FRDC ASBTIA:

Investigation of causes of mortalities in ranched SBT (southern bluefin tuna, *Thunnus maccoyii*)

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AUSTRALIAN SOUTHERN BLUEFIN TUNA INDUSTRY ASSOCIATION LTD (ASBTIA)

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OBJECTIVES:

1. To investigate causes of mortalities of ranched SBT in 2009, in particular 6-12 weeks mortalities
2. To suggest preventative measures and/or further research to reduce mortalities in the future

Non-Technical Summary

OUTCOMES ACHIEVED

This project has increased our understanding of the causes of mortalities in ranched SBT. Swimmer syndrome caused 6.5% of the investigated mortalities. Mortalities were highest during the second sampling period in early May. Mortalities and moribund SBT had a lower condition index than control fish at all sampling times, suggesting that either the cause of death is chronic, or it has a lethal effect only on compromised fish. Analysis of all industry data would be highly beneficial for the SBT industry as it would allow a much more robust statistical analysis. This project provided training to ASBTIA staff, SBT industry and research students. Future research priorities were proposed.

A total of 105 fish was examined, including 28 controls and 12 moribund/dead SBT (southern bluefin tuna, *Thunnus maccoyii*) that were examined for swimmer syndrome only. Swimmer syndrome (caused by *Uronema nigricans*) was the confirmed cause of mortality of 6.5% of the total number of moribund/dead fish. Swimmer syndrome was reported at higher water temperatures than previously observed. It is likely that there are multiple causes of morbidity, including swimmer syndrome, including tow-related factors.

On the basis of the results provided by companies that participated in the sampling, mortalities were highest during the second sampling period in early May. This was confirmed by results from the company that participated during

all sampling periods. Correlations with tow-related factors were not consistent for all sampling periods. During the first sampling in March, mortalities from 6-12 weeks were correlated with the number of mortalities in week 1. During the second sampling period in early May mortalities were correlated with the number of fish present initially in the tow pontoon, the initial condition index of the fish, the length of transfer from the tow pontoon to the grow-out pontoon and the number of fish in the grow-out pontoon. For the first two sampling periods 6-12 weeks mortalities were correlated with the number of mortalities during the tow. The lack of consistency between different sampling times for these correlations may be either due to the small datasets or different factors affecting mortalities at different times of the year, or at different temperatures. This could be tested if all tow data for the whole industry were analysed together. Analysis of all industry data would be highly beneficial for the SBT industry as it would allow a much more robust statistical analysis.

Mortalities and moribund SBT had a lower condition index than control fish at all sampling times, suggesting that either the cause of death is chronic, or it has a lethal effect only on compromised fish. The higher blood acidity of mortalities and moribunds may be related to stress and the subsequent increase in lactate level. Mortalities and moribund fish did not appear to be able to osmoregulate and maintain the balance of salt in their blood, however it is unknown if this contributed to the death or if it was a part of the death process. Preliminary analyses of cholesterol showed that moribund SBT had elevated total cholesterol and in particular nonHDL cholesterol in comparison to the control fish. This may be a significant result and should be investigated further.

The ability to analyse all industry data would make it more likely to define patterns of mortality and risk factors. Experimental manipulations may be necessary to identify factors that cause the death of SBT.

KEYWORDS:
SBT, ranching, health, mortality

Acknowledgments

This study formed part of ASBTIA research, and was supported by the Fisheries R&D Corporation. We would like to thank the SBT industry for their collaboration and support. This project would not be possible without their help. We would like to thank the SBT industry, in particular those companies that supplied SBT and data. We really appreciate their enthusiasm and persistence, even when it became apparent that no simple answers could be provided. Elizabeth Nosworthy and Clare Webber are thanked for all their help with sampling and examination of SBT. We couldn't do this project without you. David organised all the logistics and was most helpful with all the sampling. Additionally, Elizabeth and Clare are thanked for detecting all *Uronema* found in 2009 and gathering all the data for the project. Karine Cadoret and Dr Melanie Leef did many of the laboratory analyses that are included in this report. We would like to thank Dr Ryan Wilkinson for analysis of stress indicators and Dr Nathan Bott (SARDI) for PCR analysis. David Allen collected wild and end of tow SBT samples.

We would like to acknowledge expert advice from Dr Brian Jones (WA Fisheries), Prof Richard Whittington and Dr Michelle Dennis (University of Sydney) and Prof Hugh Ferguson (University of Stirling). We thank Dr Craig Hayward (SARDI) for his hospitality during our trips to Port Lincoln. We are grateful to AProf Tom Cribb (University of Queensland) and Dr Rob Adlard (Queensland Museum) for their helpful comments.

Background

In the last two years the SBT industry has experienced an increase in mortalities resulting in direct losses. In addition, SBT production has incurred changes in product quality associated with variable condition index, and growth also appears to have been compromised. It is estimated that the combined effect of all these production issues is costing the industry approximately AUD$25 million per year.

The current SBT health project aimed at improving husbandry and performance of SBT through better understanding of the relationship of fish stress and health (2006/225 - Variation) demonstrated that SBT subjected to stress had significantly increased levels of copepods *Caligus* spp. (mostly *Caligus chiastos*) and blood fluke *Cardicola forsteri* in some ranching pontoons. Recorded infection levels in some experimental SBT had increased significantly from previously identified levels by up to 4 times for blood fluke and 10 times for copepods. Although not present in season 2008, a ciliate that causes SBT mortality (swimmers disease) *Uronema nigricans* may have an adverse effect on SBT production by causing mortality in infected fish.

The SBT industry continues to experience an upward trend in mortality, with one investigation suggesting the blood fluke *Cardicola forsteri* as a cause (Landos, unpublished). This could not be confirmed in other investigations of 2008 mortalities. Furthermore, an increase in the numbers of copepods and a relationship between the high number of copepods and low condition index was reported by Hayward et al (2008). However, the exact cause or causes of mortalities have not been identified. It is possible that a combination of factors is needed to cause the mortalities. We need to define the primary cause of death as well as the contributing factors to be able to reduce SBT mortalities.

There are gaps in our knowledge of what causes mortalities and we need this information to understand what has been affecting the excellent health status of SBT for the last two to three years. We need to understand the cause of mortality as evidence suggests that whatever is causing mortalities is also having an impact on feed intake and therefore growth and possibly product quality. The scope of this study fits within objective 3 of project 2006/225 (To determine relationship between SBT health, stress and fish performance).

Recently funded project 2008/228 will improve our understanding of SBT parasites and develop management strategies to reduce their effects. However, neither that project nor project 2006/225 addresses the investigation of causes of SBT mortalities during ranching.
This project has been developed as a result of the growing concern in the industry with regard to the increase in mortalities and is consistent with the priorities defined in the Southern Bluefin Tuna Aquaculture (Wild-Capture) Strategic Plan - Towards 2012: Striving for a Profitable and Sustainable Future, and the SBT Research Program 2008 - 2012.

Need

The tuna industry in Australia is limited by catch quota. Increased competitiveness through production efficiency is now the most practical way to improve the value of the industry. Improved performance can be achieved through reduction of mortalities and optimising production. These issues will also be crucial for longer term holding, when the initial size of tuna will be smaller and the fish will be ranched for a longer time, increasing health risks to the tuna.

Since 2002 there has been a significant fall in revenue due to falling prices (down over 50%) and the strengthening Australian dollar. Worsening financial parameters, combined with the intrinsic high 'value' of each fish have placed a greater focus on all aspects of the industry and particularly on stress impacts limiting production. Reducing mortality is an obviously desirable improvement making the current, increased level of mortality even less acceptable.

While FRDC project 2008/228 investigates SBT parasites and their impact on ranched SBT, it does not include a broad approach investigation of causes of SBT mortalities. There is an urgent need for the identification of specific causes of SBT mortalities.

Objectives

1. To investigate causes of mortalities of ranched SBT in 2009, in particular 6-12 weeks mortalities

2. To suggest preventative measures and/or further research to reduce mortalities in the future

Methods

Mort sampling focused on three main periods in April, May and June 2009. These times corresponded to the mortality peaks for most companies during 8-12 weeks post-transfer mortalities. The sampling was done over 7 days each time. During that time morts (including moribund individuals) and apparently normal (harvest) fish were sampled (total 105 fish). A vessel was provided by the industry as a research platform to be positioned near where mortalities occur and any SBT fitting the sampling description (moribund or very fresh mortality) was ferried to the vessel for the taking of samples. This was essential as in our experience morts provided to ASBTIA laboratory were delayed in transit resulting in suboptimal samples. The quality of samples examined has a direct impact on the potential for diagnosis. To ensure that fresh morts and moribund fish were available we located much of our research work in the area where mortalities occurred.

Fish weight and length were measured. Gross appearance and presence of parasites were determined for each fish. Parasitology examination covered the presence of adult blood fluke *Cardicola forsteri* in heart and the presence of metazoan parasites on gills and skin. Any unknown parasites were preserved for identification. Blood samples, microbiology and histology samples were collected, processed and analysed. The following analyses were done: blood (haemoglobin content, osmolality, blood pH, glucose, lactate, cortisol, lysozyme and total immunoglobulin), histology (all organs – haematoxylin and eosin, followed up by special stains if required), microbiology (spleen and kidney, lesions – blood agar and TCBS agar, smears, growth followed by biochemical analyses – all microbiology samples were processed as soon as possible in Port Lincoln, agar plates were sent to Tasmania, analysis done by Fish Health Unit, DPIPWE). Results for morts and harvest fish for all measured variables were compared.

Detailed methods are provided in Appendix 3.

Results/Discussion

Associations between cumulative mortalities and different parameters related to tow and grow-out conditions

Cumulative mortalities of SBT–between weeks 6 and 12 of culture- grouped by grow-out pontoon, were correlated with different parameters related to culture conditions during the tow and grow-out periods, in order to investigate any possible associations between these variables. Cumulative mortalities for each sampling time were correlated with:

- Number of fish present initially in the tow pontoon**
- Initial condition index of the fish (when put in tow pontoon)**
- Number of mortalities during the tow time
- Length of transfer (in minutes) from tow pontoon to grow-out pontoon**
- Number of fish in each grow out pontoon**
- Number of mortalities in week 1*

Correlations labelled with one star * were significant during the first sampling time, while those marked with two stars ** were significant during the second sampling time. No statistically significant correlations were found during the third sampling period. These differences for each sampling period suggest that either the data sets were too limited or that different factors affect SBT at different time of the year (possibly related to temperature or other seasonal effects).

The positive correlation between cumulative mortalities and the number of mortalities during the first week, could be demonstrating that fish which show poor condition (health status) when caught and/or transferred are more likely to die during the culture period (Figure 1).

Contrary to what was observed during the mortality investigation, which showed that mortalities had lower condition index than controls, higher condition index at the beginning of the tow period was positively correlated with higher cumulative mortalities. However, this could be a spurious relationship, as the tow pontoon with SBT with highest condition index also had highest fish density and longest transfer time. Thus, it is possible that the two other factors affected mortality and not the high condition index.

On the other hand, the positive associations observed between the three factors - length of transfer, number of fish in tow and grow-out pontoons - and cumulative mortalities could be related to the level of stress fish are subjected during husbandry (Figure 2 and 3). Stressors, applied to intensive cultured fish, can have adverse effects on growth and immunocompetence of fish, resulting in an increased mortality.

