitfish, such as pilchards and herring, to improve both their condition and biomass, and are then progressively harvested meeting demands of the Japanese market. About 45 000 tonnes of baitfish, sourced both locally and overseas, are used annually. Food conversion ratios average 10–15:1 and an average size southern bluefin tuna increases in weight by 10–20 kilograms during the farming process (Love and Langenkamp 2003). Around 98 percent of the Australian southern bluefin tuna quota is now farmed in the waters off Port Lincoln (PIRSA 2000). In 2002/03, direct output generated in South Australia by tuna farms totaled almost A\$267 million.

A range of parasites are known to infect wild southern bluefin tuna (see review by Munday et al. 2003). However the parasites of farmed southern bluefin tuna and their

effects on the host have been little studied. An investigation into the risk factors facing the health of farmed southern bluefin tuna was undertaken by Nowak (2004) and a digenean sanguinicolid blood fluke, *Cardicola forsteri*, was identified as one of the more significant risks to southern bluefin tuna health, although low in terms of absolute risk. *C. forsteri* was described from farmed *T. maccoyii* in 1997 as a result of an investigation of nodular gill lesions (Colquitt 2001; Cribb et al. 2000). Mass mortalities of cultured fish in North America, Europe and Asia have been associated with sanguinicolid infections (Bullard and Overstreet 2002). Consequently Nowak (2004) suggested that further research was required on the biology and epidemiology of the blood fluke.

*C. forsteri* is a sanguinicolid trematode with a complex life cycle (Figure 1) that infects *T. maccoyii* as well as wild northern bluefin tuna, *T. thynnus* (Bullard et al. 2004). The intermediate host is unknown but is most likely a polychaete or bivalve (Smith 1997). Sanguinicolids feed on host blood and live in the vasculature of the circulatory system of both marine and freshwater fish (Smith 1972; Smith 1997). Most species of sanguiniolid establish in the heart, bulbus arteriosus, ventral aorta, or branchial vessels, although distributions within the cephalic or dorsal vessels are not uncommon (Kirk and Lewis 1994). Once established, the adult fluke lay eggs which travel to the gills where they lodge. Adult fluke may also migrate to and from the gills to lay eggs. Adult *Paracardicoloides yamagutii* have only been observed in the gills of eels (*Anguilla australis* and *Anguilla dieffenbachia*) after migrating there to lay eggs (Hine 1978). Eggs hatch and break out of the gill structure into free-swimming miracidia. These miracidia seek out an appropriate intermediate host into which they penetrate to undergo asexual reproduction as rediae and/or sporocysts to produce infective cercariae. Cercariae are shed from the intermediate host and

actively search for the definitive host, a fish. The cercariae penetrate the skin of this host and develop into juvenile fluke stages that move towards the circulatory system in which a migration to a final site occurs. Here, the juvenile fluke mature into adults (Smith 1997). For *C. forsteri* the final site of maturation is the heart (Cribb et al. 2000).

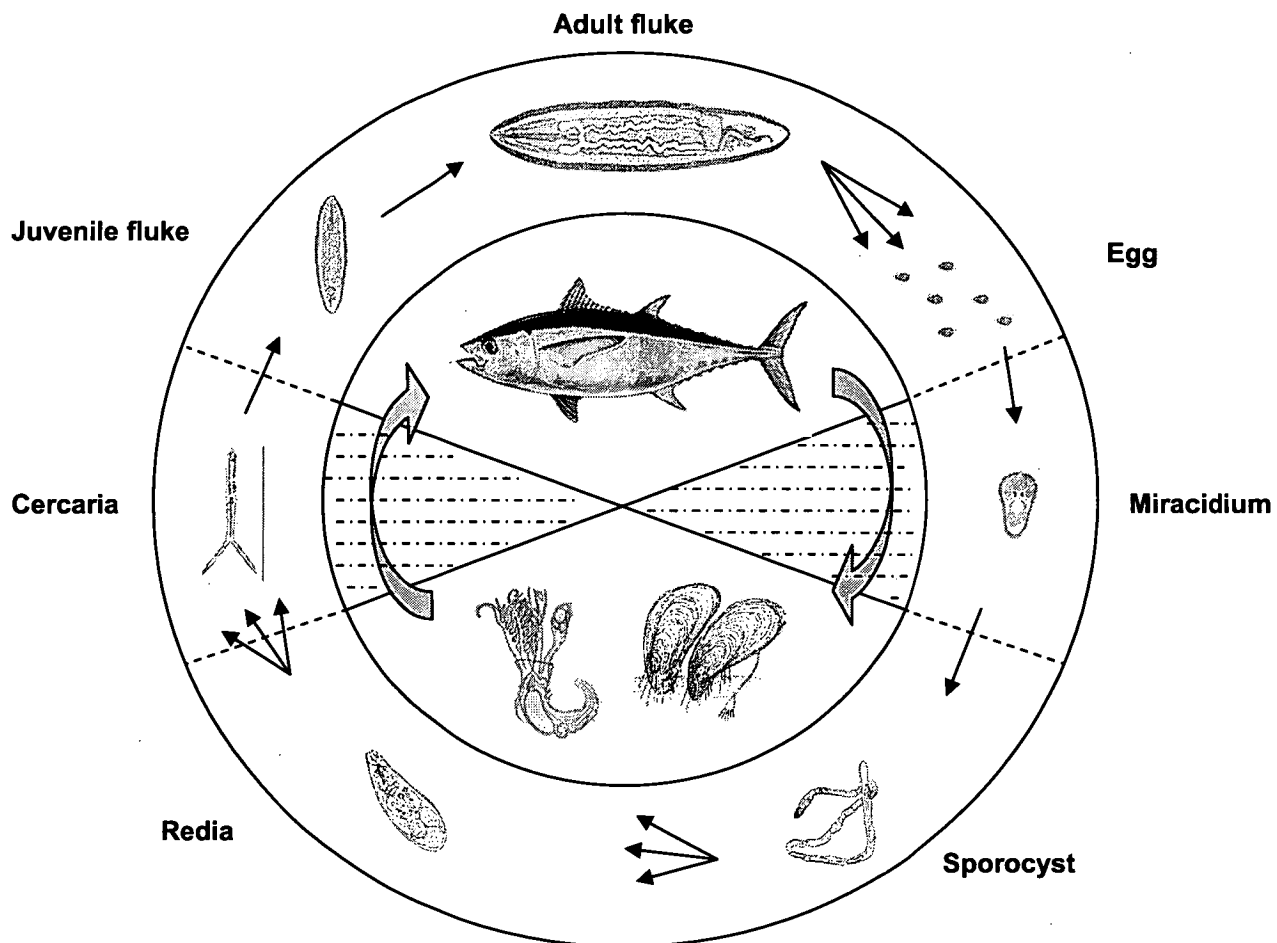


Figure 1. Hypothesised life cycle of *C. forsteri* in *T. maccoyii*. Intermediate host is unknown and may include polychaetes or bivalves. Dashed lines indicate free-living stages of *C. forsteri*. Illustrations of juvenile and adult fluke are based on a figure from Cribb et al. (2000).

Clinical signs of infection include increased mucus on the gills and respiratory distress, lethargy and slightly increased mortality (Rough 2000; Munday et al. 2003). The pathology caused by the fluke was investigated by Colquitt et al. (2001). In most cases, the changes observed were not considered to be sufficient to lead to mortalities; however they were considered to be significant (Colquitt et al. 2001).



Eggs were observed in the heart ventricle lodged in capillaries and in between individual trabeculae. Eggs were encapsulated by connective and fibrous tissue arranged in focal granulomas. It was estimated that in heavily infected fish 1.7 million eggs would have been in the entire ventricle (Colquitt 1999). In these heavily infected tuna there was a significant build up of fibrous tissue in the spongiosa. It was suggested by Colquitt et al. (2001) that the luminal space of the ventricle appeared to decrease due to the build up of fibrous tissue and the associated thickening of the myocardial fibres. Eggs accumulated in the afferent filamental arteries leading to blockages and obstructing blood flow leading to the formation of thrombi and oedema. This pathology led to parts of the gill respiratory surface being occluded by lesion formation (Colquitt et al. 2000). It is possible that the pathology observed is a result of the immune response of the host. A specific antibody response against the blood fluke in farmed southern bluefin tuna was shown using Western blot analysis (Watts et al. 2001). It is not known whether this response has a protective role or how it is affected by infection dynamics.

Little is known about the epidemiology of *C. forsteri* infection in southern bluefin tuna. Colquitt et al. (2002) observed that the prevalence and severity of infections appeared to increase after transfer of tuna from the wild suggesting that the life cycle was able to be completed in the farming zone (Munday et al. 2003). However there have been no surveys employing epidemiological methods to determine whether the life cycle is being maintained in the vicinity of the farming zone or whether infections are increasing over the growout period.

## Research objectives

The objectives of this research were to:

- Investigate the host range of *C. forsteri* in bluefin tunas
- Determine whether the life cycle of *C. forsteri* is able to be maintained in the Spencer Gulf southern bluefin tuna farming zone
- Examine the effect of the farming cycle on the prevalence and intensity of *C. forsteri* infection
- Investigate the use of serological techniques for determining *C. forsteri* infection
- Investigate the antibody response of the southern bluefin tuna against *C. forsteri* infection during the farming cycle
- Compare the current diagnostic methods for determining *C. forsteri* infection and determine the estimates of accuracy for the diagnostic methods

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## **Chapter 2. Molecular evidence for cosmopolitan distribution of platyhelminth parasites of tunas (*Thunnus* spp.)**

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## Abstract

Global distribution of platyhelminth parasites and their host-specificities are not well known. Our hypothesis was that platyhelminth parasites of large pelagic fishes are common around the world. We analysed molecular variation in three different taxa of platyhelminth parasites infecting four species of tunas: yellowfin tuna (*Thunnus albacares*, Scombridae) from Western Australia, southern bluefin tuna (*T. maccoyii*, Scombridae) from South Australia, Pacific bluefin tuna (*T. orientalis*, Scombridae) from Pacific Mexico, and northern bluefin tuna (*T. thynnus*, Scombridae) from two localities in the Mediterranean (Spain and Croatia). Comparisons of ITS2 and partial 28S rDNA demonstrated two congeneric species of blood flukes (Digenea: Sanguinicolidae) from multiple hosts and localities: *Cardicola forsteri* from southern bluefin and northern bluefin tunas, and *C. sp.* from Pacific bluefin and northern bluefin tunas; and a gill fluke, *Hexostoma thynni* (Polyopisthocotylea: Hexostomatidae), from yellowfin, southern bluefin and northern bluefin tunas. Partial 28S rDNA indicates that a second type of fluke on the gills, *Capsala sp.* (Monopisthocotylea: Capsalidae), occurs on both southern bluefin and Pacific bluefin tunas. This appears to be the first report of conspecific platyhelminth parasites of teleosts with a wide-ranging geographical distribution that has been confirmed through molecular approaches. Given the brevity of the free-living larval stage of both taxa of flukes on the gills (*Hexostoma thynni* and *Capsala sp.*), we conclude that the only feasible hypothesis for the cosmopolitan distribution of these flatworms is migrations of host tunas. Host migration also seems likely to be responsible for the widespread occurrence of the two species of blood flukes (*Cardicola spp.*), although it is also possible that these were translocated recently by the spread of infected intermediate hosts.



## 1. Introduction

The geographical distribution of parasites in marine environments is poorly known (Rohde 2005). Accordingly, the natural occurrences of parasitic platyhelminths are also poorly documented, even though some species are commonly associated with health risks to teleosts in fisheries and aquaculture. Platyhelminthes is a large and diverse phylum; worldwide, it is estimated that there may be as many as 25,000-50,000 species of endoparasitic flukes (Digenea) (Cribb *et al.* 2002) and 25,000 species of ectoparasitic flukes (Monogenea) (Whittington 1998). In addition, our understanding of the geographic ranges of platyhelminths is presently confounded by the presence of cryptic species which have been revealed recently through molecular analyses (Jousson *et al.* 2000). Furthermore, most of the recent molecular studies investigating the geographic ranges of helminths of fishes have concentrated on hosts that have relatively small geographic ranges. For example, Lo *et al.* (2001) focussed on hosts that are associated with coral reefs and which also do not exhibit migratory behaviour, and found no or negligible molecular variation. Other studies that were based on morphological characters rather than molecular analysis have concentrated on wide-ranging, epipelagic scombrid fishes and found helminths with wide-ranging distributions. For example, the polyopisthocotylean *Gotocotyla acanthura* (Gotocotylidae) is reported to occur in all three major oceans where their Spanish mackerel (*Scomberomorus* spp., Scombridae) hosts occur: the Pacific, the Atlantic, and the Indian Oceans (Hayward and Rohde 1999a). Similarly, another polyopisthocotylean, *Neothoracocotyle acanthocybii* (Thoracocotylidae) occurs on Wahoo (*Acanthocybium solandri*, Scombridae) in both the Pacific and Atlantic Oceans (Hayward and Rohde 1999b). However, because parasite identifications in such studies were based only on morphological characters, some doubt remains that these parasites may belong to cryptic species complexes that are genetically distinct

at each locality. To date, no molecular studies of platyhelminths of wide-ranging teleosts have examined whether such parasites do in fact belong to single, cosmopolitan species.

One group of teleosts that have a wide distribution are the tunas (Scombridae: Scombrinae), particularly *Thunnus* spp. Relatively little is known of the parasite fauna of wild tunas. Current knowledge was reviewed by Munday *et al.* (2003). Parasites of farmed tunas have also been surveyed in three localities: northern bluefin tuna (*T. thynnus*) in Croatia (Mladineo and Tudor 2004; Nowak *et al.* 2006), northern bluefin tuna in Spain (Nowak *et al.* 2006), and southern bluefin tuna (*T. maccoyii*) in Australia (Munday *et al.* 2003; Deveney *et al.* 2005; Nowak *et al.* 2006). Two platyhelminths were identified as possible risks to the health of farmed southern bluefin tuna: a digenean blood fluke *Cardicola forsteri* (Sanguinicolidae) and a polyopisthocotylean (Nowak 2004; Deveney *et al.* 2005). The latter species has now been identified as *Hexostoma thynni* (see Hayward *et al.* 2007). Based on morphological studies, these parasites have also been reported to be widely distributed: *C. forsteri* is known from the Southern Ocean off Australia (from *T. maccoyii*) and the western Atlantic Ocean (from *T. thynnus*) (Cribb *et al.* 2000a; Bullard *et al.* 2004), and *H. thynni* is known from the Southern Ocean (from *T. maccoyii*) and in the Mediterranean Sea (from *T. thynnus*) (Nowak *et al.* 2006). Here we use a molecular approach to investigate whether these and other parasites we collected are indeed cosmopolitan in and on various species of tunas (*Thunnus* spp.). Ribosomal DNA has been used as a taxonomic tool since the earliest works on molecular phylogenetic investigations began. The two regions used in this study, 28S and ITS2 rDNA, have been utilized in many studies of genetic differences of parasitic platyhelminths (e.g. Anderson and Barker 1998; Chisholm *et al.* 2001;

Matejusová *et al.* 2004; Nolan and Cribb 2006; Platt and Tkach 2003) and are considered sound markers for species differentiation. One exception was reported by Nolan and Cribb (2005) where two ‘good’ sister species of digeneans had identical ITS2 sequences.

## **2. Materials and Methods**

### **2.1. Parasite material**

Sources of material are listed in Table 1, and their locations mapped in Fig. 1.

Specimens of *Cardicola* spp. were collected from the heart ventricle of tuna, and specimens of *Hexostoma thynni* and capsalids were collected from the gills of tuna.

Specimens from *Thunnus maccoyii* were collected from fresh gills and heart ventricles obtained at harvest; specimens from *T. orientalis* were collected from mortalities, which were either examined fresh or had been frozen for less than one week; and samples from *T. albacares* and Spanish *T. thynnus* were collected from defrosted gills and ventricles that had been stored at -20 °C after being collected at harvest or capture. All specimens were washed extensively in physiological saline solution and some were stored at -80°C and others fixed in 100% ethanol.

Specimens from Croatian *T. thynnus* were collected fresh and fixed in 99.7-100% ethanol.

**Table 1.** Collection data for platyhelminth specimens sampled from tuna (*Thunnus* spp.) in this study.

Species	Host	Habitat	Origin	No. of hosts examined	No. of specimens sequenced	Regions sequenced
<b>A. Trematoda, Digenea</b>						
<i>Cardicola forsteri</i>	<i>Thunnus maccoyi</i>	Wild	Cabbage Patch, South Australia	11	1	ITS2, 28S
		Farm	Port Lincoln, South Australia	13	13	ITS2, 28S
	<i>T. thynnus</i>	Farm	Puerto de Mazarron, Spain	42	1	ITS2, 28S
<i>Cardicola</i> sp.	<i>T. thynnus</i>	Farm	Puerto de Mazarron, Spain	42	1	ITS2
	<i>T. orientalis</i>	Farm	Islas Coronados, Pacific Mexico	75	1	ITS2
<b>B. Monogenea, Polyopisthocotylea</b>						
<i>Hexostoma thynni</i>	<i>T. maccoyii</i>	Farm	Port Lincoln, South Australia	13	15	ITS2, 28S
	<i>T. thynnus</i>	Farm	Bay of Maslinova, Croatia	210	2	ITS2, 28S
	<i>T. albacares</i>	Wild	Geraldton, Western Australia	7	4	ITS2, 28S
<b>C. Monogenea, Monopisthocotylea</b>						
<i>Capsala</i> sp.	<i>T. maccoyii</i>	Farm	Port Lincoln, South Australia	>500	1	28S
	<i>T. orientalis</i>	Farm	Islas Coronados, Pacific Mexico	76	1	28S

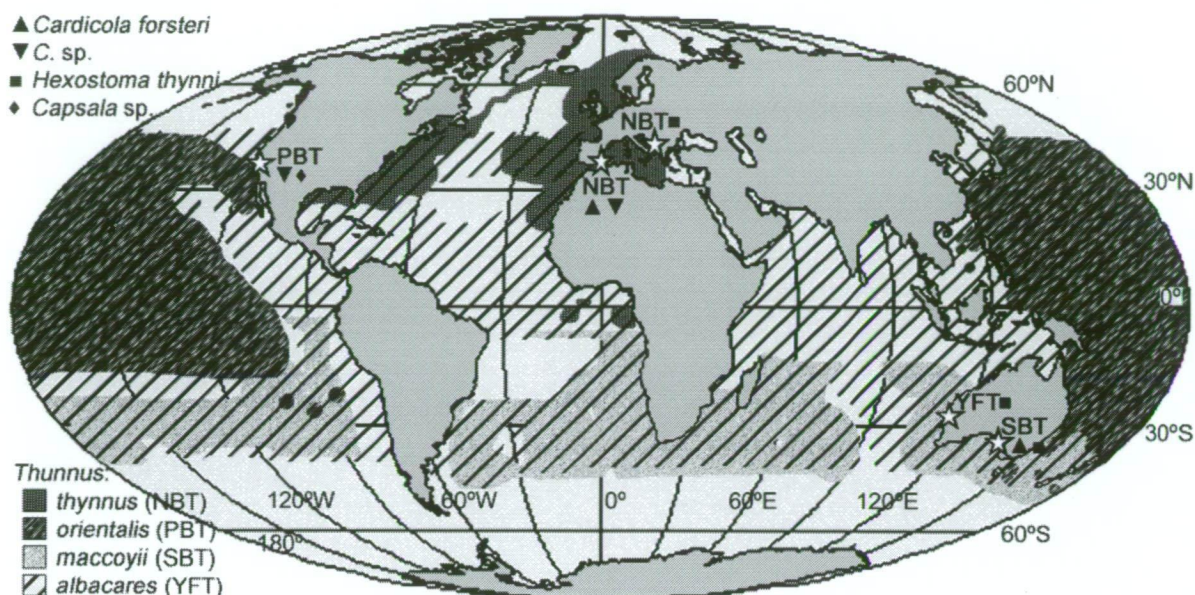


Fig. 1 Distribution of four species of host *Thunnus* spp. around the world and the occurrence

particular parasites in samples of these tunas. Tuna distributions are based either on

computer-generated 'AquaMaps' in Fishbase (Southern bluefin tuna:

<http://fishbase.sinica.edu.tw/tools/aquamaps/premap2.php?SpecID=Fis-22835&cache=1> (last accessed 12 May 2007); Northern bluefin tuna:

<http://fishbase.sinica.edu.tw/tools/aquamaps/preMap.php?cache=1&SpecID=Fis-49220> (last accessed 12 May 2007) ; yellowfin tuna:

<http://fishbase.sinica.edu.tw/tools/aquamaps/premap2.php?SpecID=Fis-22833&cache=1> (last accessed 12 May 2007)) or are represented by a composite figure (for Pacific bluefin tuna

derived from maps contained in <http://www.fao.org/DOCREP/003/W3628E/w3628e0y.htm> (last accessed 12 May 2007 and

<http://www.fao.org/DOCREP/005/T1817E/T1817E13.htm#ch10> (last accessed 12 May 2007).

## 2.2. Molecular analysis

Genomic DNA was extracted using QIAGEN DNeasy<sup>®</sup> tissue kit (QIAGEN GmbH, Hilden, Germany). For *Cardicola forsteri*, the second internal transcribed spacer (ITS2) rDNA was PCR amplified using primers and thermocycling conditions used

by Nolan and Cribb (2004a) and partial 28S D1-D2 was PCR amplified using primers and thermocycling conditions used by Bott and Cribb (2005). For *Hexostoma thynni* ITS2 rDNA was PCR amplified using two primers designed in conserved regions in the ITS2 rDNA, forward (5'-CGA TGA AGA GTG CAG CAA AC-3') and reverse (5'-TAA TGC TTA AAT TCA GCG GGT-3') performed using 1.6 µl of MgCl<sub>2</sub> (25 mM), 2 µl of PCR reaction buffer (QIAGEN, GmbH, Hilden, Germany) (10x), 0.8 µl of dNTPs (5 mM), 0.75 µl of primer and reverse primer (10 pmol), 0.25 µl Hot Star Taq polymerase (QIAGEN) (5 units/µl), 1-2 µl of DNA template (5-100 ng), made up to 20 µl with RNAase free H<sub>2</sub>O and run with the following thermocycling conditions: 95 °C for 15 min to activate Taq polymerase, preheating at 94 °C for 3 min, 30 cycles of denaturation (94 °C, 60 sec), annealing (50 °C, 3 min) and extension (72 °C, 3 min), and further elongation step of 10 min at 72 °C. For *H. thynni* and *Capsala* sp., partial 28S C1-D2 rDNA was PCR amplified using primers and thermocycling conditions used by Chisholm *et al.* (2001). All PCR products were amplified using an MJ Research PTC-200 Thermal Cycler (MJ Research, supplied by Bresatec, Watertown, U.S.A.).

PCR products were purified using Macherey-Nagel NucleoSpin® kit (Macherey-Nagel GmbH, Düren, Germany) and cycle-sequenced from both strands using ABI BigDye™ Version 3.1 (Applied Biosystems, Foster City, USA) chemistry, alcohol precipitated and automated sequences obtained. The contiguous sequences were aligned using Sequencher™ (GeneCodes Corp., Ann Arbor, U. S. A. ver. 4.2). Sequences were aligned using ClustalX (Thompson *et al.*, 1997) and further refined by eye.

For the *Hexostoma thynni* analysis, *Polystoma gallieni* (Polystomatidae) Genbank accession no. **AF382064**) was designated as the functional outgroup and for the *Cardicola forsteri* analysis, *Pearsonellum corventum* (Sanguinicolidae) Genbank accession no. **AY465873**) was designated as the functional outgroup. Table 2 shows provenance of DNA sequences used in the *H. thynni* and *Cardicola* analyses. Bayesian Inference analysis was conducted with MrBayes ver. 3.0 b4 (Huelsenbeck and Ronquist, 2001) using the following parameters: nst = 6, rates = gamma, autoclose = yes. Posterior probabilities were approximated over 2,000,000 generations (ngen = 2,000,000) via 4 simultaneous Markov Chain Monte Carlo (MCMC) chains (nchains = 4) with every 100<sup>th</sup> tree saved (samplefreq = 100). Default values were used for MCMC parameters. Consensus trees with mean branch lengths were constructed using the 'sumt' command. A 50% majority rule consensus tree was constructed from the tree output files produced in the Bayesian Inference analysis using TreeView (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>, last accessed 12 May 2007). Sequences were added to Genbank (<http://www.ncbi.nlm.nih.gov/Genbank/>, last accessed 12 May 2007), and the accession numbers of sequences obtained in this study, and previously published sequences are in Table 1.

**Table 2.** Platyhelminths used in Bayesian inference analyses in this study.

Family	Species	Host	Origin	Study/Source	Genbank
<b>A. Trematoda</b>					
Sanguinicolidae	<i>Braya jexi</i>	<i>Scarus frenatus</i> Scaridae	Heron Island, Australia	Nolan and Cribb (2006)	<a href="#"><u>DQ059624</u></a>
	<i>B. psittacus</i>	<i>S. ghobban</i> Scaridae	Heron Island, Australia	Nolan and Cribb (2006)	<a href="#"><u>DQ059625</u></a>
	<i>B. yantschi</i>	<i>Chlorurus microrhinos</i> Scaridae	Heron Island, Australia	Nolan and Cribb (2006)	<a href="#"><u>DQ059628</u></a>
	<i>Braya</i> sp. 1	<i>C. microrhinos</i> Scaridae	Heron Island, Australia	Nolan and Cribb (2006)	<a href="#"><u>DQ059626</u></a>
	<i>Braya</i> sp. 2	<i>Scarus ghobban</i> Scaridae	Ningaloo Reef, Australia	Nolan and Cribb (2006)	<a href="#"><u>DQ059627</u></a>
	<i>Cardicola forsteri</i>	<i>Thunnus maccoyii</i> Scombridae	Port Lincoln, Australia	This study	<a href="#"><u>EF661575</u></a>
		<i>T. maccoyii</i> Scombridae	Port Lincoln, Australia	Nolan and Cribb (2006)	<a href="#"><u>DQ059637</u></a>
		<i>T. thynnus</i> Scombridae	Puerto de Mazarron, Spain	This study	<a href="#"><u>EF653395</u></a>
	<i>C. bartolii</i>	<i>Siganus lineatus</i> Siganidae	Heron Island, Australia	Nolan and Cribb (2006)	<a href="#"><u>DQ059631</u></a>
	<i>C. coeptus</i>	<i>S. punctatus</i> Siganidae	Heron Island, Australia	Nolan and Cribb (2006)	<a href="#"><u>DQ059629</u></a>
	<i>C. coeptus</i>	<i>S. vulpinus</i> Siganidae	Heron Island, Australia	Nolan and Cribb (2006)	<a href="#"><u>DQ059630</u></a>
	<i>C. covacinae</i>	<i>S. punctatus</i> Siganidae	Heron Island, Australia	Nolan and Cribb (2006)	<a href="#"><u>DQ059634</u></a>
	<i>C. lafii</i>	<i>S. fuscescans</i> Siganidae	Lizard Island, Australia	Nolan and Cribb (2006)	<a href="#"><u>DQ059639</u></a>
	<i>C. milleri</i>	<i>Lutjanus bohar</i> Lutjanidae	Lizard Island, Australia	Nolan and Cribb (2006)	<a href="#"><u>DQ059640</u></a>



**Table 2.** Platyhelminths used in Bayesian inference analyses in this study (Cont.).

Family	Species	Host	Origin	Study/Source	Genbank
<b>A. Trematoda</b>					
Sanguinicolidae	<i>C. parilus</i>	<i>S. fuscescens</i> Siganidae	Ningaloo Reef, Australia	Nolan and Cribb (2006)	<u><b>DQ059638</b></u>
	<i>C. tantabiddii</i>	<i>S. fuscescens</i> Siganidae	Ningaloo Reef, Australia	Nolan and Cribb (2006)	<u><b>DQ059642</b></u>
	<i>C. watsonensis</i>	<i>S. corallinus</i> Siganidae	Lizard Island, Australia	Nolan and Cribb (2006)	<u><b>DQ059643</b></u>
	<i>Cardicola</i> sp. 1	<i>T. orientalis</i> Scombridae	Islas Coronados, Mexico	This study	<u><b>EF653396</b></u>
		<i>T. thynnus</i> Scombridae	Puerto de Mazarron, Spain	This study	<u><b>EF653397</b></u>
	<i>Cardicola</i> sp. 2	<i>Siganus fuscescans</i> Siganidae	Heron Island, Australia	Nolan and Cribb (2006)	<u><b>DQ059636</b></u>
	<i>Cardicola</i> sp. 3	<i>S. margaritifera</i> Siganidae	Nth Stradbroke Is, Australia	Nolan and Cribb (2006)	<u><b>DQ059635</b></u>
	<i>Cardicola</i> sp. 3	<i>Scomberomorus commerson</i>	Lizard Island, Australia	Nolan and Cribb (2006)	<u><b>DQ059641</b></u>
		Scombridae			
	<i>Cardicola</i> sp. 4	<i>S. munroi</i> Scombridae	Lizard Island, Australia	Nolan and Cribb (2006)	<u><b>DQ059632</b></u>
	<i>Pearsonellum corventum</i>	<i>Plectropomus leopardus</i>	Heron Island, Australia	Nolan and Cribb (2004b)	<u><b>AY465873</b></u>
		Serranidae			
<b>B. Monogenea; Polyopisthocotylea</b>					
Polystomatoinea					
Polystomatidae	<i>Polystoma gallieni</i>	<i>Hyla meridionalis</i> (frog)	France	unpublished	<u><b>AF382064</b></u>
		Hylidae			

**Table 2.** Platyhelminths used in Bayesian inference analyses in this study (Cont.).

Family	Species	Host	Origin	Study/Source	Genbank
<b>Oligonchoinea</b>					
Mazocraeinea					
Mazocraeidae	<i>Kuhnia scombri</i>	<i>Scomber scombrus</i> Scombridae	United Kingdom	Olson & Littlewood (2002)	<u><b>AF382044</b></u>
Hexostomatinea					
Hexostomatidae	<i>Hexostoma thynni</i>	<i>Thunnus albacares</i> Scombridae	Geraldton, Australia	This study	<u><b>EF653382</b></u>
		<i>T. maccoyii</i> Scombridae	Port Lincoln, Australia	This study	<u><b>EF653381</b></u>
		<i>T. thynnus</i> Scombridae	Bay of Maslinova, Croatia	This study	<u><b>EF653383</b></u>
Gastrocotylinea					
Allodiscocotylidae	<i>Metacamopia oligoplites</i>	<i>Oligoplites</i> sp. Carangidae	Brazil	Olson & Littlewood (2002)	<u><b>AF382038</b></u>
Microcotylinea					
Heteraxinidae	<i>Zeuxapta seriolae</i>	<i>Seriola lalandi</i> Carangidae	Adelaide, Australia	This study	<u><b>EF653384</b></u>
Microcotylidae	Microcotylid sp. 1	<i>Sebastes</i> sp. Sebastidae	United Kingdom	This study	<u><b>EF653385</b></u>
	Microcotylid sp. 2	<i>Argyrosomus japonicus</i> Sciaenidae	Port Lincoln, Australia	This study	<u><b>EF653386</b></u>

### 3. Results

#### 3.1. *Cardicola* spp.

Sequences of *Cardicola forsteri* from *Thunnus maccoyii* off Australia (from farmed hosts off Port Lincoln, and wild hosts from Cabbage Patch (Longitude 138° 23' 843", Latitude 34° 46' 832")), and farmed *T. thynnus* in the Spanish Mediterranean, were found to be 100% identical for ITS2 rDNA, and 100% identical 28S D1-D2 rDNA for Pt Lincoln aquaculture specimens and Cabbage Patch specimens, with the 28S D1-D2 rDNA of Spanish specimens differing by 1 nucleotide in an alignment of 714 nucleotides. Similarly, sequences of *Cardicola* sp. from *Thunnus orientalis* off Mexico and farmed *T. thynnus* in the Spanish Mediterranean were found to be 100% identical for ITS2 rDNA; no sequences of 28S were obtained. There were 20 nucleotide differences in the alignment between ITS2 rDNA sequences of *C. forsteri* and *Cardicola* sp. *C. forsteri* 28S rDNA sequences have also been submitted to Genbank under the following accession numbers EF653387 (southern bluefin tuna, Port Lincoln), EF653388 (northern bluefin tuna, Spain), EF653389 (southern bluefin tuna, Cabbage Patch), although they have not been presented in a phylogenetic analysis as there are fewer sanguinicolid 28S rDNA sequences available to us through public databases, than sanguinicolid ITS2 rDNA.

Fig. 2 depicts the results of the Bayesian inference analysis for *Cardicola* spp. ITS2 rDNA. All *C. forsteri* sequences, from *T. maccoyii* and *T. thynnus* form a clade with 100% support, and *Cardicola* sp. from *T. orientalis* and *T. thynnus* form a clade with 100% support. The two *Cardicola* spp. from *Thunnus* spp. form a clade (100%) to the exclusion of all other in-group taxa. All *Cardicola* spp. in the analysis form a clade with 78% support to the exclusion of the other taxa, and *Cardicola* spp. from siganids and lutjanids form a clade with 76% support, with the siganid species

forming a clade (100%) to the exclusion of *C. milleri* (Sanguinicolidae). The two *Cardicola* spp. from *Scomberomorus* (Scombridae: Scomberomorinae) form a clade (75%) to the exclusion of all other *Cardicola* spp. All *Braya* spp. (Sanguinicolidae) form a clade with 100% support.

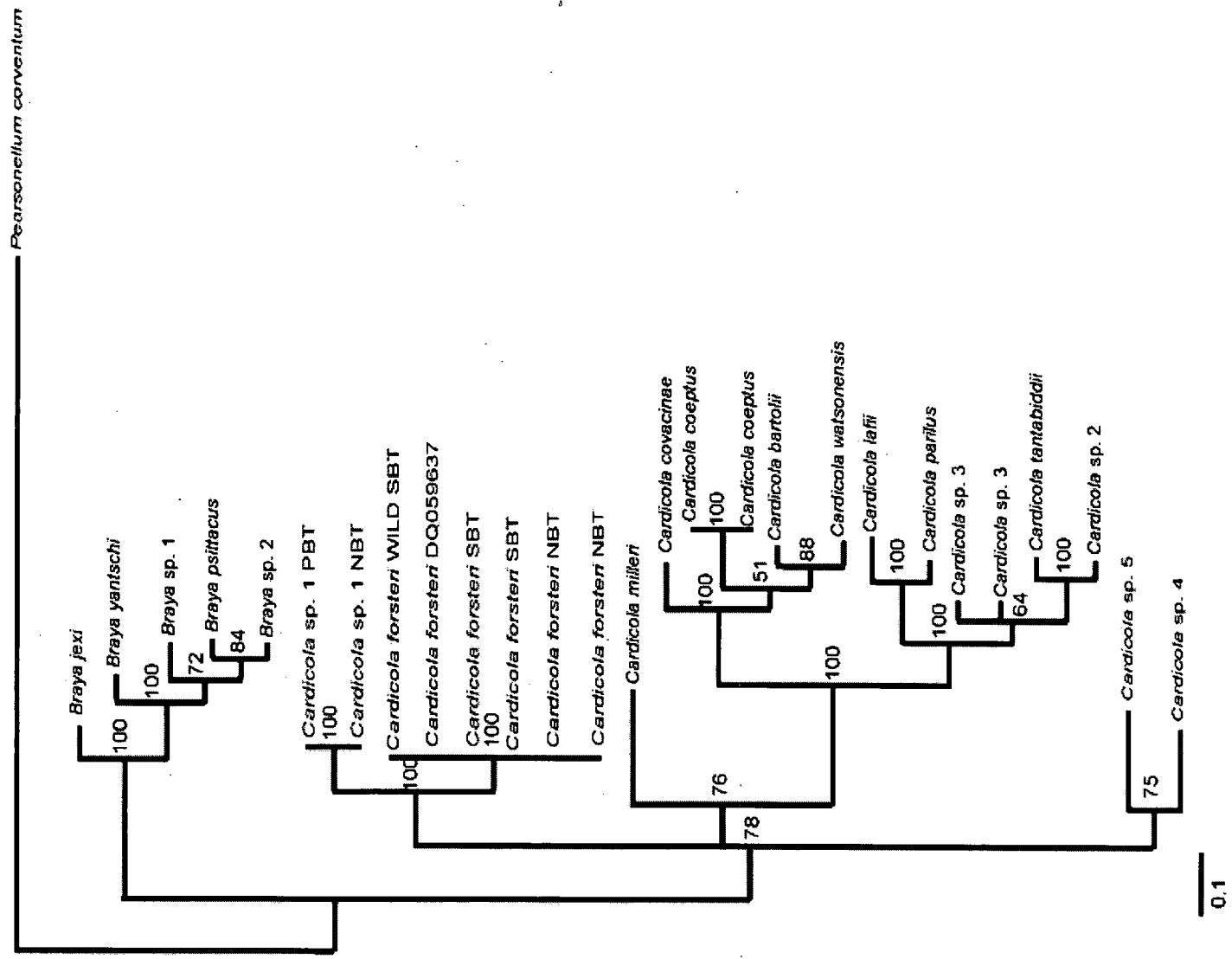


Fig. 2. Bayesian Inference analysis using ITS2 rDNA for *Cardicola* spp. *Pearsonellum corventum* designated as the functional outgroup. Bar = 0.1 substitutions/site. Abbreviations: NBT- Northern bluefin tuna, *Thunnus thynnus*, PBT- Pacific bluefin tuna, *Thunnus orientalis*, SBT- Southern bluefin tuna, *Thunnus maccoyii*, YFT- Yellowfin tuna, *Thunnus albacares*.

### 3.2. *Hexostoma thynni*

Sequences of ITS2 rDNA from all 21 worms from all three hosts were identical to each other, as were those of 28S C1-D2 rDNA. Here we present the Bayesian Inference analysis for *H. thynni* 28S C1-D2 rDNA (Fig. 3), with previously published polyopisthocotylean 28S rDNA shown in Table 2. *H. thynni* ITS2 rDNA sequences have also been submitted to Genbank under the following accession numbers EF653390 (southern bluefin tuna, Port Lincoln), EF653391 (southern bluefin tuna, Cabbage Patch), EF653392 (yellowfin tuna, Geraldton), EF653393 (northern bluefin tuna, Spain), although they have not been presented in a phylogenetic analysis as there are few polyopisthocotylean ITS2 rDNA sequences available through public databases. All *H. thynni* sequences, from *T. albacares*, *T. maccoyii* and *T. thynnus* forms a clade with 100% support to the exclusion of all other taxa in the analysis, *H. thynni* forms a clade (87%) with *Zeuxapta seriola* (Family Heteraxinidae) and two microcotylid spp. *Zeuxapta seriola* and the two microcotylid spp. form a clade with 100% support. The two microcotylid spp. form a clade with 100% support. *Metacamopia oligoplites* (Family Allodiscocotylidae) and *Kuhnia scombri* (Family Mazocraeidae) form a clade with 51% support.