An interesting observation for those fish obtained during the second sampling, is that the pontoon from Company 6, which had the highest mortalities, is also the pontoon with the longest transfer time, and the largest number of fish in the tow and grow-out pontoons. It could be possible that the high number of

mortalities could be due to the cumulative effect of a number of “poor” husbandry conditions.

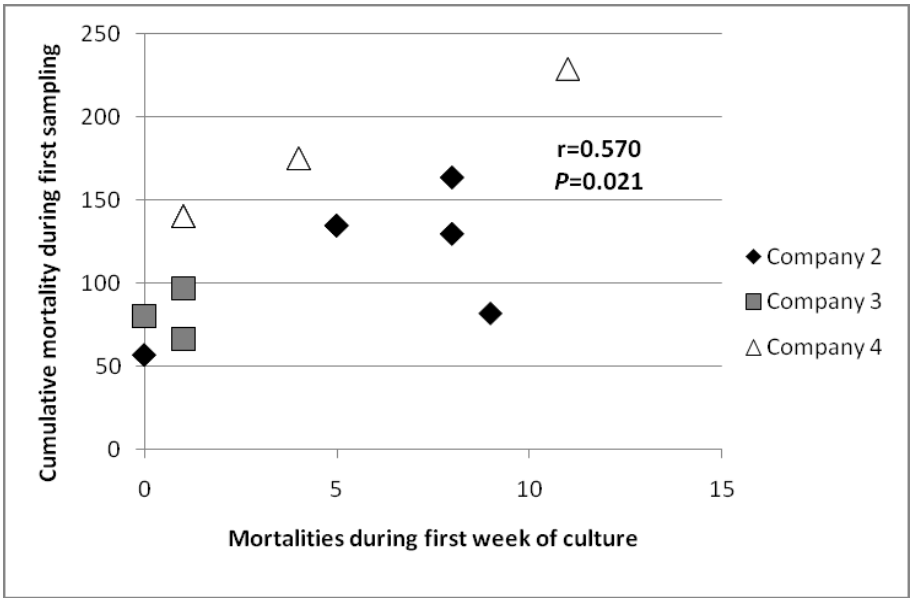

Figure 1. Cumulative mortalities of tuna per pontoon between weeks 6 and 12 post-transfer v/s mortalities during the first week of culture. Data obtained during first sampling time, from three different companies between 6 and 12 weeks of culture.

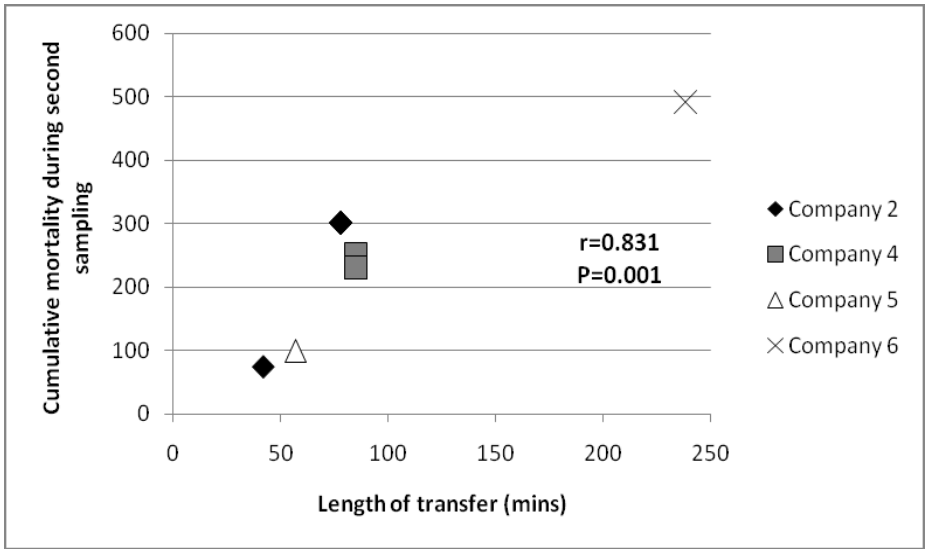

Figure 2. Cumulative mortalities of tuna per pontoon between weeks 6 and 12 post-transfer v/s length of transfer in minutes from tow pontoon into grow out pontoon. Data obtained during second sampling time, from four different companies between 6 and 12 weeks of culture.

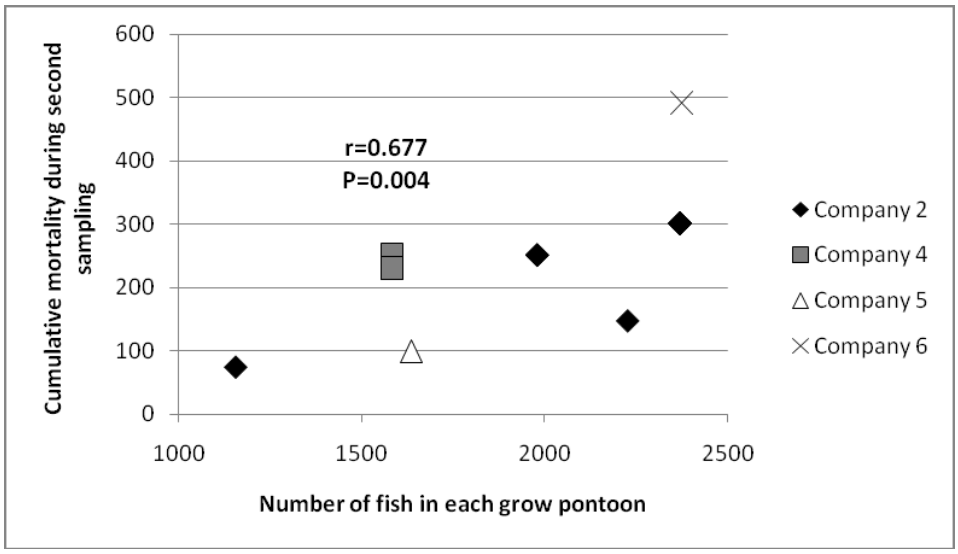

Figure 3. Cumulative mortalities of tuna per pontoon between weeks 6 and 12 post-transfer v/s number of fish in each grow-out pontoon. Data obtained during second sampling time, from four different companies between 6 and 12 weeks of culture.

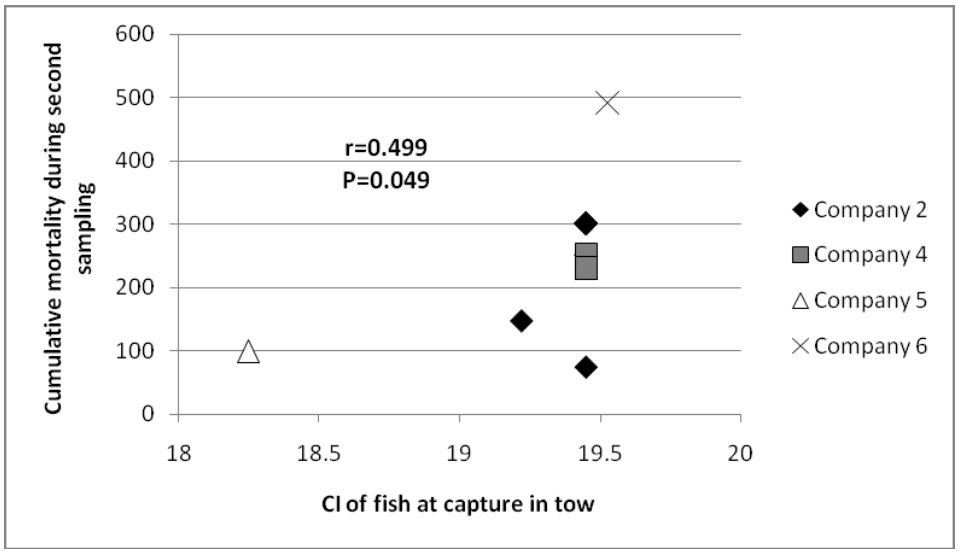

Figure 4. Cumulative mortalities of tuna per pontoon between weeks 6 and 12 post-transfer v/s condition index (CI) of fish at the moment of capture. Data obtained during second sampling time, from four different companies between 6 and 12 weeks of culture.

Cumulative mortalities between week 6 and 12 post-transfer were positively correlated with the condition index of the fish at the time of capture (Figure 4). However this relationship was only present during the second sampling time. This may be related to the small sample size, the fact that different companies provided fish each time or that there were different causes of mortalities at different times of the year.

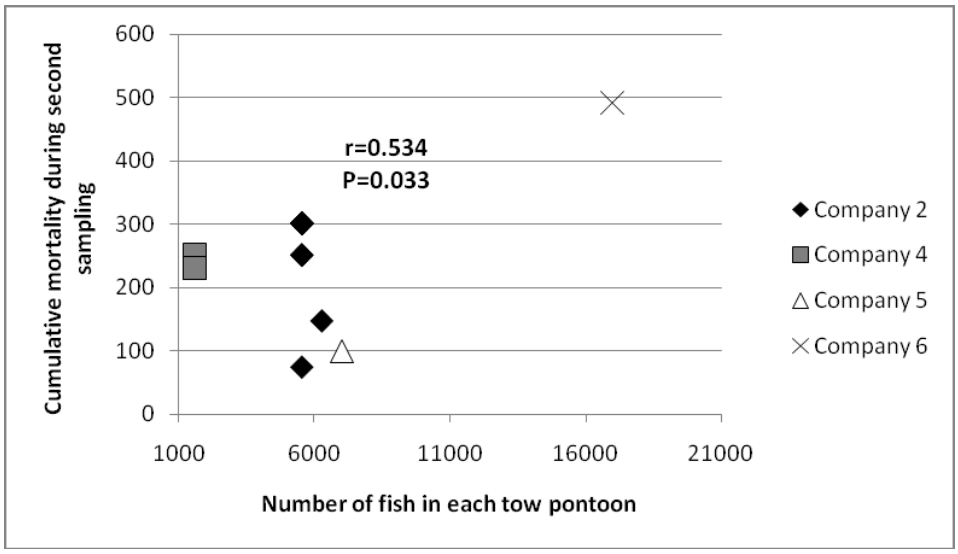

Figure 5. Cumulative mortalities of tuna per pontoon between weeks 6 and 12 post-transfer v/s number of fish in tow pontoon. Data obtained during second sampling time, from four different companies between 6 and 12 weeks of culture.

It is important to mention that if the data point from company 6 is removed from the correlation between cumulative mortalities and the number of fish in each tow pontoon (Figure 5) this correlation is no longer statistically significant (r=-0.137, P=0.626). It might be that when the density of fish is low in the tow pontoon (within a particular range), there is no effect in the mortalities observed during culture. However, when the density in the tow pontoon reaches a threshold, it might have an increasing effect on the mortalities observed later during culture.

There were significant differences in the cumulative mortalities of SBT between different companies (Figure 6). Even though all fish were sampled during weeks 6 to 10 post-transfer, it appears that the cumulative mortalities were higher for those fish examined during the second sampling time. This trend was confirmed by the results for company 2, which provided fish in all three sampling periods and had greatest mortalities in the second period.