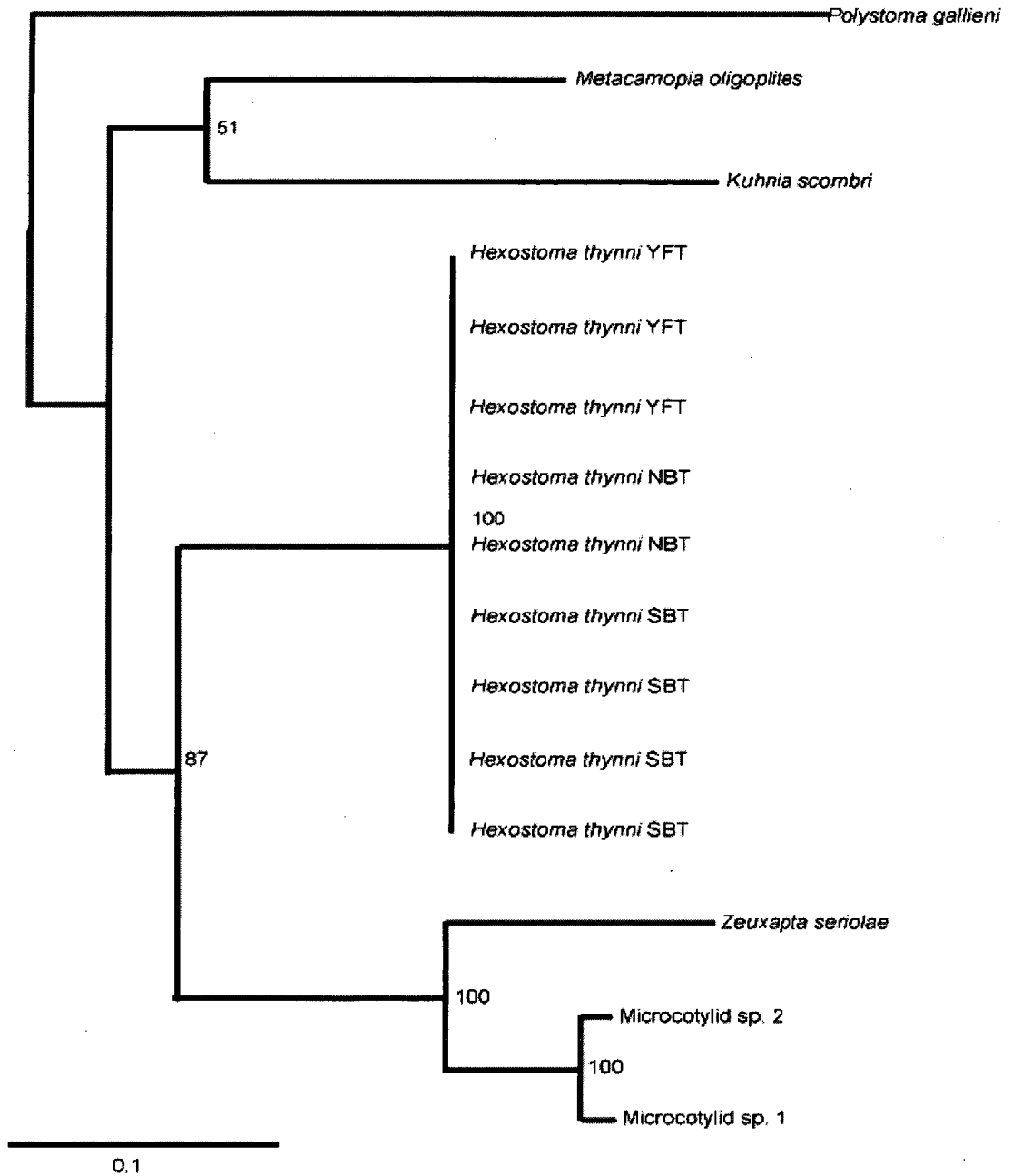


Fig. 3. Bayesian Inference analysis using 28S C1-D2 rDNA for *H. thynni*. *Polystoma gallieni* designated as the functional outgroup. Bar = 0.1 substitutions/site. Abbreviations: NBT- Northern bluefin tuna, *Thunnus thynnus*, SBT- Southern bluefin tuna, *T. maccoyii*, YFT- Yellowfin tuna, *T. albacares*. *Hexostoma thynni*, which bears four pairs of clamps, is more closely related to two representatives of a mazocraeidean suborder of Polyopisthocotylea bearing multiple pairs of clamps (Microcotylea, as represented by 2 microcotylids (*Microcotyle* spp.) and a heteraxinid, *Zeuxapta seriola*) than it is to two representatives of other mazocraeidean suborders that also bear four pairs of clamps (Mazocraeina as represented by a mazocraeid, *Kuhnia scombri*; and Gastrocotylea as represented by an allodiscocotylid, *Metacamopia oligoplites*).

### 3.3. *Capsala* sp.

The 28S D1-D2 rDNA sequences of *Capsala* sp. from farmed *T. maccoyii* off Australia and farmed *T. orientalis* off Pacific Mexico were found to be identical in an alignment of 799 nucleotides. *Capsala* sp. 28S rDNA sequences have been submitted to Genbank under the following accession numbers EF653379 (southern bluefin tuna, Port Lincoln), EF653380 (Pacific bluefin tuna, Mexico).

## 4. Discussion

### 4.1. Choice of DNA regions for taxonomy

Olson and Tkach (2005) reviewed the literature and reported many cases where multiple ribosomal DNA regions, including ITS2 and 28S, have been used successfully to determine the level of conspecificity among various platyhelminths. Nolan and Cribb (2005) reviewed the utility of ITS sequences for trematode species identification and conclude that intraspecific variation is usually low and frequently appears to be absent. These authors also noted that ITS2 has successfully discriminated differences among digeneans from many families. The current study is consistent with these conclusions and shows that ITS2 is a valid marker for distinguishing two species of *Cardicola* (*C. forsteri* and *C. sp.*) infecting northern bluefin tuna in Spain. We also found both of these ITS2 sequences of *Cardicola* in other regions of the world, without any variation, indicating that we had collected conspecific material of both species from other distant localities (*C. forsteri* in Australia and *C. sp.* in Pacific Mexico). In addition, for *Hexostoma thynni* and *Cardicola forsteri*, we have data for not one but two gene regions (ITS2 and 28S) in which no evidence of variation was found across widely separated geographic distances (except for 1 base pair difference in 28S in *Cardicola forsteri* between

Spain and Australia). Finally, for *Capsala* sp., no evidence of nucleotide variation was observed in partial 28S rDNA sequences between specimens collected from Pacific Mexico and Australian waters over a large region (799 characters). However, only small numbers of specimens of *Hexostoma thynni* and *Cardicola forsteri* were able to be collected thus increasing the chances of obtaining identical sequences by chance. We suggest that this is the first time that molecular analyses have demonstrated that marine platyhelminth species are identical on congeneric host fishes that have near global distribution.

#### 4.2. Distribution of marine digeneans

The results of previous molecular studies on the distribution of parasitic marine platyhelminths (digeneans) of coral reef fishes have shown that some cryptic species exist in different localities, but that other species are more widely distributed within particular oceans. For example, 14 *Cardicola* spp. were collected from Indo-Pacific fishes by Nolan and Cribb (2006). Based on morphological and ITS2 rDNA evidence most host species were found to harbour different species of *Cardicola* in different locations, and only one putative species (from a siganid fish, *Siganus margaritiferus*, Siganidae) was the same from different localities (Lizard Island, off North-eastern Australia and North Stradbroke Island, off central-eastern Australia). In contrast, lack of molecular variation over a relatively wide geographical range (over 6000 km) has been demonstrated previously within three digenean species infecting three coral reef fishes off French Polynesia and the Great Barrier Reef (*Schistorchis zancli*, Apocreadiidae in *Zanclus cornutus*, Zanclidae, *Preptetos laguncula*, Lepocreadiidae in *Naso lituratus*, Acanthuridae and *Neohypocreadium dorsoporum*, Lepocreadiidae in *Chaetodon vagabundus*, Chaetodontidae) (Lo *et al.* 2001). These locations were within a single ocean, the Pacific Ocean, whereas the



present study covered Pacific Ocean, Mediterranean Sea, Southern Ocean and Indian Ocean. Similarly, Chambers and Cribb (2006) showed that ITS2 rDNA sequences of a digenean (*Quadrifoliovarium pritchardae*, Lecithasteridae) in *Naso unicornis*, (Acanthuridae) were identical from Exmouth (Indian Ocean), Heron and Lizard Island (Western Pacific), and Moorea (far Eastern Indo-Pacific). Cribb *et al.* (2000b) suggested that larval coral reef fish probably contribute little to the dispersal of parasites of the adult fish, making parasite dispersal to different areas more difficult than dispersal of the fish themselves. In contrast to coral reef fishes, however, the seven species of tunas of the genus *Thunnus* have wide geographical ranges as adults and extensive regions of sympatry with each other, and very high mobility and distribution.

#### **4.3. Reasons for global distribution of tuna parasites**

It is possible that the two species of blood flukes (*Cardicola forsteri* and *C.* sp.) were translocated widely only recently, perhaps in infected intermediate hosts (which are currently unknown) transported in biofouling (attached to the hulls of ships). The outer hulls of ships provide vectors for introductions of various species as well as their parasites (Ruiz *et al.* 2000, Lafferty *et al.* 2004). Consequently, centres of shipping activity such as ports are considered hot spots for marine invasions and introductions (Cohen and Carlton 1998). The Port Lincoln farming zone can be considered particularly susceptible to bioinvasions as it receives large volumes of shipping traffic (127 vessels during 2006; Flinders Ports South Australia, see [www.flindersports.com.au](http://www.flindersports.com.au), last accessed 12 May 2007). In addition, several of these vessels are refrigeration ships, in port to load frozen tuna for export, that have previously serviced tuna farms from the Mediterranean and Mexico so the possibility of translocation of infected intermediate hosts exists.

However, high host mobility seems to be the only plausible hypothesis explaining widespread distribution of the two species of gill flukes (*Hexostoma thynni* and *Capsala* sp.). The life cycle of both polyopisthocotyleans and monopisthocotyleans involves no intermediate host. Furthermore, the majority have free-swimming larvae that are short-lived, usually surviving less than two or three days (Hayward 2005; Whittington 2005). The larvae of *H. thynni* and *Capsala* sp. are therefore unlikely to survive long-distance transport in either ballast waters or in oceanic currents.

In addition to the four platyhelminths sequenced in the present study (*Cardicola* spp., *H. thynni* and *Capsala* sp.), three copepods (*Pseudocycnus appendiculatus*, Pseudocycnidae, *Euryphorus brachypterus*, Euryphoridae and *Brachiella thynni*, Lernaepodidae) are also known, on the basis of their morphology, to be cosmopolitan on *Thunnus* spp. (see Cressey and Cressey 1980; Nowak *et al.* 2006). The distributions of *T. maccoyii* and *T. thynni* do not currently overlap; *T. maccoyii* occurs in the southern oceans, from the south-east Atlantic across the southern Indian Ocean to east Tasmania and New Zealand (Grewe *et al.* 1997, Farley *et al.* 2007); in contrast, *T. thynni* occurs primarily in the northern Atlantic Ocean and adjacent seas (Fromentin and Powers 2005) (Figure 1). A population of *T. thynni* occurs in the coastal waters off South Africa (Collette and Nauen 1983), although since the 1990s catches in the southern Atlantic Ocean have been extremely rare (Fromentin and Powers 2005). Hence, platyhelminth and copepod parasites of *T. thynni* and *T. maccoyii* are likely to have been exchanged up until modern times in this region of host sympatry. Interoceanic migration occurred in the past for many species of tuna (Ely *et al.* 2005). For example, it has been

suggested that the common ancestors of Atlantic and Pacific bluefin tuna were sympatric until they were separated by the rise of the Isthmus of Panama (Coates *et al.* 1992). Following this event, during the Tertiary there could be still mixing of species along the West African coast due to stronger currents (Alvarado Bremer *et al.* 2005). Agulhas current activity resulted in leakage of Indian Ocean fauna during Pleistocene inter-glacials (Peeters *et al.* 2004). This suggests that one possibility would be that the parasites were first distributed globally a long time ago when the ranges of different tuna species overlapped.

In the case of *H. thynni*, our molecular evidence confirms that this species also infects at least one other species of *Thunnus* – in this case *T. albacares*, a species sympatric with both *T. thynnus* and *T. maccoyii* over much of their present ranges (Collette and Nauen 1983). Studies of control nucleotide sequence in *T. albacares* did not provide any evidence of genetic differentiation between Atlantic and Pacific sub-populations (Ely *et al.* 2005). In addition, although the distributions of *T. orientalis* and *T. maccoyii* are considered largely allopatric, there are occasional anecdotal reports of individuals of *T. orientalis* in *T. maccoyii* farms in Australia. Such mixing of stocks of *Thunnus* spp. provides the opportunity for dispersal of their parasite fauna over a wide area.

*Cardicola forsteri* has been reported previously, on the basis of morphological comparisons, from southern Australia (Cribb *et al.* 2000a; Aiken *et al.* 2006 (Chapter 3)) and the Atlantic Coast of U.S.A (Bullard *et al.* 2004). We now record the species from the Mediterranean through our molecular analyses. We predict that more species of tuna with distributions that overlap with *Thunnus maccoyii* and *T. thynni* will prove to be infected with *C. forsteri*; including *T. obesus*

and *T. albacares*. Similarly, *C. sp.* has been found from the Spanish Mediterranean and from off the Pacific coast of Mexico, so it is plausible that other *Thunnus* spp. are infected by this species as well.

Unlike the five ectoparasites now known to have cosmopolitan distribution wholly or primarily on *Thunnus* spp. (*Hexostoma thynni*, *Capsala* sp., *Pseudocycnus appendiculatus*, *Euryphorus brachypterus*, *Brachiella thynni*)—each of which also has a direct life cycle—the endoparasitic *Cardicola* spp. require an intermediate host to complete their life cycles. The intermediate host (or hosts) of *Cardicola* spp. remain unknown, and their apparent cosmopolitan distributions pose some interesting questions about their possible identity or identities. Likely candidates are bivalves or polychaetes (Cribb *et al.* 2001; Bott *et al.* 2005), and the intermediate host (or hosts) may have similar or overlapping distribution with *Thunnus* spp.

Bayesian inference analysis suggests that *Cardicola* sp. is the sister taxon to *C. forsteri* and the analysis suggests that the *Cardicola* clade strongly with the host group, in this case higher scombrids, Thunninae (*C. forsteri* and *Cardicola* sp. 1), Scomberomorinae (*C. sp.* 4 and 5) and all others from siganid fishes and one lutjanid fish (Fig. 2). These relationships are similar to the distance analysis performed by Nolan and Cribb (2006) except for the separation of the two scombrid clades in the present analysis. Only one other *Cardicola* has been described from *Thunnus*, *C. ahi* Yamaguti, 1970 from *T. albacares* and *T. obesus*, and obtaining *C. ahi* specimens for molecular analysis, as well as specimens of *Cardicola* sp. for morphological analysis, will be of great interest. Bayesian inference analysis of the polyopisthocotylean gene sequence data (Fig. 3) confirms the validity of the transfer of Hexostomatidae into a new superfamily, Hexostomatinea, from Mazocraeinea by

Boeger and Kritsky (1993), which had been based upon their cladistic analyses of a large series of morphological characters.

In *Thunnus maccoyii* farmed off Port Lincoln, South Australia, the prevalence and intensities of *Cardicola forsteri* increase rapidly and peak two months after fish are transferred into cages from the wild, with 100% prevalence and an overall average of 27 flukes per infected tuna (Aiken *et al.* 2006 (Chapter 3)). Both prevalence and intensity decline over the rest of the grow-out season and this suggests that it is highly likely that the intermediate host for *C. forsteri* is in close proximity to the aquaculture leases (Aiken *et al.* 2006 (Chapter 3)). It is possible that *C. forsteri* is still present in the intermediate host population when *T. maccoyii* are not in the farming area. Marine parasites are adapted to survival in a dilute environment where encounter rates between intermediate host and definitive host are limited (Marcogliese 2002). Aiken *et al.* (2006 (Chapter 3)) also proposed that the immune response of *Thunnus* spp. may control the infection. As adult *T. maccoyii* are oceanic, and juvenile *T. maccoyii* spend only approximately 20% of the year (in summer) over shelf and shelf break waters (J. Gunn, CSIRO Hobart, personal communication) in those habitats where the intermediate hosts are likely to occur. Rapid asexual reproduction within intermediate hosts may be essential for the parasite.

#### **4.4. Discrepancies between sequences**

*Hexostoma thynni* from *Thunnus thynni* has been analysed previously using a partial sequence of 28S (C1-D1) rDNA from a specimen from Sète on the Mediterranean coast of France (Mollaret *et al.* 2000). In the present study, the partial 28S rDNA region of specimens that we also identify as *H. thynni*, from Australia and

Croatia, did differ slightly in partial 28S rDNA sequences of the species from France in that four nucleotide insertions are apparent over the alignment of 255 base-pairs with *H. thynni* from *T. maccoyii*, *T. albacares*, and Croatian *T. thynni*. We think that it is necessary for additional specimens of *H. thynni* to be sequenced from France before it can be reliably confirmed that the sequence does differ by this amount from specimens we collected from elsewhere. Similarly, Mollaret *et al.* (2000) published a partial sequence of 28S (C1-D1) rDNA from a specimen of *Capsala onchidiocotyle* from France, and in the present study *Capsala* sp. from Australia and Mexico differs from the published sequence of that species by 5 substitutions over a region of 322 base pairs (Mollaret *et al.* 2000). We suspect that the present *Capsala* sp. may in fact be conspecific with *C. onchidiocotyle*; more detailed taxonomic studies of capsalids from *Thunnus* spp. are now in progress.

#### **4.5. Geographic distribution of parasites**

We acknowledge that morphological studies would strengthen our conclusions based on molecular analysis, but due to the relative rarity of particular parasites in certain locations and hosts (Table 1), this has not yet been possible. While the parasites of this study are widespread, they were not observed at all sites which were sampled (Figure 1.) and may reflect a restricted distribution due to some influence on the host population... However, to determine whether they are truly absent from any region, large samples would need to be examined for each location. Such data have been collected only for the Port Lincoln site in Australia. The restricted distribution of the parasites to a few locations rather than all may be a result of shrinking host ranges. The abundance of many species of fishes has been adversely affected by fishing (Jackson *et al.* 2001, Myers and Worm 2003). In turn, a

fishery can fish out parasites if a stock is fished to a density below the host density threshold for transmission (Dobson and May 1987). For example, fishing apparently led to the extinction of a swim bladder nematode (*Cystidicola stigmatura*, Thelaziidae) from lake trout (*Salvelinus namaycush*, Salmonidae) in the Great Lakes (Black 1983) and significantly reduced the prevalence of a tapeworm (*Triaenophorus crassus*, Triaenophoridae) in common whitefish (*Coregonus lavaretus*, Salmonidae) (Amundsen and Kristoffersen 1990). The significant decline in the normalized reports of disease in marine fish (Ward and Lafferty 2004) was based almost entirely on data from commercially fished species, and this suggests the hypothesis that exploitation has reduced diseases in fishes by making transmission more difficult (Lafferty *et al.* 2004). On the other hand, the apparent restricted distribution of the parasites in this study may be due other factors, such as the natural distribution of the hosts themselves.

#### **4.6. Implications for tuna aquaculture and fisheries**

This is the first molecular evidence confirming that species of marine parasites are naturally cosmopolitan. We compared parasites of four species of pelagic fishes that are commercially important in fisheries and aquaculture in different regions around the world: tunas belonging to genus *Thunnus* spp. from the Pacific Ocean (Mexico), Mediterranean Sea (Croatia and Spain), Southern Ocean (South Australia) and Indian Ocean (Western Australia). The study involved extensive collaborative effort to survey parasites in each region of the world where bluefin tunas are fished and farmed. We believe that this research has two broader implications. First, it indicates that parasites (and therefore other aetiological agents associated with infectious disease as well, such as bacteria and viruses) of tunas farmed in one region of the world may have the potential to spread via wild stocks to

distant farming regions. Second, greater confidence can now be placed in the results of previous morphological studies of other particular parasites of pelagic fishes, which had indicated widespread distribution.



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### **Chapter 3. An epizootic and its decline of a blood fluke, *Cardicola forsteri*, in farmed southern bluefin tuna, *Thunnus maccoyii***

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## Abstract

The epidemiology of a blood fluke, *Cardicola forsteri*, was examined in cultured southern bluefin tuna, *Thunnus maccoyii*, over a six-month growout season in Port Lincoln, South Australia. Ten tuna were sampled from the transfer from tow cages to sea cages in March, and then ten tuna from each cage once a month until August. A total of 220 tuna were sampled. Intensities and prevalences were found to increase rapidly and peak two months after transfer with 100% prevalence and an overall average of 27 flukes per infected tuna, with one tuna having a recorded 99 worms in its heart. After this peak both prevalence and intensity declined to an average of 3.1 flukes per infected tuna and a prevalence of 35%. Condition Indices, calculated from weights and lengths and used as a measure of production, were shown to have no significant relationship with intensity or prevalence. *C. forsteri* infections declined after an initial peak and no effect was observed on tuna condition index despite high intensities being recorded. It is concluded that *T. maccoyii* is able to control blood fluke infection, despite decreasing water temperatures.

## 1. Introduction

The southern bluefin tuna, *Thunnus maccoyii*, is a commercially important fish that is distributed widely in the southern hemisphere between 30° and 50° south. Since 1991, this species has been the focus of an aquaculture industry based in Port Lincoln, South Australia. Tuna are caught in the Great Australian Bight and are towed to Port Lincoln where they are fattened in sea cages for a period of between 2-6 months before being exported as frozen or fresh product to Japan for the sashimi market. The industry is economically significant producing 7810 tonnes worth \$151 million in 2004 (T.B.O.A., pers. com.)

In comparison to other farmed species, tuna health has been little studied (see review by Munday *et al.* 2003). An investigation into the risk factors facing the health of southern bluefin tuna was undertaken by Nowak (2004) and a digenean sanguinicolid blood fluke, *Cardicola forsteri*, was identified as one of the more significant risks, though low in absolute terms, to southern bluefin tuna health. *C. forsteri* was discovered in farmed southern bluefin tuna in 1997 as a result of an investigation of nodular gill lesions (Colquitt, 1999; Cribb *et al.* 2000). The pathology of the fluke was investigated by Colquitt, Munday and Daintith (2001). In most cases, the pathology observed was not considered to be sufficient to lead to mortalities, however it was considered to be significant (Colquitt *et al.* 2001). Mass mortalities of cultured fish in North America, Europe and Asia were associated with blood fluke infections (Bullard, 2002). Consequently Nowak (2004) suggested that further research was required on the biology and epidemiology of the blood fluke. The aim of this study was to investigate the epidemiology of the blood fluke and to determine if there was a loss of production in farmed southern bluefin tuna over one grow-out season in 2004.

## 2. Materials and Methods

A single cohort of tuna was examined over a six-month period. Ten tuna were sampled during the transfer from a tow cage to four experimental sea cages (set apart from normal commercially operated sea cages) on the 17<sup>th</sup> March 2004. Tuna were then sampled from the experimental sea cages at approximately monthly intervals until August. Forty tuna were sampled on 18<sup>th</sup> April (10 tuna from each of the 4 experimental sea cages), thirty tuna were sampled on 19<sup>th</sup> May (10 tuna from 3 of the 4 experimental sea cages), forty tuna were sampled on 16<sup>th</sup> June (10 tuna from each of the 4 experimental sea cages), fifty tuna were sampled on 18<sup>th</sup> July (10 tuna from each of the 4 experimental sea cages plus ten tuna from a commercially operated sea cage) and forty tuna were sampled on 19<sup>th</sup> August (10 tuna from each of the 4 experimental sea cages). To determine whether different feed regimes had an impact on worm burdens, five cages were used throughout the trial; Cages A and B were normal commercial cages that were fed pilchards, Cage C was fed pilchards with a high vitamin coating, Cage D was fed a standard pellet and Cage E was fed a high vitamin pellet. A total of 220 tuna were examined. Sampling took place during normal commercial harvesting operations. Systematic random sampling was used by selecting southern bluefin tuna at five minute intervals during harvest operations. Gills and hearts were obtained during the harvest, stored on ice, and taken to the laboratory. Hearts were dissected open 2-4 hours after removal from the carcass and flushed with physiological saline to dislodge any adult flukes. Flushes were then poured into petri dishes and were then examined for the presence of adults using a dissection microscope. Prevalences, the number of host infections as a proportion of the population at risk, and intensities, the number of individual worms in each host (Bush *et al.* 1997), were recorded. Gills were examined for grossly visible lesions. Weights and lengths for each tuna sampled were also obtained, and a Condition

Index was calculated using the South Australian tuna industry formula, whole weight / length<sup>3</sup> (Hayward et al. 2008). Temperature was obtained using a probe. Two-way ANOVA's were used to determine if there was any statistical differences between months and regression/correlation tests were used to determine if there was any relationship between Condition Index and fluke intensities for each month and also for the overall 6-month period. SPSS 12.0.1 was used for all statistical analyses. An alpha level of 0.05 was set for all statistical analyses.

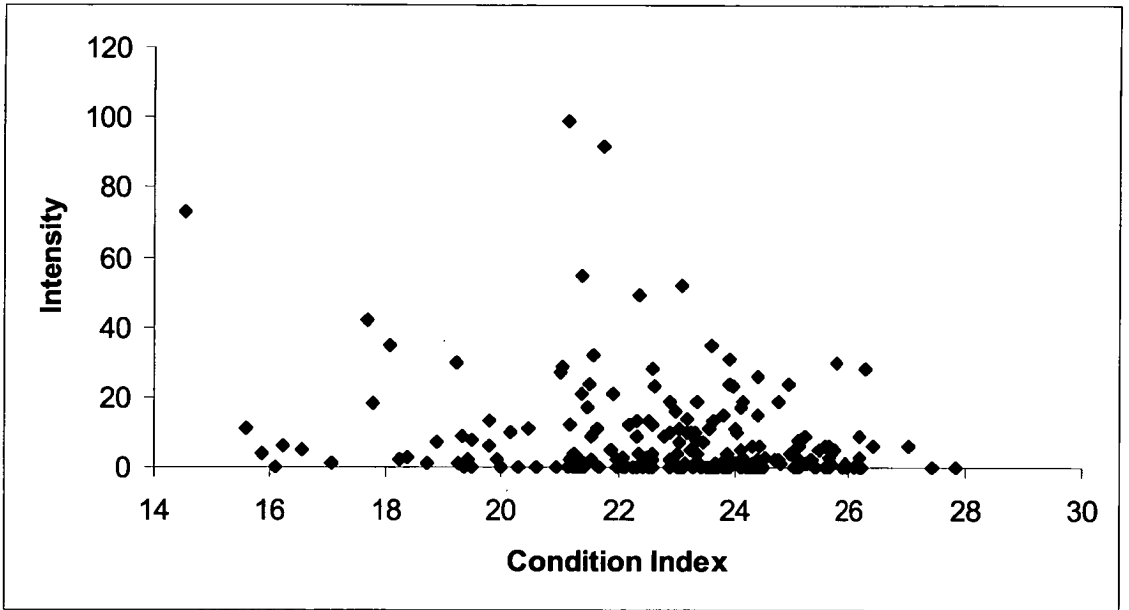
### 3. Results

A range of adult fluke intensities (0-99) were observed within the ventricle of tuna. No statistical correlation between fluke intensity and Condition Index was observed for any month (Figure 1, Table 1.). Most individuals with high intensities of blood fluke infection had similar Condition Indices to those with low intensities.

**Table 1.** Results of correlation analysis examining the relationship between the intensity of blood fluke infection and condition index in each month for southern bluefin tuna farmed in Port Lincoln.

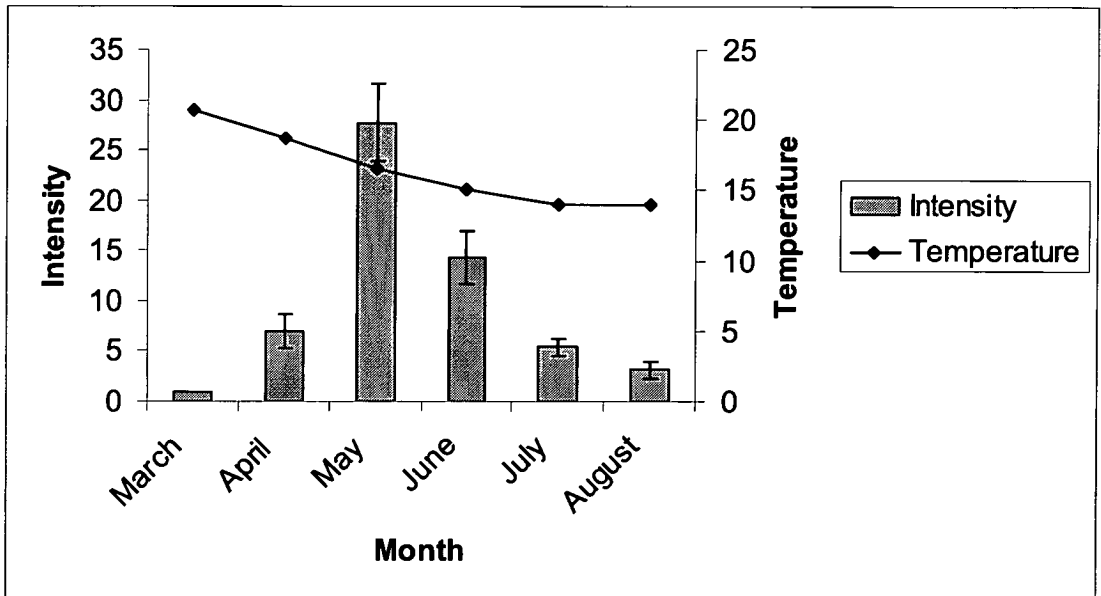
Month	<i>F-value</i>	<i>d.f.</i>	<i>R</i> <sup>2</sup>	<i>P-value</i>
March	0.403	9	0.048	0.543
April	0.365	39	0.026	0.347
May	0.117	29	0.092	0.117
June	0.101	39	0.069	0.102
July	0.690	49	0.003	0.69
August	0.114	39	0.064	0.114



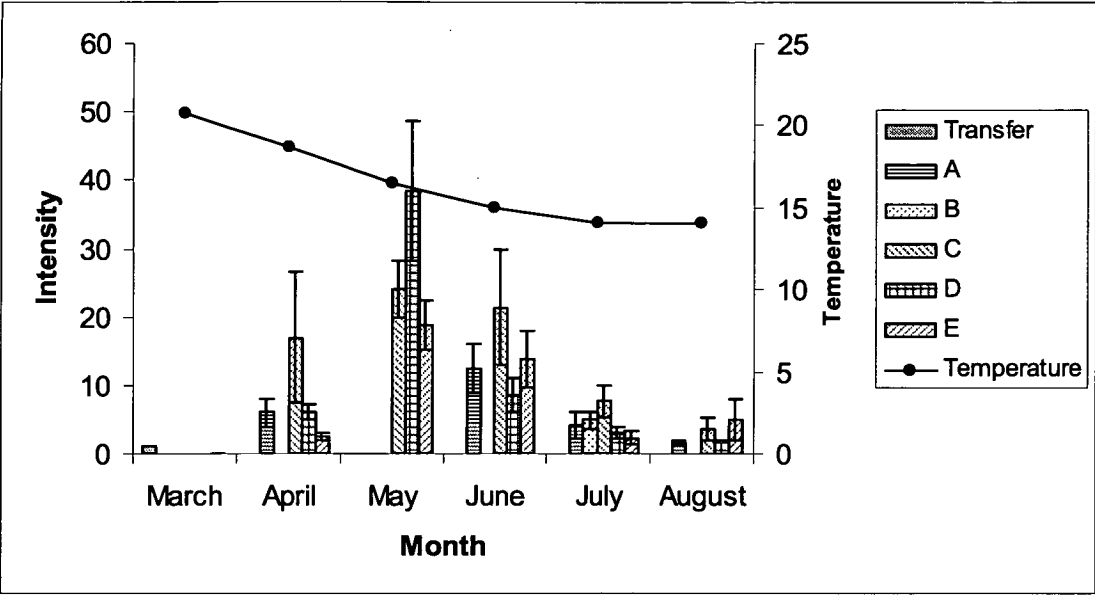


**Figure 1.** Intensity of flukes (*Cardicola forsteri*) recorded in *Thunnus maccoyii* cultured over a six-month period off Port Lincoln, for each Condition Index recorded.

Intensities of flukes were observed to be very low initially with just a single worm in one of ten fish at the time of transfer. Within two months, the intensities increased rapidly, peaking in May with an average of 27 flukes per tuna (Figure 2). It was during this month that a single tuna was observed to be infected with 99 adults in the ventricle, in a cage where the average was 38 flukes in each tuna (Figure 3).

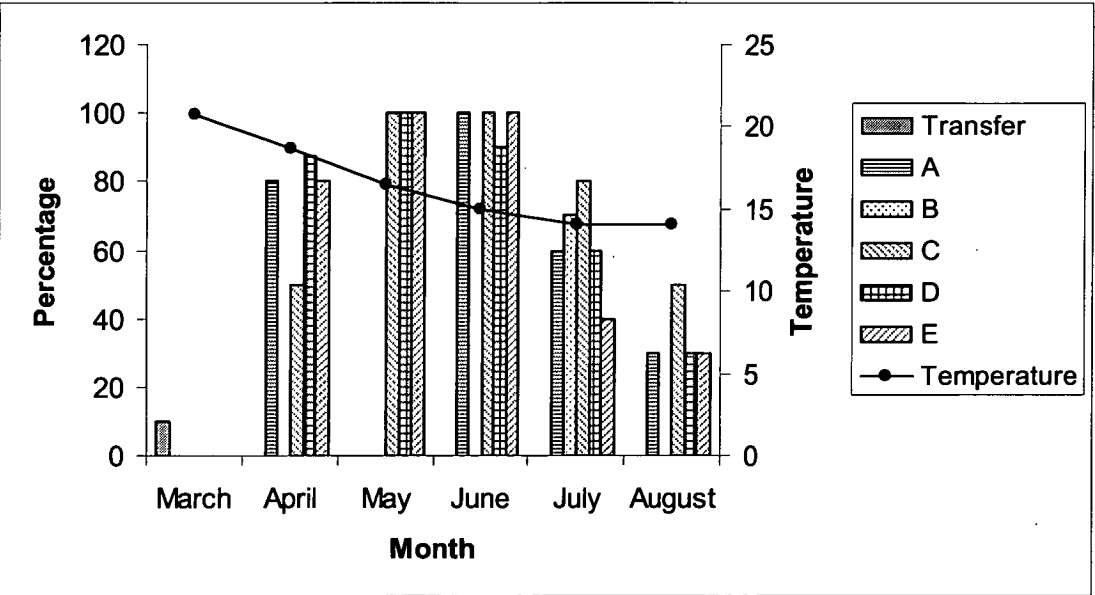


**Figure 2.** Average intensity of *Cardicola forsteri* in *Thunnus maccoyii* cultured over a six-month period off Port Lincoln, each month including temperature (°C) recorded for that month.

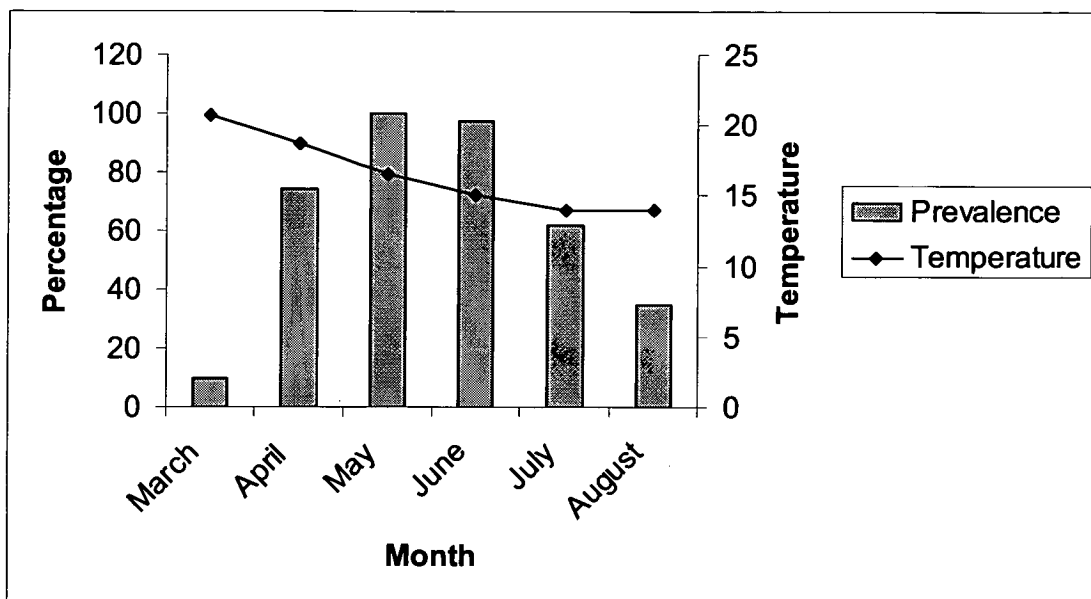


**Figure 3.** Average intensities of *Cardicola forsteri* in *Thunnus maccoyi* cultured over a six-month period off Port Lincoln, for each cage per monthly harvest including temperature (°C) recorded for that month.

Intensities then tapered off over the next three months, declining to an average of 3.14 adults per infected tuna (Figure 2). A similar pattern was observed in the monthly prevalences, with a rapid increase to peak in May (reaching 100% prevalence in all cages) (Figure 4), followed by a decline to 35% (Figure 5). This decline, however, was more gradual than the decline observed for the fluke burdens.