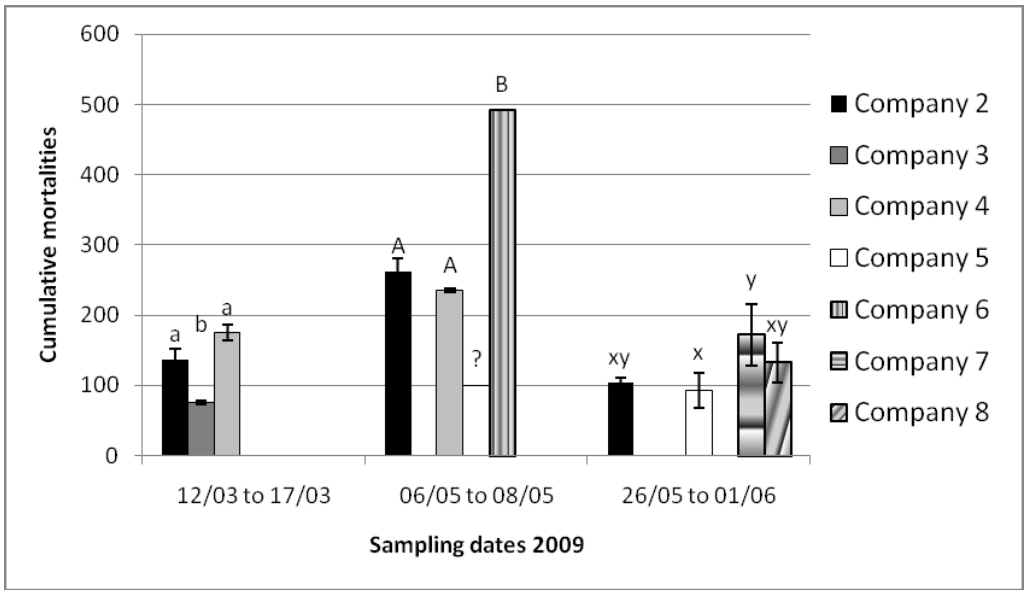

Figure 6. Cumulative mortality ± standard error of tuna in different companies between 6 and 12 weeks of culture, for three different sampling dates. Means with different letters within each sampling time were significantly different from one another (one-way ANOVA; 1st sampling: F=19.125, *df* 2,29, P<0.001; 2nd sampling: F=26.152, *df* 3,26, P<0.001; 3rd sampling: F=3.047, *df* 3,27, P=0.046. Company 5 had to be removed from the post-hoc analyses for the second sampling due to lack of replication).

Differences in tow temperatures and their correlations with cumulative mortalities

Five companies that participated in the mortality investigation provided the temperature data for their tows. Temperatures were compared with cumulative mortalities between 6-12 weeks post transfer.

The average temperature during the tow time was significantly different among the companies (F=5.581, df 4,90 P<0.001). The company with the largest cumulative mortality was also the one with the highest average temperature (20.15^{o}C) during the tow and the highest initial tow temperature (20.6^{o}C). However, this was not observed inversely: companies with low cumulative mortalities did not always have the lowest temperature (Figure 7). A correlation analysis was run in order to establish any relationship between these variables. No significant association was found between cumulative mortalities and average tow temperature (r=0.587, P=0299), temperature at the beginning of the tow (r=0.252, P=0.682) or at the end of the tow (r=-0.136, P=0.827).

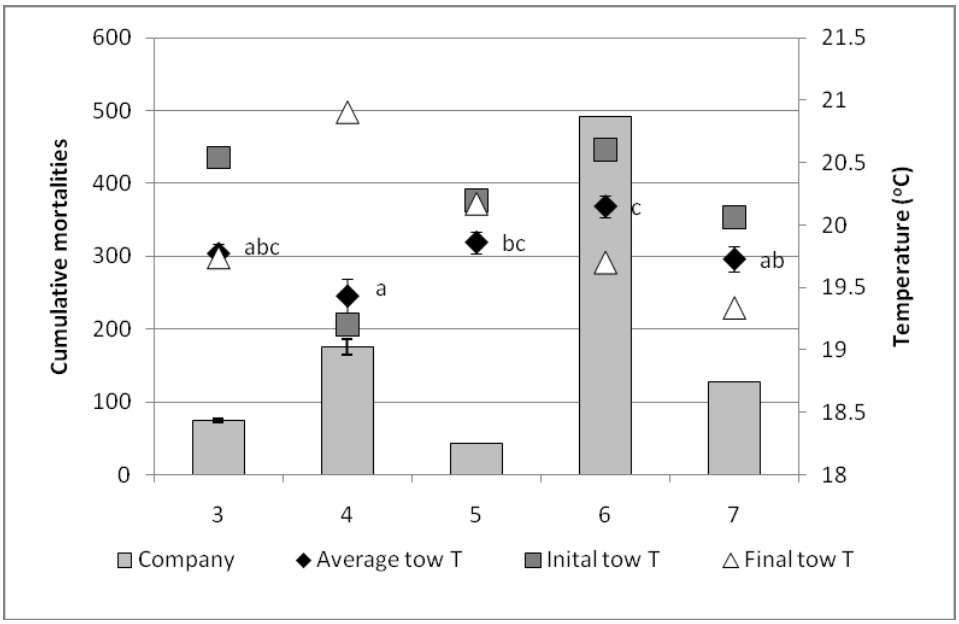

Figure 7. Average cumulative mortalities ± standard error of tuna per pontoon between weeks 6 and 12 post-transfer by company and average, initial and final tow temperatures for each company and average temperature during tow ± standard error. Average tow temperatures with different letters are statistically different from one another (F=5.581, df 4,90 P<0.001).

Additionally, differences between the max and the min temperature for the whole tow period and differences within daily temperatures were calculated for each company.

Cumulative mortalities were not significantly correlated with any of the three parameters evaluated:

- Difference between max and min temperature for the whole tow period
- Average of daily temperature differences
- Max daily temperature difference

However, a trend was observed showing an increase in cumulative mortalities when the average difference and maximum difference in daily temperatures both decreased (Figure 8).

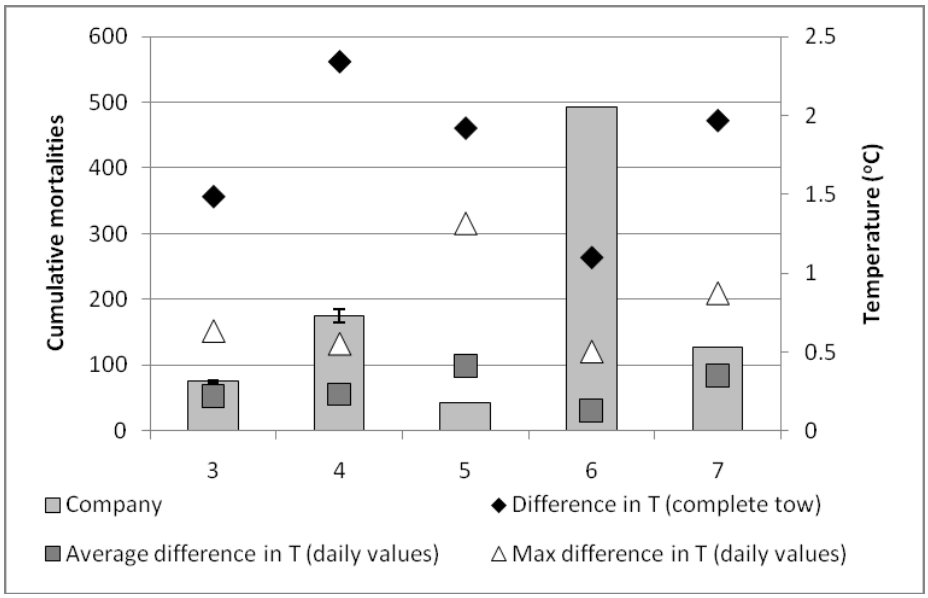

Figure 8. Average cumulative mortalities ± standard error of tuna per pontoon between weeks 6 and 12 post-transfer by company, average difference between minimum and maximum tow temperature (daily and for the whole tow) and maximum daily difference in temperature of tow period for each company.

Condition Index

During all three sampling times, the mean condition index (CI) was significantly greater for control fish than for M/D fish (Table 1, 1st Sampling: $F=8.211$, df 1,30, $P=0.001$; 2nd sampling: $F=22.987$, df 1,28, $P<0.001$; 3rd sampling: $F=31.176$, df 1,29, $P<0.001$). During the first sampling time in late March, three control fish had condition index below 18 and during the early May sampling only mortalities (7) and a moribund had condition index below 18. During the sampling conducted in late May/early June, only one mort had a condition index below 18 and all controls presented condition indices above 22. This difference suggests that the mortalities and moribund fish were compromised. In any of the three sampling times, the CI of the mortalities and the moribunds fish were significantly different. The death was either a result of a longer term problem (which resulted in lower CI) or a problem which affected only already compromised fish (which already had lower CI).

Table 1. Average condition index ± standard error (SE) of control and moribund/dead (M/D) tuna sampled during 2009. Average values with different letters within each sampling time were statistically different.

	1st Sampling			2nd Sampling			3rd Sampling		
	n	Average	±SE	n	Average	±SE	n	Average	±SE
Control	10	20.85^{a}	2.90	8	21.25^{A}	0.526	10	24.26^{X}	0.456
M/D	22	18.30^{b}	2.04	22	18.09^{B}	0.348	21	19.89^{y}	0.491

Parasitology

Prevalence (or percentage of infected fish from the total number of fish) and intensity (or average number of parasites in the infected fish) of parasitic infections appeared to be consistent with those reported in the literature for SBT at similar period of time post-transfer for control and M/D fish during all the sampling periods, with the exception of the gill fluke *Hexostoma thynni* during the third sampling, which showed intensity slightly higher than that previously reported. Other gill parasites were at low prevalence and intensity at all times.

With the exception of the second sampling, the percentage of SBT infected with gill fluke *H. thynni* was higher in controls than in the M/D group. Prevalence and intensity for *H. thynni* was higher during the third sampling in both, the control and the M/D groups. Intensity and abundance were lower in the M/D group when compared to the controls during the first two samplings, but this tendency reversed over the third sampling time.

While intensity and abundance of gill copepod *Pseudocycnus appendiculatus* remained constant for the control group throughout the 2009 samplings, the M/D group showed the highest mean intensity during the third sampling. In contrast, prevalence of this gill copepod for both control and M/D SBT was higher during the first sampling period. No considerable differences in prevalence and intensity were observed within each sampling time between controls and M/D fish.

The gill copepod *Euryphorus brachypterus*, infected the greatest percentage of M/D during the second sampling, while the largest percentage of control fish was affected during the third sampling period. The copepod infected notoriously more M/D fish than controls during the first two sampling times, while the proportion of infected control fish reached similar magnitude than that of M/D fish during the third sampling. Across all sampling periods, intensity and abundance seemed to be higher during the first and second samplings for control and M/D fish, respectively. M/D appeared to present more parasites per infected fish than controls in all three samplings.

The sea lice, *Caligus* spp., were found in control fish only during the first sampling – in relative low prevalence- and in the third sampling – with the highest prevalence reported during 2009. With the exception of the third sampling, sea lice infected a greater percentage of M/D than control fish, with half or more of the SBT affected during the first two samplings. For all three samplings, the number of sea lice on infected SBT (intensity of infection) was greater in M/D group. The highest intensity of infection for control fish was observed during the third sampling time, while in the M/D group, the highest intensity was found during the first sampling period.

Presence of blood fluke, *C. forsteri* was examined by heart flushes and counts of adults. Prevalence of blood fluke infection was almost double in M/D fish of that in control fish during the second sampling, however the prevalence was greater in control fish during the first and third sampling times and in this last one, prevalence of *C. forsteri* in control fish was the highest from all 3 times.

Prevalence of infection in M/D fish was very consistent throughout 2009, with values ranging from 56% to 67%. However, the control group showed a marked decay in the blood fluke prevalence during the second sampling time. The intensity of infection of the blood fluke (or number of parasites in the infected SBT) was lower in the M/D group when compared to the controls in all three sampling times. The intensity seemed to reach a peak for control fish during the second sampling, but in the M/D group, the highest intensity value was observed in the first sampling and then decreased over the next two periods. Two parasites (presumably *C. forsteri*) were found lodged in the afferent artery of the primary filament in one mort when gills were flushed, during the second sampling. Gill flushes from additional harvest fish were examined for the presence of adult blood flukes. Some of the harvest SBT had adult blood flukes in their gills.

Table 2. Number of fish sampled (n), prevalence (%), intensity and abundance of the sea lice, blood fluke and three gill parasites in tuna during three sampling periods of 2009. Definitions for prevalence, intensity and abundance are provided in Appendix 3 (Bush et al 1997).

		1st Sampling				2nd Sampling				3rd Sampling			
		n	Prevalence	Intensity	Abundance	n	Prevalence	Intensity	Abundance	N	Prevalence	Intensity	Abundance
Caligus spp (sea lice)	Control	10	10	1	0.1	8	0	0	0	10	70	1.28	0.9
	M/D	22	56	3.2	2.7	22	50	2.18	1.1	21	19.04	3.12	2.5
C. forsteri (blood fluke)	Control	10	70	5.4	3.1	8	37.5	6.33	2.4	10	80	3.12	2.5
	M/D	22	56	4.7	2.7	22	63.6	3.42	2.2	21	57.14	1.75	1.0
H. thynni (gill fluke)	Control	10	10	2	0.2	8	0	0	0	10	20	7	1.4
	M/D	22	8.6	3.5	0.3	22	4.5	1	0.04	21	19.04	4.25	0.8
P. appendiculatus (gill copepod)	Control	10	20	1	0.1	8	12.5	1	0.1	10	10	1	0.1
	M/D	22	17.4	1.5	0.3	22	13.6	1	0.1	21	14.28	2	0.3
E. brachypterus (gill copepod)	Control	10	10	2	0.2	8	0	0	0	10	20	1	0.2
	M/D	22	30.4	2.7	0.9	22	40.9	3.2	1.3	21	19.04	1.25	0.2

The mean intensity of infection (average number of parasites/infected SBT) was overall very low, much lower than reported in the experimental pontoons

in 2008, and it is unlikely that the presence of any of these parasites could be the primary cause of the SBT mortality.

U. nigricans was observed only in one moribund fish (suspected swimmer – confirmed by microscopic examination of cerebrospinal fluid, Figure 9) during the second sampling time. However, during the time the investigation was carried out, two companies submitted a total of 12 fish to be examined, 3 in April from one company and 9 in July from another company, which were suspected swimmers. All of the fish from April and one from July were positive to the presence of the ciliate as determined by examination of cerebrospinal fluid under a microscope, and later the infection was confirmed by positive PCR results for olfactory rosettes from the fish submitted in April (N. Bott pers. comm.). It is important to mention that the swimmer in April was in higher water temperature than previously reported (18.7^{o}C). The confirmation of infection means that 6.5% of the total number of M/D fish died due to swimmer syndrome.

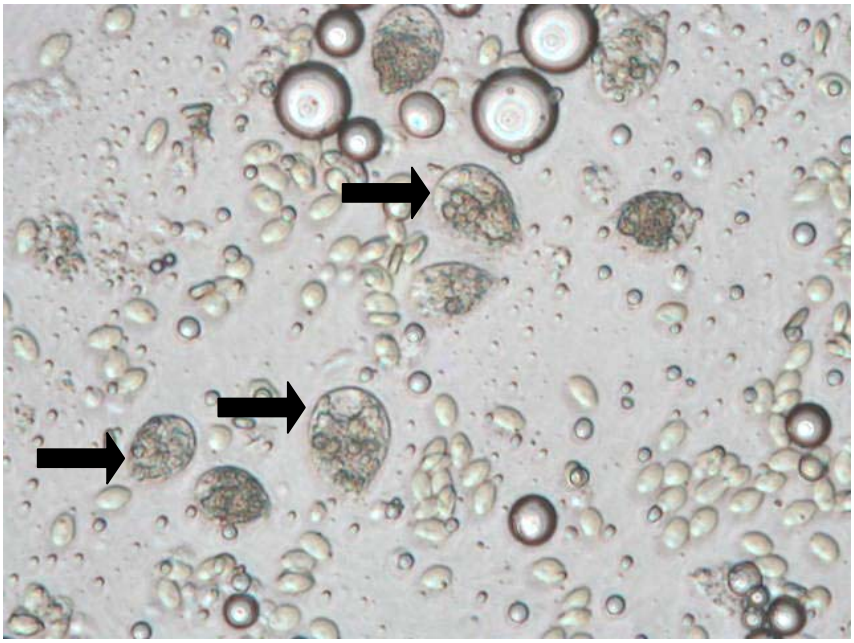

Figure 9. The ciliate *Uronema nigricans* (arrows) present in the cerebrospinal fluid of a "swimmer" tuna examined in April 2009 (photo Elizabeth Nosworthy).

Didymozoids were found in SBT during all three sampling times. 22.7% of the M/D fish and 20% of the controls presented didymozoids in the olfactory rosette during the first sampling. During the second sampling period, 25% of control fish presented them in the intestinal tract and in 40.9% of the M/D fish in various internal organs as diverse as heart, pyloric caecae and olfactory rosette. Finally, didymozoids were observed in 46.8% of all fish during the third sampling, corresponding to 60% of the olfactory rosettes of all control fish - an increase from the numbers observed in the early May 2009 sampling

- and in 40.9% M/D fish - percentage similar to that observed during the previous sampling - in organs such as olfactory rosette, skin and gills arches.

Gross Pathology

No major lesions were observed on the external surface of both normal and M/D fish sampled during 2009. No corneal opacity or eye damage was observed in any of the SBT collected (except one fish had one white pinpoint size lesion in one eye during the second sampling), and only minor damage to the skin was observed in a few M/D animals, probably related with handling and rubbing of the bottom of the net.

The main observation of concern was the dark banding in the gills as shown in Figure 10, which affected 68.1% and 59.1% of M/D fish during the second and third samplings, respectively. In addition, during the second sampling, two gill lesions were sampled from a particular fish for histology. One was characterised as a decolouration of an area approximately 5 cm^2 (Figure 11). The second one was a white focal lesion, which appeared to be scar tissue (Figure 12).

Small bivalves were observed lodged between the gill lamellae of 4 fresh mortalities during the second sampling and in 1 moribund and 2 fresh mortalities, during the third sampling (Figure 13). These bivalves, common in net biofouling, seemed to separate the filaments and inflammatory response could be observed in the gills (Figure 13). At the time when the bivalves were found, the companies reported that they did not clean nets on the pontoons affected at the time of sampling or immediately before.

Similarly, no significant lesions were observed in the internal organs of tuna. During the first sampling period, only one fish showed considerable congestion in the liver and spleen, but it could have been a post-mortem change or a non-specific sign of acute stress. Additionally, another mortality presented small white lesions on the external surface of the heart, which were sampled for histology. During the second sampling, one fish showed changes in the appearance of the internal organs. Liver and serosa as well as intestinal content appeared extremely yellow in colour, and it was suspected that the fish presented jaundice. This finding was later associated with a low haemoglobin content and high cortisol levels for this individual.

An increase in opacity in the swim bladder tissue, which gave the appearance of a white “net” was observed in 2 controls and 8 M/D fish during the second sampling and in a few SBT during the third sampling (Figure 14). This was caused by blood vessels drained of blood, possibly a post-mortem change. No accumulation of fluid in pericardial sac (as reported in 2008 by Dr Landos) was found of any of the fish sampled during 2009. However, this may be due to a different dissection method.

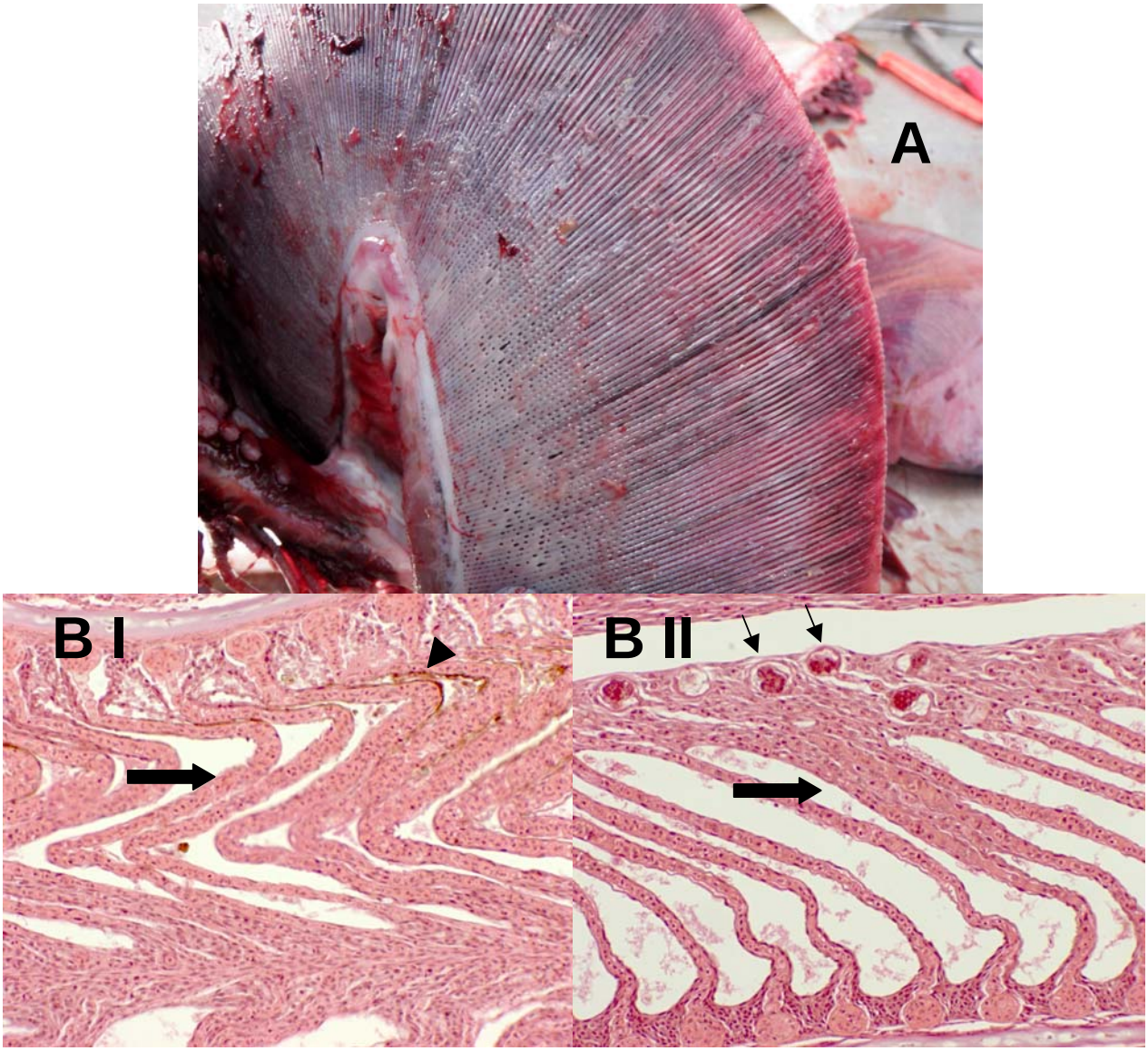

Figure 10. Dark banding of gills (A) was sometimes associated with the presence of blood fluke eggs (BII). Eggs seemed to be located in the outer marginal channel of the lamellae (BII). Dark brown precipitate was observed on the margin of the gill lamellae (BI, arrow head), it was negative for iron by Perls staining (not shown).