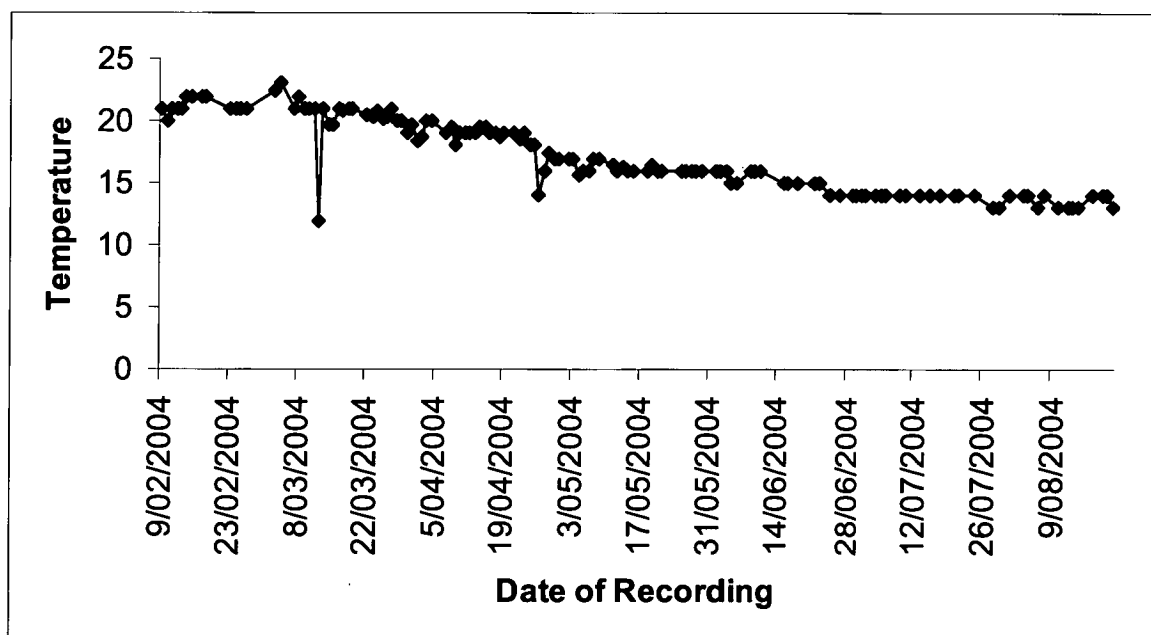


**Figure 4.** Prevalence of *Cardicola forsteri* in *Thunnus maccoyi* cultured over a six-month period off Port Lincoln, for each cage per month including temperature (°C) recorded for that month.



**Figure 5.** Monthly prevalence of *Cardicola forsteri* in *Thunnus maccoyi* cultured over a six-month period off Port Lincoln including temperature (°C) recorded for that month.

The water temperature of the area in which the tuna were being cultured was shown to decline gradually over the period of sampling (Figure 6.). The temperature was highest in March at 20.75°C and the lowest temperatures that were recorded in July and August at 14.00°C.



**Figure 6.** Temperature (°C) of water in which cultured *Thunnus maccoyi* were located off Port Lincoln.

No statistical correlation was observed between Condition Index and fluke intensity in any month (Table 1.). No significant difference in intensity was observed between groups of tuna fed different diets (ANOVA,  $F=1.66$ ,  $d.f.=3$ ,  $P<0.178$ ). Mean intensity was significantly affected by months (ANOVA,  $F=26.89$ ,  $d.f.=4$ ,  $P<0.001$ ). May and June had different mean intensities to all other months and to each other (Fisher LSD test,  $P<0.05$ ). There was no significant difference between cages ( $F=1.31$ ,  $d.f.=4$ ,  $P=0.266$ ). Month was shown to significantly exert an influence on cage with regards to mean fluke intensity ( $F=1.898$ ,  $d.f.=11$ ,  $P=0.042$ ) (Table 1.).

Table 2. Summary of statistics illustrating the effect of month of intensity of *C. forsteri* infection in farmed *T. maccoyii* in Port Lincoln.

Month	Mean	Median	Range	Interquartile range
March	1.0	1	0	0
April	6.6	4	41	4.5
May	26.3	23.5	97	16.5
June	14.3	10	91	10
July	4.7	3	18	5
August	3.1	2	10	1.75

Levene's test of equality of error variances was shown to be significant ( $F=7.44$ ,  $P<0.001$ ), therefore the results of the analysis should be treated with caution (Underwood, 1981).

#### 4. Discussion

The intensity and prevalence of blood fluke infection of southern bluefin tuna declined after an initial peak. Similar declines in intensity and/or prevalence following a peak has been observed in other cultured fish species infected by sanguinicolids. Ogawa and Fukudome (1994) investigated a *Seriola dumerili* mass mortality in Japan, May 1993, caused by blood fluke *Paradeontacylix spp.* and observed an increase and peak in mortalities and suggested that the blood fluke infection has an annual cycle; cercarial invasion starts in September, eggs

accumulate in the gills and heart from November, and mortality, occurring in the winter months from December to March, decreases with increasing water temperature. Ogawa *et al.* (1993) suggested that cercariae started to emerge with decreasing water temperature. Some blood flukes exhibit seasonal development driven by an interacting set of abiotic factors (Bullard and Overstreet, 2002). However the way in which each of those factors affects the miracidium, schistosomule, and adult for any species, is not well understood. Water temperature and salinity are two factors that would most likely influence the behaviour and physiology of blood fluke miracidia and cercariae as well as the host (Bullard and Overstreet, 2002).

In our study as water temperature decreased the intensity and prevalence of *C. forsteri* infection decreased after an initial peak. One might expect that with decreasing temperature infection would rise due to the immunosuppressive effects of temperature (Bly *et al.* 1997). Immune responses to sanguinicolids have been documented in fish and have also been shown to be influenced by temperature (Richards *et al.* 1996). The immune system of *Thunnus maccoyi* is not affected by ambient temperature, due to their endothermic and homeothermic abilities enabling their bodies to have elevated body temperatures above that of ambient (Watts *et al.* 2002). Antibody response to *C. forsteri* by southern bluefin tuna has been demonstrated (Watts *et al.* 2001). This may be why the infection tapered off after the initial peak and also the reason why the Condition Index of the tuna is not associated statistically with fluke intensity. The antibody response may also explain the overdispersed distribution of intensity. Parasite distributions in host populations are almost always overdispersed, in that many hosts are infected with a few parasites and only a few hosts are infected with a large number of parasites (Cox 1993). Variation

in host response may lead to differing infection levels in the host population leading to an overdispersed distribution. Rubio-Godoy et al. (2003) suggested that host response was a factor that led to an overdispersed distribution of *Discocotyle sagittata* in *Oncorhynchus mykiss*, therefore it is possible that the tuna antibody response against blood fluke is leading to a similar distribution of the parasite population.

Based on the pattern for prevalence and intensity of infection, it is unlikely, that wild tuna re-introduce the fluke at the start of each season. Therefore, the tuna are being exposed to infective stages when they are introduced to the sea cages. Colquitt *et al.* (2001) also suggested that the tuna were being infected post capture as their study failed to detect infections in wild fish. This would also indicate a short time for the penetrating cercariae to develop into mature adults. Kirk and Lewis (1993) found that *Sanguinicola inermis* larvae mature into adults approximately 30 days post exposure whilst Sommerville and Iqbal (1991) reported sexual maturity at 60 –90 days. This difference may be due to temperatures. The hosts used by Sommerville and Iqbal (1991) were kept at lower temperatures (15°C-18°C) than those reported by Kirk and Lewis (1993) (20°C). As tuna are able to maintain higher temperatures than ambient, which was between 18.67°C and 20.75°C in the first two months of this study, one would expect a relatively quick maturation.

The rapid initial increase in fluke burdens may indicate that the asexual stages of *C. forsteri* aestivate in the intermediate host over the summer when the tuna are not present in the area. This aestivation may be a response to the absence of tuna over the summer period. A similar situation has been observed in other digeneans where the definitive host is not present throughout the whole year and an over-wintering period

is apparent. Vayrynen *et al.* (2000) observed the larval digenean fauna of lymnaeid snail populations from two lakes in northern Finland and found that the annual parasite species composition within the two lakes was relatively stable despite a long dormant winter in which the avian definitive host were not present, thus causing a break in the transmission cycle of the parasites each year. Parasite infections are gradually lost when the birds migrate to their wintering area. Vayrynen *et al.* (2000) found clear evidence the larval digeneans were over wintering in their snail hosts thereby maintaining infections within the mollusc population which then act as a source for re-infection of the birds returning to breed in the spring. Vayrynen *et al.* (2000) concluded that the complete re-establishment of parasite life-cycles each spring via the adult birds must therefore be a rapid process as with our observations for *C. forsteri*.

The intermediate host of *C. forsteri* is currently unknown, but other marine sanguinicolid are known to be hosted by polychaetes or bivalves (Smith, 1997). This intermediate host must be in close proximity to the tuna for so many tuna to become infected in a short period, and their location is likely to be in the sediment below the cages or attached to the sea cages. Presently there is only one life cycle known for a marine sanguinicolid. *Aporocotyle simplex* uses a polychaete intermediate host, *Artacama proboscidea*, a sedentary tube-dwelling terebellid polychaete common on the muddy sea floor of Oresund, Denmark (Køie, 1982).

Munday *et al.* (2003) suggested that the *C. forsteri* may also have a deleterious effect on its intermediate host as Port Lincoln tuna farmers have reported more severe infections at new cage sites. This is supported by Køie (1982) who observed atrophication of the gonads and free sexual products in infested *A. proboscidea* during

growth and multiplication of the rediae, possibly a result of competition by the rediae for available nutrients. Consequently all *A. proboscidea* harbouring rediae became sterile due to the castration by the parasites. Infested polychaetes also showed a decreased life span compared to uninfested polychaetes (Køie, 1982).

The apparent lack of an effect of the blood fluke on southern bluefin tuna is in contrast to other investigations of blood fluke infected fish. In the mass mortality of 1993 in *S. dumerili* in Japan the cumulative mortality rate in one month ranged from 50% to more than 80%. Typical signs of suffocation such as an opened mouth and opercula, were apparent on dead fish. Examination of the gills of dead fish revealed that eggs of *P. grandispinus* and/or *P. kampachi* had accumulated in the afferent arteries (Ogawa and Fukudome, 1994). The mortalities reported occurred during winter at temperatures that were not optimal for *Seriola* and in consequence may have been a result of immunosuppression. If a host immune response is operating in southern bluefin tuna, there may be no or little energy consumed by such a process, as we have been unable to demonstrate an effect on growth as measured by Condition Indices. However, one negative effect is apparent, as white lesions commonly occur on the gills, and this is presumed to be caused by the lodgement and hatching of the eggs of *C. forsteri* (Colquitt, 1999). Nevertheless, this pathology may not be significant at the levels of infection seen over the reported harvest season, as the lesions do not occlude all of the gills. Herbert and Shaharom (1995) found no pathology in cultured *Lates calcarifer* when infected by *Parasanguinicola vastispina*. However *P. vastispina* was not abundant in the sea bass, with an average intensity of 2.5 adults in the branchial arteries, dorsal aorta, mesenteric venules and renal arteries. Another sanguinicolid found in sea bass is *Cruoricola lates* and although pathology due to this parasite was reported, Herbert *et al.* (1995) found



levels that were insufficient to cause mortality (3 – 13). Herbert *et al.* (1995) suggests that it was the low intensity of infection that was the direct cause of the lack of mortality and minor pathological changes. The results from this study disagree with this suggestion as relatively high intensities were observed with no significant pathology and also condition indices that were higher than the average for the cage from which the tuna had come from. Despite there being no significant pathology in the tuna this does not mean that the infection could not combine with other factors to cause severe illness or death (Colquitt *et al.* 2001). It is generally believed that a disease assists in secondary infections. First infection might compromise host biodefence mechanisms. Kumon *et al.* (2002), for example, found that blood fluke infested yellowtail *Seriola quinqueradiata*, when challenged with the bacterial fish pathogen *Lactococcus garvieae* had a significantly higher final cumulative mortality than fish uninfested with blood fluke.

Currently no significant gross pathological changes or mortalities have been attributed to *C. forsteri*. However the infection may combine with other agents to cause such effects, and further investigations into the epidemiology of this parasite are warranted. Furthermore, examination of the immune response to *C. forsteri* could provide insight into why there is no apparent pathological effect and may prove to be informative in providing information about other cultured fish species that are adversely affected by blood fluke infections.

## **5. Acknowledgments**

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## **Chapter 4. Simulating blood fluke, *Cardicola forsteri*, infection in farmed southern bluefin tuna, *Thunnus maccoyii*, using stochastic models**

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Aiken, H. M., Hayward, C. J., Cameron, A., Nowak, B. F. 2009. Simulating blood fluke, *Cardicola forsteri*, infection in farmed southern bluefin tuna, *Thunnus maccoyii*, using stochastic models. *Aquaculture* 293(3-4), 204-210.

## Abstract

Two stochastic models were developed to describe the infection pattern of *Cardicola forsteri* in farmed southern bluefin tuna, *Thunnus maccoyii*. Observed field data on the lengths of flukes over a growout season were used as the basis for the models and biological data from *C. forsteri* and other blood flukes infecting fish were used as parameters. Monte Carlo sampling techniques were used in the simulations. The first model simulated the lengths of fluke according to the month when they were sampled. The output of this model was then compared to the observed field data. A second model was developed to determine stochastically the age of the observed fluke using their lengths. The output of the first model was shown to be similar to the observed lengths of the flukes. An estimated time of infection was produced from the second model and it was shown that there were two major infection events early in the growout season that contributed to the majority of infections by *Cardicola forsteri*. These peaks of infection were shown to occur at 14 days (S.D.=10.2) and 55 days (S.D.=10.1) post-transfer. Stochastic modeling has not been used before to describe the infection period of a helminth in cultured fish, and was shown to be useful here.



## 1. Introduction

*Cardicola forsteri* Cribb, Daintith and Munday, 2000 (Digenea: Sanguinicolidae), the object of this study, is a parasite of farmed southern bluefin tuna, *Thunnus maccoyii*. The industry is based on the capture of wild tuna from the Great Australian Bight. Southern bluefin tuna are towed from the Bight to the offshore tuna farming zone near Port Lincoln and transferred into sea cages during summer and early autumn. Harvesting occurs 2 – 6 months after transfer once the southern bluefin tuna have been fattened on a diet of baitfish. A risk assessment examining the effect of husbandry on the health of southern bluefin tuna was conducted (Nowak, 2004) and identified *C. forsteri* as a moderate risk to Port Lincoln southern bluefin tuna industry, not for mortality but possibly compromising growth. The impact of *C. forsteri* on southern bluefin tuna has been investigated as well as its epidemiology (Colquitt et al., 2004; Aiken et al., 2006 (Chapter 3)). Aiken et al. (2006 (Chapter 3)) observed an epizootic in southern bluefin tuna without any evident adverse production effects such as a decrease in condition indices. However it was suggested that more investigation was warranted in light of this epizootic and that sublethal production effects were uncertain at the time.

*Cardicola forsteri* is a blood fluke with a complex life cycle that infects southern bluefin tuna as well as wild and farmed northern bluefin tuna, *T. thynnus* (Bullard et al., 2004; Nowak et al., 2006). It is a sanguinicolid trematode and its intermediate host is unknown but is most likely a polychaete or bivalve (Smith, 1997).

Sanguinicolids are parasites of marine and freshwater fish (Smith, 1997). Most species establish in the heart, bulbus arteriosus, ventral aorta, or branchial vessels, although distributions within the cephalic or dorsal vessels are not uncommon (Kirk and Lewis 1994). Once established, the adult fluke lay eggs which travel to the gills where they lodge. Adult fluke may also migrate to and from the gills to lay eggs.

Adult *Paracardicoloides yamagutii* have only been observed in the gills of eels (*Anguilla australis* and *Anguilla dieffenbachia*) after migrating there to lay eggs (Hine 1978). Free swimming miracidia hatch from the eggs and break out of the gill structure into the external environment. These miracidia will seek out an appropriate intermediate host into which they will penetrate to undergo asexual reproduction as rediae and/or sporocysts to produce infective cercariae. Organisms observed to be infected with marine sanguinicolid cercariae have included bivalves and polychaetes (Smith, 1972; Smith, 1997). Cercariae are shed from the intermediate host and actively search for the definitive host, a fish. The cercariae will penetrate the skin of the host and juvenile flukes will attempt to reach the circulatory system in which they will undergo a migration to a final site where they will mature (Smith, 1997). For *C. forsteri* the final site is the heart (Cribb et al., 2000). Aiken et al. (2006 (Chapter 3)) observed very low levels of *C. forsteri* in southern bluefin tuna at transfer to sea cages, suggesting that the majority of infection and the occurrence of the life cycle occur in the Port Lincoln farming area. New methods that do not require destructive techniques are needed to elucidate aspects of the ecology and life cycle of this parasite due to the complexities of working with the highly valued southern bluefin tuna.

One method is to develop a stochastic simulation model using observed field data. Although models are commonly used to explore host-parasite systems, they are rarely used to examine marine fish-helminth systems and few of these have incorporated observed field data as a basis for deriving the model (des Clers, 1990; Langlais and Silan, 1995; Bouloux et al., 1998). Using such information a simulation can be derived describing the time that the southern bluefin tuna are infected and how long flukes remain in the host. Although this biological model is not intended to

be predictive it can be used to describe the patterns of infection over a single growout season in 2004. The benefits of this model over a descriptive analysis is that modelling will provide information about actual infection events by *C. forsteri* cercariae over the growout period whereas a descriptive analysis is only able to provide a picture of infection at the time the tuna is sampled. Additionally a good understanding of the growth of the fluke inside the host will assist in understanding the life cycle of the parasite, and therefore, support any measures to contain its effect on farmed southern bluefin tuna if ever warranted.

## **2. Methods and Materials**

### **2.1. Life cycle elucidation**

Bivalves and polychaetes were collected from the nets and pontoons of sea cages and from the sediment beneath cages for identification of the intermediate host. Samples were dissected and analysed under a dissecting microscope (Olympus SZX12) for the presence of *C. forsteri* asexual stages.

Investigations into the organs of fifty-seven southern bluefin tuna, sampled in 2005, were undertaken to determine if the heart is the primary site of location for *C. forsteri*. Random samples of southern bluefin tuna were taken from normal commercial operations throughout the 2005 growout season. Systematic random sampling was used by selecting southern bluefin tuna at five minute intervals during harvest operations. The liver, posterior and head kidney, branchial arteries, gut, and spleen were examined for adult flukes through dissection under a dissecting microscope (Olympus SZX12).

The size the of adult flukes when reaching maturity in southern bluefin tuna was estimated by mounting a selection of *C. forsteri* specimens, stored in 10% neutral

buffered formalin, over a size range between 295  $\mu\text{m}$  to 3931  $\mu\text{m}$  taken from southern bluefin tuna sampled in 2004. Whole-mounts were stained with Mayer's haematoxylin, cleared with cedarwood oil and mounted in Canada balsam. Measurements were made with the Digitize program. Maturity was determined through the presence of eggs in the uterus. These measurements were not used in the models.

## **2.2. Data collection**

A single cohort of tuna, originating from the Great Australian Bight fishing grounds (map reference 33 27S, 132 04E), was examined over a six month period in 2004 (Aiken et al. 2006 (Chapter 3)). Ten tuna were sampled during the transfer from a tow cage to four experimental sea cages (set apart from normal commercially operated sea cages) on the 17<sup>th</sup> March 2004. Tuna were then sampled from the experimental sea cages at approximately monthly intervals until August. Forty tuna were sampled on 18<sup>th</sup> April (10 tuna from each of the 4 experimental sea cages), thirty tuna were sampled on 19<sup>th</sup> May (10 tuna from 3 of the 4 experimental sea cages), forty tuna were sampled on 16<sup>th</sup> June (10 tuna from each of the 4 experimental sea cages), fifty tuna were sampled on 18<sup>th</sup> July (10 tuna from each of the 4 experimental sea cages plus ten tuna from a commercially operated sea cage) and forty tuna were sampled on 19<sup>th</sup> August (10 tuna from each of the 4 experimental sea cages). A total of 220 tuna were sampled. Sampling took place during normal commercial harvesting operations. Systematic random sampling was used by selecting southern bluefin tuna at five minute intervals during harvest operations. Sampling involved the collection of gills and hearts during harvest, which were then stored on ice and taken to the laboratory. Hearts were dissected 2-4 hours after removal from the carcass and flushed with physiological saline to dislodge any

adult flukes. Flushes were poured into Petri dishes and examined for the presence of flukes using a dissection microscope (Olympus SZX12) at 6× magnification.

### **2.3. Data records**

All flukes were stored in 10% neutral buffered formalin and labelled according to what fish, cage and month they had been sampled from. Flukes were then pipetted out onto a Petri dish where they were viewed under a light microscope (Olympus BH2) at 25× magnification and images taken (Leica DC300f, Wetzlar Germany). Images were analysed using image analysis software (Sigma Scan Pro, Version 5.0; SPSS Science, Chicago, USA) and lengths recorded in Microsoft Office Excel (2003).

### **2.4. Observed data analysis**

Distributions of lengths for each month were graphed and normal distributions fitted to them so that comparisons could be made to the output of the first model. Categories of 100  $\mu\text{m}$  were used to group flukes into different length classes and frequencies of lengths for each month were calculated. Normal distributions were produced by calculating the means and standard deviations of the observed distributions which were then optimised to obtain the best fit of the normal distribution to the observed data. Values were then multiplied by a scaling factor. The scale parameter was a representation of the relative number of flukes observed at each month and was used to obtain the mean number of fluke per fish for the range of fluke lengths.

## 2.5. Models

Two stochastic simulation models were used to explore the period of infection of southern bluefin tuna by *C. forsteri*. Both models were written in Microsoft Office Excel (2003) and simulations were run using Palisade @Risk (Version 4.5) with Monte Carlo sampling. The lengths of individual flukes were used as the basis for both models. Copies of the models are attached in Appendix 1. The purpose of the models is to describe the infection pattern during the 2004 growout season and is not intended to be predictive. There is no level of detail to take into account annual variability and the effect of temperature. An optimal situation for data collection would have been to repeat the collections from the field many times recording data and parameters for the models to explain variation in the observed data. However this was not possible due to the nature of the southern bluefin tuna industry. Serial testing at different times of culture is unusual as southern bluefin tuna harvests are subject to market demand for product and usually occur only from June to August. Consequently the 2004 serial sampling represented an unique opportunity to collect such data. These biological models are based on field data and are not biased by experimental constraints and are aimed at capturing the aspects which drive the relationship between *C. forsteri* and *T. maccoyii*.

The models were to examine the patterns of *C. forsteri* infection in farmed southern bluefin tuna and also to determine when southern bluefin tuna were being infected. The first model simulated the lengths of flukes using the parameters that would be used for both models. The model was validated by comparing the fitted distributions of the observed data to distributions of flukes sizes by month as predicted by the stochastic model. The second model incorporated the observed data into a stochastic model to determine when southern bluefin tuna were being infected with *C. forsteri*

over the 2004 growout season. Once these key parameters were determined, they were used in the development of a model which could describe the time of infection. In order to describe the variability, resulting from the large number of repetitions of flukes lengths measured within each monthly sampling point, a stochastic model was used.

#### *2.5.1. Model parameter values*

The parameters used in the models in this paper have been derived from data obtained from known life cycles of other sanguinicolids (Sommerville and Iqbal, 1991; Kirk and Lewis, 1992; Kirk and Lewis, 1996; Kua et al. 2002) as well as being the result of having been fitted to the observed data of the model. Parameters used include a mean time of infection in days after tuna are brought into the farming zone, a value for the time in days it takes for migration to the heart after cercarial penetration, an initial size at cercarial penetration, a growth rate, a minimum and a maximum size at death. Variability for the life span parameters was calculated through a best fit approach.

#### *2.5.2. Model development*

Using model parameters the growth and life span of 10,000 individual flukes at each day of southern bluefin tuna captivity were stochastically simulated using @Risk (Version 4.5) in model 1. Initially, parameters were calculated to describe fluke dynamics. Tuna are brought into the southern bluefin tuna farming zone on a particular day and may be infected by flukes at any time. Therefore a parameter describing the time of infection in relation to the days in captivity for each tuna was calculated from a normal distribution (normal: scale, mean, SD). At the moment of infection cercariae penetrate the skin at a particular size. Using results from previous

studies on sanguinicolids and biological information from *C. forsteri*, a size of fluke at time of entry to host was determined as a fixed value. Having penetrated the host at this size the fluke undergoes a migration to the heart. This time from infection to reaching the heart is defined by the migration parameter as calculated by a normal distribution (normal: scale, mean and SD). On reaching the heart, a fluke will grow at a certain rate until death. The growth rate parameter is described by an exponential distribution (exponential, A (scale), Lambda (power)). This development in the heart will take place within a period that is determined by the life span of the fluke. The life span parameter is described by a beta distribution (Pert (minimum, most likely, maximum)). Predicted distributions using these parameters were fitted to the observed distributions (at days 32, 63, 91, 123, 155) by varying the inputs, thus optimizing the parameters.

The optimized parameters were applied to the observed fluke population to calculate likely time of infection in model 2. For each of the observed fluke the relationship of length to age was determined. Age was calculated for each fluke given its length, with variability taken into account using model parameters. These simulated data were then organized by length to find the mean and standard deviation of age for each length. Using these distributions for each length class, an age for each observed fluke was stochastically calculated based on its observed length. The age of the flukes was subtracted from the days in captivity to calculate the date of infection.

## **2.6. Wild southern bluefin tuna survey**

Thirty-one wild southern bluefin tuna were caught from two areas in the Great Australian Bight, Cabbage Patch (March 2005) and the commercial fishing grounds much further west (map reference 33 27S, 132 04E) (January 2005 and January 2006). Hearts were examined for the presence of adult flukes.



### 3. Results

No asexual stages of *C. forsteri* were found in bivalve and polychaete samples (Table 1).

**Table 1.** Specimens of bivalves and polychaetes sampled for the asexual stages of *C. forsteri* located in or around sea cages of southern bluefin tuna farmed off Port Lincoln, South Australia, with the months and year in which they were sampled during the growout seasons of 2004 and 2005.

Polychaeta			Bivalvia		
Family/Genus	No. Dissected	Month	Species	No. Dissected	Month
Terbellidae	10	Feb (2005)	<i>Pinna bicolor</i>	11	May (2005), Aug (2005)
Oweniidae	1	Feb (2005)	<i>Pinna sp.</i>	4	Aug (2005)
Trichobranchidae	1	Feb (2005)	<i>Mimaachlamys asperima</i>	9	Aug (2005), May (2006)
Magelona	1	Feb (2005)	<i>Pecten fumatus</i>	8	Aug (2005)
Peocilochaetus	4	Feb (2005)	<i>Chlamys bifrons</i>	7	Aug (2005)
Phyllodocidae	1	Feb (2005)	<i>Ostrea angasi</i>	16	Aug (2005), Sep (2006)
Dorvilleidae	7	Feb (2005)	<i>Electroma georgiana</i>	638	Aug (2005), Sep (2006)
Cirratulidae	2	Feb (2005)	<i>Mytilus edulis</i>	355	Aug (2005), May (2006), Sep (2006)
Syllidae	2	Feb (2005)			
Oenidae	2	Feb (2005)			
Capitellidae	5	Feb (2005)			
Ampharetidae	6	Feb (2005)			
Scalibregmatidae	1	Feb (2005)			
Sabellidae	5	Feb (2005)			
Eunicidae	22	Feb (2005)			
Glyceridae	2	Feb (2005)			
Nereidae	2	Feb (2005)			
Non Polychaetes					
Sipuncula	1	Feb (2005)			
Nemertea	1	Feb (2005)			

*C. forsteri* adults were observed only in the branchial arteries and the ventricle of the tuna. No adults were observed in the ventral aorta, bulbus arteriosus, atrium, liver, kidney, stomach or spleen. The majority of flukes were observed in the ventricle and only a small number of southern bluefin tuna (16%) were observed to have *C. forsteri* in the branchial arteries (Table 2).

**Table 2.** Intensities and prevalences of *C. forsteri* infection in 51 southern bluefin tuna farmed off Port Lincoln, South Australia, 2005, where both branchial arteries (BA) and ventricles (V) have been checked including the days the southern bluefin tuna have been in captivity (Days in Captivity, DIC). S.D. = standard deviation.

DIC	n	Prevalence (%)	Intensity $\pm$ S.D.	Prevalence (%)	Intensity $\pm$ S.D.
		V	V	BA	BA
80	5	80	$3.5 \pm 3.8$	20	1
86	1	100	2	100	4
94	12	75	$3 \pm 2.2$	16	$3 \pm 2.8$
99	6	16	1	0	0
102	6	50	$4 \pm 2.6$	16	2
105	6	33	$1.5 \pm 0.7$	16	2
108	6	33	$1.5 \pm 0.7$	0	0
118	6	100	$2.2 \pm 1.9$	33	1
135	1	1	5	0	0
142	2	0	0	0	0
Total	51	57	2.8	16	2.1

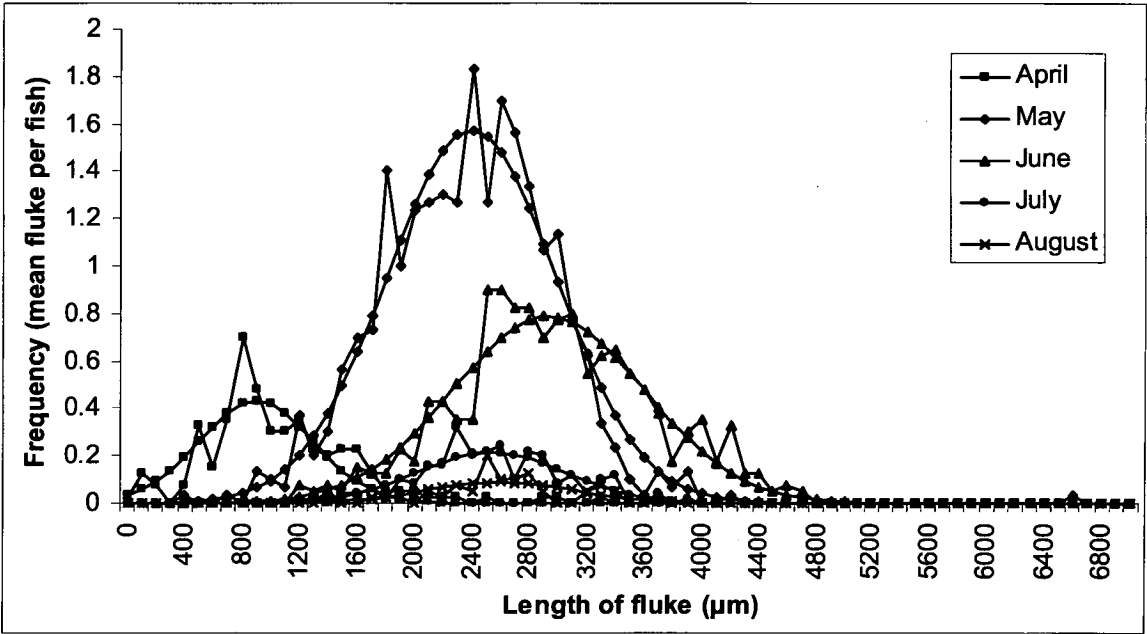
A total of 40 flukes were measured. Fluke above a length of 1790  $\mu\text{m}$  appeared to be gravid. One fluke was observed without eggs at 1772  $\mu\text{m}$ .

The parameters used in the model are summarized in Table 3. For the initial size parameter at which *C. forsteri* penetrates the host as cercariae, 50  $\mu\text{m}$  was used. The mean date of infection parameter, infection (Days in Captivity, DIC), that the cercariae penetrate the host was 33 days with a standard deviation of 8 days and was the most likely value to produce the distributions in model 1 that best fit the observed distributions (the time from this estimated cercarial infection moment will be considered days post infection in the text). Six days with a standard deviation of 3 days was used as the amount of time taken for migration. A most likely maximum life span was chosen to be 95 days and a most likely minimum life span to be 33 days.

**Table 3.** Summary of parameters used in the two stochastic models.

Infection (DIC) (days)			
Mean	33		
SD	8		
Migration to Heart (days)			
Mean	6		
SD	3		
Growth ( $\mu\text{m}$ )	Minimum	Most Likely	Maximum
Power	0.033	0.04	0.041
Scale	2000	3200	6000
Initial size at infection	48	50	51
Death (days)	Minimum	Most Likely	Maximum
Minimum age at death	30	33	37
Maximum age at death	90	95	150

A relationship was shown to occur between the average size of flukes and the month in which they were collected (Figure 1.). Normal curves were fitted to the observed distribution of lengths ( $\mu\text{m}$ ) by month of collection.



**Figure 1.** Observed distribution of lengths of *C. forsteri* ( $\mu\text{m}$ ) by month of collection during the 2004 growout period in Port Lincoln South Australia. Smooth curves show fitted normal distributions.

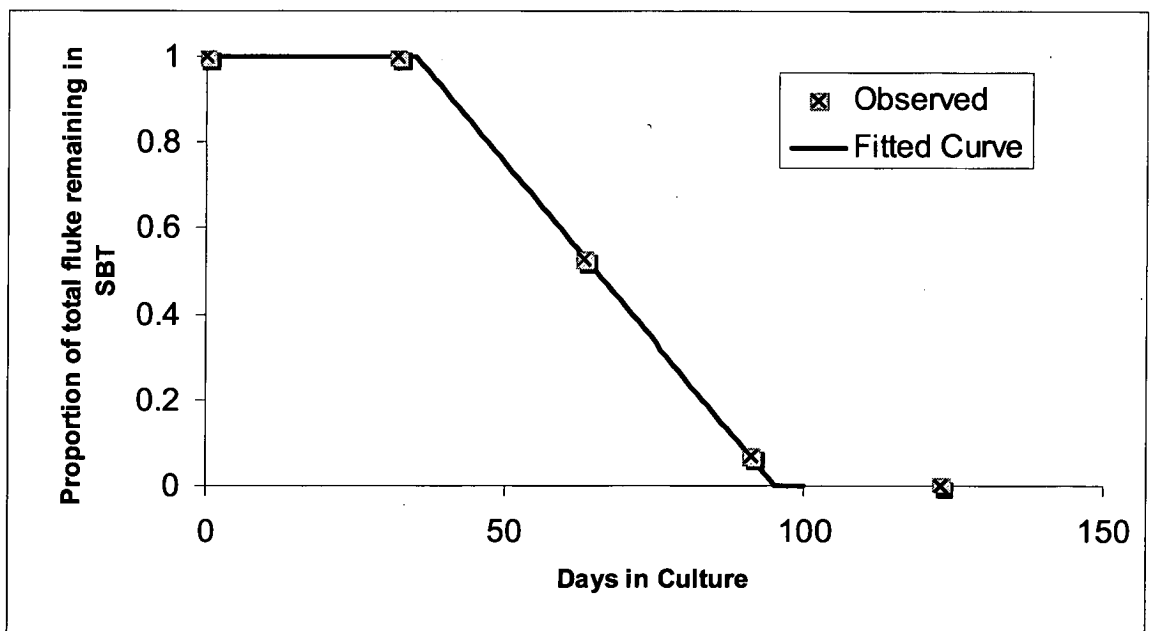
The parameters of the fitted distributions are shown in Table 4. At 32 days, the mean was constrained to 900 $\mu\text{m}$ , to remove the effect of the right tail of that distribution, representing a small number of flukes that were likely to have infected the fish prior

to transfer. The smallest flukes were observed in April, flukes observed in the other months were approximately the same length (Table 4.).

**Table 4.** Parameters of the fitted distributions of observed *C. forsteri* lengths by month of collection from southern bluefin tuna farmed off Port Lincoln, South Australia, 2004, where DIC= Days in Culture, SBT= southern bluefin tuna.

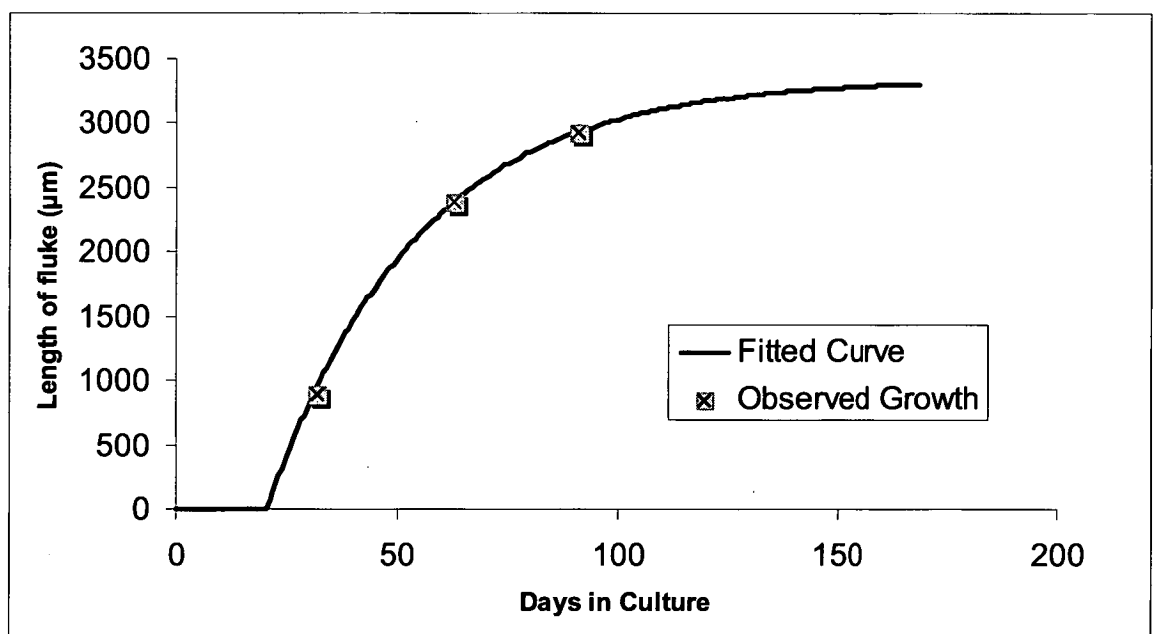
Sampling time	April	May	June	July	August
No. of SBT sampled	40	30	40	50	40
Days in Captivity (DIC) (days)	32	63	91	123	155
Mean ( $\mu$ m)	900.0	2394.8	2932.3	2543.9	2600.4
SD ( $\mu$ m)	396.4	590.0	665.7	512.0	512.7
Scale ( $\mu$ m)	427.6	2329.3	1314.2	276.6	114.8

Flukes were shown to start to disappear from the heart approximately 30 days post infection and almost all flukes that had infected southern bluefin tuna during the growout were gone by day 90 (Figure 2.). A persistence curve was fitted to the observed proportion of flukes remaining in the heart. The fitted curve is a linear decrease commencing at 35 days and continuing to 95 days. At day 63, an intermediate point on the curve, 53.3% flukes remained in the heart.



**Figure 2.** Observed proportion of *C. forsteri* remaining in the heart at different times, and fitted persistence curve over the period that southern bluefin tuna were in culture (Days in culture) during 2004, Port Lincoln, South Australia. No *C. forsteri* were observed in March.

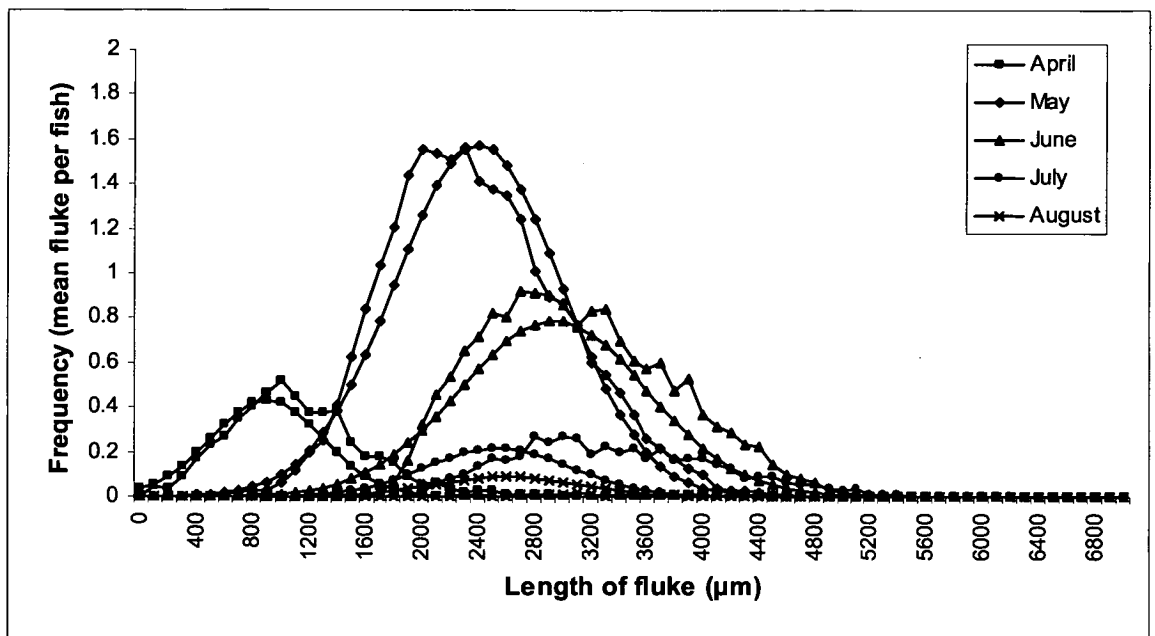
The growth of a blood fluke follows an exponential curve with a lambda of 0.0298, a y-shift of 20.79 and a scaling factor of 3344.8 (Figure 3.). Growth parameters were derived from the fitted curve on the observed growth of blood flukes. Observed growth was calculated from the means of the fitted distributions from the observed lengths for each month. The fitted and observed curves show that the flukes grow more rapidly in the early stages of infection rather than later. At day 32 flukes were observed to have grown to 900  $\mu\text{m}$ , day 63 they had grown to 2394  $\mu\text{m}$  and by day 91 flukes had grown 2932  $\mu\text{m}$ .



**Figure 3.** Observed and fitted *C. forsteri* growth curves. Observed data points represent mean length of flukes at each sampling point during the 2004 growout period in Port Lincoln, South Australia.

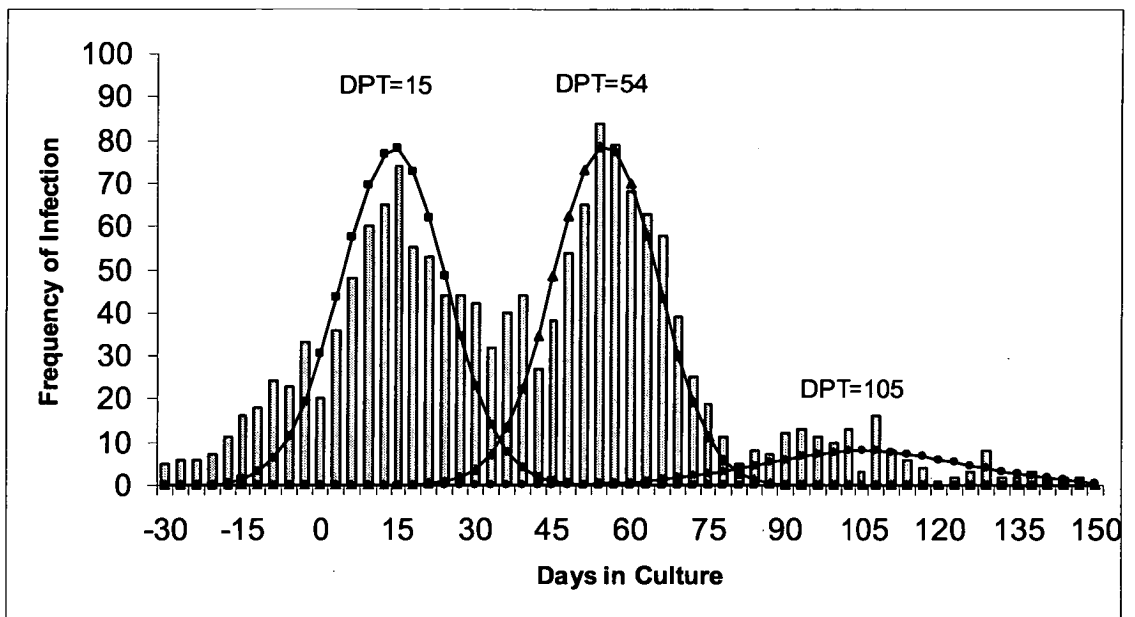
The distribution of fluke sizes as predicted by the stochastic model was compared to the fitted distributions of the observed data (Figure 4.). Although the outputs of the model are broadly similar in pattern to the observed, there are some differences. The number of flukes present in April was larger according to the model than the observed. This was adjusted by including a time delay for migration from skin to heart. The lengths of flukes in June and July to some degree shifted to the right and

were expected to be longer than they were observed to be. The number of flukes modeled in August was much lower than that observed in the August distribution.



**Figure 4.** Distribution of *C. forsteri* sizes by month as predicted by the stochastic model, compared to the fitted distributions of the observed data from flukes collected during the 2004 growout period in Port Lincoln, South Australia.

Based on the growth curve an estimated time of infection was determined (Figure 5.). Two peaks are shown at 14 days (S.D.=10.2) and 55 days (S.D.=10.1). A smaller peak of ongoing infection is shown at 110 days (S.D.=20.0).



**Figure 5.** Frequency of infection by *C. forsteri* in southern bluefin tuna over the 2004 growout season, in Port Lincoln, South Australia, based on estimated growth curve with fitted normal curves for each peak with days post transfer (DPT) for each peak occurrence.

Twenty southern bluefin tuna were caught in the commercial fishing grounds and eleven southern bluefin tuna were caught at the Cabbage Patch fishing grounds. Blood flukes were only observed from the hearts of two fish from Cabbage Patch, one fish with one fluke and the second fish with two blood flukes.

#### 4. Discussion

Two peaks of *C. forsteri* infection, at around 14 and 55 days after transfer of southern bluefin tuna, were demonstrated, with some lower level of continuing infection later with a peak at around 110 days. Although it appears that southern bluefin tuna are being infected throughout the growout season there are two major infection points. It is most likely that these points represent periodicity in shedding of cercariae from the intermediate host. Yacoubi et al. (1999) demonstrated the existence of successive periods of high production of *Schistosoma haematobium* cercariae in its intermediate host *Planorbarius metidjensis*. The same rhythm in daily

cercarial production was reported in three different populations of the intermediate host with the periodicity being 18-20 days. For the intermediate host of *C. forsteri* it maybe that the periodicity is between 40-42 days which is reflected in the two major peaks of infection of southern bluefin tuna.

Another possible explanation for the two peaks of infection is that they represent periodical excysting of schistosomules from cercarial infection in the musculature of southern bluefin tuna. The musculature of the southern bluefin tuna was unable to be checked as the carcass is highly valued and therefore not available for dissection. Encysted sanguinicolid schistosomules have recently been described by Montero et al. (2009) in a study of *Paradeontacylix ibericus* infections in *Seriola dumerili* with schistosomula observed in the muscles more than 100 days after transfer from sea cages to landbased tanks. Koie (1982) also demonstrated small juvenile *Aporocotyle simplex* in the muscles of *Limanda limanda* up to 94 days post infection. If excysting of *C. forsteri* schistosomula is occurring in southern bluefin tuna then it may be that the majority of infections in southern bluefin tuna are due to wild tuna with current infections being transferred into the farming zone rather than a locally infected intermediate host population. However, this is unlikely as other evidence suggests that the infection occurs in the Spencer Gulf farming zone as *C. forsteri* intensities and prevalences are very low in the wild (Aiken et al. 2007 (Chapter 2)), and at transfer (Aiken et al. 2006 (Chapter 3)), and an antibody response against *C. forsteri* is only initiated after transfer of southern bluefin tuna into the farming zone from the wild (Aiken et al. 2008 (Chapter 6)). The two peaks may also be explained by a cyclical pattern of movement of flukes into the gills such as that observed in infections of *Paracardicoloides yamagutii* in the gills of *Anguilla australis* and *Anguilla dieffenbachii* (Hine 1978). However, this is unlikely to have occurred in this



study as the models are based on observations of fluke in the heart and a deduction of the day the cercariae infected the host based on their length. The bimodal distribution represents the number of cercariae infecting a host per day over the growout period. Thus any movement of fluke into the gills occurs after this period and is not taken into account by the model and therefore is not a causal factor in the shape of the distribution. The two peaks distribution is also unlikely to be the result of increasing host resistance on fluke growth rates as the model used constant parameters, e.g. growth rate, for all fluke in the model. As there was no difference in growth rates for fluke in the models there would be no effect by some influence on growth rate, e.g. host resistance, on the shape of the bimodal distribution.

After the two peaks the number of infections taper off to an extremely low level. One possibility for this decline is that the cercariae are not present in the water column during this period. The decreasing water temperatures may be inducing an overwintering period in the intermediate host when the cercaria are not being shed (Väyrynen et al., 2000). Alternatively the intermediate host may be going through some mortality throughout the year either due to the parasite or some external influence. Køie (1982) showed that castration caused by *Aporocotyle simplex* was occurring in the populations of the intermediate host *Artacama proboscidea*. Kirk and Lewis (1992, 1993) showed that *Sanguinicola inermis* infected snails dies 12 weeks after exposure to miracidia.

Another reason for the decline in infections is that the juvenile migrating stages may be being killed before reaching the heart due to some host response. Many *S. inermis* flukes are unable to complete migration to the blood system of carp suggesting that it is most likely that most of these flukes are being killed by host defence mechanisms

during migration (Kirk and Lewis, 1996). The extracts of the cercarial and adult stages of *S. inermis* induce *in vitro* proliferation of carp lymphocytes and polarization of leucocytes (Richards et al., 1996a). These leucocytes were shown to cause tegumental damage to the cercaria and only minimal damage to the adult suggesting the presence of an immune evasion system (Richards et al., 1996b). *C. forsteri* juveniles may be being killed in this way leading to a decline in adult numbers in the heart as there is no recruitment to replace naturally dying adult flukes.

*C. forsteri* adults appeared to die at a linear rate. Gradual decreases in fluke numbers have been observed in *S. inermis* as well. Kirk and Lewis (1996) analysed serial sections of carp for parasites. It was shown that approximately one third of the cercarial dose penetrated the fish and survival of the flukes gradually decreased throughout the course of migration and maturation. Smith (1984) also showed that the natural death rate of *Fasciola hepatica* is constant and gradual.

The growth of the flukes in the host was represented by an exponential curve which described the flukes as growing more rapidly earlier rather than later. The growth of *S. inermis* was shown by Kirk and Lewis (1996) to be more rapid during the earlier juvenile stages than later when the flukes are mature. If the size of *C. forsteri* at maturity is taken to be around 1800  $\mu\text{m}$ , as determined by the analysis of the stained and mounted flukes, the growth to this length represents the steepest part of the growth curve. However, this measurement must be treated with caution when comparing to the results of the models as it is possible that some shrinking of fluke may have occurred due to the staining and mounting method. Koie (1982) also showed that for many *A. simplex* the most rapid growth was that experienced in the early stages of development once the fluke had undergone its migration through the lymphatic system and muscles of its dab fish host to reach the branchial arteries.

The lengths simulated by the first model were shown to be similar to the observed data, although there were some differences. In June and July fluke lengths were expected to be longer than they were observed to be. This difference could be due to the earlier mortality of the longer worms so the worms which persist tend to be the shorter ones. As well, in August a low persisting infection may have led to a lower number of worms in the output of the model compared to the observed. Despite these differences the model was mostly similar to the observed data, and the model and the assumptions used were validated by this comparison. Model 2 could not be validated. However with the development of a new ELISA for *C. forsteri* infection in southern bluefin tuna the output of model 2 could be validated against serological results (Aiken et al. 2008 (Chapter 6)). There is evidence that this ELISA is detecting penetrating cercariae and immature migrating blood flukes (Aiken et al. 2008 (Chapter 6)). Serial non-destructive sampling using the ELISA could be used to determine the intensity and time of infection of immature flukes invading the southern bluefin tuna host. Another possible method of validating the model would be to collect a second independent dataset from the field. A second dataset including the use of any parameters needed, e.g. water temperature, annual variation, would be able to explain variation in the observed data. However serial testing at different times of culture is difficult as southern bluefin tuna harvests usually occur only from June to August and over a short period of time.

There is a possibility that these two peaks of infection are an artifact from the way the data were gathered, as the two peaks are roughly a month apart and may therefore represent flukes at the first collection and then the second. The model assumed constant growth rate regardless of the time of infection. This assumption may have

been incorrect and the two peaks may be being observed because growth rate is not constant in which case the peaks should merge into one. Flukes that infect later may have been growing more rapidly so that all flukes reached the same size after two months. Growth rate may be influenced by external factors outside the host. Water temperature is a significant factor in the maturation of adult trematodes in fish hosts (Chubb, 1979). In carp infected by *S. inermis* it has been shown that there are differences in the amount of time it takes for the blood flukes to reach maturity due to temperature of the water. Time to maturity was shown to take longer the lower the water temperature (Sommerville and Iqbal, 1991; Kirk and Lewis, 1996). However growth rate is most likely to be constant and independent of the time of infection or ambient temperature for *C. forsteri* in southern bluefin tuna. The homeothermic tuna provides an environment where the temperature is maintained above ambient water temperature. Additionally if any change in growth rate was to be expected it would be that growth rate would be slowed in response to decreasing water temperature and not increased. The same would be true if there was some intraspecific competition for space in the heart caused by the existing blood flukes slowing the growth rate in the newly recruiting flukes. Density dependent survival and reproduction as a result of space constraints has been shown in the case of the digenean *Transversotrema patialense* on the fish host *Brachydanio rerio* (Mills et al., 1979). Competition for space is unlikely for *C. forsteri* as the blood flukes are extremely small compared to the large size of the heart of the tuna.

Parameters for the models were derived using other sanguinicolid life cycles. *A. simplex* is the only marine sanguinicolid life cycle that has been determined (Koie, 1982). Extensive work on the life cycle of *S. inermis* infecting freshwater cyprinids has also been completed (Sommerville and Iqbal, 1991; Kirk and Lewis, 1993; Kirk

and Lewis, 1996). A small cercarial size was chosen as small *C. forsteri* were observed in the heart, one specimen measuring 93.78  $\mu\text{m}$ . In other sanguinicolids the cercarial stage is much larger. In *A. simplex* the body of living, slightly flattened cercariae is 140 – 170  $\mu\text{m}$  with an average of 10 cercariae at 160  $\mu\text{m}$  (Koie 1982). In *S. inermis* the average length of cercariae were observed to be 95.7  $\mu\text{m}$  and the cercarial length for *Sanguinicola armata* is 67.2  $\mu\text{m}$  (Kirk and Lewis, 1993; Kua et al., 2002). As flukes of small size are found in the heart it can be assumed that the migration from the skin to the ventricle is rapid and six days was chosen for the value of this parameter. A short migration time (12 days) and minimal growth (67.2  $\mu\text{m}$  – 206.0  $\mu\text{m}$ ) for *S. armata* has been observed while a longer migration time (94 days) and more growth (160  $\mu\text{m}$  – 1000  $\mu\text{m}$ ) has been observed in *A. simplex* (Koie, 1982; Kua et al. 2002).

The life span of blood flukes is variable between species. Maximum life span for *S. inermis* was reported to be 56 -70 days (Kirk and Lewis, 1996). (Koie, 1982) observed *A. simplex* that were at least 180 days old. Taking into account the decline in numbers over a three month period observed in *C. forsteri* (Aiken et al. 2006 (Chapter 3)) we chose a most likely maximum life span to be 95 days and a most likely minimum life span to be 33 days (Table 2). Maximum life span was chosen on account of the decrease in fluke numbers three months after southern bluefin tuna transfer observed in (Aiken et al. 2006 (Chapter 3)). Additionally it is unlikely that blood flukes will die before they are sexually mature and (Kirk and Lewis, 1996) reported that *S. inermis* produced eggs 28 – 42 days post infection. Therefore 35 days was chosen as a minimum life span value.

Small numbers of flukes were observed in the branchial arteries of southern bluefin tuna. However there seemed to be no relationship between the numbers of fluke observed in the ventricle and those observed in the branchial arteries (data not shown). Similarly *S. inermis* adults are observed in the afferent branchial arteries, ventral aorta, bulbous arteriosus, ventricle and the atrium (Kirk and Lewis, 1996). During the later stages of the infection period (42-56 days post infection) degrading flukes are found lodged in the afferent branchial vessels proximal to the gills and amongst the muscular trabeculae of the atrium (Kirk and Lewis, 1996). It could be that in southern bluefin tuna the small number of flukes observed in the branchial arteries may have been dislodged from the heart due to morbidity of the flukes. Degraded flukes have been observed in the branchial arteries from tuna that had been in captivity for 80 days (Aiken Unpublished results). Due to the small numbers involved in the infection of the branchial arteries and the likelihood that these flukes have been dislodged from the ventricle, use of the heart as the basis for diagnosing *C. forsteri* infection for the model is justified. Cribb et al. (2000) observed *C. forsteri* only in the heart.

As the data used in the models were taken from only one site in one year (2004), assessment of natural variability and therefore an evaluation of how generic the models might be is limited. However, these models are not intended to be predictive and despite the limitations the results are significant in that they provide evidence for *C. forsteri* infection in southern bluefin tuna as a localized occurrence, in which the majority of cases are due to infections that have occurred in the southern bluefin tuna farming zone in the Spencer Gulf rather than tuna bringing in infections from the wild. This is evident from the two estimated major infection periods after transfer and the low prevalence and intensity of infections in wild southern bluefin tuna.

Therefore if any action were needed to control infection the farm sites would be the focus rather than southern bluefin tuna entering the farming region.

## **5. Acknowledgements**

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**Chapter 5. Factors affecting abundance and prevalence of blood fluke, *Cardicola forsteri*, infection in farmed southern bluefin tuna, *Thunnus maccoyii*, in Australia.**

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## **Abstract**

A survey of blood fluke, *Cardicola forsteri*, infection in farmed southern bluefin tuna was undertaken from March 2004 to September 2006. Analyses of covariance and logistic regression were used to explore the effects of company, year, season, time in culture, and condition index on intensity, abundance and prevalence of blood fluke infection. Average prevalence of blood fluke infection was observed to be 62.64% over the period of the survey. Average intensity was demonstrated to be 6.20 ( $\pm 0.57$ ) fluke per infected host and 3.70 ( $\pm 0.57$ ) fluke per host. Year did not influence mean intensity or abundance although a significant decrease in prevalence in 2005 was evident. Tuna harvested in winter have a significantly greater abundance and prevalence of blood fluke than the tuna harvested in autumn. No effect of intensity or abundance of infection was observed on the condition of tuna. A universal factor in explaining variation in *C. forsteri* intensity, abundance and prevalence was company. Differences in infection levels between companies may be related to different 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 each company.

## 1. Introduction

The blood fluke, *Cardicola forsteri* was first detected and described from farmed *Thunnus maccoyii* in Spencer Gulf, South Australia in 1997 (Cribb et al. 2000). Since this time *C. forsteri* has been detected in wild northern bluefin tuna in the Atlantic (Bullard et al. 2004) and in farmed northern bluefin tuna in Spain (Aiken et al. 2007 (Chapter 2)). *C. forsteri* appears to be able to complete its life cycle in the farming zone of the Spencer Gulf (Aiken et al. Submitted (Chapter 4)). However the blood fluke was most likely introduced into this area through the translocation of wild infected tuna into the zone or through local shipping activity acting as a vector for infected intermediate hosts (Aiken et al. 2007 (Chapter 2)). The intermediate host is unknown, but may include polychaetes or bivalves (Smith 1997).

As a result of the concern caused by its presence in farmed *T. maccoyii* stocks, *C. forsteri* has been the focus of a targeted surveillance system since 2003 (Nowak et al. 2003; Deveney et al. 2005). A pattern of infection in farmed tuna includes an initial increase in intensity and prevalence after the wild tuna are brought in for farming. This increase is then followed by a decrease in both intensity and prevalence (Aiken et al. 2006 (Chapter 3)). This pattern of infection was observed first in 2004 (Aiken et al. 2006 (Chapter 3)) and again in 2005 (Aiken et al. 2008 (Chapter 6)). It is not known, however, what factors are responsible for this pattern of infection. Factors influencing infection patterns are usually investigated individually in fish parasitic diseases without consideration of the interactions between factors that may be generating confounding effects (Lefebvre et al. 2002). The aim of this study is to simultaneously assess the relative contribution of various spatial and temporal factors on blood fluke intensity, abundance and prevalence in farmed southern bluefin tuna over a three-year period (2004-2006).



## **2. Materials and Methods**

Southern bluefin tuna were sampled from commercial farms located in Spencer Gulf, South Australia. Nine companies participated, of which three were targeted for intensive sampling. Sampling was conducted over three consecutive years from March 2004 to September 2006. In each year samples were collected in the months March, April, May, June, July, August, and September. Sampling took place during normal commercial harvesting operations. Systematic random sampling was used by selecting southern bluefin tuna at five minute intervals during harvest operations. Gills and hearts were obtained during harvest, stored in individual containers on ice, and taken to the laboratory. Hearts were dissected open 2-4 hours after removal from the carcass and flushed with physiological saline to dislodge any adult flukes. Flushes were then poured into Petri dishes and were then examined for the presence of adults using a dissection microscope. They were then counted and prevalence (the number of infected tuna divided by the number of tuna examined  $\times 100$ ), mean abundance (the total number of parasites divided by total number of tuna examined), and mean intensity (the total number of parasites divided by the number of infected tuna), were determined, as described by Bush et al. (1997). Weights and lengths for each tuna sampled were also obtained, and a Condition Index was calculated using the South Australian tuna industry formula, whole weight / length<sup>3</sup>. A number of companies also submitted mortalities over the three years. Hearts were removed from the carcass and processed as above for the presence of adult blood flukes.

The effects of company, year, season, time in culture, and condition index on intensity, abundance and prevalence of blood fluke infecting southern bluefin tuna from three companies (Company A, B and C) were explored using SPSS 13.0. These companies were selected as they were represented in every year of the study. Months

in which southern bluefin tuna were sampled were grouped into seasons as data were missing for some months. An alpha level of 0.05 was set for all analyses.

### **2.1. Blood fluke intensity and abundance analysis**

The effects of the various factors on blood fluke intensity and abundance were explored using analysis of covariance. Company (A, B and C), year (2004, 2005, 2006), and season (autumn, winter) were entered as categorical predictors. Time in culture (days) and condition index were entered as continuous predictors. Since time in culture might have made a difference in exposure to parasitic infections, the influence of the variable, days in culture, was taken into account as a covariate. A three-way Analysis of Covariance (ANCOVA) was applied to the intensity data and separately to the abundance data for this purpose. ANCOVA requires that the relationship between the response variable and each covariate is the same for each group within a factor, that is, the slopes of the regression lines fitted for each group are parallel (Sokal and Rohlf, 1995). Interactions between the covariate and factors were tested to determine whether covariate values were similar across groups.

### **2.2. Blood fluke prevalence analysis**

As the response variable is binomial (infected vs. non-infected) for prevalence data, logistic regression was used for analysis of the infection status of individual fish. Factors (company, year, season, time in culture, and condition index) were first analysed individually through univariable logistic regression and then incorporated into multivariate models using a forward stepwise approach. All factors identified as having a  $P$ -value < 0.25 through the univariate analysis were retained and used to build the multivariable regression model. Factors included in the models were examined for multicollinearity through correlation. We used the Hosmer and

Lemeshow goodness-of-fit test to assess the model fit (Hosmer and Lemeshow 1989).

### **2.3. Condition index analysis**

To examine the effect of intensity and abundance of infection by blood fluke on body condition, we analysed condition index by linear regression. To determine if there was any difference in condition indices of tuna between companies, a Three-way ANCOVA was also used. Company, year, and season were entered as categorical predictors and time in culture (days) was entered as a continuous predictor and taken into account as a covariate. Interactions between the covariate and factors were again tested to determine whether covariate values were similar across groups.

## **3. Results**

### **3.1. Temporal infection dynamics**

A total of 771 southern bluefin tuna were examined during the three year survey. A total of 2561 *Cardicola forsteri* were counted in the lumen of ventricles from 486 infected tuna. From March 2004 to September 2006, blood fluke intensity varied from  $1.34 \pm 0.34$  to  $12.78 \pm 3.17$  blood fluke per infected host with an overall mean intensity  $5.30 \pm 0.32$  (n=486) (Fig. 1).

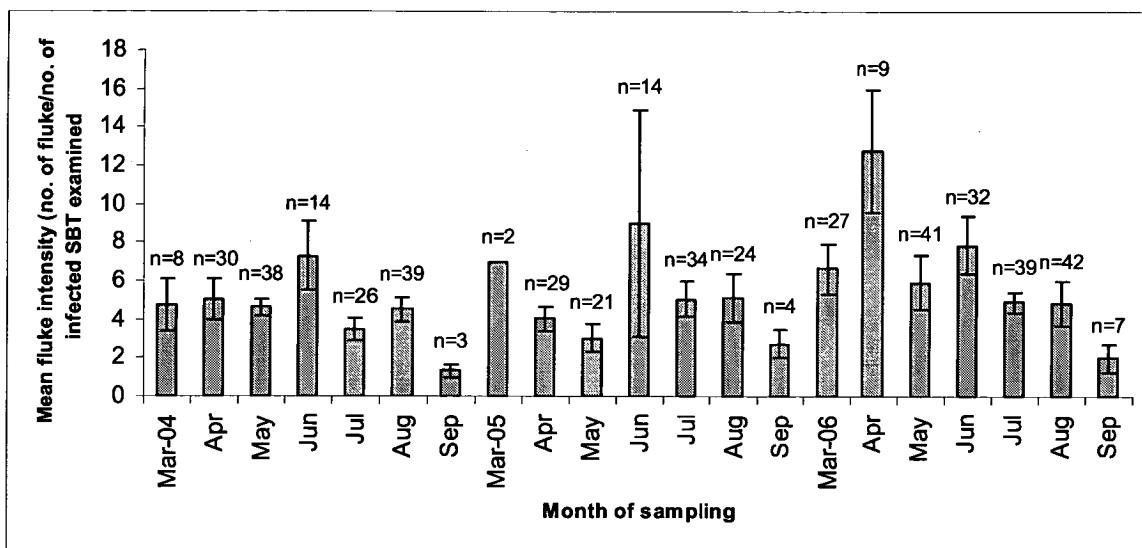


Figure 1. Mean ( $\pm$  SE) intensity of *Cardicola forsteri* as a function of month during sampling of farmed southern bluefin tuna, *Thunnus maccoyii*, between 2004 and 2006 from the Spencer Gulf, South Australia.

Monthly mean abundance varied from  $0.53 \pm 0.28$  to  $6.39 \pm 2.19$  with an overall abundance of  $3.33 \pm 0.21$  ( $n=771$ ) (Fig 2). Mean monthly prevalence varied between 29.2% and 90.9% ( $n=771$ ) (Fig 3). Overall prevalence for the three years was 62.64% ( $n=771$ ). For statistical analysis of the influence of company, year, season, time in culture, and tuna condition on intensity, abundance and prevalence, 503 tuna from three companies, A, B and C were selected. These companies were selected as the remaining 268 tuna came from companies where data was missing for some years and seasons.

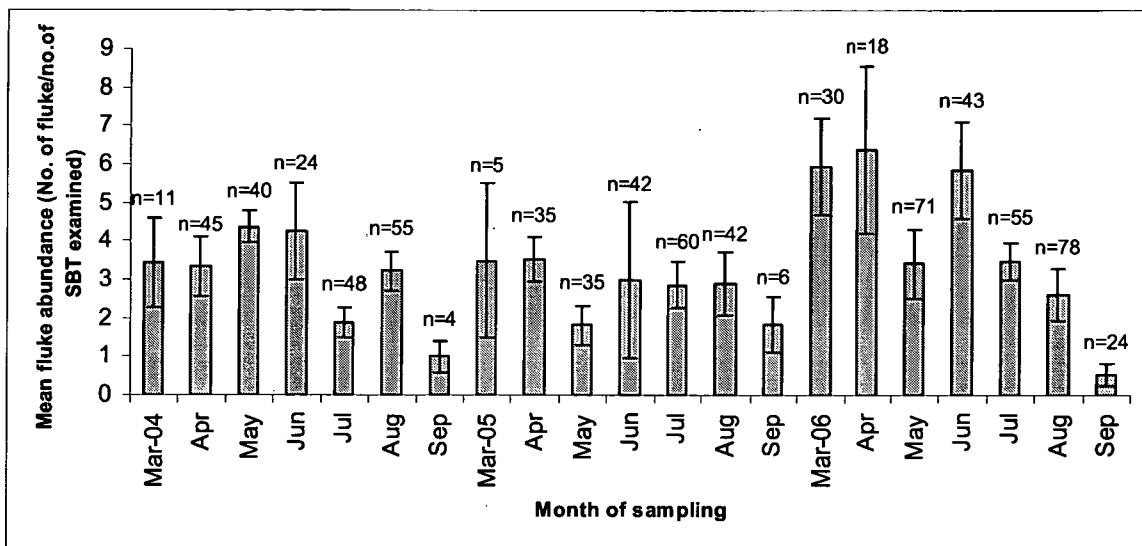


Figure 2. Mean ( $\pm$  SE) abundance of *Cardicola forsteri* as a function of month during sampling of farmed southern bluefin tuna, *Thunnus maccoyii*, between 2004 and 2006 from the Spencer Gulf, South Australia.

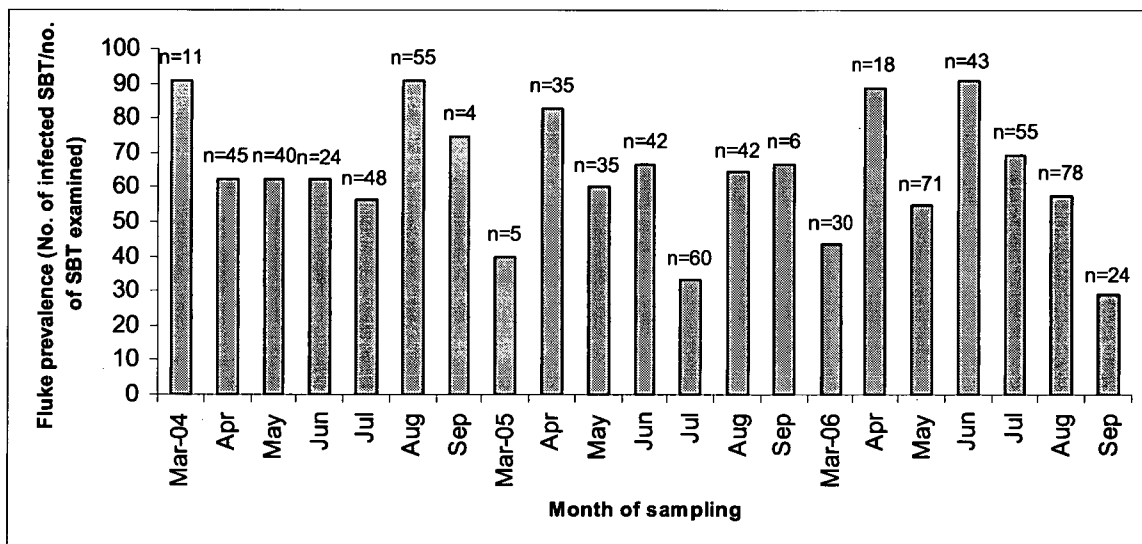


Figure 3. Prevalence of *Cardicola forsteri* as a function of month during sampling of farmed southern bluefin tuna, *Thunnus maccoyii*, between 2004 and 2006 from the Spencer Gulf, South Australia.

### 3.2. Factors affecting blood fluke abundance

Time in culture was shown to have a significant effect on blood fluke abundance (ANCOVA,  $W=11.765$ ,  $d.f.=1$ ,  $P=0.003$ ) in tuna and therefore mean abundance had to be corrected by days in culture (Fig 4.).

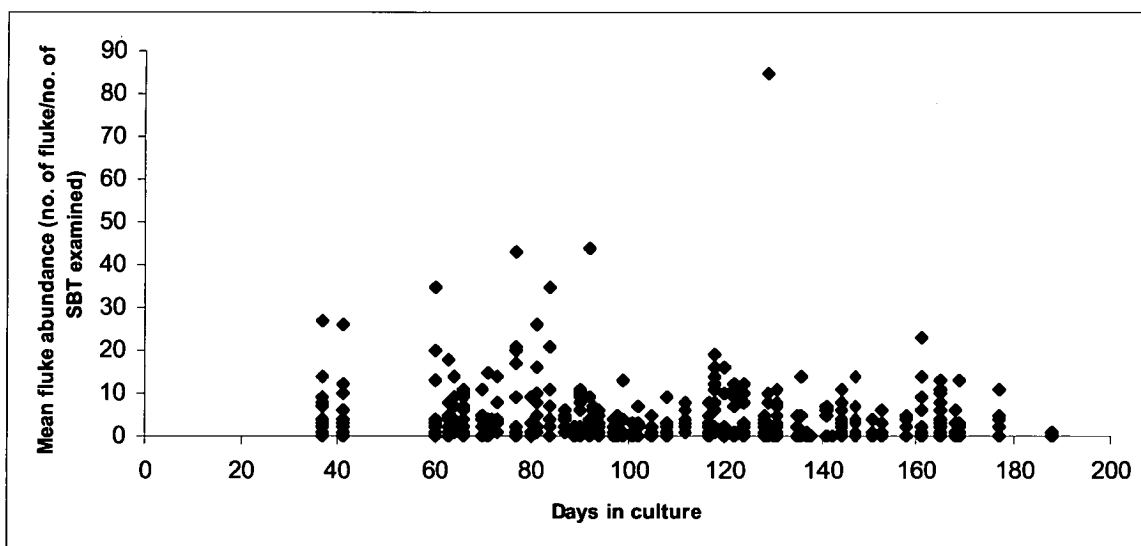


Figure 4. Mean abundance of *Cardicola forsteri* as a function of the days in culture since transfer for farmed southern bluefin tuna, *Thunnus maccoyii*, sampled between 2004 and 2006 from the Spencer Gulf, South Australia.

Significant differences were shown between blood fluke abundances from different companies (ANCOVA,  $F=3.577$ ,  $d.f.=2$ ,  $P=0.029$ ). Company C ( $4.944 \pm 0.592$  (SE) fluke per host) had a significantly greater abundance of fluke than Company B ( $2.780 \pm 0.535$  (SE) fluke per host). No significant differences occurred between Company A and Company B ( $P=0.456$ ) and between Company A and Company C ( $P=0.091$ ). Season was shown to have an influence on abundance (ANCOVA,  $F=6.237$ ,  $d.f.=1$ ,  $P=0.013$ ). A greater abundance of fluke was observed in tuna harvested in winter ( $5.046 \pm 0.636$  (SE) fluke per host) than in autumn ( $2.357 \pm 0.614$  (SE) fluke per host) irrespective of different companies harvesting at different times of the year. No significant difference in abundance was observed between years (ANCOVA,  $F=1.338$ ,  $d.f.=2$ ,  $P=0.263$ ). A significant first-order interaction was observed between company and year (ANCOVA,  $F=3.533$ ,  $d.f.=4$ ,  $P=0.007$ ). A significant second order interaction was observed between year, season and company (ANCOVA,  $F=3.175$ ,  $d.f.=4$ ,  $P=0.14$ ) (Fig. 9).

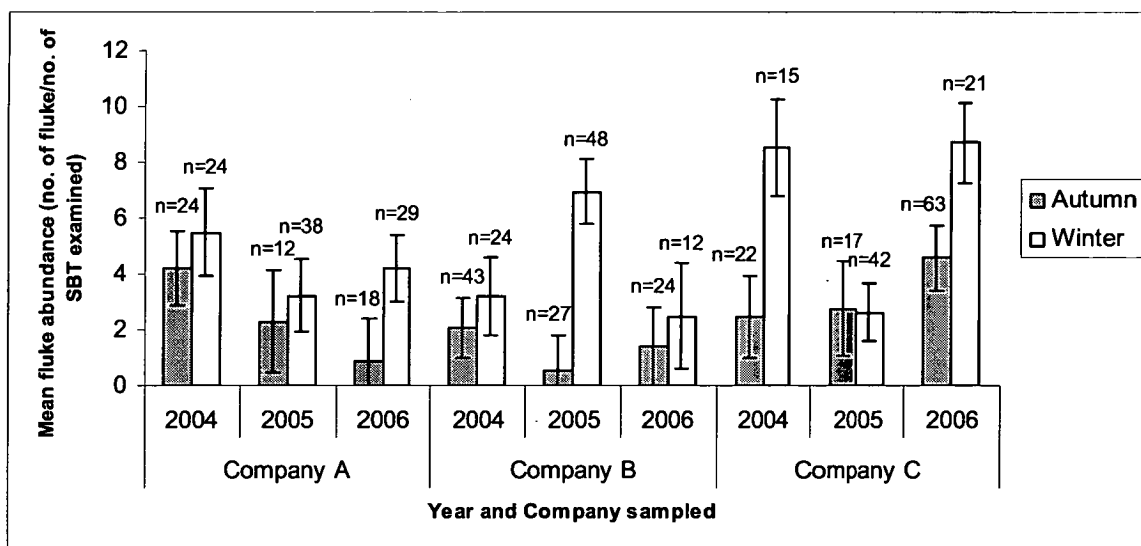


Figure 9. Adjusted mean ( $\pm$  SE) *Cardicola forsteri* abundance in farmed southern bluefin tuna, *Thunnus maccoyii*, for each company yearly and divided seasonally sampled between 2004 and 2006 from the Spencer Gulf, South Australia.