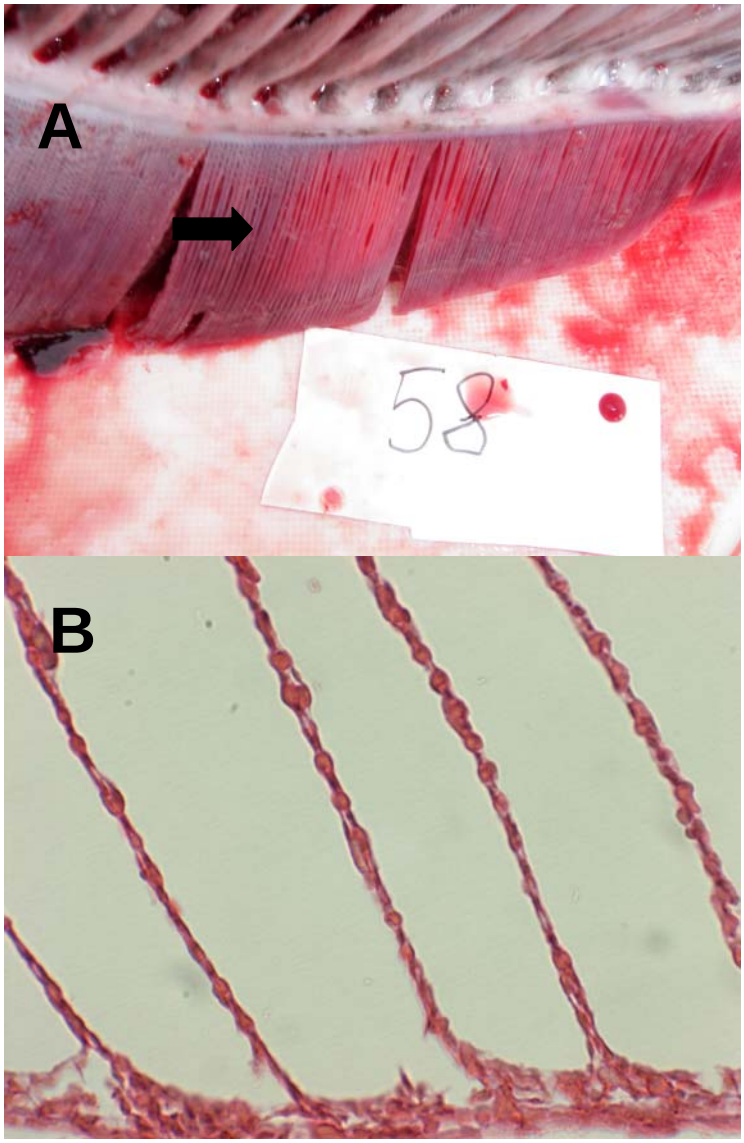

Figure 11. A: Macroscopic lesion in the gill of tuna during second sampling. B: Gill lamellae showing drainage of the blood from the lamellae, reduced thickness and an epithelial and mucous cell atrophy, with apparent erosion of respiratory epithelium.

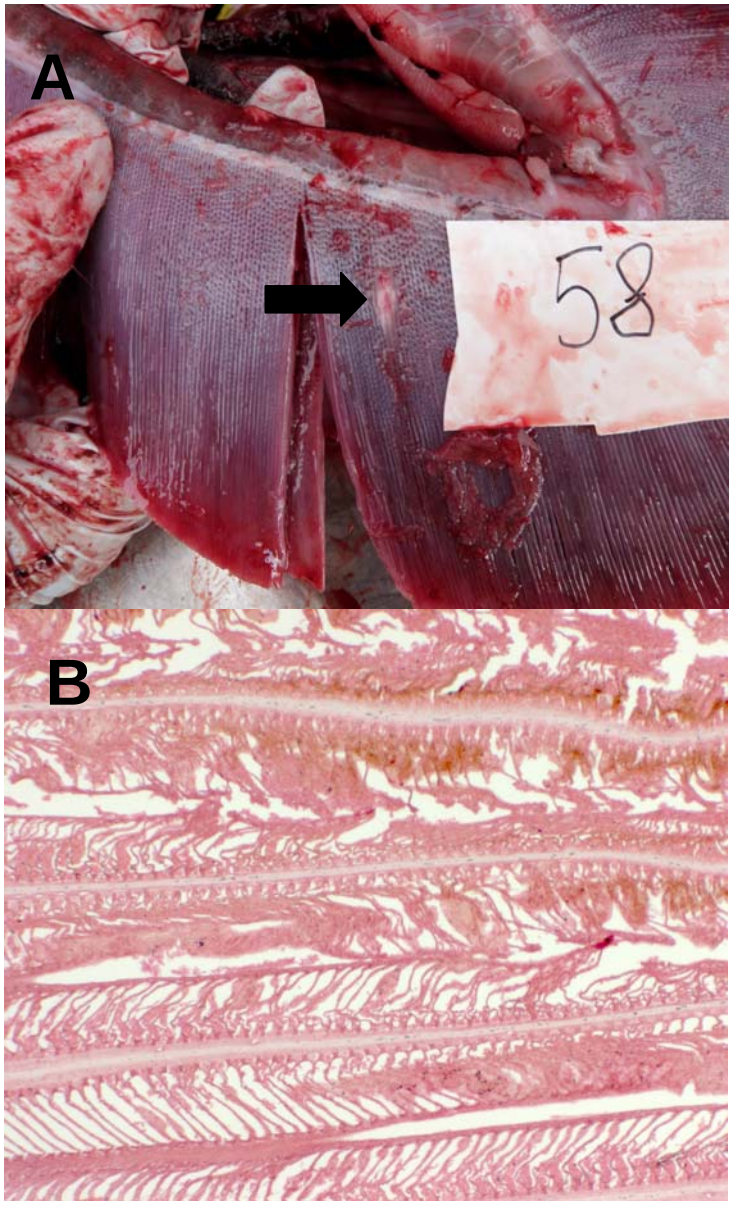

Figure 12. A: Macroscopic white focal gill lesion. B: Gill lesion showing an almost complete loss of lamellar structure, with extended areas of lamellar fusion and necrosis (upper half of image). The lower half of the image shows a more normal gills structure.

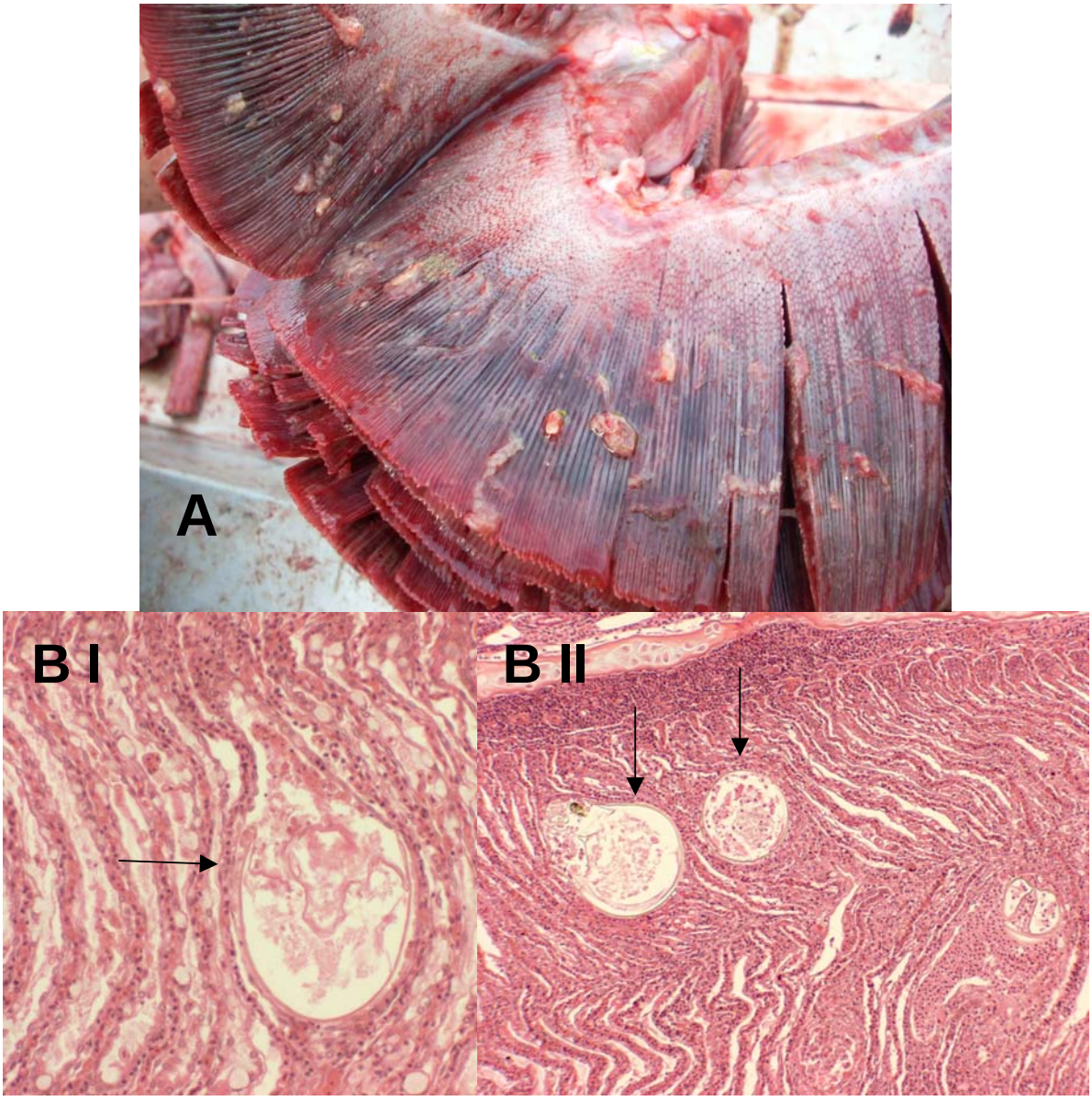

Figure 13. A: Gills showing the presence of bivalves between the filaments . B: Unidentified bivalve (arrows) in the gills of tuna. Gill responses to these parasites varied from non-existent (I) to haemorrhage and filamental/lamellar thickening (II), which was associated with attachment of the parasite or its presence in larger numbers.