The total adjusted mean abundance for all samples taken was  $3.702 \pm 0.317$  (SE) fluke per host. Covariate means did not significantly differ between groups for each factor. The relationships were tested and found to be linear, and the slopes of the regression lines did not differ significantly.

### 3.3. Factors affecting blood fluke intensity

Time in captivity was not shown to have a significant effect on blood fluke intensity (ANCOVA,  $F=1.809$ ,  $d.f.=1$ ,  $P=0.18$ ) and therefore mean intensity did not have to be corrected by 'days in culture' and a 3-way ANOVA was used for analysis. Levene's test of equality of error variances was shown to be significant ( $F=2.371$ ,  $P<0.001$ ), therefore the results of the analysis should be treated with caution (Underwood 1981). Significant differences were not observed between winter and autumn (ANOVA,  $F=1.512$ ,  $d.f.=1$ ,  $P=0.220$ ), or between different years (ANOVA,  $F=0.117$ ,  $d.f.=2$ ,  $P=0.889$ ). A significant difference was observed between companies (ANOVA,  $F=3.743$ ,  $d.f.=2$ ,  $P=0.025$ ) (Fig. 12). Company C ( $8.311 \pm$

0.856 (SE) fluke per infected host) had a significantly greater intensity of fluke than Company A ( $4.795 \pm 1.047$  (SE) fluke per host) (Fig 12). No significant differences occurred between Company B and Company A ( $P=0.475$ ) or between Company B and Company C ( $P=0.069$ ). No first or second order interactions were significant. Linear regression analysis demonstrated that there was no relationship between intensity and condition index ( $F=0.10$ ,  $d.f.=1$ ,  $P=0.922$ ,  $R^2<0.001$ ). The total mean intensity for all samples was  $6.319 \pm 0.569$  (SE) fluke per infected host.

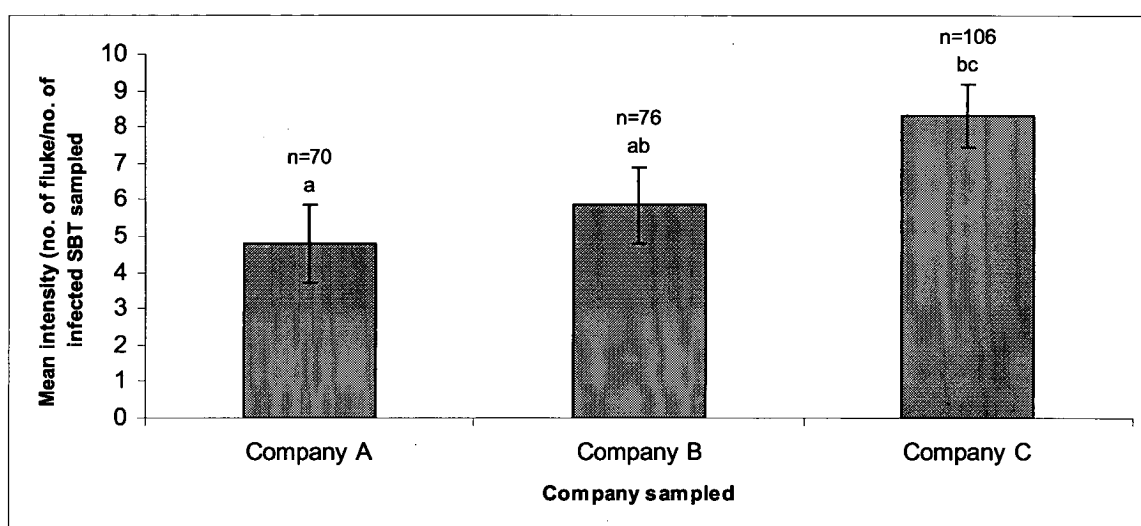


Figure 12. Mean ( $\pm$  SE) *Cardicola forsteri* intensity in farmed southern bluefin tuna, *Thunnus maccoyii*, for each company sampled between 2004 and 2006 from the Spencer Gulf, South Australia. Means with different letters are significantly different from one another.

### 3.4. Factors affecting blood fluke prevalence

Five variables, company, year, season, time in culture, and tuna condition, were examined for their effect on blood fluke prevalence. Logistic regression univariate analysis reduced the number of potential candidate variables for consideration in the multivariate model from five to four using the cut-off  $P<0.25$  (Table 1). Prevalence of blood fluke was shown to have no effect on Condition Index ( $W=0.10$ ,  $d.f.=1$ ,  $P=0.921$ ). Of the candidate variables, two were found to show multicollinearity with each other and could not be included together in the logistic regression model. These were 'Season' and 'Days in Culture', which were strongly correlated ( $R^2=0.613$ ,



$F=795.121$ ,  $d.f.=1$ ,  $P<0.001$ ). In these cases, only the most statistically significant factor in the univariate analysis, 'Days in Culture', was included in the multivariate analysis. The three remaining candidate variables were analysed through multivariate logistic regression. 'Days in Culture' and 'Year' were shown to be statistically significant (Table 2). The model shows that for each day of culture the odds of being an infected tuna are decreased by 0.09%. The model also showed that the odds of being infected in 2005 were decreased when compared to 2004 or 2006. The model was shown to fit the data satisfactorily as the Chi-square (Hosmer-Lemeshow) test of goodness of fit was not significant ( $\chi^2=10.090$ ,  $d.f.=8$ ,  $P=0.259$ ).

Table 1. Factors examined for their effect on *Cardicola forsteri* infections, through univariate logistic regression analysis of prevalence data of blood fluke from southern bluefin tuna, *Thunnus maccoyii*, sampled between 2004 and 2006 from the Spencer Gulf, South Australia (n=503). OR=Odds ratio, CI= Condition index,  $W$ = Wald statistic,  $d.f.$ = degrees of freedom.

Variable	Crude OR	95% CI	$W$	$d.f.$	$P$ -value
Season: Autumn* vs. Winter	1.655	1.163-2.354	7.851	1	0.005
Condition Index (continuous)	0.997	0.938-1.059	0.100	1	0.921
Days in Culture (continuous)	0.989	0.984-0.994	20.219	1	$P<0.001$
Year			19.942	2	$P<0.001$
Year: 2004* vs. 2005	0.404	0.260-0.627	16.260	1	$P<0.001$
Year: 2004* vs. 2006	0.887	0.568-1.384	0.278	1	0.598
Year: 2005 vs. 2006*	0.455	0.297-0.698	12.992	1	$P<0.001$
Company			9.570	2	0.008
Company: Company A <sup>1</sup> vs.					
Company B	1.253	0.806-1.947	1.003	1	0.317
Company: Company A <sup>1</sup> vs.					
Company C	0.652	0.419-1.012	3.629	1	0.057
Company: Company B vs.					
Company C <sup>1</sup>	0.52	0.342-0.792	9.306	1	0.002

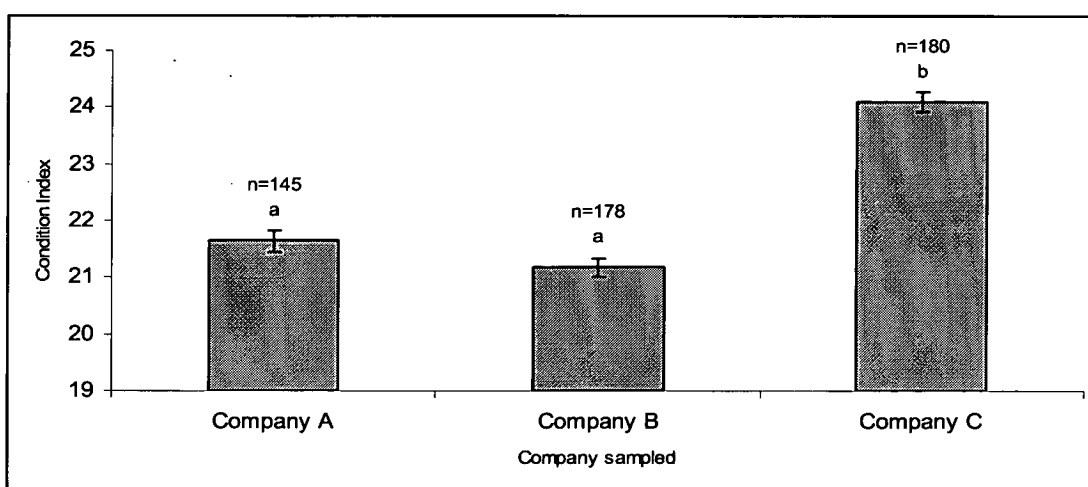
<sup>1</sup> Reference group used in comparison between variables.

Table 2. Results from multivariate logistic regression of prevalence data of *Cardicola forsteri* infection in southern bluefin tuna, *Thunnus maccoyii*, sampled between 2004 and 2006 from the Spencer Gulf, South Australia (n=503). OR=Odds ratio, CI= Condition index, *W*= Wald statistic, *d.f.*= degrees of freedom.

Variable	Odds ratio	95% CI	<i>W</i>	<i>d.f.</i>	<i>P</i> -value
Year			11.472	2	0.003
Year: 2004 <sup>1</sup> vs. 2005	0.46	0.293-0.722	11.420	1	0.001
Year: 2004 <sup>2</sup> vs. 2006	0.716	0.448-1.142	1.969	1	0.161
Year: 2006 <sup>2</sup> vs. 2005	0.643	0.401-1.029	3.392	1	0.066
Company			5.754	2	0.056
DIC	0.991	0.985-0.996	12.040	1	0.001
Constant			14.194	1	<i>P</i> <0.001

### 3.5. Condition index analysis

A significant difference in mean condition indices were observed between companies (ANCOVA,  $F=72.183$ ,  $d.f.=2$ ,  $P<0.001$ ). Tuna originating from Company C had a significantly greater mean condition index than tuna from Company A or B (Figure 13). Levene's test of equality of error variances was shown to be significant ( $F=1.699$ ,  $P=0.04$ ), therefore the results of the analysis should be treated with caution (Underwood 1981).



**Figure 13.** Mean ( $\pm$  SE) condition index of farmed southern bluefin tuna, *Thunnus maccoyii*, for each company sampled between 2004 and 2006 from the Spencer Gulf, South Australia. Means with different letters are significantly different from each other.

<sup>2</sup> Reference group used in comparison between variables. DIC = Days in Culture.

### 3.6. Mortalities

A total of 21 southern bluefin tuna mortalities from 8 companies were submitted for *C. forsteri* examination. Four southern bluefin tuna (19%) were shown to have *C. forsteri* infection with the highest burden being 15 flukes in an individual sampled on 20-Jun-06 (Table 3.).

Table 3. Number of *C. forsteri* observed in the heart of southern bluefin tuna mortalities submitted between 2006 and 2006 from the Spencer Gulf, South Australia. n = number of tuna mortalities examined on each date.

Date	Company	No. of <i>C. forsteri</i>	n
3-Mar-04	Company 1	0	1
18-Mar-04	Company 2	0	1
19-Mar-04	Company 3	0	1
24-Mar-04	Company 4	0	1
30-Mar-04	Company 5	0	1
6-Apr-04	Company 6	0	1
30-Apr-04	Company 7	0	1
3-May-04	Company 6	6	1
11-May-04	Company 6	9	1
26-Jan-05	Company 7	0	2
26-Jan-05	Company 7	0	1
8-Feb-05	Company 8	0	1
3-Mar-05	Company 5	0	1
4-Mar-05	Company 7	0	1
18-Mar-05	Company 3	1	1
23-Mar-05	Company 8	0	1
16-May-06	Company 1	0	2
16-May-06	Company 1	0	1
20-Jun-06	Company 6	15	1

### 4. Discussion

No particular trend in the development of *Cardicola forsteri* infection could be detected across the 3 years of the survey when parasite predictors were examined as a function of month. After almost a decade since the first record of *C. forsteri* in the southern bluefin tuna farming zone of the Spencer Gulf (1997: Cribb et al. 2000) blood fluke infection has since reached a constant infection rate of approximately 60% in tuna at harvest. An equilibrium of infection levels in harvested tuna may have been attained. This is also reflected in blood fluke intensity and abundance where, although peaks in intensity and abundance did occur, infection levels rarely increased

above ten flukes per infected tuna at harvest. Annual means of intensity were fixed around six flukes per infected host and yearly means of abundance between three and five flukes per host. Stabilisation of infection levels have previously been observed in fish-parasite systems. The nematode *Anguillicola crassus* infection in European wild eels, *Anguilla anguilla*, in the Rhone River delta, has reached a constant infection rate of 50% and annual intensity means of between 3 and 4 parasites per eel, a decade after its introduction (Lefebvre et al. 2002). Density-dependent effects, host adaptation, and mortality of heavily infected individuals have been suggested to explain the equilibrium (Lefebvre et al. 2002). Immune response in *T. maccoyii* has been suggested as being able to control *C. forsteri* re-infection and this host response may partly be responsible for the maintenance of an infection equilibrium towards the end of the growout season (Aiken et al. 2008 (Chapter 6)).

A universal factor in explaining variation in *C. forsteri* intensity, abundance and prevalence was company. Although company was not a significant factor in the multivariate analysis of prevalence ( $P=0.056$ ) it was a significant factor in the univariate analysis and its effect cannot be overlooked. Company C had significantly greater counts of blood fluke intensity and abundance than company A and a greater prevalence than company B. Differences in site location or husbandry measures used in production may account for the variation between companies. Company C may be located on or near more infected intermediate hosts than the other two companies. Husbandry effects that may influence the occurrence of blood fluke include the frequency of sea cage net cleaning, if the intermediate host is located on the net, and also the frequency and duration that sea cage sites are fallowed if the intermediate host is located in the sediment beneath cages. Control of sanguinicolid infections in farmed fish systems depends primarily on separation of the intermediate host and

definitive host (Bullard and Overstreet 2002). However, this measure is based on knowledge of the identity of the intermediate host which is currently not known for *C. forsteri*. Identification of the intermediate host should be a priority for further research into mitigating the effects this parasite has on farmed *T. maccoyii*.

Another possibility for differences between companies may be in the different sizes of tuna held by each company. Company C had significantly larger tuna at harvest than Company A and Company B. The general trend in fish-parasite systems is for larger fish to harbour more parasites and for smaller fish to harbour fewer parasites (Poulin 2000). The larger sized tuna held by Company C may account for the greater level of infection observed. Differences in tuna sizes between companies may be a reflection of the different populations captured in the wild, differences in the amount of time taken to tow tuna from the wild to the farming zone, or possibly different growth rates as a result of the unique husbandry techniques employed by each of the companies.

Different years did not influence intensity or abundance although a decrease in prevalence in 2005 is evident. It was expected that infections may be increasing in time due to the naïve wild hosts being brought into the farming environment where infected intermediate hosts are most likely located. A lack of annual variation in numbers of adult flukes may be a result of similar infection patterns occurring each year. Aiken et al. (2006) (Chapter 3) and Aiken et al. (2008) (Chapter 6) observed a similar pattern of infection in different cohorts of research tuna in 2004 and 2005. Tuna are brought into the farming zone with no or few adult *C. forsteri* and after two months experience an increase in intensity and prevalence followed by a decrease over the next three months to low levels of both infection parameters. As a similar infection cycle occurred in both years this may have resulted in lack of variation

between years. Some density dependent factor in the intermediate host population may be occurring with this population not being able to support more infected hosts. Blood fluke are known to castrate and cause significant pathology in the intermediate hosts (Koie 1982). A threshold level of cercarial production may have been reached, beyond which the intermediate host population begins to decrease due to mortality from the increasing numbers of miracidia in the environment resulting from the influx of naïve hosts annually. Although intensity and abundance did not vary between years, a decrease in prevalence was observed in 2005. Differences between cohorts towed to the farming zone annually may account for this. The 2005 cohort may have been a healthier group better able to resist infection by blood fluke than the cohorts from 2004 and 2006.

Tuna harvested in winter have a significantly greater abundance and prevalence of blood fluke than those harvested in autumn. This may be a reflection of the seasonality of cercarial release from the intermediate host. Increased cercarial emergence associated with decreasing water temperature has been observed in other blood flukes, *Paradeontacylix* spp., infecting cultured amberjack, *Seriola dumerili*. Cercaria begin to invade amberjack in September, the month corresponding with the beginning of water temperature decrease (Ogawa et al. 1993). Decreasing water temperatures in the Spencer Gulf tuna farming zone may also be associated with increasing cercarial emergence from the intermediate host resulting in greater numbers of fluke being observed in winter.

Interestingly, the period of time that tuna are in captivity does not significantly influence intensity of infection even though it does affect blood fluke abundance and prevalence. Previous studies on blood fluke infection during the tuna growout season

have shown that after an initial increase, intensity and prevalence decline with time, possibly a result of a specific immune response against the blood fluke (Aiken et al. 2006 (Chapter 3); Aiken et al. 2008 (Chapter 6)). The lack of effect of time in captivity on intensity may be a result of a longer time that tuna need to be able to clear infections. In the previous studies on non-commercial (research) tuna in 2004 and 2005, where decreases in intensity were observed over approximately three months, tuna were being held in considerably lower stocking densities than the tuna of this study (Aiken et al. 2006 (Chapter 3); Aiken et al. 2008 (Chapter 6)). Lower stress levels as a result of the difference in stocking density may account for a better ability to clear infections. Alternatively, at high stocking densities and if infections are acquired from proximity to the bottom, it may take most tuna longer to acquire flukes as not all tuna can reach the bottom of the cage. This would explain why some infections are observed later in the season compared with previous surveys.

Although there was no significant decrease in blood fluke intensity during growout, no effect of intensity or abundance was observed on the condition of tuna. This supports previous results where no association has been observed (Aiken et al. 2006 (Chapter 3)). The infection levels observed in this study may be too low to cause any significant decrease in the condition of tuna. Hebert et al. (1995) has suggested that low intensity of infection in cultured sea bass, *Lates calcarifer*, infected with the blood fluke *Cruoricola lates* is responsible for the lack of pathological changes. The intensities observed in this study may be too low to cause pathology to result in production loss through decreased condition. The low intensities observed in this study are also unlikely to have been responsible for the mortalities that were submitted by industry as mortalities that were shown to be infected with *C. forsteri* were observed to have similar burdens as tuna sampled at harvest. Additionally, the

majority of mortalities did not have *C. forsteri* infections. However these results must be treated with caution as they do not take into account any post mortem migration or deterioration of flukes.

Whilst there was variation between companies in parasite predictors no significant increase in blood fluke intensity, abundance or prevalence was observed to be associated with the length of time in culture. In addition, the low levels of intensity and the lack of effect on the condition of tuna indicate that the numbers of adults of this parasite are not associated with a significant risk to the health of tuna. However, as a result of the high prevalence observed and the ability of the flukes eggs to cause gill pathology, continual monitoring of parasite levels is warranted.



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## **Chapter 6. Serological evidence of an antibody response in farmed southern bluefin tuna naturally infected with the blood fluke *Cardicola forsteri***

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## Abstract

In this study, adaptive immune response was investigated in farmed southern bluefin tuna, *Thunnus maccoyii*, infected with a sanguinicolid *Cardicola forsteri*. A cohort (Cohort<sub>2005</sub>) of southern bluefin tuna was sampled between March 2005 and August 2006. Samples were taken at the transfer of wild caught tuna to sea cages and then at regular intervals. Parasite intensity, abundance and prevalence data were recorded. An ELISA was developed to detect and quantify an antibody response against the blood fluke in southern bluefin tuna serum. Intensity and prevalence of the blood fluke were shown to peak in May 2005 at 10.9 fluke per infected fish (SE=1.72) and 97.5% prevalence and then decreased to low prevalence (10%) and intensity (1.0). There were no significant changes in prevalence or intensity in 2006. Antibody titres and seroprevalence increased from 1.37 U  $\mu\text{l}^{-1}$  and 10% at transfer in March 2005 to reach a peak in December 2005 of 25.86 U  $\mu\text{l}^{-1}$  (SE=6.26 U  $\mu\text{l}^{-1}$ ) and 66.66%. No significant changes were observed in antibody titres for the same cohort of fish during 2006. Parasitological and serological values from Cohort<sub>2005</sub> were compared to a 2006 cohort (Cohort<sub>2006</sub>) in March 2006 and August 2006 to determine if prior infection in Cohort<sub>2005</sub> elicited any protection against infection in 2006. Although significant differences were not observed in intensities between cohorts it was shown that Cohort<sub>2005</sub> had significantly lower abundances and prevalences of blood fluke infection than Cohort<sub>2006</sub>. Although there was no significant difference in mean antibody titres between cohorts in March 2006, the mean antibody titre of Cohort<sub>2006</sub>, was significantly greater than that of Cohort<sub>2005</sub> in August 2006. No significant differences were observed in seroprevalence. This is one of the few studies to demonstrate the development of acquired resistance in fish against a parasite in an aquaculture environment under natural infection conditions.

## 1. Introduction

Southern bluefin tuna, *Thunnus maccoyii*, is a commercially important aquaculture species. The industry is based on the capture of 2-3 year old wild fish which are purse-seined in the Great Australian Bight, moved into towing pontoons and towed to the farming zone in the Spencer Gulf of South Australia where they are transferred into sea cages. On-growing in sea cages occurs for a period between 2-8 months before southern bluefin tuna are exported as frozen or fresh product to Japan for the premium sashimi market. The southern bluefin tuna farming industry is economically significant, producing 9993 tonnes worth A\$160 million in 2006 (T.B.O.A., pers. com.).

Although wild southern bluefin tuna are infected with a range of parasites (see review by [1]) heavy infections are rarely observed in southern bluefin tuna aquaculture [2]. This could be a consequence of a well developed host immune system in southern bluefin tuna [3]. Of the parasites that are of concern, a sanguinicolid *Cardicola forsteri*, is a cause of significant gill pathology [4,5]. Sanguinicolids are parasites of marine and freshwater fish [6]. Most species establish in the heart, bulbus arteriosus, ventral aorta, or branchial vessels, although distributions within the cephalic or dorsal vessels are not uncommon [7]. Once established, the adult fluke lay eggs which lodge in the gills. Here the eggs hatch and break out of the gill as free living miracidia. These miracidia infect an intermediate host into which they penetrate to undergo asexual reproduction as rediae and/or sporocysts to produce infective cercariae. The intermediate host of *C. forsteri* is as yet unknown. Bivalves and polychaetes have been reported to be intermediate hosts for some species of marine sanguinicolids [6,8]. Cercariae emerge from the intermediate host and actively search for the definitive host, a fish. The cercariae penetrate the skin of the host and juvenile fluke attempt to reach the circulatory

system in which they undergo a migration to a final site where they mature [6]. For *C. forsteri* the final site is the heart [9]. In southern bluefin tuna this parasite can reach 100% prevalence with heavy burdens in the first two months of growout [10]. However, toward the end of the growout season (6-8 months) low intensities and prevalences are observed. A previous study has shown a specific response against the blood fluke in farmed southern bluefin tuna using Western blot analysis [11]. It is not known whether this antibody response in the tuna has a protective role or how it is affected by infection dynamics. Little is known about specifics of the immune responses directed at marine sanguinicolids [12].

In this study, an enzyme-linked immunosorbent assay (ELISA) was developed to detect and quantify antibodies against the blood fluke. ELISA is believed to be the best method to measure specific antibody titers in fish and is a widely used, sensitive and reliable monitoring tool for the detection and quantification of specific humoral antibody responses to a variety of fish pathogens [13, 14, 15, 16]. The development of such serological tests is important for risk assessment in disease management strategies [17]. Serology has a number of advantages over direct detection of parasite pathogens. Diagnostically, serological assays offer the potential to demonstrate exposure to given parasites long after the parasite may be detected [18].

The aim of this study was to investigate the antibody response of southern bluefin tuna against *C. forsteri* infection. We approach this issue by addressing the following questions: (1) What is the relationship over time between parasite burden and antibody titres? (2) Is there an antibody response that could lead to resistance against re-infection in an aquaculture environment?



## **2. Methods and Materials**

### **2.1. Experimental fish and study design**

A cohort of southern bluefin tuna (Cohort<sub>2005</sub>) was sampled (hearts and blood) at various stages over a sixteen month period during 2005 and 2006 from one company's lease site. Wild *T. maccoyii* were captured by purse-seine in the Great Australian Bight (map reference 33 27S, 132 04E) on 19<sup>th</sup> February 2005 and towed to the Spencer Gulf farming zone over a period of approximately six weeks in a towing pontoon. Tuna were transferred from the tow pontoon to four sea cages for farming on 5<sup>th</sup> April 2005. Two hundred and twenty tuna were transferred into each sea cage and ten tuna were sampled at this time representing 4.5% of the total population for that sea cage. During the growout period, ten tuna were sampled from each of the four cages on 30<sup>th</sup> May, 11<sup>th</sup> July, and 22<sup>nd</sup> August. Following a harvest in August, all remaining tuna of the four cages were moved to a single cage. Thirty tuna were then sampled from this cage on 6<sup>th</sup> December, ten tuna sampled on each on the 7<sup>th</sup> March, 14<sup>th</sup> March, 31<sup>st</sup> March and then 30 tuna sampled on the 15<sup>th</sup> August. A total of 220 research tuna were examined. In addition, tuna from 2006 intake of wild fish (Cohort<sub>2006</sub>) were sampled for comparison with Cohort<sub>2005</sub> to determine if prior infection in the remaining southern bluefin tuna from Cohort<sub>2005</sub> elicited any protection against re-infection in 2006. Twenty tuna from a different company were examined on the 24<sup>th</sup> March (37 days post transfer) and ten tuna on 28<sup>th</sup> March (41 days post transfer). Twenty tuna from the same company farming the Cohort<sub>2005</sub> were sampled on 18<sup>th</sup> August (154 days post transfer). A total of 50 Cohort<sub>2006</sub> tuna were examined. The sampling times and number of fish were determined in collaboration with the industry. The number of southern bluefin tuna sampled was limited due to the high cost of individual tuna. Samples taken and dates are summarized in Table 1.

**Table 1.** Number of farmed southern bluefin tuna *Thunnus maccoyii* sampled at different times during 2005 (Cohort<sub>2005</sub>), including from different cages during May, July and August 2005, and 2006 (Cohort<sub>2005</sub> and Cohort<sub>2006</sub>) for blood fluke *Cardicola forsteri* infection. Samples were not taken for serological analysis on 7<sup>th</sup> March 2006 and 14<sup>th</sup> March 2006.

Cohort	Sampling time	Cage	n	n (Serology)
2005	Transfer 5-Apr-05		10	10
	30-May-05	1	10	40
		2	10	40
		3	10	40
		4	10	10
	11-Jul-05	1	10	10
		2	10	10
		3	10	10
		4	10	10
	22-Aug-05	1	10	10
		2	10	10
		3	10	10
		4	10	10
	06-Dec-05		30	30
	07-Mar-06		10	0
	14-Mar-06		10	0
	31-Mar-06		10	10
	15-Aug-06		30	30
2006	24/28-Mar-06		30	30
	18-Aug-06		20	20

## 2.2. Sample collection

### 2.2.1. Parasitology

Blood flukes were collected according to the method of [10]. Due to time constraints tuna were not screened for the presence of blood fluke eggs. Hearts were obtained during harvest, stored on ice, and taken to the laboratory. Hearts were dissected 2-4 h after removal from the carcass and flushed with physiological saline to dislodge any adult flukes. Flushes were poured into Petri dishes and examined for the presence of flukes using a dissection microscope (Olympus SZX12) at 6× magnification. They were then counted and prevalence (i.e. the number of infected tuna divided by the number of tuna examined × 100), mean abundance (i.e. the total number of parasites divided by total number of tuna examined), and mean intensity (i.e. the total number

of parasites divided by the number of infected tuna), were determined, as described by [19]. All flukes were frozen at -20°C for approximately one week and then transferred to a -80°C freezer where they were stored.

#### *2.2.2. Fish serum samples*

Southern bluefin tuna were bled during harvest by cutting into the pectoral artery and collecting blood in 50 ml falcon tubes which were then stored on ice until transfer to the laboratory approximately 3-4 h later. Blood was allowed to clot overnight, centrifuged at 5000 g for 10 min, after which serum was aliquoted and stored at -80°C.

#### **2.3. *C. forsteri* antigen preparation**

Adult flukes collected from tuna during 2005 and 2006 and stored at -80°C were used as antigen in Western blotting analyses and enzyme-linked immunosorbent assay (ELISA) analysis. Flukes were transferred to a 15 ml tube and suspended in carbonate buffer (2.93 g NaHCO<sub>3</sub>, 1.59 g Na<sub>2</sub>CO<sub>3</sub>, in 1 l of distilled water, pH 9.6). The buffer was then subjected to ten pulses of a Branson sonicator for approximately 10 s a pulse. A tissue homogenizer was then used to further break down the flukes in ten pulses of approximately 15 s each. The buffer was then transferred to 1.5 ml tubes and centrifuged at 3000 g for 10 minutes at 4°C. Supernatant was aliquoted and stored at -80°C. Protein concentration of antigen in the solution was determined by BCA Protein Assay Kit.

## **2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and preliminary enzyme-linked immunosorbent assay (ELISA)**

*C. forsteri* antigen solution was partially characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine approximate weights of antigen proteins. *C. forsteri* antigen solution was diluted 2:1 in reducing buffer (0.35 M Tris, pH 6.8, 30% glycerol, 10% SDS, 9.3% DL-Dithiothreitol, 0.05% bromophenol blue), heated to 100°C for 4 minutes and placed on ice. SDS-PAGE was carried out according to [20]. Ten µl of reduced fluke antigens were loaded into wells of a 5% stacking gel over 10% resolving gels. Biotinylated molecular weight standards (Biorad) and Wide Range Protein Standards (Mark 12™ Novex™) were used for molecular weight calibration. Electrophoresis was carried in a Mighty Small SE 250 vertical electrophoresis unit (Hoefer) for 1 h in tris buffer (25 mM tris, 192 mM glycine, 0.1% SDS, pH 8.3) at a constant current of 40 mA and 200 V. Protein bands on gels were visualised after staining with silver nitrate following procedures modified from those of [21]. Briefly, gels were fixed with 50% ethanol and 10% glacial acetic acid for at least 30 min, followed by 15 min in 5% ethanol and 1% glacial acetic acid, rinsed three times in distilled water then washed in 0.2% sodium thiosulfate for 1 min. Gels were exposed to 0.2% silver nitrate with 0.15% formalin solution, rinsed in distilled water then developed in a solution of 6% sodium carbonate, 0.05% formalin and 0.2% sodium thiosulfate. Development was stopped with 1.5% sodium EDTA.

A preliminary ELISA using fluke antigen and a range of tuna serum samples was employed to identify seropositive and seronegative tuna sera for later use as controls for a quantitative ELISA. This method was used as it was not possible to inject tuna with fluke extracts to produce a positive serum standard. As *T. maccoyii* is a valuable

species it is difficult to acquire specimens for experimental studies. Furthermore, appropriate tank systems for holding tuna are not available for research purposes. Transfer tuna were used as possible negative standards as they were not infected with adult fluke as determined by parasitological examination. A flat bottom 96-well microplate (IWAKI) was coated overnight at 4°C with *C. forsteri* antigen solution diluted with carbonate buffer (2.93 g NaHCO<sub>3</sub>, 1.59 g Na<sub>2</sub>CO<sub>3</sub>, in 1 l of distilled water, pH 9.6) to 20 µg ml<sup>-1</sup> protein. Plates were blocked with 0.3% casein in tris buffered saline (TBS; 20 mM Tris, 500mM NaCl, pH7.2) for 1 h at 37°C. The plates were then washed three times with TBS. Multiple tuna serum samples were screened for any activity and were applied at 1:100 single dilutions. After 1 h incubation at 37°C, plates were washed three times in TBS then rabbit anti-tuna heavy chain immunoglobulin (RATH) (Watts et al. 2001), diluted 1:100 in 0.3% casein in TBS, was added and incubated for one h. Following another wash cycle sheep anti-rabbit IgG alkaline phosphatase conjugate (Sigma, Castle Hill, NSW, Australia), diluted to 1:8000 in 0.3% casein in TBS, was added. All reagents were added as 50 µl volume. A final wash in TBS was performed immediately prior to adding 200 µl of the detection reagent, *p*-nitrophenyl phosphate (PNPP) (Sigma, Castle Hill, NSW, Australia). Colour was allowed to develop for 30 min at room temperature and the optical density was measured at 405 nm using a Spectra Rainbow Thermo microplate reader (TECAN Trading AG, Switzerland). Controls for the initial ELISA consisted of duplicate wells where each reagent (antigen, tuna serum, RATH and conjugated sheep anti-rabbit IgG) was in turn omitted.

## **2.5. Western blotting**

Western blotting was used to investigate seropositive and seronegative samples, determined by the preliminary ELISA, in order to visualize the specific antibody

against fluke antigen and to choose a positive and negative control for the quantitative ELISA. Gels from SDS-PAGE analysis to be used in protein transfer were equilibrated in transfer buffer (48 mM tris, 39 mM glycine, 20% methanol, pH 9.2) for 5 minutes. After equilibration, antigen proteins were electro-transferred from gels onto 0.45 µm nitrocellulose membrane (Biorad) in transfer buffer using a Hoefer Semi-Phor® semi-dry transfer unit with a maximum setting of 50 V and 100 mA for 1.5 h. Following transfer a temporary total protein stain (0.1% Ponceau, 0.5% glacial acetic acid v/v; Sigma, Castle Hill, NSW, Australia) was applied to membranes to confirm transfer. Various lanes were then cut from membranes as strips to enable individual staining or probing with a range of serum samples. Temporary stains were removed by rinsing in TBS for 5 min. Molecular weight standards were stained with amido black (0.1% amido black 10B, 25% isopropanol, 10% glacial acetic acid) for 1 min then destained (25% isopropanol, 10% glacial acetic acid) for 30 min. Residual binding sites on membrane strips were blocked for 1 h in 0.3% casein in TBS then washed with TBS, TBS with 0.05% Tween-20 (TBST), then TBS again (5 min for each wash). Membrane strips were probed with seropositive or seronegative serum from tuna at a dilution of 1:100 in 0.3% casein in TBS for 1 h. After repeating the washing step, membrane strips were then incubated for 1 h in RATH diluted to 1:100 in 0.3% casein in TBS followed by another washing step. Bound antibodies were detected by incubation for 1 h with sheep anti-rabbit IgG alkaline phosphatase conjugated antiserum, used at a dilution of 1:8000 in 0.3% casein in TBS. The washing step was repeated (10 min each wash) before membranes were developed in BCIP/NBT (Biorad kit) then washed in distilled water to stop the reaction. All incubations were carried out at ambient temperature on a shaker. Negative controls for the Western blot consisted of probing with: seropositive serum without RATH, seronegative serum, seronegative serum without RATH, *Salmo salar* serum

substituted for *T. maccoyii* serum, *Salmo salar* serum without RATH, RATH only, and conjugated sheep anti-rabbit IgG only. A strong seropositive sample (May 2005 sampling) and a seronegative sample (sampled in 2005 at the time of transfer to sea cages) were chosen as positive and negative controls for the quantitative ELISA.

## **2.6. Enzyme-linked immunosorbent assay**

A standard indirect ELISA was used to detect and quantify specific serum antibodies in tuna against *C. forsteri*. Assay conditions were optimized empirically and reagent concentrations determined by chequerboard titrations [22]. The same protocol used for the preliminary ELISA was utilized except multiple dilutions were used for each sample. Tuna serum samples were diluted in 0.3% casein in TBS, and added in duplicate wells across rows in a twofold serial dilution beginning at 1:100 through to 1:3200. Positive and negative standard sera, previously chosen from the preliminary ELISA and Western blot analysis, were titrated in duplicate on each plate.

Antibody activities were determined according to [23] with a method which expresses titers as units or antibody activity per volume of serum. The positive standard serum was titrated against *C. forsteri* antigen starting at a dilution of 1:100 to calculate a serum volume equating to 0.5 unit of antibody activity. In this study one unit of antibody activity was defined as the volume of the positive standard which gave 50% of the maximum OD, thus the reciprocal of this volume gives units of activity per  $\mu\text{l}$  of serum. The volume of serum giving 50% of the OD is determined from the dilution factor. Thus specific antibody activities of samples were expressed as units of antibody activity per volume of serum and were determined relative to the immune standard included on all plates and calculated as follows:

Activity of sample = (activity of standard)  $\times$  (50%volume of standard/50%volume of sample)

where activity of the standard is the reciprocal of the volume of the immune standard which gives 50% maximum OD, as determined from a titration curve and is expressed as units of activity per  $\mu$ l of serum, 50% volume of the standard is the volume of the immune standard giving 50% maximum OD and 50% volume of the sample is the sample volume giving 50% maximum OD. The activity of the standard is a constant in the equation, determined from its full titration, whereas the other terms are derived from individual plates and thus account for variations between plates. All samples and standards, both immune and non-immune, were titrated in duplicate from dilutions of 1:100 to 1:3200. Samples that displayed a maximum OD that was less than 50% of the maximum OD of the immune standard were designated as having no anti-*C. forsteri* activity. Using the ELISA quantification method of [23] antibody activities for samples that have a maximum OD which is less than 50% of the maximum OD of the standard cannot be calculated - and are therefore designated negative. In practice the 50% level is arbitrary and depends on the difference in activity between the samples and the positive control. The 50% level was chosen in this study as OD differences between samples that were positive and the positive control were in general small and those designated negative were in general well below the 50% cut off mark. The percentage of tuna which were seropositive out of the total number sampled was expressed as seroprevalence.

## **2.7. Statistical analysis**

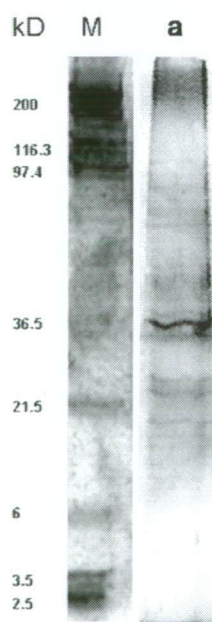
Data from cages were pooled at each sampling time point where  $p > 0.15$  due to the small sample sizes for each cage ( $n=10$ ). Fluke intensities, abundances and antibody



titres were compared using one-way analysis of variance (ANOVA) and the homogeneity of the variances was tested with Levene's test. Fisher's Least Significant Difference test was used for post-hoc comparisons. Prevalences of blood fluke infection and seroprevalences were compared using Pearson Chi-square tests. Pearson correlations between parasite abundance or intensity and antibody titres (with a time lag for antibody titre) were calculated. Calculations were carried out with SPSS for Windows 13.0; in all analysis, the significance level adopted was 95% ( $\alpha=0.05$ ).