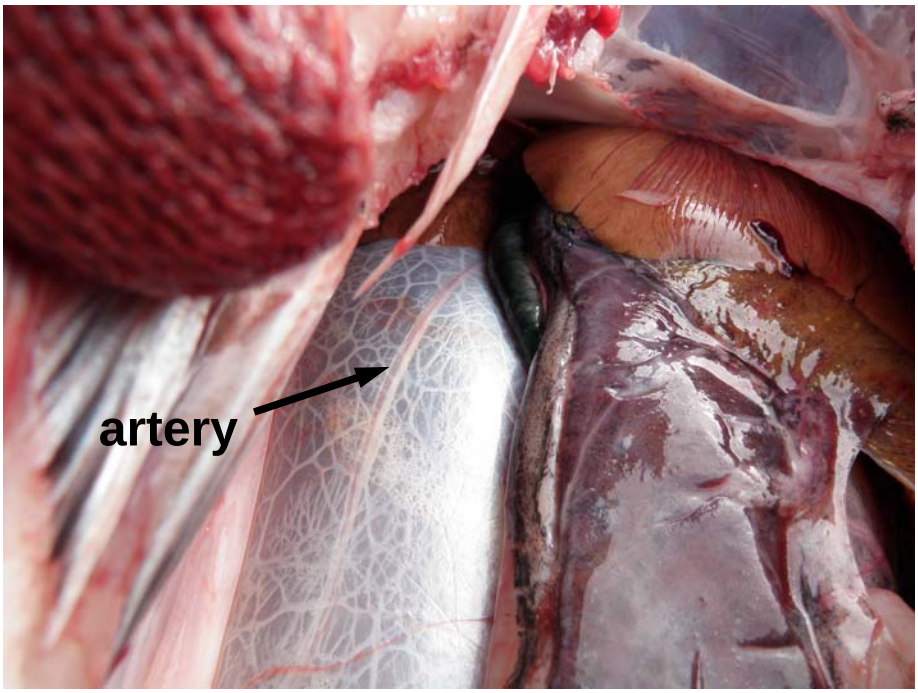

Figure 14. Swim bladder showing prominent blood vessels, which are drained of blood (possibly post-mortem change).

Histopathology

The macroscopic dark banding observed during sampling was associated with hyperperfusion of gills with erythrocytes, which showed associated epithelial lifting/oedema, occasional necrosis and haemorrhage, variable inflammation and hyperplasia. These finding were also associated with the presence of *C. forsteri* eggs (though they were not always found in histological sections), which were observed occasionally within lamellae but usually within the lamellar outer marginal channels.

This hyperperfusion of gills was observable as multiple lamellae frequently congested with erythrocytes and a collapse of the pillar cell structural network within congested lamellae. Sinusoidal spaces within the filaments aligning the cartilage were often congested with erythrocytes. These findings associated the banding observed macroscopically in the gills with a certain degree of hyperperfusion and congestion in the gills. However, the origin of this pathology is not clear. Congestion, hyperplasia and hyperperfusion were not always observed in the presence of eggs and the same was observed conversely (Figure 10). A dark brown precipitate was observed lining the margin of lamellae. Pearls-Prussian blue stain, which identifies ferric iron, fail to show presence of iron.

The two macroscopic lesions observed during the second sampling corresponded to, in the case of lesion showed in Figure 11, drainage of the blood from the lamellae, associated with reduction of the lamellae thickness, epithelial and mucous cell atrophy and possible erosion of the respiratory epithelium (Figure 11). The white focal lesion (Figure 12) was associated with

a total loss of lamellar structure, with extended areas of lamellar fusion and necrosis.

In addition, it was observed that fish from the second and third samplings– both control and M/D- were affected with an unidentified metazoan organism, which corresponded to the mini-bivalves observed macroscopically, similar to bivalves found in biofouling. Gill responses to these parasites varied but their presence was associated with haemorrhage, telangiactasis, filamental/ lamellar thickening, and epithelial and mucous cell hyperplasia. The latter three signs were more often associated with larger numbers of the parasite and where attachment appeared to have been prolonged as the parasite's integrity diminished (Figure 13).

Prevalence appeared to be higher in M/D fish than in controls for all the sampling times. The number of fish that showed the presence of *C. forsteri* eggs in hearts and gills was not consistent throughout the sampling times. During the first sampling time, eggs and granulomas were found in histological sections of heart of 64% of M/D fish and 40% of control fish. 91% of M/D fish and 25% of control fish presented eggs and granulomas during the second sampling, while over 86% of M/D fish and 20% of control SBT presented eggs in the heart muscle during the third sampling (Figure 15). Prevalence appeared to decrease in the control fish.

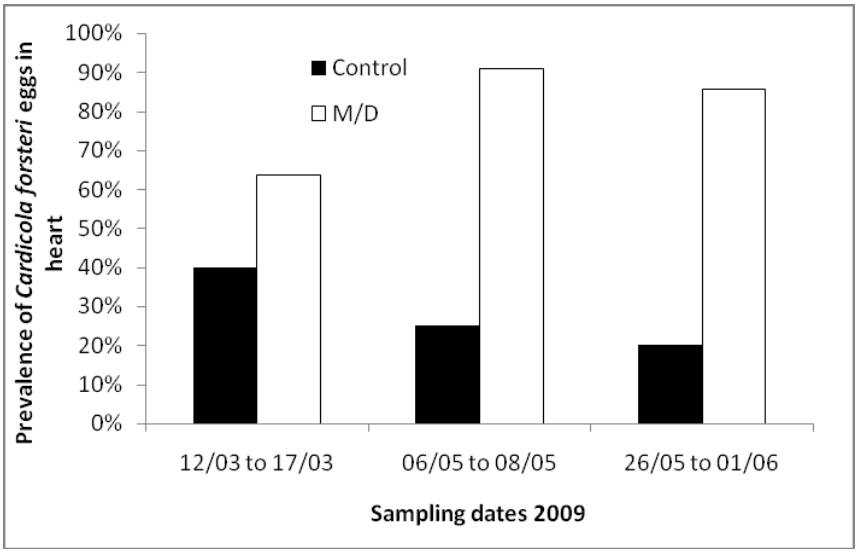

Figure 15. Prevalence of blood fluke (*Cardicola forsteri*) eggs and associated granulomatous reaction in heart muscle of tuna during three samplings in 2009.

The number of eggs in heart sections was also very low (usually fewer than 10 eggs in 2-3 sections during the first sampling time, with the highest count being just over 30 eggs during the second sampling). In the majority of cases, eggs were associated with a granulomatous reaction. No difference in the intensity of infection between control and M/D fish were found during the first ($F=3.276$; df 1,16; $P=0.089$), second ($F=0.177$; df 1,21; $P=0.678$) and third ($F=0.030$; df 1,18; $P=0.865$) samplings (Figure 16). Similarly, no difference in

the abundance of infection between control and M/D fish were found in any of the sampling times (First: F=4.186; df 1,30; P=0.050; Second: F=2.453; df 1,28; P=0.120 and Third: F=0.547; df 1,29; P=0.466). The degree of pathology found was similar throughout the samplings of 2009 and not as marked as that observed during 2008 (Figure 17).

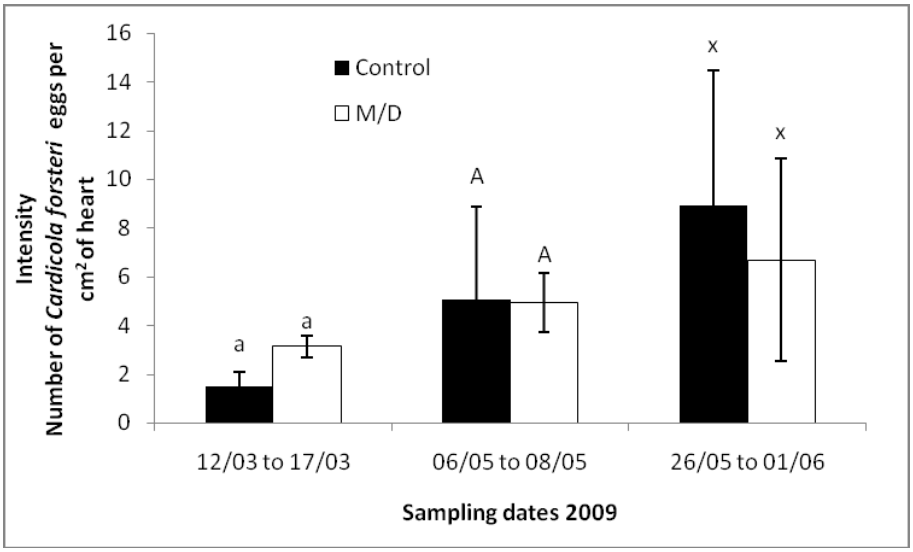

Figure 16. Intensity of infection ± standard errors of blood fluke (*Cardicola forsteri*) eggs and associated granulomatous reaction in heart muscle of tuna during three samplings in 2009. Values with different letters within each sampling time were statistically different using one way ANOVA.

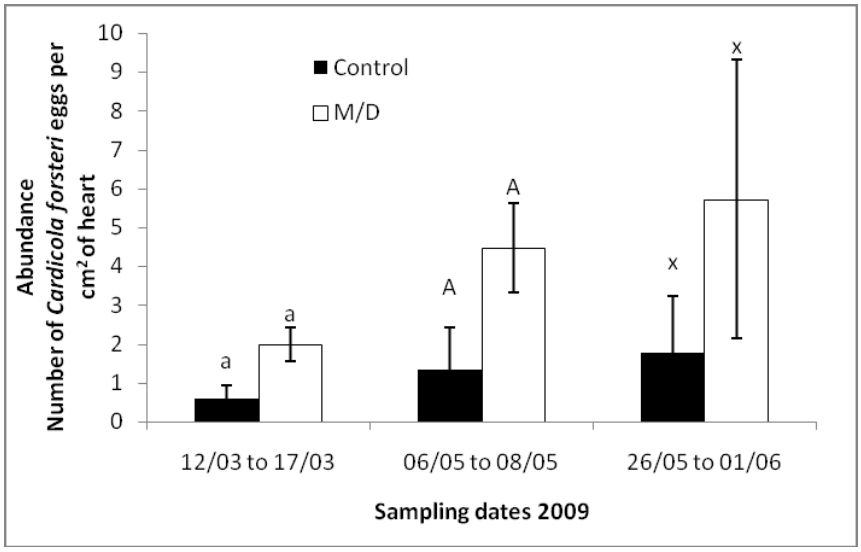

Figure 17. Abundance of infection ± standard errors of blood fluke (*Cardicola forsteri*) eggs and associated granulomatous reaction in heart muscle of tuna during three samplings in 2009. Values with different letters within each sampling time were statistically different using one way ANOVA.

During the third sampling time, all hearts displayed a small degree of perivascular inflammation. No histological differences were found between hearts that were soft during the dissection and gross examination and those characterised as normal.

In contrast to the blood fluke eggs in hearts, blood fluke eggs were present in the gills of control SBT only during the first sampling time (Figure 18). In the M/D group, percentages of fish presenting the blood fluke eggs during the first and second samplings were relatively low (22% and 4.5%, respectively) but they increased to over 75% by the third sampling. The numbers of eggs found within a single section of gill tissue varied greatly, from less than 10 to more than 400 eggs in one case.