### 3. Results

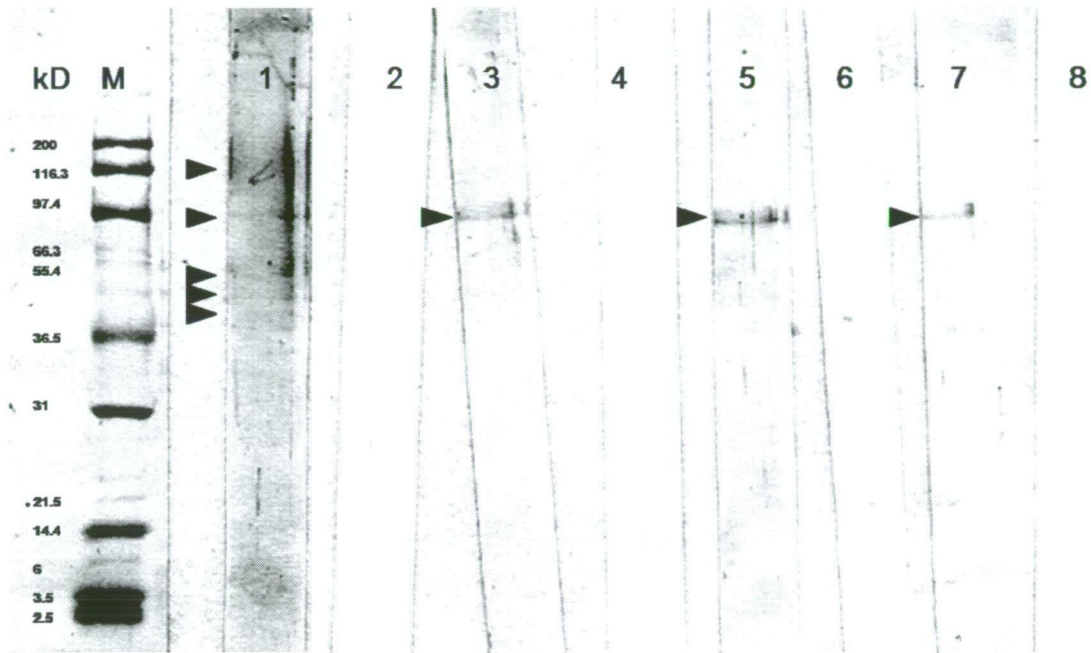
The preliminary ELISA identified serum samples that were positive and negative for anti-*C. forsteri* antibody activity. Electrophoretic analysis of sonicated and reduced blood fluke showed protein bands ranging between 21kD and 200kD (Figure 1.).



**Figure 1.** *Cardicola forsteri* protein bands (a) following electrophoresis in 10% reducing gels. M: markers. Molecular weight is given in kilodaltons (kD).

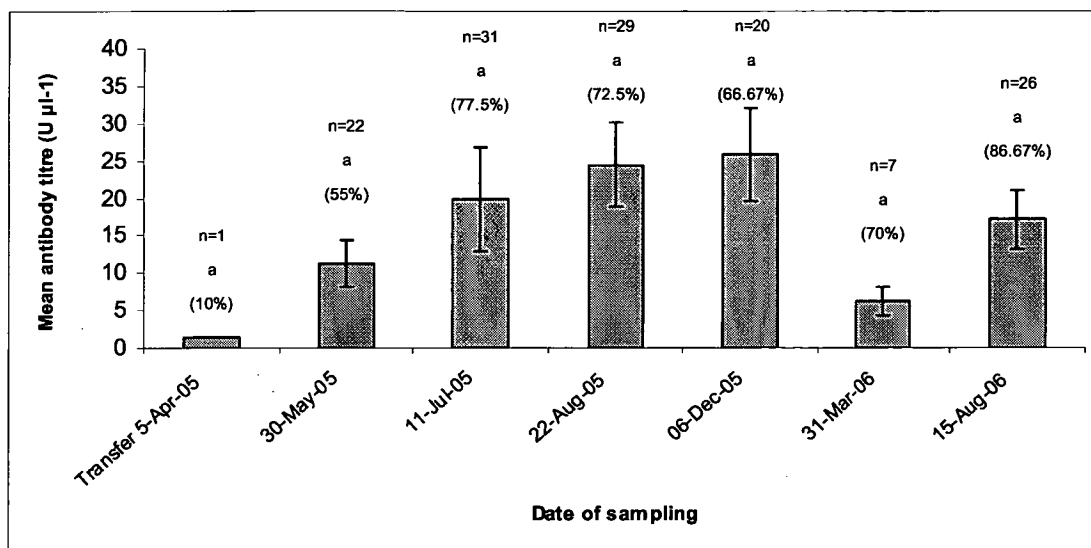
Western blot analysis of positive samples, as determined by the preliminary ELISA, showed the specific antibody of southern bluefin tuna serum was reactive to 5 fluke

bands (Figure 2.). Rabbit anti-tuna Ig antiserum was cross-reactive with *C. forsteri* proteins but only with one band of approximately 100 kD. The four remaining bands were not observed in any of the negative controls.



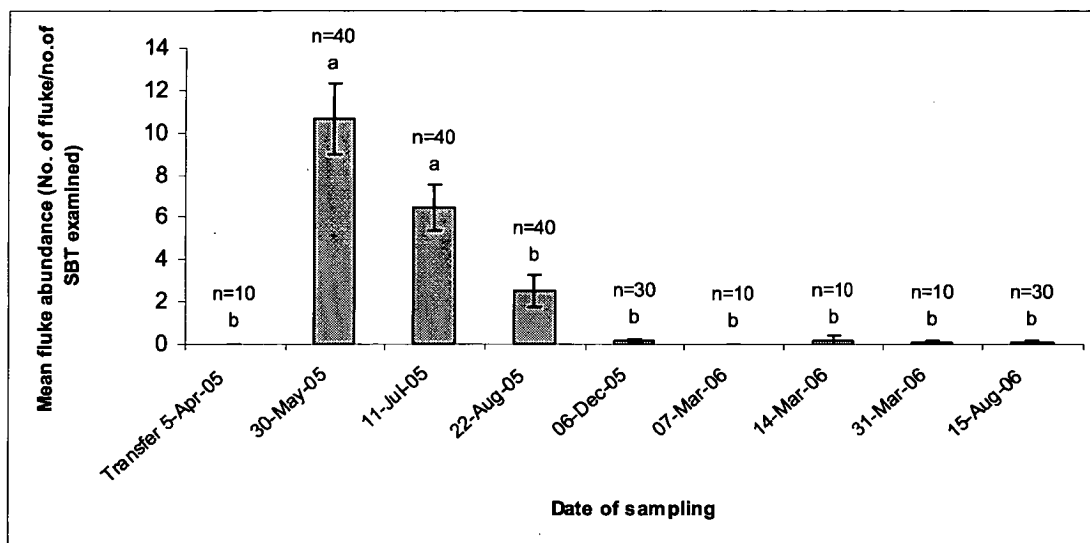
**Figure 2.** Analysis of *Cardicola forsteri* protein probed with *Thunnus maccoyii* sera, identified as seropositive and seronegative by enzyme-linked immunosorbent assay, *Salmo salar* sera and no sera; positive control *Thunnus maccoyii* serum (1), positive control *T. maccoyii* serum without rabbit anti-tuna heavy chain immunoglobulin (RATH) (2), negative control *T. maccoyii* serum (3), negative control *T. maccoyii* serum without RATH (4), *Salmo salar* serum (5), *Salmo salar* without RATH (6), no serum (7), and no serum and no RATH (8) against blood fluke *C. forsteri* antigen. All serum was diluted at 1:100 in 0.3% casein in TBS, Arrows show bands stained with antiserum in membrane (1) and a cross reactive band is shown in membranes (3, 5, 7). M: markers. Molecular weight is given in kilodaltons (kD).

One tuna sampled at transfer demonstrated a positive result in the ELISA analysis with an antibody titre of  $1.37 \text{ U } \mu\text{l}^{-1}$ , resulting in a seroprevalence of 10%. Transfer fish were not infected with adult blood fluke in the heart. Mean antibody titres increased after transfer and a peak of  $25.86 \text{ (S.E.= 6.26) } \text{U } \mu\text{l}^{-1}$  was reached in December 2005 (Figure 3a.).



**Figure 3a.** Mean antibody titres ( $\text{U } \mu\text{l}^{-1}$ ) against blood fluke *Cardicola forsteri*, including standard errors, for each sampling of Cohort<sub>2005</sub> southern bluefin tuna, *Thunnus maccoyii*, April 2005 – August 2006. No significant differences were observed between means. Seroprevalences (no. of seropositive tuna/no. of tuna sampled serologically) of blood fluke *Cardicola forsteri* infection are included in brackets for each sampling harvest.

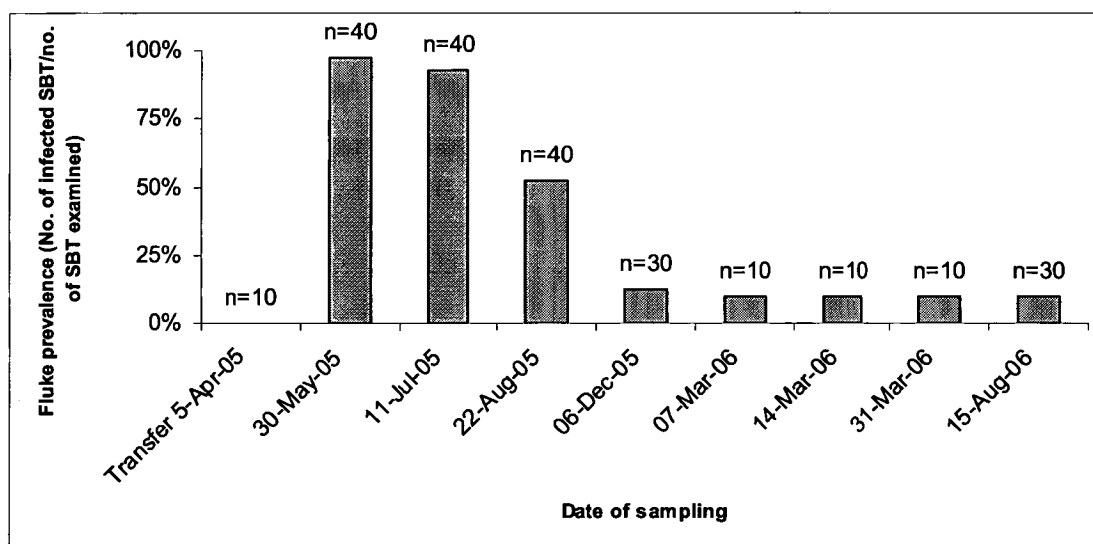
Fluke intensity and abundance (Figures 3b) also increased after transfer to reach a peak of 10.92 (S.E.= 1.72) fluke per infected fish in May and 10.65 (S.E.= 1.7) mean fluke per fish examined in May (Figure 3b). The maximum number of flukes observed during the study was 42 from a fish harvested in May 2005.



**Figure 3b.** Mean blood fluke abundance (no. of flukes/no. of tuna examined), including standard errors, for each sampling harvest of Cohort<sub>2005</sub> southern bluefin tuna (SBT), *Thunnus maccoyii*, April 2005 – August 2006. Means with different letters are significantly different from one another.

No significant differences were shown between mean antibody levels of different cages for May, July and August 2005 and therefore data were pooled for these months (ANOVA<sub>May</sub>,  $F=0.56$ ,  $d.f.=3$ ,  $P=0.66$ , ANOVA<sub>July</sub>,  $F=1.9$ ,  $d.f.=3$ ,  $P=0.15$ , ANOVA<sub>Aug</sub>  $F=1.37$ ,  $d.f.=3$ ,  $P=0.27$ ). Blood fluke intensity and abundance also did not differ between cages for any month (Intensity: ANOVA<sub>May</sub>  $F=0.70$   $d.f.=3$ ,  $P=0.56$ , ANOVA<sub>July</sub>  $F=0.45$ ,  $d.f.=3$   $P=0.71$ , ANOVA<sub>Aug</sub>  $F=0.31$ ,  $d.f.=3$ ,  $P=0.82$ , Abundance: ANOVA<sub>May</sub>  $F=0.461$ ,  $d.f.=3$ ,  $P=0.71$ , ANOVA<sub>July</sub>  $F=3.7$ ,  $d.f.=3$   $P=0.78$ , ANOVA<sub>Aug</sub>  $F=1.36$ ,  $d.f.=3$ ,  $P=0.27$ ) and data from cages were pooled. Mean antibody titres decreased after the peak in December 2005 to  $6.30$  (S.E.=  $1.88$ )  $U \mu l^{-1}$  in March 2006 and then increased again to  $17.17$  (S.E.= $3.95$ )  $U \mu l^{-1}$  in August 2006 (Figure 3a.). However, no significant differences were observed between mean antibody titres from different months, most likely due to high individual variability. Fluke intensity and abundance decreased after the peak in May 2005 to one fluke per infected fish in December 2005 and  $0.17$  (S.E.= $0.07$ ) mean fluke per fish examined in December 2005 (Figure 3b) after which intensity and abundance did not increase

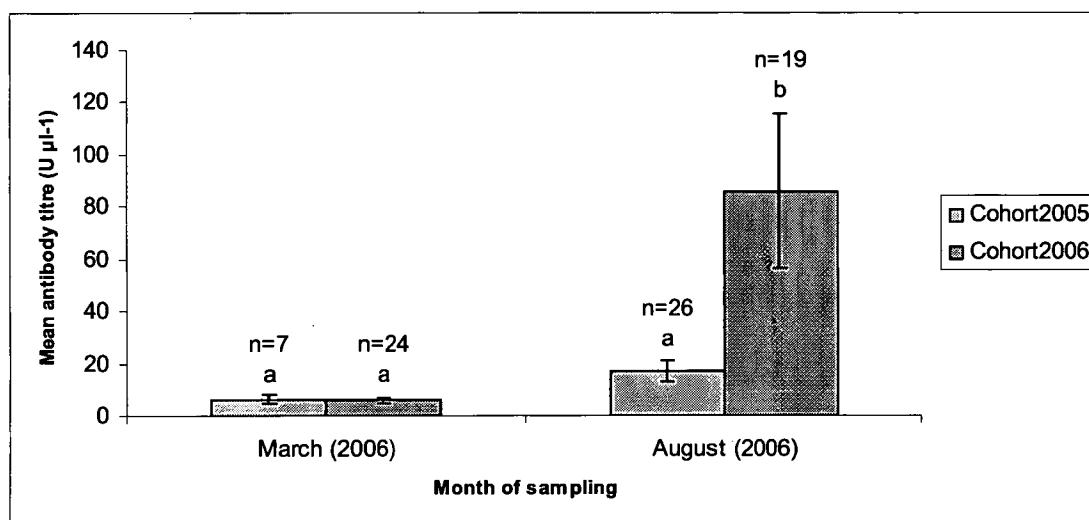
significantly. Intensity in May 2005 was greater than all other sampling points (ANOVA, Fisher LSD test,  $P<0.05$ ) and abundance in May and July 2005 was significantly greater than all other sampling points (ANOVA, Fisher LSD test,  $P<0.05$ ) (Figure 3b.). Seroprevalence increased after transfer to 77.5% in July 2005, decreased to 66.67% in December and then increased to 86.67% by August 2006 (Figure 3a.). Pearson Chi-square analysis showed a significant relationship between time in culture and seroprevalence ( $\chi^2=21.19$ ,  $d.f.=1$   $P=.002$ ). Fluke prevalence peaked in May 2005 at 97.5% and then declined to reach a plateau from December 2005 (12.5%) to August 2006 (10%) (Figure 3c.).



**Figure 3c.** Prevalence (no. of infected tuna/total tuna sampled) of blood fluke *Cardicola forsteri* infection for each sampling harvest of Cohort<sub>2005</sub> southern bluefin tuna (SBT), *Thunnus maccoyii*, April 2005 – August 2006.

Serological and parasitological results were compared between cohorts to determine if prior infection in Cohort<sub>2005</sub> elicited any protection against re-infection in 2006. Although there were no significant differences in mean antibody titres between cohorts in March 2006 (ANOVA,  $F=0.83$ ,  $d.f.=1$ ,  $P=0.833$ ), the mean antibody titre of Cohort<sub>2006</sub>, 85.68 (S.E.=29.55) U  $\mu\text{l}^{-1}$ , was significantly greater than that of

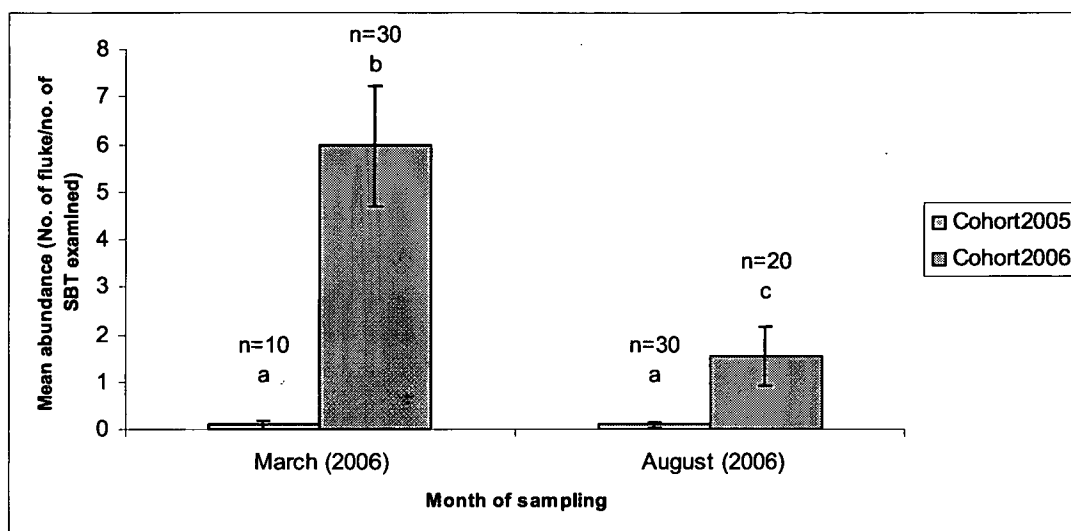
Cohort<sub>2005</sub> in August 2006 (ANOVA,  $F=7.17$ ,  $d.f.=1$ ,  $P=0.01$ ) (Figure 4a.). Further analysis of the antibody titres of Cohort<sub>2006</sub> during the August 2006 revealed that titres were significantly greater than Cohort<sub>2005</sub> at any other sampling point during 2005 and 2006 (ANOVA, Fisher LSD Test,  $P<0.001$ ). Levene's test of equality of error variances was shown to be significant ( $F=33.54$ ,  $P<0.001$ ), therefore the results of the analysis should be treated with caution (Underwood, 1981).



**Figure 4a.** Mean antibody titres ( $\text{U } \mu\text{l}^{-1}$ ) against blood fluke *Cardicola forsteri*, including standard errors, for Cohort<sub>2005</sub> southern bluefin tuna (SBT), *Thunnus maccoyii*, and Cohort<sub>2006</sub> tuna sampled at the same time, March 2006 and August 2006. Means with different letters are significantly different from one another.

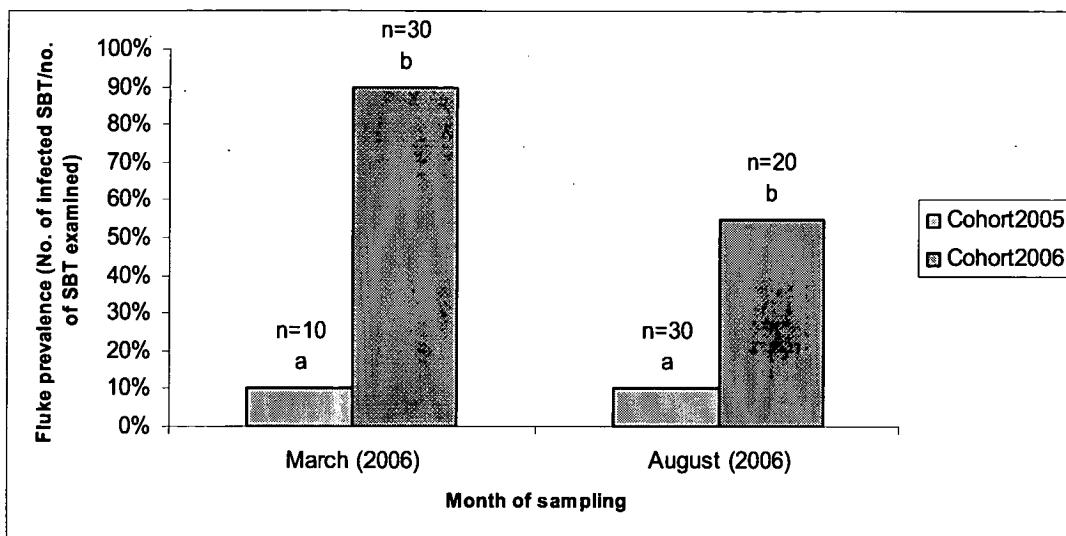
Fluke abundance was significantly greater in Cohort<sub>2006</sub> than Cohort<sub>2005</sub> in March 2006 (ANOVA,  $F=7.06$ ,  $d.f.=1$ ,  $P=0.011$ ), and in August 2006 (ANOVA,  $F=8.09$ ,  $d.f.=1$ ,  $P=0.007$ ) (Figure 4b.). However, significant differences were not observed between fluke intensities of the two cohorts in March 2006 and in August 2006. Mean antibody titres of Cohort<sub>2006</sub> were significantly greater in August 2006 than in March 2006 (ANOVA,  $F=9.26$ ,  $d.f.=1$ ,  $P=0.004$ ). Levene's test of equality of error variances was shown to be significant ( $F=33.54$ ,  $P<0.001$ ), therefore the results of the analysis should be treated with caution [24]. Fluke abundance of Cohort<sub>2006</sub> was

significantly greater in March 2006 than in August 2006 (ANOVA,  $F=7.32$ ,  $d.f.=1$ ,  $P=0.009$ ) (Figure 4b.).



**Figure 4b.** *Cardicola forsteri* abundance (no. of flukes/no. of tuna examined), including standard errors, for Cohort<sub>2005</sub> southern bluefin tuna (SBT), *Thunnus maccoyii*, and Cohort<sub>2006</sub> tuna sampled at the same time, March 2006 and August 2006. Means with different letters are significantly different from one another.

No significant differences were shown in seroprevalences between either cohort or within each cohort during the sample periods March and August 2006. Pearson Chi-square analysis showed that fluke prevalence was significantly greater in Cohort<sub>2006</sub> than Cohort<sub>2005</sub> in March 2006 ( $\chi^2_{March}=22.86$ ,  $d.f.=1$ ,  $P<0.001$ ) and in August (2006) ( $\chi^2_{August}=12.05$ ,  $d.f.=1$ ,  $P=0.001$ ) (Figure 4c.)



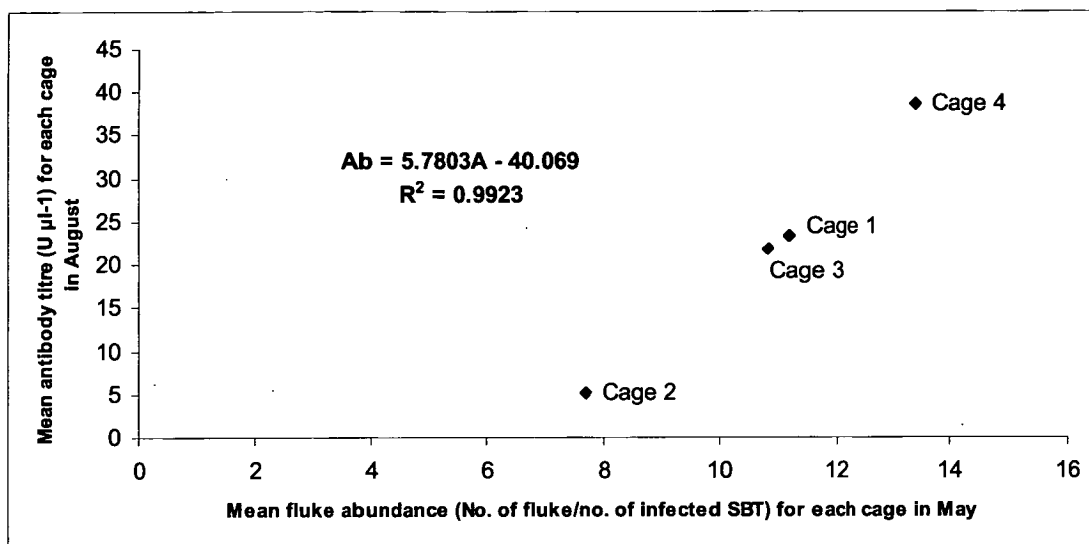
**Figure 4c.** Prevalence (no. of infected tuna/no. of tuna examined) of blood fluke *Cardicola forsteri* for Cohort<sub>2005</sub> southern bluefin tuna (SBT), *Thunnus maccoyii*, and Cohort<sub>2006</sub> tuna sampled at the same time, March 2006 and August 2006. Frequencies with different letters are significantly different from one another.

A significant correlation was found between fluke abundance in May 2005 and mean antibody levels in the respective cages in August 2005 (Figure 5.) (ANOVA,  $F=257.86$ ,  $d.f.=1$ ,  $P=0.004$ ). The correlation was described by the following equation:

$$Ab = 5.7803A - 40.069 \quad (r^2 = 0.992)$$

Where: Ab is the average antibody response for cages in May 2005, expressed as antibody units; A is the abundance for respective cages in August 2005. No significant correlation was observed between parasite intensity and antibody titre.





**Figure 5.** Correlation between blood fluke abundance (no. of flukes divided/no. of sampled tuna) for cages in May 2005 and mean antibody titres (U  $\mu$ l<sup>-1</sup>) against blood fluke infection for respective cages in August 2005. Ab is the average antibody response for cages in May, expressed as U  $\mu$ l<sup>-1</sup>; A is the abundance for respective cages in August.

#### 4. Discussion

Southern bluefin tuna are able to mount a specific humoral response against a macroparasite, *Cardicola forsteri*, in an aquaculture system under natural infection conditions, as detected in both the Western blot and ELISA analyses. Although immune responses of fish to sanguinicolids have been documented previously, this study is the first to demonstrate a specific humoral response against blood fluke under natural infection conditions. Previous studies have been undertaken in environments with controlled conditions [25] or have utilized in vitro experiments [26, 27].

Our study has shown that the antibody response of tuna to *C. forsteri* is initiated, for the most part, after the transfer of wild caught tuna into sea cages and suggests that infection occurs after this period as demonstrated by the lack of blood flukes observed at transfer in 2005. *T. maccoyii* sampled at transfer in 2004 showed low

infection prevalence of 10% and an intensity of one fluke per infected fish [10].

Parasitological surveys of wild southern bluefin tuna have shown low intensities and prevalences of infection (Aiken et al. Submitted (Chapter 4))<sup>1</sup> and histological [4] and serological [11] examinations of wild southern bluefin tuna have previously failed to find evidence of *C. forsteri* infection. In addition, a computer simulation based on the observed lengths of blood fluke has been used to determine the most likely time of infections of blood fluke in farmed southern bluefin tuna (Aiken et al. Submitted (Chapter 4))<sup>1</sup>. The simulations showed that the majority of infections occur after transfer into sea cages. Results from the same study showed that most fluke reach maturity at a size above 1790  $\mu\text{m}$  which takes approximately 46 days. As this time is significantly less than the time between transfer and the first sampling (55 days) it seems reasonable to conclude that the majority of infections observed occurred in the farming zone.

Mean antibody levels increased gradually in 2005 and remained elevated in 2006 despite the significant decrease in fluke numbers after the peak in intensity, abundance and prevalence of fluke in May 2005. The decrease in fluke prevalence and burden may be a result of some external affect on the prevalence of the intermediate host thus reducing parasites within the water column. However it is more likely that there is no decrease of parasites in the water column due to the increase of antibody that was observed during the study period. The gradual increase of antibody levels over the year and the fact that antibody levels remained elevated despite decreasing parasite loads suggests that some kind of antigenic stimulation is keeping antibody titres at an elevated level. In most host-pathogen systems, specific humoral factors decline unless the host is re-exposed to antigens [28]. A reaction to

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<sup>1</sup> Aiken. H. M., Hayward, C. J., Cameron, A., Nowak, B. F. 2009. Simulating blood fluke, *Cardicola forsteri*, infection in farmed southern bluefin tuna, *Thunnus maccoyii*, using stochastic models. Aquaculture, 293(3-4):204-210.

entrapped parasite eggs may be responsible for ongoing antibody production.

However it is more likely a continuing presence of immature or adult fluke that is responsible for the ongoing antibody response observed [25]. [25] has shown that for carp inoculated with live *S. inermis* cercariae antibody levels will decline to control levels at 56 days post inoculation. The constant elevated antibody titres demonstrated in this study may be a result of ongoing cercarial penetration. The antibody response may be directed at these larval stages resulting in almost nil recruitment of adult stages in the heart and causing the decrease in parasite numbers observed. The immune system of southern bluefin tuna may be initially stimulated by secretory/excretory products that are released into the blood by adult worms.

Secretory/excretory products appear to stimulate an effective host response against challenge infection by blood fluke in rhesus monkeys and hamsters [29]. In addition to exposure to antigens released by adult fluke, immunity to the incoming larval stages may accumulate with exposure to larval challenge itself. Exposure to larval blood fluke seems particularly important in the stimulation of a host response in rats [30] and appears to be central to the concomitant immunity found in lymphatic filariases [31]. Larval exposure may be responsible for the elevated antibody levels in southern bluefin tuna observed later in the growout season when adult fluke are not found in the host.

To determine whether southern bluefin tuna are able to develop resistance against reinfection, fluke intensity, abundance, prevalence and antibody titres of the Cohort<sub>2005</sub> and Cohort<sub>2006</sub> were compared to each other during the March and August 2006 sampling points. The mean antibody titre of Cohort<sub>2006</sub> tuna in August (2006) was shown to be significantly greater than that of Cohort<sub>2005</sub> tuna at any sampling point during 2005 and 2006. Annual variability between the two cohorts entering the

farming zone in 2005 and 2006 may account for this difference. This variability may be a result of biological factors that influence the rate and intensity of a specific immune response against the blood fluke. In particular, the origin of the tuna and their history prior to capture may be responsible for the variation observed. Tow conditions and husbandry may also be factors in the observed variability as each cohort was subjected to different stocking densities and harvest frequencies. Although blood fluke intensities did not differ significantly between the two cohorts, Cohort<sub>2005</sub> tuna had significantly lower blood fluke infection abundance and prevalence than the Cohort<sub>2006</sub> tuna in March 2006 and also in August 2006. Cohort<sub>2005</sub> tuna may have acquired some specific immunity having already been infected previously during 2005. It was assumed that cercariae were in the water of the Cohort<sub>2005</sub> sea cages and that no site specific effects were occurring, which is reasonable considering that infection prevalence for this cohort reached 100% in 2004 [10] and almost 100% in 2005. Cohort<sub>2006</sub> sampled at the same site as Cohort<sub>2005</sub>, and farmed by the same company, also had a high prevalence of 55% and seroprevalence of 95% after 154 days of farming indicating that tuna were being infected by cercariae at the same site that Cohort<sub>2005</sub> were being held. Thus, it is likely that Cohort<sub>2005</sub> tuna were being penetrated by cercariae and possible that the already active antibody response was targeting juvenile stages so that no recruitment of adults in the heart was observed. Due to the difficulty of obtaining juvenile fluke from the large and valuable carcasses, cross reactivity of tuna antibody with these life stages could not be checked. However, similar patterns of infection and protection have been observed in other sanguinicolids. Recent investigations on *S. inermis* in *C. cyprinus* have revealed that there is a significant decrease in flukes recovered in fish that have previously been infected with blood fluke and then exposed to more cercariae compared with fish that have been exposed for the first

time [32]. [7] showed that many *S. inermis* are unable to complete migration to the blood system, and that most likely the flukes were killed by host defence mechanisms during migration. Individuals of cage-farmed less than 1-year old amberjack had a relatively high number of eggs in cardiac muscle and gill, suggesting that older fish acquired some level of immunity to infections after receiving an initial exposure to flukes a year earlier [33]. Resistance has also been demonstrated in fish infected with other digenean groups. Naïve rainbow trout have been demonstrated to become infected with significantly more *Diplostomum spathaceum* than rainbow trout that have already been exposed, indicating that the latter had developed resistance against the parasite as a result of previous infections [34].

The results of the Western blot analysis showed that tuna antibody was directed against additional bands to those demonstrated by [11] where pooled serum from fluke infected southern bluefin tuna bound only those molecular weight bands at around 145 and 136 kD. In our study five distinct bands were stained, only one of which corresponded to the bands (136 kD) demonstrated by [11]. A possible reason for more bands being observed is in the different methodologies used to produce antigen. In this study fluke were sonicated and homogenised in carbonate buffer in contrast to the previous study of [11] in which fluke were ground in an Eppendorf microtube with a glass rod in sample buffer. Different reducing buffers were also utilized; in this study DL-Dithiothreitol was added to the buffer whereas [11] used 2-mercaptoethanol. In addition, different concentrations of fluke protein in the antigen solution may have also produced the different results.

One band was found to be reactive with rabbit anti-tuna immunoglobulin and may be a result of using polyclonal antibodies which due to their multiple specificity, often show cross reactivity [11]. One of the reasons fish serology has not been widely used in infectious disease diagnosis is because fish immunoglobulins are predominantly of the IgM isotype, which is generally of relatively low specificity [35]. This may have been the reason for the sero-positive tuna that was observed in the transfer group where sero-positive tuna were not expected. Helminths share epitopes and as a relatively crude antigen preparation was used the ELISA may have been cross-reacting with another helminth parasite of the tuna [36]. Although the negative control sera showed a weak reaction with the cross reactive antigen, the intensity of the reaction differed significantly between sera from positive and negative controls. Further purification and characterization of the blood fluke antigens might reveal more specific results [15]. Further work on the sensitivity and specificity of the ELISA is being carried and will be reported in another study.

Despite decreasing water temperatures (approximately from 20°C to 12°C) antibody levels still continued to increase and parasite loads still decreased for Cohort<sub>2005</sub> during 2005, and for Cohort<sub>2006</sub> in 2006. As tuna are able to conserve metabolic heat and maintain their body temperature at around 25°C, regardless of ambient temperature [37], the low temperature immunosuppression demonstrated for other fish species [26] may not be present in the endothermic tuna [3]. Captive southern bluefin tuna have previously demonstrated significantly higher levels of serum immunoglobulin, lysozyme and complement in winter than in summer [3]. In contrast, temperature has been shown to be immunosuppressive in carp infected with *S. inermis*. Antibody levels against the blood fluke are greater and maintained at higher levels for a longer period in carp kept at 25°C compared to fish maintained at

20°C [25]. Juvenile southern bluefin tuna production, currently under commercial trial, may also be more susceptible to blood fluke infection. As juveniles do not migrate from the tropical spawning grounds until their countercurrent heat exchangers are fully developed, very young fish, with immature heat exchangers, cultured in the cooler waters of South Australia may show the same low temperature immunosuppression observed in other aquaculture species [3].

This study shows that southern bluefin tuna under natural aquaculture conditions develops an antibody response against *C. forsteri*. Although these burdens decrease over time and there is no apparent mortality associated with the blood fluke, infection at the levels observed could result in sublethal production effects. Additionally, the elevated level of immune response observed may not be sustainable under long term culture. In mammalian systems nutrients are directed away from normal metabolism to support host defence mechanisms and this is also likely to be true for fish [3].

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## **Chapter 7. A comparison of gross pathology, histopathology and parasitology for the diagnosis of blood fluke, *Cardicola forsteri*, infection in farmed southern bluefin tuna**

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In Preparation for Journal of Fish Diseases

## **Abstract**

The agreement between a gross pathological test and a histopathological test for detecting infection of farmed southern bluefin tuna with *Cardicola forsteri* was evaluated by testing 140 southern bluefin tuna from 5 South Australian tuna farming companies. The results were compared with those obtained by dissection of the tuna heart to count adult fluke. The sensitivity of gross gill pathology was 0.85 (CI:0.76-0.91) and the specificity was 0.34 (CI:0.20-0.51). The sensitivity of gill histopathology was 0.52 (CI:0.41-0.62), significantly lower ( $P<0.001$ ) than the sensitivity of gross pathology, and specificity was 0.59 (CI:0.42-0.74). The kappa value for agreement beyond chance between gross pathology and histopathology was calculated as 0.49, indicating a moderate amount of agreement. Although the other estimates of accuracy are low, the high sensitivity of gross gill pathology suggests that it may be a useful tool for future epidemiology studies.



## 1. Introduction

Blood fluke, *Cardicola forsteri*, infection is a common parasitosis of farmed southern bluefin tuna (Nowak et al. 2004; Aiken et al. 2006 (Chapter 3)). Infection results in gill pathology that may be a limiting factor in the production of southern bluefin tuna (Colquitt et al. 2001). The life cycle of the blood fluke is indirect and involves most likely a polychaete or bivalve intermediate host, presently unknown (Aiken et al. Submitted (Chapter 4)), in which asexual reproduction occurs (Smith 1997). Cercariae break free from this host to penetrate the tuna definitive host where they mature in the heart (Smith 1997; Cribb et al. 2000). Eggs from the adult are transported with blood and may cause the pathology seen in the gills (Colquitt et al. 2001). Southern bluefin tuna aquaculture represents an important and valuable finfish culture industry in Australia and reliable diagnostic techniques are needed for the detection of parasites. To date no evaluation of any diagnostic technique has been undertaken for blood fluke nor any other parasite or pathogen infecting southern bluefin tuna.

Infection of the southern bluefin tuna host by adult blood fluke is usually diagnosed by heart dissection and flushes followed by an observation and counts of the adult fluke in the heart (Colquitt et al. 2001; Aiken et al. 2006 (Chapter 3)). This method is time consuming and labour intensive and may be not suitable for large scale screening programmes. This method is also unable to detect other life stages such as invading immature fluke in the musculature and eggs lodged in the gills. Alternative methods of diagnosing infection, including histopathology (Colquitt et al. 2001), serology (Aiken et al. 2008 (Chapter 6)) and gross pathology have been explored; however, there has been no attempt to evaluate the accuracy of these tests for screening farmed southern bluefin tuna. This paper compares gross pathology of the gills and gill histopathology as two diagnostic methods in a population of farmed

southern bluefin tuna randomly chosen from farms located in Boston Bay, South Australia. Serology as a diagnostic method is considered in another study (Aiken et al. In Prep (Chapter 8).

## **2. Methods and Materials**

### **2.1. Population**

A total of 140 southern bluefin tuna were selected from six companies between March 2005 and August 2006. Tuna were randomly selected for sampling during commercial harvest operations.

### **2.2. Gross Pathology**

A gross pathological assessment for each southern bluefin tuna sampled was made immediately when gills were removed from the carcass the harvest vessel. Each gill arch was visually checked for gill lesions. southern bluefin tuna exhibiting multifocal, white to yellow, demarcated lesions involving the gill filaments, which are indicative of blood fluke infection (Colquitt et al. 2001) and that were not the result of pathology caused by grossly visible gill ectoparasite attachment were categorized as positive. Southern bluefin tuna without these lesions were classified as negative.

### **2.3. Histopathology**

Gill samples for histology were collected aboard the harvest vessel after the gross gill pathology had been assessed. A 2 cm long piece of gill arch with filaments was removed from the gill of each fish. In cases where macroscopic lesions were present a sample for histology was taken from the area of the lesion. In cases where the tuna were considered negative, 2 cm of gill was removed from an area without any macroscopic pathology. Tissue samples were immediately placed in 10% neutral

buffered formalin. Standard histological techniques were used to produce 5 µm thick sections, which were then stained with haemotoxylin and eosin (H & E). After staining, sections were examined under a light microscope (Olympus BH2) and images were photographed (Leica DC300f, Wetzlar, Germany). Southern bluefin tuna with sections demonstrating blood fluke eggs in the gills were classified as positive and those southern bluefin tuna with sections showing no eggs were classified as negative.

#### **2.4. Parasitology**

Hearts were obtained during harvest at the same time that gills were removed, stored individually in containers on ice, and taken to the laboratory. Hearts were dissected open 2-4 hours after removal from the carcass and flushed with physiological saline to dislodge any adult flukes. Flushes were then poured into petri dishes and were then examined for the presence of adults using a dissection microscope. Southern bluefin tuna were classified as positive if adult blood flukes were observed in the heart and negative if no flukes were observed in the heart.

#### **2.5. Statistical analysis**

Presence of adult blood fluke in the heart was considered the gold standard for this study. The accuracy of gross pathology and histopathology was determined by comparing the diagnostic test results with those of parasitology. Values for sensitivity, specificity, positive predictive value (the proportion of cases with positive test results which have the parasite), negative predictive value (the proportion of cases with negative test results which do not have the parasite) and their 95% confidence intervals were determined. Differences were analysed using the

$\chi^2$  test. The kappa statistic was used to assess agreement between gross gill pathology and gill histopathology (Thrusfield 2005).

### 3. Results

Gross pathological gill lesions were demonstrated in 111 (79.29%) of the 140 southern bluefin tuna (Figure 1.) Lesion severity ranged from small localised patches to large lesions covering the majority of filaments on all gill arches including some petechial haemorrhaging. Histopathological analysis revealed blood fluke eggs in the gills of 68 (48.57%) of the 140 southern bluefin tuna (Figure 2.) whilst adult blood fluke were dissected from 99 (70.71%) of the 140 southern bluefin tuna (Figure 3.).

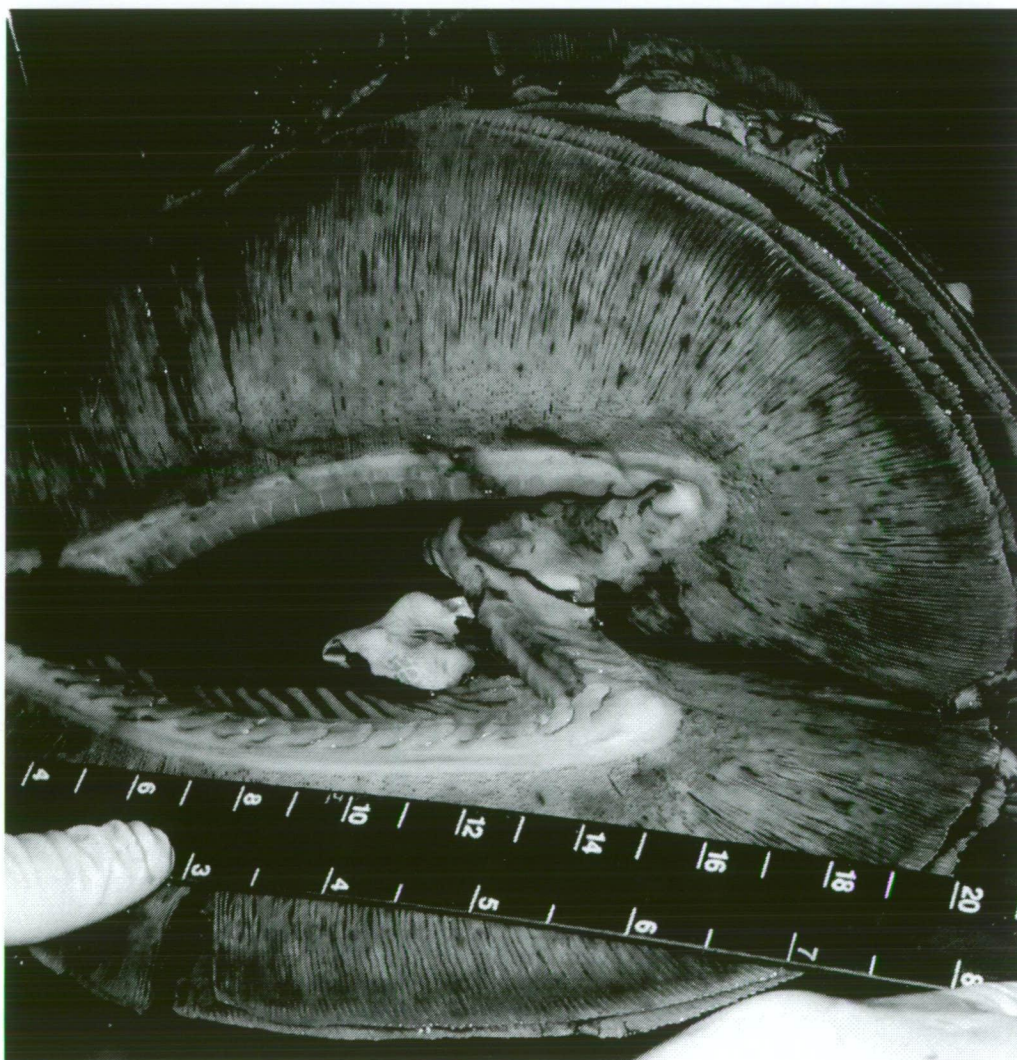


Figure 1. Gills of a farmed southern bluefin tuna, sampled from the waters of Boston Bay, South Australia, showing lesions (white patches) caused by eggs of the blood fluke, *Cardicola forsteri*.



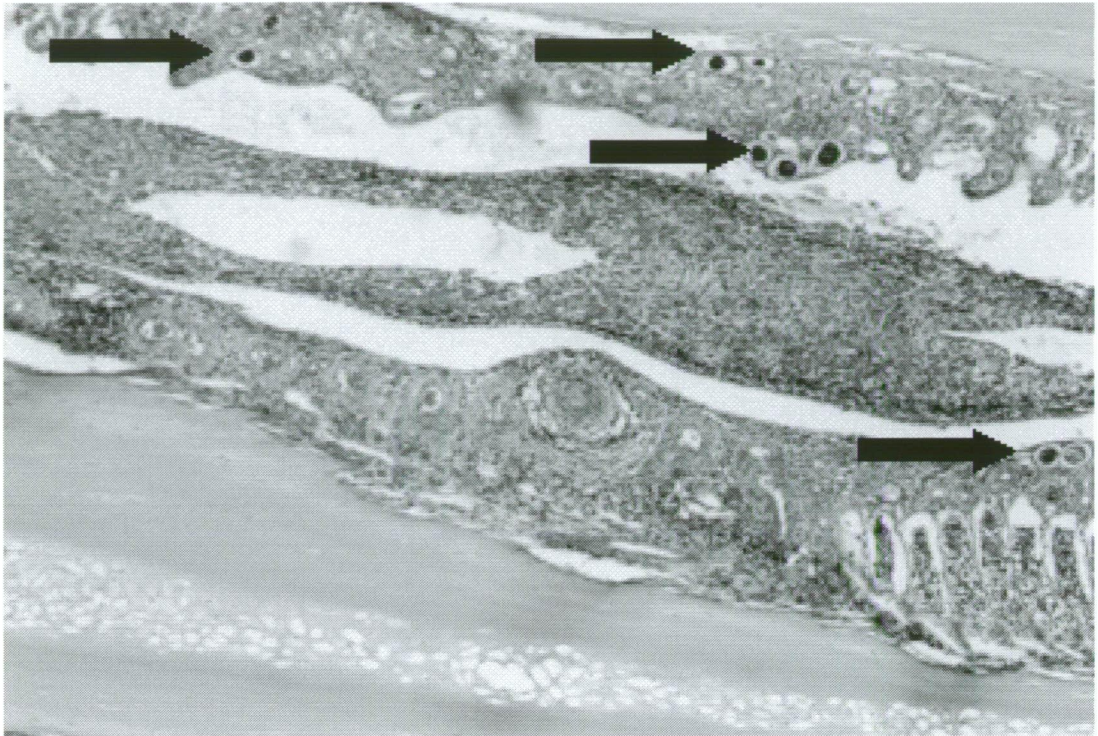


Figure 2. Histological section of a farmed southern bluefin, sampled from the waters of Boston Bay, South Australia, tuna gill showing blood fluke, *Cardicola forsteri*, eggs and associated pathology. Arrows indicate location of blood fluke eggs.

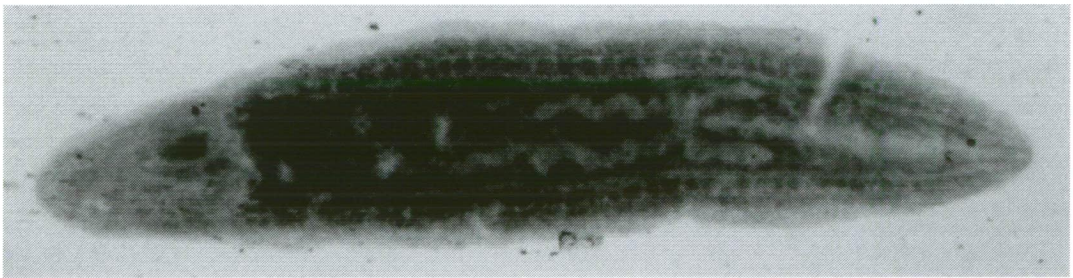


Figure 3. Adult blood fluke, *Cardicola forsteri*, dissected from hearts of farmed southern bluefin tuna, sampled from the waters of Boston Bay, South Australia.

In 56 (40%) of the 140 southern bluefin tuna, for which there were complete results for gross pathology, histopathology, and parasitology, the 3 tests agreed on the blood fluke status of an southern bluefin tuna: 46 were disease positive and 10 disease negative by all 3 tests (Table 1.).

Table 1. Comparison of gross pathology and histopathology to parasitology. + = positive, - = negative.

Gross Pathology	Histopathology	Parasitology	Total
+	+	+	46
+	+	-	13
+	-	+	38
+	-	-	14
-	+	+	5
-	+	-	4
-	-	+	10
-	-	-	10
111*	68*	99*	140

\*Total positive results for each diagnostic test.

The sensitivity of gross pathology was 0.85 (CI:0.76-0.91) and the specificity was 0.34 (CI:0.20-0.51) (Table 2). The sensitivity of histopathology was 0.52 (0.41-0.62) and specificity was 0.59 (CI:0.42-0.74) (Table 3.).

Table 2. Comparison of gross pathology to parasitology with sensitivity , specificity, positive predictive value (PPV), and negative predictive value (NPV), and 95% confidence intervals, of gross pathology. + = positive, - = negative.

Gross Pathology	Parasitology	Total
+	+	84
+	-	27
-	+	15
-	-	14
Sensitivity (95% CI)	0.85 (0.76-0.91)	
Specificity (95% CI)	0.34 (0.20-0.51)	
PPV (95% CI)	0.76 (0.67-0.83)	
NPV (95% CI)	0.48 (0.29-0.67)	

Table 3. Comparison of histopathology to parasitology with sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), and 95% confidence intervals, of histopathology. + = positive, - = negative.

Histopathology	Parasitology	Total
+	+	51
+	-	17
-	+	48
-	-	24
Sensitivity (95% CI)	0.52 (0.41-0.62)	
Specificity (95% CI)	0.59 (0.42-0.74)	
PPV (95% CI)	0.75 (0.63-0.85)	
NPV (95% CI)	0.33 (0.23-0.45)	

The positive predictive values (PPV) for both tests were moderately high. The PPV for gross pathology was 0.76 (CI:0.67-0.83) (Table 2) and for histopathology was 0.75 (CI:0.63-0.85) (Table 3). The negative predictive values (NPV) for both tests were low. The NPV for gross pathology was 0.48 (CI:0.29-0.67) (Table 2) and for histopathology was 0.33 (CI:0.23-0.45) (Table 3). The kappa values for agreement beyond chance between gross pathology and histopathology was calculated as 0.49 indicating a moderate amount of agreement. Histopathological analysis of gross lesions demonstrated eggs in 59 (53%) cases of the 111 southern bluefin tuna that were exhibiting gross lesions. Gross lesions were present in 59 (86%) cases of the 68 southern bluefin tuna that were positive for blood fluke eggs as demonstrated by histopathological analysis.

#### **4. Discussion**

Histopathological analysis of gross lesions showed that in 53% of cases blood fluke eggs were present and that they were the most likely cause of pathology. The pathology observed is very similar to a previous study examining the histopathological response of farmed southern bluefin tuna to blood fluke eggs in the gills (Colquitt et al. 2000). Eggs were shown to accumulate in the afferent filamental arteries leading to blockages and obstructing blood flow leading to the formation of thrombi and oedema (Colquitt et al. 2000). Adult blood flukes were also recovered from hearts of a large number of southern bluefin tuna (71%). Previous studies of blood fluke intensity and prevalence have shown high prevalences of blood fluke infection in farmed southern bluefin tuna. Aiken et al. (2006 (Chapter 3)) showed that prevalence can reach 100% during a growout season.

Gross pathology was shown to have a moderately high sensitivity which was the highest point estimate for both tests. The high sensitivity is expected as the presence

of blood fluke in the heart would mean that there are eggs travelling to the gills where they will cause pathology. However, the converse would occur in the very early period of the growout when southern bluefin tuna are infected. Blood fluke completing migration to the final site within the host would require a period of time to mature and begin producing eggs (Kirk and Lewis 1993). *Paradeontacylix* spp. mature and start to deposit eggs in host fish approximately two months after cercarial invasion (Ogawa et al. 1993). A similar period may exist in this case and blood fluke could be observed in the host without the presence of eggs in the gills resulting in a lower sensitivity. Surprisingly, the sensitivity of gross pathology was higher than the sensitivity of histopathology. The difference may be a result of the inspection of the whole gills for gross lesions whereas only one section from one block representing only a few filaments was examined histopathologically for each tuna. However, it was expected that the estimates of sensitivity would correlate as both tests detect the presence of eggs in the gills either directly, in the case of histopathology, or indirectly, in the case of gross pathology. A higher sensitivity for histopathology was also expected as gross lesions were being targeted for histological examination as opposed to routine removal of a predetermined location of the gill (Adams et al. 2004). The low sensitivity may be a reflection of the histopathology case definition limiting what constitutes a positive animal. The case definition used in this study means that the presence of eggs was required to be observed in the histology section for a southern bluefin tuna to be considered positive for infection. However there were southern bluefin tuna that exhibited the histopathological signs of blood fluke egg presence but without eggs being present in the section. Histopathological indications of blood fluke egg presence included localised hyperplasia of the epithelium and connective tissue cells of the gill filaments, which is consistent with previous histopathological findings (Colquitt et al. 2001). Eggs may have been



present in the gills but not detected in that section, or eggs may have been previously there, causing pathology, but when the miracidia had hatched and the eggs degraded, the pathology remained despite the lack of egg presence. It has been demonstrated in other fish host species parasitized by sanguinicolids that eggs lodged in the gill tissue are encapsulated by the granulomatous inflammation response of the fish host and consequently degrade (Kirk and Lewis 1993). The pathology would still be observed without the presence of the blood fluke eggs.

The specificities of both tests were low. A possible reason for the high number of apparent false positives is that adult blood fluke may have been cleared from the host whilst the eggs are still present in the gills causing pathology. It has been shown previously that adult blood fluke are cleared from the host over a period of 4 months (Aiken et al. 2006 (Chapter 3)). It is possible that eggs may be still causing pathology even though there are no adult blood flukes in the tuna. This would result in the high number of false positives that were observed. A high number of false positives was also seen when histopathology was used as a diagnostic test. This high number may be due to similar reasons as that seen when using gross pathology. Eggs may be present in the gills after adult fluke was in the heart, which means that there may be a discrepancy in time of detection of different life stages. A histopathological study of cultured *Lates calcarifer* infected with the sanguinicolid *Cruoricola lates* demonstrated that whilst eggs may be present in the gills, a lack of infection by juvenile or adult worms may be demonstrated depending on the location in the host examined (Herbert et al. 1995). Presence of eggs in the gills and the absence of juvenile or adult blood fluke was also shown in a histopathological study of *Sparus aurata* infected with sanguinicolid trematodes (Padros et al. 2001). In this study, 34 of the fish examined revealed the presence of large numbers of sanguinicolid eggs,

however of these fish only 4 demonstrated the presence of an adult blood fluke. It was concluded that other organs in the host need to be examined especially where there is the possibility of influences related to seasonal and individual variability due to age. A higher specificity than that observed when gross pathology was used as a diagnostic test may be related to the histopathology being a more direct indication of blood fluke egg presence. The gross gill lesions may indicate a current egg infection, a previous egg infection, or possibly a previous infection by other parasites, for example ectoparasites. Ectoparasites are commonly observed on the gills of southern bluefin tuna and include two species of copepods and one species of monogenean (Hayward et al. 2007). It is possible that these ectoparasites may be dislodged during the harvesting process leaving the lesion behind resulting in a misdiagnosis as a lesion caused by blood fluke eggs.

The positive predictive values of both tests were shown to be moderately high, whilst negative predictive values were shown to be low. By targeting gross lesions for histopathology, the positive predictive value would be maximised. For an animal to be considered positive histopathologically two criteria were required to be met, a tuna must have gross lesions and also demonstrate blood fluke eggs in the histological section, therefore only severely affected animals at a later stage of infection were considered positive for comparison to parasitology; thus minimising the false positive rate (Rohonczy et al. 1996). Additionally the high positive predictive values and the low negative values may be a reflection of the high prevalence of infection that was observed. The effect of prevalence on predictive values is considerable. As prevalence of infection in a population increases, the positive predictive value increases and the negative predictive value decreases (Thrusfield 2005).

The estimates of sensitivity, specificity, positive predictive value, and negative predictive value of gross pathology and histopathology demonstrate that these tests are not accurate in determining the adult blood fluke infection status of southern bluefin tuna. This is in part due to the tests detecting the presence of blood fluke eggs and this being compared with the presence of adult fluke in the heart.

Epidemiological surveys of experimental fish demonstrate that adult blood fluke decline in numbers during the growout season and it is likely that there are situations where there are no adult fluke left in the host but eggs are still remaining in the gills (Aiken et al. 2006 (Chapter 3); Aiken et al. 2008 (Chapter 6)). It is also possible that the adult fluke have moved to a different organ of the host and are not being detected. *Paradeontacylix kampachi*, a blood fluke parasite of *Seriola dumerili* is routinely observed in the girdles, cephalic kidney, sinus venosus, kidney and branchial arteries (Montero et al. 2003). Adult fluke may also be migrating to the gills to lay eggs thereby creating a situation where eggs are being observed in the gills but no fluke are observed in the heart. Adult *Paracardicoloides yamagutii* have only been observed in the gills of eels (*Anguilla australis* and *Anguilla dieffenbachia*) after migrating there to lay eggs (Hine 1978). However it is unlikely that the majority of fluke are being missed due to their location in other organs as Aiken et al. (Submitted) (Chapter 4) has examined other organs of tuna and has not observed any fluke other than a small percentage found in the branchial arteries of the gills.

Despite the low estimates of accuracy for both tests in determining the presence of adult fluke there was moderate agreement between tests. As opposed to sensitivity and specificity, kappa measures the agreement in the positive as well as the negative

categories. It has been suggested that a kappa of 0.40-0.75 indicates a moderate to good level of agreement between tests and over 0.75 indicates excellent agreement between tests (Fleiss 1981). The presence of gross lesions typical of blood fluke infection at post-mortem inspection showed moderate agreement beyond chance, with the histopathological observation of blood fluke eggs. Disagreement between the tests was mainly in the situation where a positive gross pathology disagreed with a negative histopathology result. A number of reasons could be responsible for this; most likely the eggs are in the gills but not in the section which was examined, or the lesions may have been the result of a previous blood fluke infection, or the lesions may have been caused by a previous ectoparasite infection. A disadvantage in the use of kappa is that the magnitude of the kappa statistic is dependent on the true prevalence of the trait being measured (Feinstein and Cicchetti 1990). As true prevalence approaches 0 or 1 the value of kappa decreases (Thompson and Walter 1988). Therefore, the moderate value of kappa may also be a reflection of the high prevalence of blood fluke infection that was observed in the southern bluefin tuna. Thus, the values of kappa and the predictive values reported in this study must be treated with caution when applied to surveys of southern bluefin tuna carried out early (2 months post transfer) or late in the growout season (6 months post transfer). Early during the season infection is very high and late in the season it is very low (Aiken et al. 2006 (Chapter 3); Aiken et al. 2008 (Chapter 6)) and this may affect the accuracy of any diagnostic test applied. Other methods of assessing diagnostic tests are available and can also be utilised when there is no gold standard available (Enoe et al. 2000). Bayesian analyses are one such approach and are increasingly being used in the assessment of aquatic disease diagnostic tests (Aiken et al. In Prep. (Chapter 8)). These approaches are sometimes preferred over using the kappa statistic which is influenced by the prevalence resulting in kappa values rarely being

compared across studies or populations (Thompson & Walter 1988; Feinstein & Cicchetti 1990).

The estimates of accuracy for the tests evaluated in this study may be applied to surveys where these tests have been used and a conversion from apparent prevalence to true prevalence is required. Additionally, the relatively high sensitivity of the gross pathology means that this diagnostic method may be a useful onboard test that can be used to quickly determine the number of infected tuna that are being harvested. It is very unlikely that the eggs causing these lesions are from any other sanguinicolids as surveys incorporating molecular analysis have failed to identify any other species of sanguinicolid infecting *T. maccoyii* (Aiken et al. 2007 (Chapter 2)). As a result wet preparations of gill material to identify the presence of eggs as the cause of gill lesions could be used as a more specific onboard test that would not require any significant extra amount of time to perform. However, parasitology is still the preferred method of detecting sanguinicolids, as this method directly detects the presence of adult fluke (Cribb et al. 2000; Montero et al. 2003; Bullard et al. 2004; Hutson and Whittington 2006).

## **5. Acknowledgements**

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## **Chapter 8. Bayesian estimation of diagnostic test parameters and prevalence of blood fluke in farmed southern bluefin tuna in the absence of a gold-standard**

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## Abstract

Bayesian and classical approaches were used to determine the prevalence of *Cardicola forsteri* infection in farmed southern bluefin tuna and to determine the test properties of the two diagnostic procedures used. Samples for analysis were obtained from tuna that had been farmed in 2005 and 2006. Samples were examined using both parasitological examination (PE) and enzyme-linked immunosorbent assay (ELISA) detecting specific antibody response against the parasite. Classical analysis indicated a prevalence of 75% (CI: 67%-82.37%) in 2005 and 76% (CI: 61%-86.66%) in 2006 using PE and a prevalence of 41.86% (CI: 33%-50.87%) in 2005 and 61.22% (CI: 46%-74.8%) in 2006 using the ELISA. Based on the results of the two tests, Bayesian analysis indicated a true prevalence of 88.77% (BCI: 73.33%-99.26%) in 2005 and 84.79% (BCI: 68.12%-98.28%) in 2006. The Bayesian analysis also indicated that PE was the more sensitive and specific technique (Se: 84% and Sp: 99%) than the ELISA (Se: 35% and Sp: 37%). The results of the Bayesian analysis demonstrate that the prevalence of *C. forsteri* infection has been underestimated in previous surveys due to consideration of PE as a perfect diagnostic test. This is the first known study to use Bayesian analysis to estimate the prevalence of a helminth parasite in fish and to estimate test characteristics of diagnostic procedures used for the detection of helminth parasites infecting fish.

## 1. Introduction

*Cardicola forsteri* is a blood fluke parasite of southern bluefin tuna *Thunnus maccoyii* and northern bluefin tuna *Thunnus thynnus* (Cribb et al. 2000; Bullard et al. 2004; Aiken et al. 2007 (Chapter 2)). It is the cause of significant gill pathology in farmed tuna from Australia (Colquitt et al. 2001). Nodular lesions on the gills result from an inflammatory response to *C. forsteri* eggs trapped in the afferent filamental blood vessels (Colquitt et al. 2001). *C. forsteri* has a complex life cycle and its intermediate host is currently unknown but it is most likely a polychaete or bivalve (Smith 1997). Infective cercariae are shed from this intermediate host and actively search for the final host, in this case a southern bluefin tuna. On penetrating the skin of the host the juvenile fluke will undergo a migration to the heart where they will mature (Smith 1997; Cribb et al. 2000). Once established, the adult fluke lay eggs, which travel to the gills where they lodge. Adult fluke may also migrate to the gills to lay eggs. Adult *Paracardicoloides yamagutii* have only been observed in the gills of eels (*Anguilla australis* and *Anguilla dieffenbachia*) after migrating there to lay eggs (Hine 1978). Free swimming miracidia hatch from the eggs and break out of the gill structure into the external environment. These miracidia will seek out an appropriate intermediate host into which they will penetrate to undergo asexual reproduction as rediae and/or sporocysts to produce infective cercariae (Smith 1972; Smith 1997).

A surveillance program targeting *C. forsteri* in farmed southern bluefin tuna has been continuing since 2003 (Nowak et al. 2003; Deveney et al. 2005; Aiken et al. 2006 (Chapter 3); Aiken et al. 2008 (Chapter 6)). Prevalence of infection estimated during a growout season has varied from 31% in 2003 (Deveney et al. 2005) to 68.2% in 2004 (Aiken et al. 2006 (Chapter 3)) and 76.33% in 2005 (Aiken et al. 2008 (Chapter 6)). In these surveys two diagnostic procedures, parasitological examination and

enzyme-linked immunosorbent assay (ELISA), have been used to diagnose infections and to estimate the prevalence of *C. forsteri* in tuna, although the primary method of detection has been parasitological examination to observe the adult fluke in the heart as the ELISA has been developed only recently (Aiken et al. 2008 (Chapter 6)). Parasitology has historically been the method of diagnosis of parasites in wild and cultured fish although ELISA is increasingly being used to detect fish parasites as this technique can be used in non-destructive sampling methods (Richards et al. 1996; Knopf et al. 2000; Taylor and Jones 2005). Neither the sensitivity nor the specificity of these two *C. forsteri* detection tests is known, yet reliable estimates of these parameters are needed to estimate true prevalence in farmed tuna populations (Guerden et al. 2004).

One problem in the validation of the diagnostic tests, particularly for diseases of aquatic animals, is the lack of an appropriate gold standard. A recent study of *C. forsteri* in southern bluefin tuna considered the use of parasitological detection as a gold standard (Aiken et al. In Prep. (Chapter 7)). However there is no accepted gold standard for the diagnosis of *C. forsteri* in southern bluefin tuna and therefore the accuracy assessment of any new diagnostic test developed may be biased if parasitology, which may be an imperfect reference test, is used as a gold standard for comparison (Guerden et al. 2004). When a gold standard is not available it is possible to obtain estimates of test sensitivity and specificity using latent class methods in which the unknown (latent) variable is the true infection status (Enoe et al. 2000). A variety of methods have been developed to fit these latent class models (Enoe et al. 2000). In this study we used a Bayesian technique. This approach requires that two or more conditionally independent tests be applied to tuna derived from two or more groups with different prevalence of infection (Nerette et al. 2005).

The Bayesian approach and other 'no gold standard' approaches are increasingly being used in diagnostic test evaluation and prevalence estimation of diseases of aquatic animals (Nerette et al. 2005, Thebault et al. 2005, Gustafson et al. 2008). In this study, the prevalence of *C. forsteri* in farmed southern bluefin tuna in the Spencer Gulf, South Australia, was estimated by Bayesian analysis based on data obtained from 2005 and 2006 using two independent diagnostic techniques. A secondary objective was to determine the sensitivity and the specificity of an ELISA, at various cut-off levels. To account for the absence of a gold-standard, we used Gibbs sampling to compare the ELISA to parasitological examination.

## **2. Methods and Materials**

### **2.1. Population**

Southern bluefin tuna were sampled (hearts and blood) at various stages over a five month period (April – August) during 2005 and also in March and August 2006. Tuna from each year represented two distinct populations as wild southern bluefin tuna are introduced to the farming zone annually and are harvested before the end of that year. For both years wild *T. maccoyii* were captured by purse-seine in the Great Australian Bight (map reference 33 27S, 132 04E) in February and towed to the Spencer Gulf farming zone over a period of approximately six weeks in a towing pontoon. In 2005 tuna were transferred from the tow pontoon to four sea cages for farming on 5<sup>th</sup> April 2005 and nine tuna were sampled at this time. During the growout period, ten tuna were sampled from each of the four cages on 30<sup>th</sup> May, 11<sup>th</sup> July, and 22<sup>nd</sup> August. A total of 129 tuna were examined in 2005. Tuna from 2006 intake of wild fish were sampled on the 24<sup>th</sup> March (37 days post transfer) and ten tuna on 27<sup>th</sup> March (41 days post transfer). A total of 49 tuna were examined in 2006. The sampling times and number of fish were determined in collaboration with the

industry. The number of southern bluefin tuna sampled was limited due to the high cost of individual tuna. Samples taken and dates are summarized in Table 1.

Table 1. Number of farmed southern bluefin tuna *Thunnus maccoyii* sampled at different times during 2005 (Cohort<sub>2005</sub>), including from different cages during May, July and August 2005, and 2006 (Cohort<sub>2005</sub> and Cohort<sub>2006</sub>) for blood fluke *Cardicola forsteri* infection. Samples were not taken for serological analysis on 7<sup>th</sup> March 2006 and 14<sup>th</sup> March 2006.

Cohort	Sampling time	Cage	n
2005	Transfer 5-Apr-05		9
	30-May-05	1	10
		2	10
		3	10
		4	10
	11-Jul-05	1	10
		2	10
		3	10
		4	10
	22-Aug-05	1	10
		2	10
		3	10
		4	10
2006	24/28-Mar-06		29
	18-Aug-06		20

## 2.2. Diagnostic tests

### 2.2.1. Parasitological examination (PE)

The presence of blood flukes was assessed according to Aiken et al. (2006) (Chapter 3). Hearts were obtained during harvest, stored on ice, and taken to the laboratory. Hearts were dissected 2-4 h after removal from the carcass and flushed with physiological saline to dislodge any adult flukes. Flushes were poured into Petri dishes and examined for the presence of flukes using a dissection microscope (Olympus SZX12) at 6× magnification. Prevalence (i.e. the number of infected tuna divided by the number of tuna examined ×100) was determined, as described by Bush et al. (1997). All flukes were frozen at -20°C for approximately one week and then transferred to a -80°C freezer where they were stored.



## 2.2.2. Enzyme-linked immunosorbent assay (ELISA)

### 2.2.2.1. Sample collection

Southern bluefin tuna were bled during harvest by cutting into the pectoral artery and collecting blood in 50 ml falcon tubes which were then stored on ice until transfer to the laboratory approximately 3-4 hours later. Blood was allowed to clot overnight, centrifuged at 5000 g for 10 min, after which serum was aliquoted and stored at -80°C.

### 2.2.2.2. *C. forsteri* antigen preparation

Adult flukes collected from tuna during 2005 and 2006 and stored at -80°C were used as antigen in the ELISA. Flukes were transferred to a 15 ml tube and suspended in carbonate buffer (2.93 g NaHCO<sub>3</sub>, 1.59 g Na<sub>2</sub>CO<sub>3</sub>, in 1 l of distilled water, pH 9.6). The buffer was then subjected to ten pulses of a Branson sonicator for approximately 10 s a pulse. A tissue homogenizer was then used to further break down the flukes in ten pulses of approximately 15 s each. The buffer was then transferred to 1.5 ml tubes and centrifuged at 3000 g for 10 minutes at 4°C. Supernatant was aliquoted and stored at -80°C. Protein concentration of antigen in the solution was determined by BCA Protein Assay Kit to determine concentration for the chequerboard titration.

### 2.2.2.3. ELISA

A recently developed standard indirect ELISA (Aiken et al. 2008 (Chapter 6)) was used to detect and quantify specific serum antibodies in tuna against *C. forsteri*. Assay conditions were optimized empirically and reagent concentrations determined by chequerboard titrations (Crowther 1995). A flat bottom 96-well microplate (IWAKI) was coated overnight at 4°C with *C. forsteri* antigen solution diluted with carbonate buffer (2.93 g NaHCO<sub>3</sub>, 1.59 g Na<sub>2</sub>CO<sub>3</sub>, in 1 l of distilled water, pH 9.6) to 20 µg ml<sup>-1</sup> protein. Plates were blocked with 0.3% casein in tris buffered saline (TBS; 20 mM Tris, 500mM NaCl, pH7.2) for 1 h at 37°C. The plates were then washed

three times with TBS. Tuna serum samples were then added to plates. Tuna serum samples were diluted in 0.3% casein in TBS, and added in duplicate wells across rows in a twofold serial dilution beginning at 1:100 through to 1:3200. Positive and negative standard sera, previously chosen from Aiken et al. (2008) (Chapter 6), were titrated in duplicate on each plate. After 1 h incubation at 37°C, plates were washed three times in TBS then rabbit anti-tuna heavy chain immunoglobulin (RATH) (Watts et al. 2001), diluted 1:100 in 0.3% casein in TBS, was added and incubated for one h. Following another wash cycle sheep anti-rabbit IgG alkaline phosphatase conjugate (Sigma, Castle Hill, NSW, Australia), diluted to 1:8000 in 0.3% casein in TBS, was added. All reagents were added as 50 µl volume. A final wash in TBS was performed immediately prior to adding 200 µl of the detection reagent, *p*-nitrophenyl phosphate (PNPP) (Sigma, Castle Hill, NSW, Australia). Colour was allowed to develop for 30 min and the optical density was measured at 405 nm using a Spectra Rainbow Thermo microplate reader (TECAN Trading AG, Switzerland).

Antibody activities were determined according to Arkoosh & Kaattari (1990) with a method which expresses titers as units or antibody activity per volume of serum. The positive standard serum was titrated against *C. forsteri* antigen starting at a dilution of 1:100 to calculate a serum volume equating to 0.5 unit of antibody activity. In this study one unit of antibody activity was defined as the volume of the positive standard which gave 50% of the maximum OD, thus the reciprocal of this volume gives units of activity per µl of serum. The volume of serum giving 50% of the OD is determined from the dilution factor. Thus specific antibody activities of samples were expressed as units of antibody activity per volume of serum and were determined relative to the immune standard included on all plates and calculated as follows:

Activity of sample = (activity of standard)  $\times$  (50%volume of standard/50%volume of sample)

where activity of the standard is the reciprocal of the volume of the immune standard which gives 50% maximum OD, as determined from a titration curve and is expressed as units of activity per  $\mu$ l of serum, 50% volume of the standard is the volume of the immune standard giving 50% maximum OD and 50% volume of the sample is the sample volume giving 50% maximum OD. The activity of the standard is a constant in the equation, determined from its full titration, whereas the other terms are derived from individual plates and thus account for variations between plates. All samples and standards, both immune and non-immune, were titrated in duplicate from dilutions of 1:100 to 1:3200.

### **2.3. Statistical analysis**

Two different approaches were used during the study to determine prevalence of infection for each year/population and test characteristics. In a classical approach, presence of adult flukes as detected by examination of flushes using dissecting microscope, parasitological examination (PE), was considered as the reference method ('gold standard') where sensitivity and specificity were assumed to be perfect. Specific antibody serum titre was determined by ELISA for each individual and compared to parasitological examination. The results were plotted in a two-graph receiver operating characteristic (TG-ROC) curve as described by Frossling et al. (2003). A test's sensitivity and specificity ( $y$ -axis) are plotted for each cut-off ( $x$ -axis) in a TG-ROC. This plot demonstrates changes in test accuracy with increasing/decreasing cut-off. The cut-off where sensitivity and specificity were equal, point of equivalence, was reported. A sample was considered positive if its antibody activity was greater than this cut-off value.

A second means of data analysis was based on Bayesian estimation by comparing the antibody titre of the ELISA to the presence of adult flukes observed during PE using a Gibbs sampler, an iterative Markov-chain Monte Carlo (MCMC) technique, as described by Frossling et al. (2003). The Gibbs sampler approximates the marginal posterior densities of the parameters of interest (sensitivity, specificity and prevalence) in the absence of a gold standard. In order to use the Gibbs sampler it is necessary to have the results of two tests simultaneously applied to individuals from two populations with different prevalences of disease (Frossling et al. 2003). To meet this requirement samples were arranged into the two years (2005 and 2006) in which different groups of tuna were examined. The 2006 year samples were from the early part of the season and therefore would expect to have a different level of infection prevalence to 2005 where samples were collected during the entire growout season. Independence was assumed between the two tests, PE and the ELISA, and sensitivity and specificity were assumed to be constant between both populations. A burn in phase of 5,000 iterations was used and inferences were made based on a subsequent 20,000 iterations.

In the Gibbs sampling, prior probabilities (“priors”) of the parameters of interest are incorporated into the simulation. These probabilities are specified as beta distributions described by two variables ( $\alpha$  and  $\beta$ ) presented in the form Beta ( $\alpha, \beta$ ) (Gelman et al., 1995). Priors can be non-informative, a uniform distribution of between 0 and 1, or informative, a distribution achieved by using past data, or by using expert opinion, or a combination of both. In this study, the Gibbs-sampler simulations were performed with non-informative priors (uniform distributions set to Beta (1,1)) for both population prevalences, both test sensitivities, and the ELISA

specificity. Uninformative priors were used for the ELISA due to its recent development and no information of test accuracy. Additionally there is a lack of information about the sensitivity of PE when diagnosing blood fluke infections in fish. An informative prior of PE specificity was estimated to be greater than 98%, with a most probable value of 99%, which gave a prior of Beta (560.72, 6.65). This high specificity is warranted due to the requirement of viewing the actual parasite under the microscope. Using the non-informative and informative priors, point estimates (medians of posterior distributions) and 95% Bayesian credible intervals (2.5% and 97.5% percentiles of posterior distributions) of sensitivity and specificity for both the ELISA and PE were calculated. Point estimates and 95% credible intervals of prevalence of infection for both populations were also calculated simultaneously.