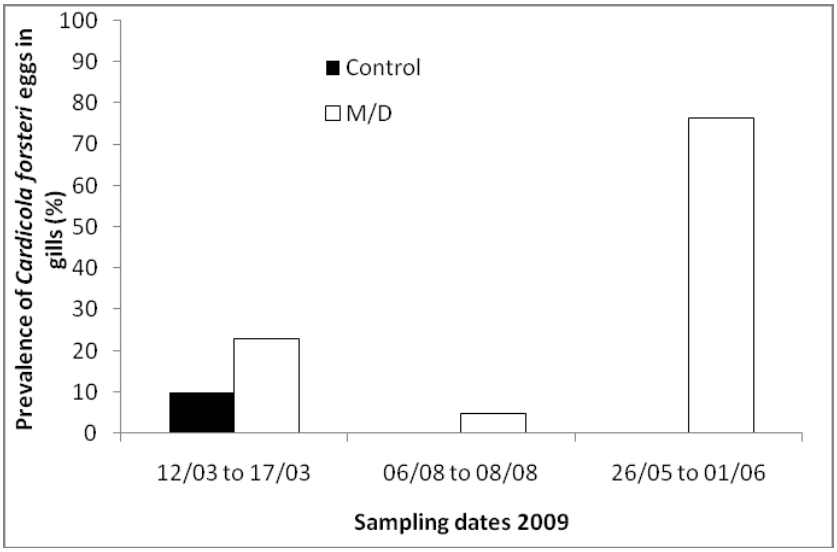

Figure 18. Prevalence of blood fluke (*Cardicola forsteri*) eggs in gills of tuna during three samplings in 2009

The low number of blood fluke eggs in heart and gills and the percentage of fish affected is in contrast to the mortalities investigated from another company earlier in 2009 (February), where all fish sampled had high numbers of blood fluke eggs in hearts, and most (with the exception of two fish) had high numbers of blood fluke eggs in gills.

No eggs were observed in anterior (head) kidney in any fish during 2009, which is in contrast to 2008. Non-specific granulomas were found in head kidneys of some fish, but they cannot be directly related to the presence of eggs, since fish that presented kidney granulomas did not always show eggs in gills and heart. The extra sections obtained from head and tail kidney did not showed the presence of blood fluke eggs in any of the sampled fish during the second and third samplings. Two M/D had a single blood fluke in a blood vessel in kidney during the first sampling.

Varying degrees of perivasculitis (inflammation associated with blood vessels) was present in liver (100% of SBT affected in first sampling; 54.5% of M/D fish

and 100% of control fish in second sampling; 14.2% of M/D and 40% of controls during third sampling), and this finding did not correspond to the presence or absence of eggs in either gills or heart. Some fish showed vascular changes usually associated with chronic condition. However, none of the fish was affected to the extent as presented by Dr Landos in August 2008 (the histopathology results were compared to the photographs included in the 2008 presentation). Necrosis was present in many organs, particularly in kidney, intestine and pyloric ceacae, at least in some individuals it was most likely a post mortem change. Intestine sections often appeared quite different within each fish, sometimes appearing to display marked inflammation and poor villi structure.

The results for control and M/D fish were compared to histology samples from wild SBT provided by Marnikol. Varying degrees of perivascular serositis and gastroenteritis were present in the wild SBT. The perivascular inflammation and necrosis was at similar severity as in the controls and M/D fish. While no parasites were seen in the histological sections from the wild SBT, this could be due to small number of fish sampled (n=4) and low prevalence and intensity of infection. Five SBT were sampled by Marnikol at the end of the tow. Their histology was similar to all groups of fish, including those sampled during the investigation of mortalities and wild SBT. Imprints of some intestines from control fish were examined for presence of intestinal mucosal parasites, however there were no significant findings. Additionally some blood smears from control fish were examined for presence of haemoparasites but none was detected. Mucosal smears and cytological preparations should be included in future investigation.

Didymozoids were found in histological sections of olfactory rosettes (10% of control, 18% M/D) and pyloric ceacae (14% M/D). Unidentified parasites were found in liver (bile duct and melanomacrophage centres) of one mort.

Two fish from a company obtained during the third sampling showed inflammatory reactions in pancreatic tissue and a reduction in the adipose reserves surrounding this tissue. These findings correlated to the low condition indices of these fish. This inflammatory reaction affected walls of the ceacae and the pancreatic tissue and its origin is uncertain. Additionally, one fish from the third sampling obtained from a pontoon undergoing a pellet trial, showed a low degree of inflammation in the PC, and this was also associated with a relatively low condition index. It would be recommended to follow up this apparent spread of inflammatory reaction in pancreatic tissue of SBT, since it might be compromising their ability to properly digest food and hence their growth.

Gross pathology and histopathology of white lesions found in epicardium of SBT hearts

Heart from Company 4

One of the hearts sampled from Company 4 presented gross white lesions of approximately 1mm diameter on the epicardium (Figure 19 A). Microscopic examination of these lesions revealed the presence of granulomatous reaction around debris and structures consistent with digenean parasites (Figure 19 B). These lesions were confirmed as calcified using Von Kossa stain, which presents calcium salts as structures of dark or brown black colour (Figure 19 C). It is possible that the structures represent old digenean parasites that have been encapsulated by the SBT.

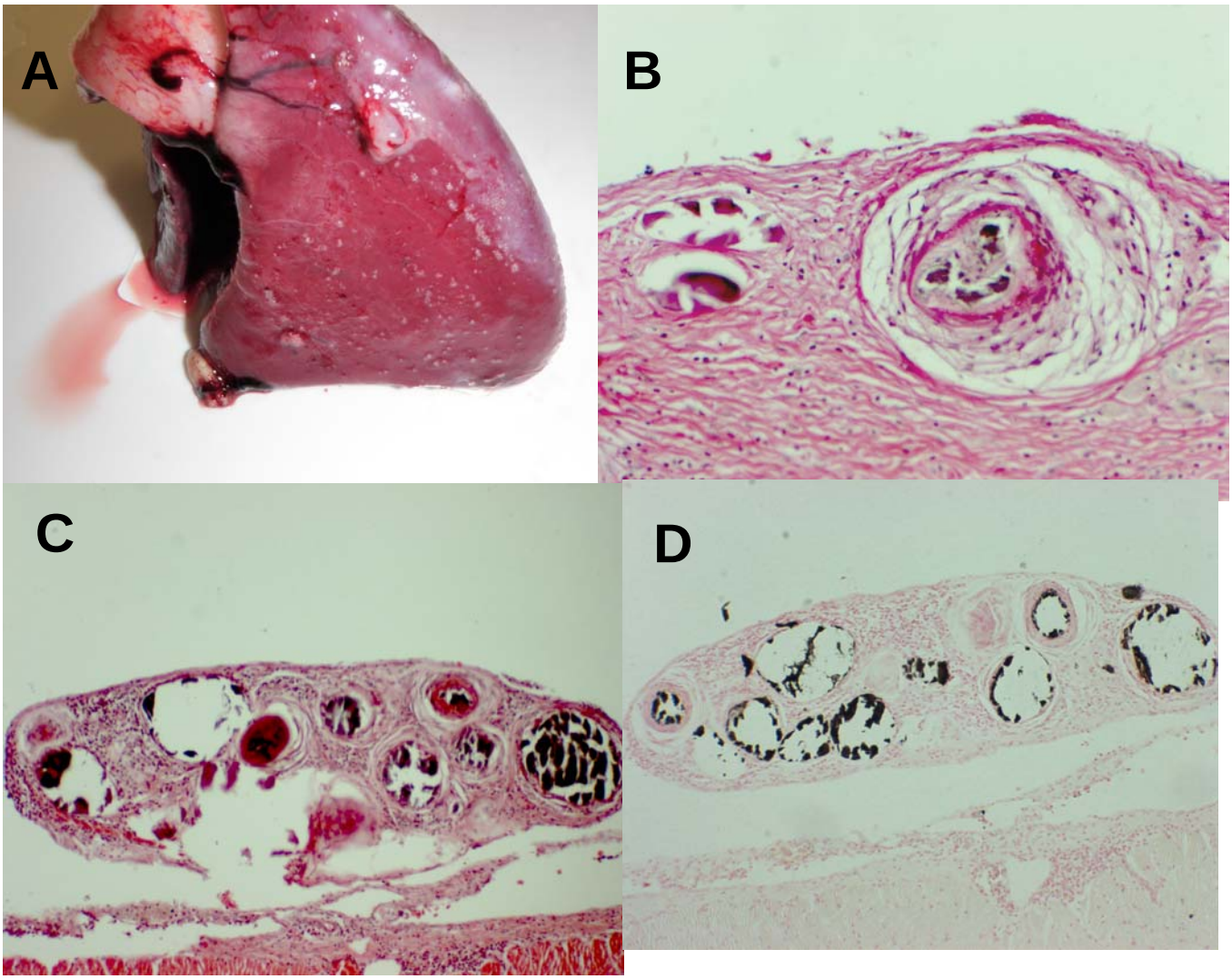

Figure 19. A: A: Macroscopic lesions in epicardium of tuna heart, note multiple white dots on surface. B: Microscopic appearance of white round lesions observed in epicardium of heart, high magnification. Granulomatous reaction surrounding debris and structures consistent with digenean parasites. C: Microscopic appearance of white round lesions observed in epicardium of heart, low magnification.
D: Structures confirmed as calcified lesions by Von Kossa stain.

A particular heart obtained from a mortality presented a similar type of small, round, white gross lesion in epicardium, of approximately 1 mm diameter. When this lesion was examined histologically, the presence of a large amount of adipose tissue underlying the epicardium was observed, with associated vascularisation of the adipose layer and reduction of the epicardium thickness (Figure 20). Another heart obtained from a control SBT during this sampling, presented a larger pale ovoid lesion of approximately 1 cm in diameter. This lesion was microscopically characterised by an increase in the epicardium thickness, with associated vascularisation and an underlying layer of what appeared to be loose connective tissue (Figure 21). None of these lesions appeared to compromise the function of the SBT hearts sampled. In fact, two of them were obtained from apparently healthy fish.

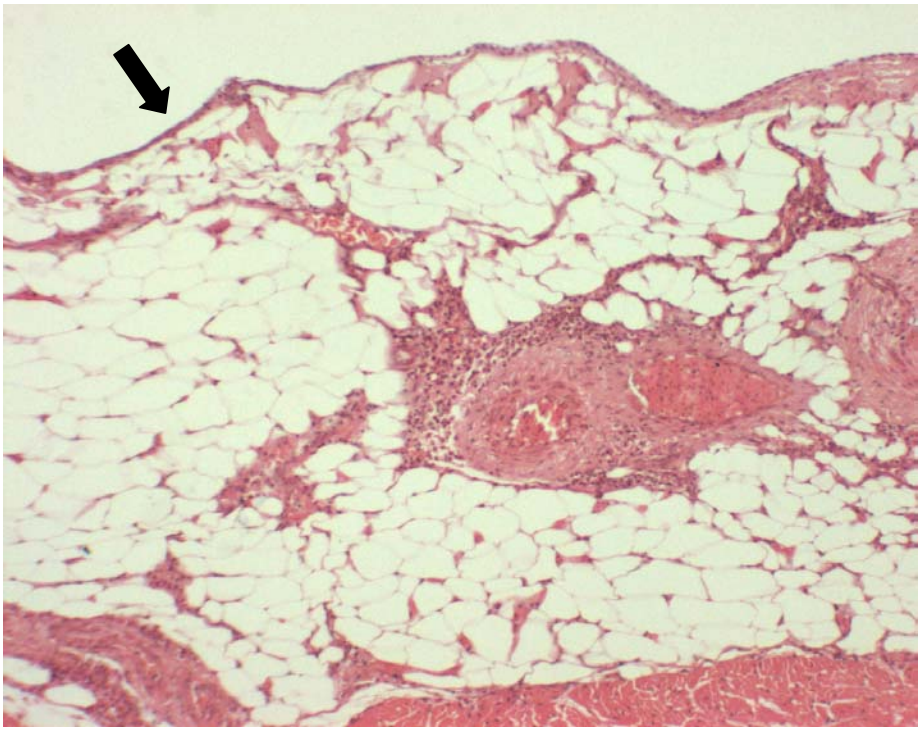

Figure 20. Adipose deposit underlying epicardium, presenting vascularisation and a thin epicardium (arrow) in a tuna heart.