Estimates of sensitivity and specificity for the ELISA at antibody titre values ranging from 0 to 20 (by increments of 1) were calculated and plotted in a TG-ROC. A cut-off value was derived from the TG-ROC where sensitivity and specificity were equal. Further data points were added near the equivalence point to aid in resolution of the optimum cut-off value.

#### **2.4. Computer software**

Data management and descriptive statistics were performed using SPSS 13.0 and Microsoft Excel 2003. Beta distributions were calculated using online software (<http://www.ausvet.com.au/epitools/content.php?=&BetaParams1>) provided by AusVet Animal Health Services (Sergeant 2004). The Gibbs sampler was run using WinBUGS software (Imperial College and Medical Research Council) (Spiegelhalter et al. 1996) version 1.4.

### 3. Results

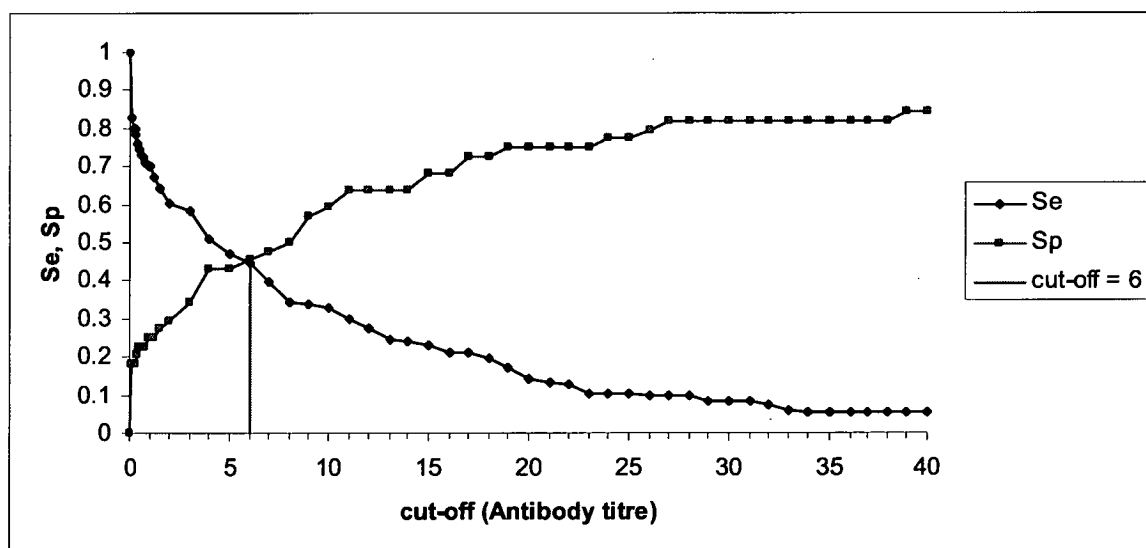
#### 3.1. Classic analysis

##### 3.1.1 Estimation of prevalence using parasitological examination (PE)

The prevalence of *C. forsteri* in the examined samples of 178 southern bluefin tuna was 76% (CI: 69%-81.75%). Prevalence of blood fluke infection in the examined samples of 129 southern bluefin tuna for 2005 was estimated at 75% (CI: 67%-82.37%) through using the gold standard method of PE. Prevalence of blood fluke infection in the examined samples of 49 southern bluefin tuna for 2006 was estimated at 76% (CI: 61%-86.66%) through using the gold standard method of PE.

##### 3.1.2. Classical estimation of ELISA test characteristics

Sensitivity decreased rapidly whilst specificity increased slowly over the range of cut-off values (Figure 1.). A cut-off antibody titre value of 6 was determined from the TG-ROC analysis. At this equivalence point sensitivity and specificity was equal to 45% (Se CI: 27%-41.16%; Sp CI: 30%-61.15%) (Figure 1.)



**Figure 1.** TG-ROC plot with sensitivity (Se), specificity (Sp) and cut-off value =6 of a *C. forsteri* ELISA (Aiken et al. 2008) (178 sera from two annual cohorts of tuna, 2005 and 2006).

### 3.1.3. Estimation of prevalence using ELISA

The cut-off value as determined by equivalence point was used to estimate prevalence of infection. The prevalence of infection amongst all southern bluefin tuna was 47% (CI: 40%-54.8%). Prevalence of infection in 2005 was 41.86% (CI: 33%-50.87%) and in 2006 was 61.22% (CI: 46%-74.8%).

## 3.2. Bayesian analysis

### 3.2.1. Estimation of test characteristics

The TG-ROC plot of sensitivity and specificity as calculated through Bayesian analysis showed that sensitivity decreased rapidly initially and then gradually whilst specificity increased gradually (Figure 2.). A cut-off value of 8.59 was chosen as being closest to the equivalence point with a sensitivity of 34.9% (BCI: 27.16%-42.98%) and specificity of 36.96% (BCI: 2.7%-72.25%). Estimates of PE sensitivity and specificity across the range of ELISA cut-off values did not vary considerably, 0.82-0.86 for sensitivity and 0.9882-0.9883 for specificity.

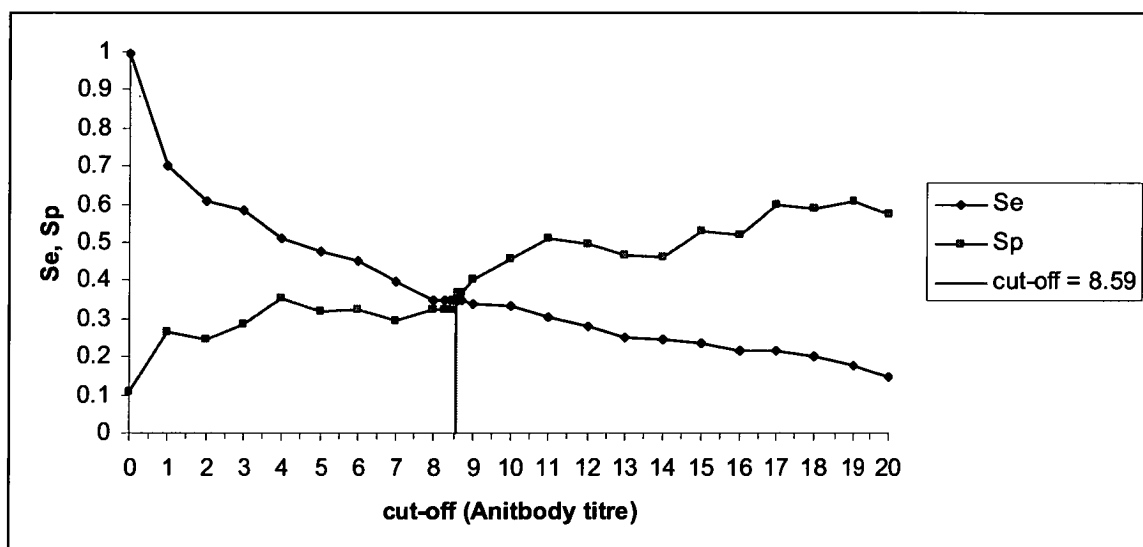


Figure 2. TG-ROC plot with sensitivity (Se), specificity (Sp) and cut-off value =8.59 of a *C. forsteri* ELISA (Aiken et al. 2008) as estimated by Gibbs sampling using non-informative priors for sensitivity and ELISA specificity and an informative prior for PE specificity of Beta (560.72, 6.65) (178 sera from two annual cohorts of tuna, 2005 and 2006).

### 3.2.2. Bayesian estimation of prevalence

Using the cut-off estimated in section 3.2.1 prevalence of infection in southern bluefin tuna in 2005 was shown to be 88.77% (BCI: 73.33%-99.26%) and in 2006 was shown to be 84.79% (BCI: 68.12%-98.28%).

### 3.3. Comparison of test and prevalence estimates

Results shown in Table 2 compare sensitivity, specificity and prevalence estimates between the two methods of test evaluation and prevalence determination. Bayesian analysis demonstrated a higher prevalence of infection than classical analysis. Additionally, the estimates of test accuracy analysed through Classical means were higher than those estimates of test accuracy measured through Bayesian analysis.

Table 2. Comparison of estimation of sensitivity (Se) and specificity (Sp) of parasitological examination (PE) and *C. forsteri* enzyme-linked immunosorbent assay (ELISA) (Aiken et al. 2008) by different statistical approaches and prevalence estimates (Prev.) for each test and each statistical approach. MCMC: Markov Chain Monte Carlo.

Analysis	Test	Se (%)	Sp (%)	Prev (%)		
				2005	2006	Total
Classical	PE	100	100	75	76	76
	ELISA	45	45	42	61	47
MCMC	PE	85	99	89	85	-
	ELISA	35	37	89	85	-

## 4. Discussion

The results presented give important information regarding the prevalence of *C. forsteri* in farmed southern bluefin tuna and the performance of diagnostic procedures for the detection of infection. Bayesian estimation of prevalence in this study has demonstrated that the prevalence of infection is higher than previously thought (85-89%). Earlier estimates have suggested that the prevalence of *C. forsteri* infections over one growout season is 31-68.2% (Deveney et al. 2005; Aiken et al.



2006 (Chapter 3)). However in the study of Deveney et al. (2005) sampling was initiated in July and so the prevalences estimate only represents the growout season from that point of time. In another study examining spatial and temporal patterns of infection, information on prevalence of infection in commercially harvested tuna were collected over a three year period 2004-2006 and was shown to be 62.6% (Aiken et al. In Prep. (Chapter 5)). Previous studies examining the intensity and prevalence of blood fluke infection in southern bluefin tuna have used parasitological examination as the only diagnostic method. This method has been shown to be only a moderately sensitive technique leading to underestimation of prevalence as sensitivity is affected significantly due to the entire carcass requiring examination for the presence of these internal parasites which may be located in many locations (Montero et al. 2003). The sensitivity of parasitological examination of metazoan parasites of fish has not been estimated previously. However, sensitivity values for parasitological examination have been estimated for terrestrial and human metazoan parasites. Bayesian estimation of the sensitivity of stool examination for *Strongyloides* parasites in humans has been shown to be 31% (Joseph et al. 1995). The sensitivity of microscopical examination for *Giardia duodenalis* cysts in calf faeces has been estimated to be 56% (Guerden et al. 2004). The sensitivity of liver dissection for *Fasciola hepatica* flukes has been estimated to be 63.2% through Bayesian analysis (Rapsch et al. 2006).

Both tests using classical techniques of prevalence estimation were shown to underestimate the prevalence of blood fluke infection that was demonstrated by the Bayesian analysis. Parasitological examination underestimation of prevalence was due to the occurrence of false negatives. False positives are not a significant problem in parasitological examination as the process usually involves the visual detection of

the presence of the parasite. Parasitological methods have a high degree of specificity generally as the actual parasite needs to be observed for a positive result to be recorded. A decrease in specificity would only occur through the misidentification of the parasite (Rapsch et al. 2006). It is very unlikely that another species of blood fluke was present in the tuna examined in this study as previous surveys have failed to identify any other blood fluke species (Cribb et al. 2000; Nowak et al. 2003; Deveney et al. 2005; Nowak et al. 2006) and a molecular analysis of blood fluke in farmed southern bluefin tuna was unable to demonstrate any cryptic species (Aiken et al. 2007 (Chapter 2)).

Although the sensitivity of parasitological examination was shown to be moderately high, a number of false negative results were demonstrated. False negatives may possibly be occurring as a result of the presence of blood fluke in other organs of the tuna. Blood flukes of other fish species are known to inhabit other organs of the host. Adult blood fluke infecting the sea bream *Sparus aurata* have been observed in the kidney (Padros et al. 2001). The blood fluke *Cruoricola lates* has been observed in the gills, eyes, pericardial blood vessels, gut, caudal kidney, liver and spleen of infected *Lates calcarifer* (Herbert et al. 1995). *Paradeontacylix kampachi*, a blood fluke infecting *Seriola dumerili*, has been observed in the girdles, cephalic kidney, sinus venosus, kidney and branchial arteries of infected fish (Montero et al. 2003). Adult *Paracardicoloides yamagutii* have been observed in the gills of eels (*Anguilla australis* and *Anguilla dieffenbachia*) after migrating there to lay eggs (Hine 1978). In an earlier survey of blood fluke infection in southern bluefin tuna, samples of heart, liver, posterior and head kidney, branchial arteries, gut, and spleen were examined for *C. forsteri* infection (Aiken et al. Submitted (Chapter 4)). Adult flukes were observed in the heart and in a small number of branchial arteries. No flukes

were observed in other organs of the tuna and it is unlikely that there are significant populations of *C. forsteri* inhabiting other organs of the tuna. This is supported by the results of a histopathological study which was unable to demonstrate presence of blood fluke eggs in the posterior kidney, liver, spleen or intestine of infected southern bluefin tuna (Colquit 1999).

False negatives when utilising parasitological examination are likely to occur early in the growout period when tuna are being invaded by cercaria and immature stages which have yet to migrate to the heart. Screening for invading immature stages is impossible due to the size of the carcass and its high value making it unavailable for dissection. During migration to the heart Parasitological examination of the heart will not be able to detect infected tuna. The period of migration is not known for tuna but has been suggested to be short due to the size of some of the fluke that have been observed in the heart (Aiken et al. Submitted (Chapter 4)). A short migration time (12 days) for *Sanguinicola armata*, a blood fluke infecting grass carp, *Ctenopharyngodon idella*, has been observed while a longer migration time (94 days) has been observed in *Aporocotyle simplex*, a blood fluke infecting dab, *Limanda limanda* (Køie 1982; Kua et al. 2002).

The ELISA significantly underestimated the prevalence of infection. A large number of false negatives and false positives resulted in poor estimates of sensitivity and specificity thus affecting the estimation of prevalence. False negative results may have occurred early in the growout period when it was too early for an immune or serological response to be developed to an antigenic stimulation. Most tuna entering the farming zone would be naïve hosts having no experience of *C. forsteri* infection previously (Aiken et al. 2008 (Chapter 6)). There is always a delay between infection

and detectable specific antibody levels and, therefore, false negative results are typical for antibody detection systems (Voller and De Savigny 1981). Another explanation for the false negative results is that the burdens observed in this study may be too low for a serological response to occur. The burdens observed in the tuna are quite variable with many tuna having only minor worm burdens which may have an effect on the level of antibody the ELISA is able to detect. The ability of serological tests to detect helminth infections is known to be affected by the size of the burden of the infection. Venturiello et al. (1998) demonstrated that low burdens of porcine trichenellosis resulted in a high number of false negative results in various serological assays including an ELISA that had been developed for use as a diagnostic test.

Also a significant number of false positive results resulted from use of the ELISA. A common issue in the use of serological diagnostic tests is that tests for antibodies cannot discriminate between current and past infections as detectable levels of specific antibodies may persist following recovery (Voller and De Savigny 1981). This is quite probable in the case of southern bluefin tuna. It has been shown previously that antibody levels persist despite blood fluke having been cleared from the heart (Aiken et al. 2008 (Chapter 6)). False positives would be most common later in the growout season when tuna have cleared infections of the adult fluke but there is still a remaining measurable antibody level present. Another possibility is that the ELISA is detecting antibodies to invading cercaria which are not able to complete the migration to the heart due to the host immune response (Aiken et al. 2008 (Chapter 6)).

Western Blot analysis of tuna serum against fluke antigen has demonstrated some cross-reactivity that may result in false positives. Polyclonal antibodies, used in this study, often show cross reactivity due to their multiple specificity (Watts et al. 2001). One of the reasons fish serology has not been widely used in infectious disease diagnosis is because fish immunoglobulins are predominantly of the IgM isotype, which is generally of relatively low specificity (Denzin and Staak 2000). Helminths share epitopes and as a relatively crude antigen preparation was used the ELISA may have been cross-reacting with another helminth parasite of the tuna (Tsuji 1975).

The results presented in this study show that, on its own, the ELISA is not applicable for use as a diagnostic test to detect blood fluke infection. This is not surprising, taking into account the persisting uncertainty in the serodiagnoses of human parasitoses (Knopf et al. 2000). Parasitological examination demonstrated a moderately high sensitivity and almost perfect specificity. This method provides a reliable and low cost method of determining *C. forsteri* infections and will most likely be the preferred diagnostic test for surveillance programs. However, Parasitological examination requires destructive sampling whereas samples for serological tests can be taken from live animals. Additionally, immunoserological tests are essential in investigating the immune response of tuna against the blood fluke and this ELISA test has been used successfully in a longitudinal study investigating such a response (Aiken et al. 2008 (Chapter 6)). This study has also demonstrated the usefulness of the ELISA in determining an accurate estimate of prevalence through Bayesian analysis when used in conjunction with another diagnostic test. This approach to estimating the true prevalence of infection, and also estimates of test accuracy, is the preferred method when a gold standard test is not available (Georgiadis et al. 2001).



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## Chapter 9. General Discussion

This research provided new information about the epidemiology of blood fluke, *Cardicola forsteri*, infection in farmed southern bluefin tuna, *Thunnus maccoyii*, in South Australia. The results suggest that the majority of *C. forsteri* infections are due to infections occurring in the farming zone of the Spencer Gulf rather than tuna bringing in infections from the wild. However it is possible that the infections observed in the farming zone are a result of tuna having been infected in the wild as *C. forsteri* infections have been observed in wild southern bluefin tuna (Aiken et al. 2007 (Chapter 2)). Some of these infections may lie dormant as encysted schistosomula similar to *Paradeontacylix ibericus* infections in *Seriola dumerili* (Montero et al. 2009). *C. forsteri* schistosomula may excyst post transfer to account for the infections observed during the growout season. However, the prevalence and intensity of adult *C. forsteri* infections observed in the wild were very low (Aiken et al. 2007 (Chapter 2)) suggesting that juvenile fluke infections are low as well. Additionally, much of the evidence in this thesis points towards a local pattern of infection as parasites with complex life cycles are most prevalent in locations where intermediate hosts are abundant (Padros et al. 2001). The evidence for a local infection pattern includes the demonstration of two major infection periods after transfer in the model (Aiken et al. Submitted (Chapter 4)), a low prevalence and intensity of infections in *T. maccoyii* at transfer (Aiken et al. 2006 (Chapter 3)), and only a minor antibody response in one tuna (out of 10) at the beginning of the growout season (Aiken et al. 2008 (Chapter 6)). This has ramifications for the farming zone in that there may be a build up of infection over time by either more intermediate hosts acquiring infection or more heavy infections being experienced by the intermediate host and as a consequence more cercariae being released into the water column increasing both the intensity and prevalence of infections in *T.*



*maccoyii*. It is highly likely that once an intermediate host is infected with *C. forsteri* it is infected for life (T. Cribb, University of Queensland, personal communication). There is no risk to wild *T. maccoyii* populations from the build-up in infections in the intermediate host population as tuna no longer naturally inhabit the area where the farming occurs.

Despite this potential for an increase in *C. forsteri* infections the surveys undertaken in this study demonstrated that prevalence and intensity decreased over the growout period. It is possible that the specific humoral response, demonstrated by the ELISA and Western Blot, is responsible for this decrease by targeting invading and migrating cercariae after the first infection period resulting in an almost nil recruitment of adult stages in the heart (Aiken et al. 2008 (Chapter 6)). A similar host response against invading and migrating sanguinicolid cercariae has been suggested to occur in *Cyprinus carpio* against *Sanguinicola inermis* as it has been observed that many flukes were unable to complete migration to the blood system of *C. carpio* (Kirk and Lewis 1996). Tuna re-exposed to cercariae in the second growout season had a lower prevalence and intensity of *C. forsteri* infection (Aiken et al. 2007 (Chapter 2)). This pattern of infection has been observed in *C. cyprinus* re-exposed with *Sanguinicola inermis* cercariae (Hoole et al. 2003) and *Seriola quinqueradiata* re-exposed with *Paradeontacylix* spp. cercariae (Ogawa et al. 1989). In both studies, fish re-exposed to cercariae demonstrated lower intensities of infection than naïve hosts exposed for the first time. It would have been preferable to have demonstrated under experimental laboratory conditions that there is protection against re-infection with *C. forsteri*. However this type of research is impossible with *C. forsteri* as the intermediate host is unknown and therefore there is no available source of cercariae

to use in such an experiment. Additionally, appropriate tank systems for holding tuna are not available for research purposes.

Although there is evidence that specific humoral response is causing the decrease in intensity and prevalence other factors may also explain this infection pattern. It is possible that mortalities in the intermediate host population may be responsible for the decrease in fluke numbers. It was suggested that *C. forsteri* may have a deleterious effect on its intermediate host (Munday et al. 2003). Pathological effects by the sanguinicolid *Aporocotyle simplex* resulting in castration of the intermediate host, *Artacama probosidea* have been reported (Køie 1982). Intermediate hosts parasitised by cercariae almost invariably exhibit lower fecundity due to castration (see Mouritsen and Poulin 2002 for review). Sterility and a decreased life span may be experienced by the intermediate host of *C. forsteri* resulting in decreasing infections in the *T. maccoyii* population. However, this is unlikely as this would most likely lead to a decline in infections in tuna year-to-year due to the declining intermediate host population which was not observed (Aiken et al. In Prep. (Chapter 5)).

Parasite-induced mortality of the most heavily infected individuals in fish populations has been used to explain observed parasite prevalence and intensity patterns (Knudsen et al. 2002). It is possible that the decrease in intensity and prevalence observed in the surveys of this thesis was due to mortality of tuna with the highest intensities of *C. forsteri*. However, this is an unlikely explanation for the observed decrease as the surveys occurred on farms where divers recovered all mortalities, the number of which was low and could not explain the reduction in

prevalence. Additionally, the number of flukes observed in the hearts of mortalities was the same as in normal fish at the same time (Aiken et al. In Prep. (Chapter 5)).

No relationship was observed between the intensity of *C. forsteri* infection and the condition of the tuna despite the presence of gill pathology in many infected tuna. This apparent lack of an effect of the *C. forsteri* on *T. maccoyii* is in contrast to other investigations of blood fluke infected fish. *Paradeontacylix* spp. have caused mass mortality of *Seriola dumerili* in Japan with dead fish displaying signs of suffocation resulting from an accumulation of eggs in the afferent arteries (Ogawa and Fukudome 1994). However, in a histopathological study of a blood fluke, *Cruoricola lates*, in *Lates calcarifer* it was suggested that low intensities of *C. lates* did not cause mortality and were only responsible for minor pathological changes (Herbert 1995). The inflammatory response of *Sparus aurata* against sanguinicolid eggs has been observed to be lower than that of *Seriola purpurascens* against *Paradeontacylix* spp. and it has been suggested that this was a result of the lower intensity of sanguinicolid infection in *Sparus aurata* (Padros et al. 2001). In general only low intensities of *C. forsteri* were observed in *T. maccoyii* with annual means of intensity fixed around six fluke per infected host (Aiken et al. In Prep. (Chapter 5)). This level of intensity may not be enough to produce serious pathological effects or mortality. Additionally, it was shown that there was no year-to-year increase of infection intensity suggesting that some equilibrium has been reached (Aiken et al. In Prep. (Chapter 5)). However, despite the lack of major pathology and low levels of infection this does not mean that the infection could not contribute to severe illness or death by acting together with other factors (Colquitt et al. 2001). Kumon et al. (2002) found that blood fluke infested yellowtail *Seriola quinqueradiata*, when challenged with *Lactococcus garvieae*, a bacterial pathogen of fish, had a significantly higher final cumulative

mortality than fish uninfected with blood fluke. Another factor that may combine with *C. forsteri* infection includes husbandry which differs according to the company holding the tuna. This study identified company as factor in explaining variation in *C. forsteri* intensity, abundance and prevalence. Husbandry effects that may influence the occurrence of *C. forsteri* include the frequency of sea cage net cleaning, if the intermediate host is located on the net, and also the frequency that sea cage sites are fallowed if the intermediate host is located in the sediment beneath cages. Additionally, stress resulting from poor management can lead to immunocompromised fish resulting in higher parasite loads and possibly more clinical disease (Murray and Peeler 2005). *T. maccoyii* fingerlings, currently under commercial trial, may also be more susceptible to *C. forsteri* infection than wild fish which are currently farmed. Juvenile *T. maccoyii* may not be able to migrate from the tropical spawning grounds into waters of higher latitudes until they become endothermic (Dickson et al. 2000), therefore very young fish, with immature heat exchangers, cultured in the cooler waters of South Australia may show the same low temperature immunosuppression observed in other aquaculture species (Watts et al. 2002).

A range of epidemiological methods was used to understand *C. forsteri* infection in farmed tuna. The methods used in this thesis were constrained by some industry specific and general issues related to undertaking surveys in aquaculture. An analysis of risk factors for disease and infection using case-control or cohort surveys was not possible in this study due to the industry direction as well as due to technical reasons. Therefore, observational studies using cross-sectional surveys were used to describe patterns of infection. Despite the industry and technical constraints the data generated from the observational studies provided valuable information. Temporal-

spatial studies have been used previously to provide information on the occurrence and modes of transmission of an infectious disease (Georgiadis et al. 2001). In order to evaluate transmissions patterns of white sturgeon iridovirus (WSIV) in a sturgeon hatchery Georgiadis et al. (2000) described and analyzed data on the temporal-spatial distribution of disease in individual fish tanks to effectively provide information that could be used to manage disease outbreaks (Georgiadis et al. 2000).

Some issues with methods arose in these studies that have been identified in previous epidemiological studies in an aquaculture environment. Sampling of confined fish populations to obtain a representative sample is a challenge in any observational study. In the longitudinal surveys (Aiken et al. 2007 (Chapter 2); Aiken et al. 2008 (Chapter 6)) sampling was constrained by industry demands on how the fish were selected. Instead of undertaking systematic random sampling to select fish, tuna were haphazardly selected by a diver in the pontoon catching the closest fish and swimming to the sampling deck. Because fish sometimes have strong social hierarchies, they tend to stratify in the water column (Georgiadis et al. 2001). Therefore selection of fish from the surface will not yield a representative sample of the population. Crowding fish into the net and then taking a sample has been suggested as the best way of taking a random sample of fish from a pontoon (Cameron 2002). However, this is not possible for farmed *T. maccoyii* due to the size and cost per individual tuna and the potential for significant damage and stress to occur during crowding. Alternatively all fish should be removed from the cage and sample chosen randomly. However, this is possibly only during a harvest of all fish from one cage or grading of all fish from one the cage, either of which are rare in the tuna industry.

Another problem in trying to obtain a random sample was that often commercial production was prioritised over sampling protocols. This situation often occurred in the sampling of commercial fish (Aiken et al. In Prep. (Chapter 5)) where some harvests only targeted larger or smaller fish driven by the requirements of the buyer of the tuna. However, the commercial harvests represented a better opportunity to attempt a random sample from the harvested tuna as the sampled tuna were selected from the processing line at 5 minute intervals rather than have an individual tuna selected for sampling as was the case in the longitudinal surveys (Aiken et al. 2007 (Chapter 2); Aiken et al. 2008 (Chapter 6)).

Investigation of mortalities as a possible outcome of *C. forsteri* infection was problematic and the question of whether *C. forsteri* causes mortalities remains unanswered. Only a small number of mortalities were available for examination for *C. forsteri* and any associated pathology. Small numbers were examined as only a small number of dead tuna were submitted by industry. Additionally farms provided very little information about the history of the submitted fish. As such no significant inferences based on statistical analyses could be made about mortalities and *C. forsteri* infection. However, those mortalities examined were shown to have the same or fewer numbers of adult flukes as harvest fish (Aiken et al. In Prep. (Chapter 5)).

A limitation of many aquatic epidemiological studies is the use of diagnostic tests with unknown sensitivity and specificity. Assuming that a test has perfect sensitivity and specificity (accuracy) when this is not true, will bias the inferences of epidemiological investigations that use the test results (Georgiadis et al. 2001). Additionally in the case of pathogens with more than one life stage in the infected host, such as sanguinicolids, the diagnostic test usually is only able to detect one stage of that life history (Figure 1).

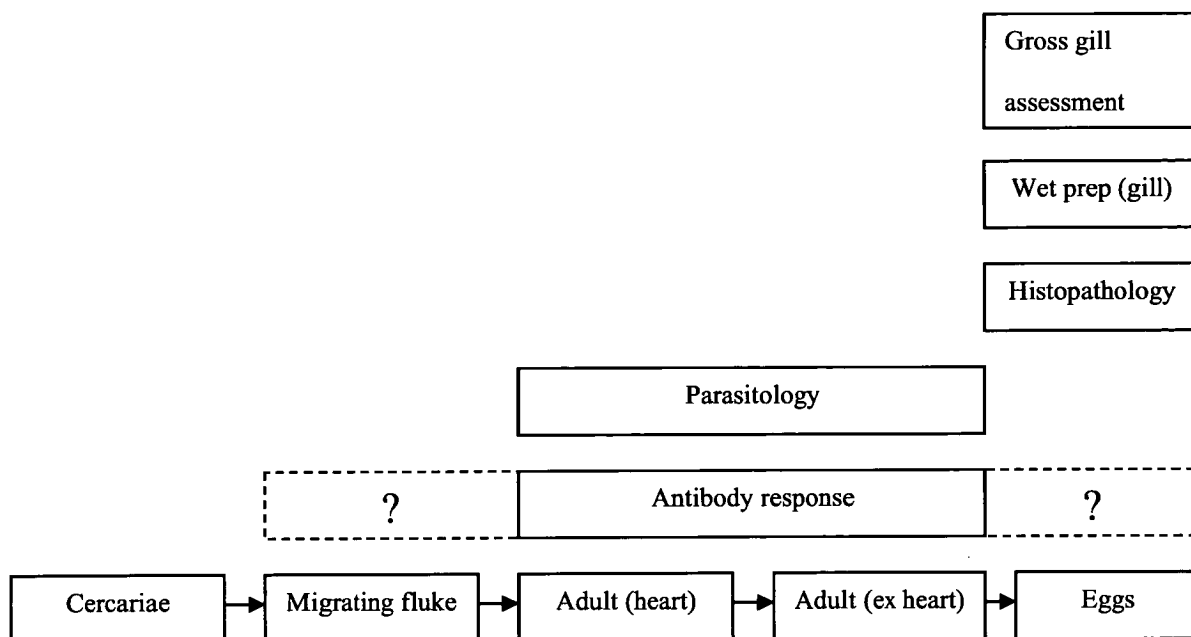


Figure 1. Life stages of *C. forsteri* in a naïve *T. maccoyii* and the various diagnostic tests that are able to detect the respective life stages. Parasitological test is the dissection technique used in this thesis to demonstrate adult *C. forsteri* from the heart. It is possible for this technique to be used in identifying adult fluke from other organs. Antibody response represents the ELISA and Western Blot tests used in this thesis. It is assumed that an antibody response can be elicited against adult fluke as adult fluke extracts were used to develop the ELISA and Western Blot (Aiken et al. 2008 (Chapter 6)). Dashed lines indicate lack of knowledge about ability of antibody responses test to detect migrating fluke and egg life stages.

As a result it is difficult to determine the true infection status of a tuna when using a diagnostic test that is only able to detect one life stage of *C. forsteri*. This study demonstrated the low accuracy of several diagnostic tests used to detect *C. forsteri* infection in *T. maccoyii*. There is some argument that detection of *C. forsteri* from the heart is not representative and that fluke migrate when tuna die and possibly also migrate later in the season resulting in low levels of infection being observed. However the Bayesian analysis demonstrated that the parasitological examination is a relatively sensitive technique with almost perfect specificity. It provides a reliable and low cost method of determining *C. forsteri* infections and will most likely be the

preferred diagnostic test for surveillance programs, where hearts can be obtained, as this method requires destructive sampling. Another low cost method that was not used in this study that could also be undertaken is the analysis of wet preparations of gill material. *C. forsteri* eggs could be detected in this way, however, as with the histology there would be a lack of sensitivity and in contrast to histopathology it would be difficult to assess pathological changes as a result of egg presence. However, this method would be faster and cheaper than histopathology and could detect a stage of infection when adults are no longer present in the heart (Figure 1). Gill wet preparations have been demonstrated to be more sensitive than parasitological detection of adult sanguinicolids in *Sparus aurata* due to the ability of wet preparations being able to detect infection when adults are not present in the fish host or when the adult infected organs are not targeted for dissection (Padros et al. 2001). The ELISA should also be considered as a diagnostic tool in further research despite the lack of accuracy of the test's parameters (Aiken et al. In Prep. (Chapter 8)). Immunoserological tests are the only tools capable of investigating the immune response of tuna against *C. forsteri* and this ELISA test was used successfully in the longitudinal study investigating such a response (Aiken et al. 2008 (Chapter 6)). The serological test is also the only test that can be used in non-destructive sampling.

Identification of the intermediate host should be a high priority for any further research on *C. forsteri* in *T. maccoyii*. Management actions are based on separating the intermediate host from that definitive host (Bullard and Overstreet 2002) and thus information is needed about the intermediate host and this is an identified priority for further *T. maccoyii* health research. Marine sanguinicolid cercariae have been detected from a number of intermediate hosts (Table 1.), however, only one complete life cycle is known; *Aporocotyle simplex* uses a polychaete intermediate host,



*Artacama proboscidea*, a sedentary tube-dwelling terebellid polychaete common on the muddy sea floor of Oresund, Denmark (Køie 1982).

Table 1. Sanguinicolid cercariae from polychaete (P) and bivalve (B) intermediate host species.

Adapted from Smith (1997) and Cribb et al. (2008).

Cercaria	Host	Locality	Reference(s)
"Aporocotylidae"	<i>Tagelus divisus</i> (B)	Biscayne Bay, Florida, USA	Fraser (1967)
<i>Cercaria amphicteis</i>	<i>Aphicteis gunneri floridus</i> (P)	Florida	Oglesby (1961)
<i>Aporocotyle simplex</i>	<i>Artacama proboscidea</i> (P)	Oresund, Denmark	Koie (1982)
<i>Cercaria hartmanae</i>	<i>Lanicides vayssierei</i> (P)	Ross Island (Antartica)	Martin (1952); Koie (1982)
<i>Cercaria loossi</i>	<i>Eupomatus dianthus</i> (P)	Woods Hole, Massachusetts, USA	Linton (1915a); Martin (1944); Rankin (1946); Stunkard (1983)
<i>Cercaria martini</i>	<i>Pecten irradians</i> (B)	Woods Hole, Massachusetts, USA	Linton (1915b); Stunkard (1983)
<i>Cercaria asymmetrica</i>	<i>Donax variabilis</i> (B)	Florida	Holliman (1961)
<i>Cercaria cristulata</i>	<i>Chione cancellata</i> (B)	Florida	Holliman (1961)
<i>Cercaria mercenariae</i>	<i>Mercenaria campechiensis</i> (B)	Galveston, Texas, USA	Wardle (1979)

It is highly likely that the intermediate host in Boston Bay and the intermediate host in Spain are either the same species or very closely related (Aiken et al. 2007 (Chapter 2)) as the prevailing parasitological paradigm is that each digenean species usually infects just a single first intermediate host species (Gibson and Bray 1994; Nunez and De Jong-Brink 1997), with both host ecology and host-parasite physiological compatibility driving this specificity (Adamson and Caira 1994; Sapp and Loker 2000a; Sapp and Loker 2000b).

Risk factor analysis in a case-control or cohort study would be an additional avenue of further research to provide information on *C. forsteri*. However for this type of analysis to be undertaken there would be a need for intensive monitoring of daily activities as well as a need for information on daily husbandry practices (Georgiadis et al. 2001). This information is considered by the tuna industry to be highly sensitive and confidential and therefore is hard to access. Some information needed

would include stocking densities, feed used, feed operations, management practices etc. In some sectors of the tuna industry there was an unwillingness to participate in epidemiological surveys as it was perceived that the industry was not being affected by any tuna health issues. This attitude must be addressed before undertaking any risk factor analysis as a close working relationship is needed in order for the study to produce meaningful results. Farm personnel would be needed during a risk factor study to help in procedures involving monitoring of the populations as well as recording any daily activities. However, production is always the first priority for any industry and determination of a final study design should be done in close consultation with the farm managers and personnel. Furthermore, accessibility of farming sites is sometimes limited and this is another area where help might be needed from farm personnel (Georgiadis et al. 2001). This is especially the case of research in the southern bluefin tuna industry where pontoons are located offshore and a close working relationship is needed to access the tuna.

There are also some technical difficulties in undertaking a risk factor analysis of *C. forsteri* in tuna. Primarily the adoption of the most appropriate case definition would be problematic. There would need to be a decision on whether infection, disease or subclinical infection would be the case definition. Infection is easily diagnosed through the observation of the adult fluke, however, tuna obviously have differing intensities of infection and the parasitological technique used to diagnose *C. forsteri* infection is unable to detect any migrating stages of *C. forsteri*. Therefore presence or absence of adult fluke may not be a true indication of the pathology experienced by the tuna. The same problem would occur for any diagnosis of the infection; absence or presence of gill lesions may not represent the degree of pathology experienced. As well, diagnosis based on clinical signs often has only moderate

sensitivity and specificity compared with laboratory diagnostic tests (Aiken et al. In Prep. (Chapter 7)). These lower accuracy estimates may be a result of differential misclassification occurring due to the diagnosticians becoming more familiar with disease manifestations over time and inadvertently biasing the result (Vågsholm et al. 1994; Kleinbaum et al., 1982). This possibility should be anticipated when designing aquatic epidemiological studies and strategies such as “blinding” of data collectors and laboratory personnel should be used to prevent differential misclassification.

Despite the technical difficulties a risk analysis is worth undertaking if supported by the industry. Although only a few such studies have been conducted in an aquaculture environment (Thorburn 1987; Thorburn 1993; Corsin 2002) this type of analysis can help define risk factors for infectious diseases in fish farms. Other risk factors, apart from the presence of a pathogen, may be associated with the disease such as stocking density or feed or other management practices but these have not been evaluated critically. In the absence of knowledge of such risk factors, it will be difficult to design management approaches to prevent or control their occurrence.

A risk factor analysis and identification of the intermediate host should be high priorities for future research. The major findings from this thesis will be useful in designing studies to investigate these research priorities. These findings provided the tuna industry with information about the infection patterns of *C. forsteri* and the immune response of *T. maccoyii* against *C. forsteri*. New diagnostic tool have been developed to aid in diagnosing the different life stages of *C. forsteri*. Additionally the tools use to diagnose *C. forsteri* infection have been assessed for accuracy. These

estimates of accuracy are now able to be used to determine the true prevalence of *C. forsteri* in farmed *T. maccoyii*.

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**Appendix 1. Electronic copies of stochastic models used in Chapter 4. Simulating blood fluke, *Cardicola forsteri*, infection in farmed southern bluefin tuna, *Thunnus maccoyii*, using stochastic models**