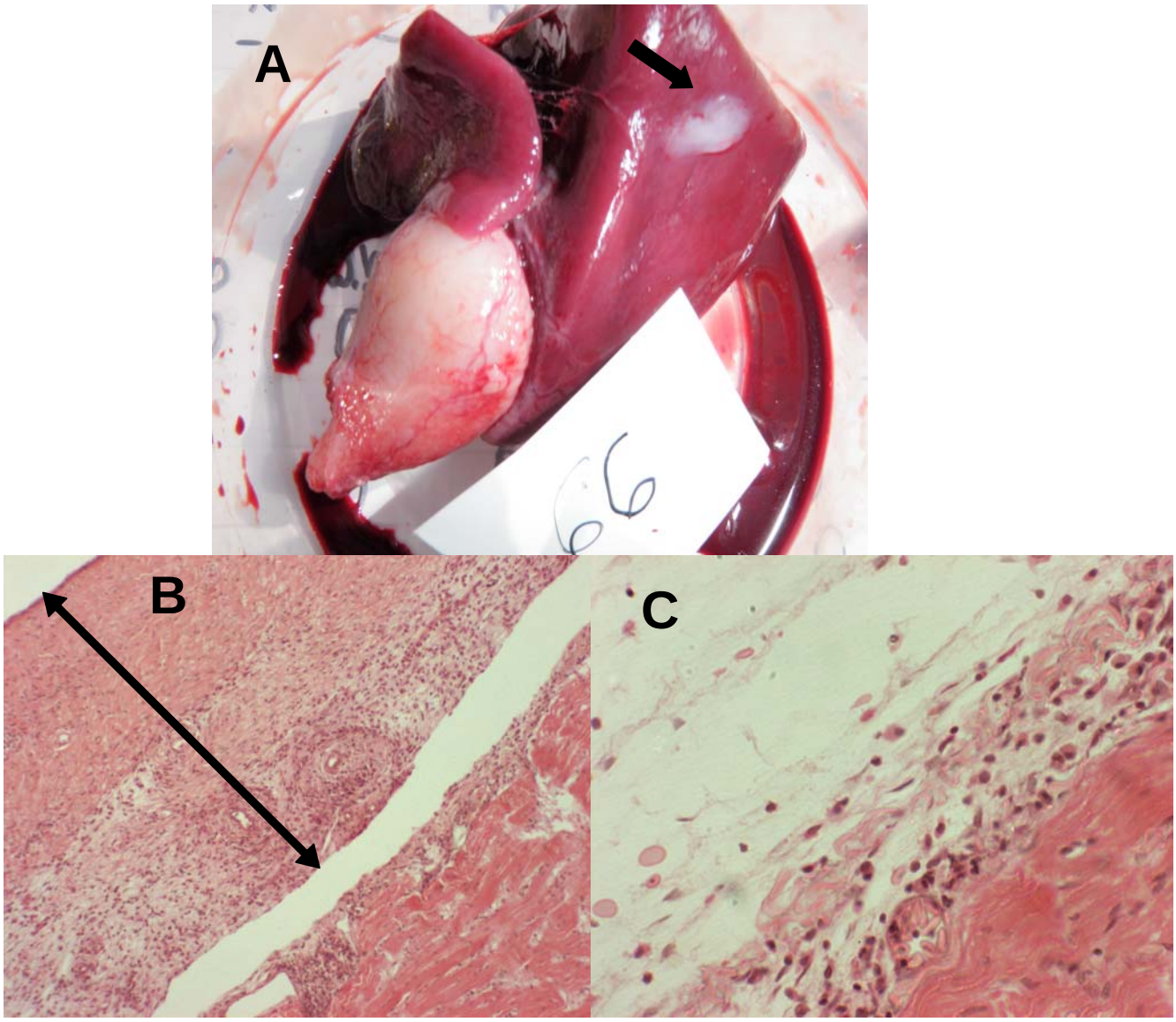

Figure 21. A: Gross pathology of a tuna heart showing a large white lesion B: Thickening of epicardium (arrow) with vascularisation. C: Loose connective tissue underlying the thickened epicardium.

Differences in number of eggs of *C. forsteri* in different areas and depths of SBT heart tissue

Multiple SBT hearts were sampled during 2009 from fish obtained during the mortality investigation and another group obtained from 1 particular company. The purpose of this sampling was to compare the number of eggs of *C. forsteri* found in different areas of the myocardium and from different depths within each area, in order to establish the best sampling area for histological analysis. Samples from the heart were obtained from 5 different areas (A→E) and in those hearts obtained from Company 4, samples were also obtained from three different depths (external, intermediate and internal sections (Figure 22).

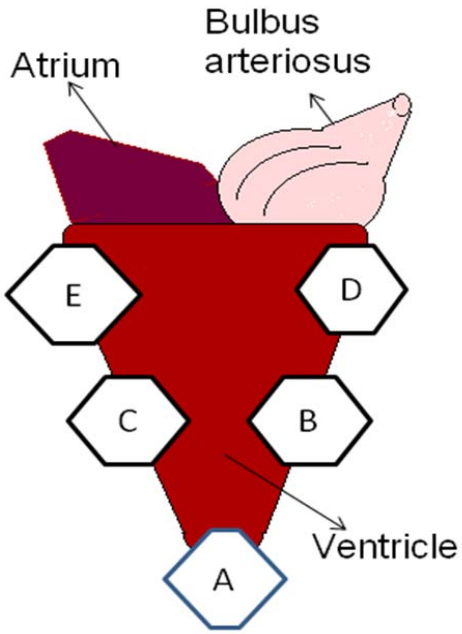

Figure 22. Heart areas compared for the presence of blood fluke eggs using histology.

Samples obtained during mortality investigation – early May.

There was no significant difference ($F=1.765$, df 4,30, $P=0.162$) in the number of eggs of *C. forsteri* present in different areas of the SBT hearts. The average number of eggs varied between 1.08 eggs per cm^2 in area "C" to 4.77 eggs per cm^2 in area "D" (Table 3). These results indicate that the number of eggs should be similar between different areas sampled.

Hearts from Company 4 – late May

It was noticeable that these SBT hearts obtained from Company 4 had a higher mean density of eggs per cm^2 (52 eggs) than hearts from the mortality sampling obtained during late May – early June (4 eggs per cm^2). The hearts sampled in late May were from fish belonging to one single company, which had been in the grow-out pontoons for almost 18 weeks, while the hearts

sampled in early May were obtained from fish of 3 different companies, which have been in culture for only 6 to 9 weeks

The number of eggs and granulomas of *C. forsteri* in the SBT hearts from company 4 was significantly greater (F=8.481; df 2,30; P=0.001) in sections obtained from the intermediate and internal layers of the hearts, and lower counts were observed in the external section. However, it is important to consider that sections obtained from the external layer of the heart comprised mostly the compact layer, where eggs have not been found previously. No significant difference (F=0.372, df 4,30, P=0.827) was observed in the number of eggs found between sampled areas (A-E), with number of eggs varying from 47.23 eggs per cm^2 to 55.81 eggs per cm^2 (Table 3). The effects of depth in the tissue and the area sampled on the number of eggs found in the myocardium were independent of one another (F=0.648; df 8,30; P=0.731). These results indicate that as long as samples are collected with enough depth into the tissue, the number of eggs should be similar between areas.

Table 3. Average number of eggs per cm^2 of heart muscle from tuna. Samples obtained during the second sampling of the mortality investigation and from hearts donated by Company 4.

	Average number of eggs per cm^2 of myocardium	
	Mortality Sampling Early May	**Company 4**
Area A	2.42^{a}	47.65^{A}
Area B	3.47^{a}	54.39^{A}
Area C	1.09^{a}	55.81^{A}
Area D	4.78^{a}	47.23^{A}
Area E	1.78^{a}	54.90^{A}
External section	N/A	34.09^{x}
Intermediate section	N/A	61.13^{y}
Internal section	N/A	60.77^{y}

Differences in number of eggs of *C. forsteri* over time – immunostimulant trial

SBT hearts were obtained during the three immunostimulant trial sampling times (at transfer, 8 weeks and 19 weeks post-transfer) and assessed for the presence of blood fluke eggs, then the values compared between sampling times.

SBT hearts were obtained from fish that came from 2 different tows. However, the effect of tow and the effect of the interaction between tow and the date of sampling were not significant for either intensity of infection (Tow effect: F=1.211; df 1, 53; P=0.276; Interaction two*date: F=3.178; df 2,53; P=0.050) or abundance of infection (Tow effect: F=3.196; df 1, 86; P=0.077; Interaction two*date: F=0.439; df 2, 86; P=0.646) using a two-way ANOVA, and therefore the data from tow 1 and 2 were combined.

Prevalence, intensity and abundance did not change between fish sampled at transfer and those sampled 8 weeks post-transfer (Figures 23, 24 and 25). However, prevalence increased more than twice during the third sampling

time, reaching 100% (Figure 19). Similarly, intensity (F=15.134; df 2,56; P>0.001) and abundance (F=46.846; df 2,89; P>0.001) were more than ten times higher in the third sampling time than in the first two (Figure 24 and 25). These results demonstrate an increase in the number of eggs and granulomas observed in SBT hearts, which occurs between 8 and 19 weeks post-transfer. It appears that the number of eggs in SBT hearts does not change during the first weeks post-transfer.

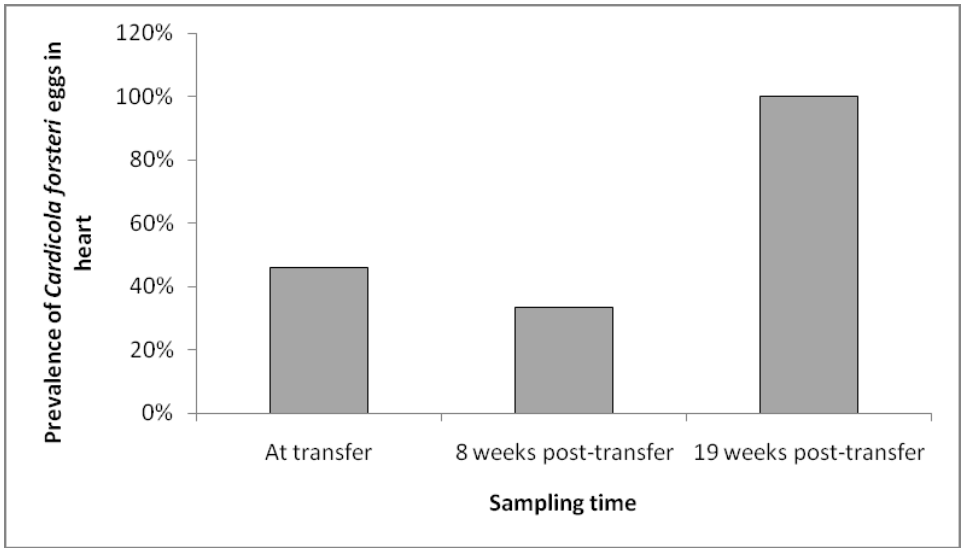

Figure 23. Prevalence ± standard error of blood fluke (*Cardicola forsteri*) eggs and associated granulomatous reaction in heart muscle of tuna from immunostimulant trial during three samplings in 2009.

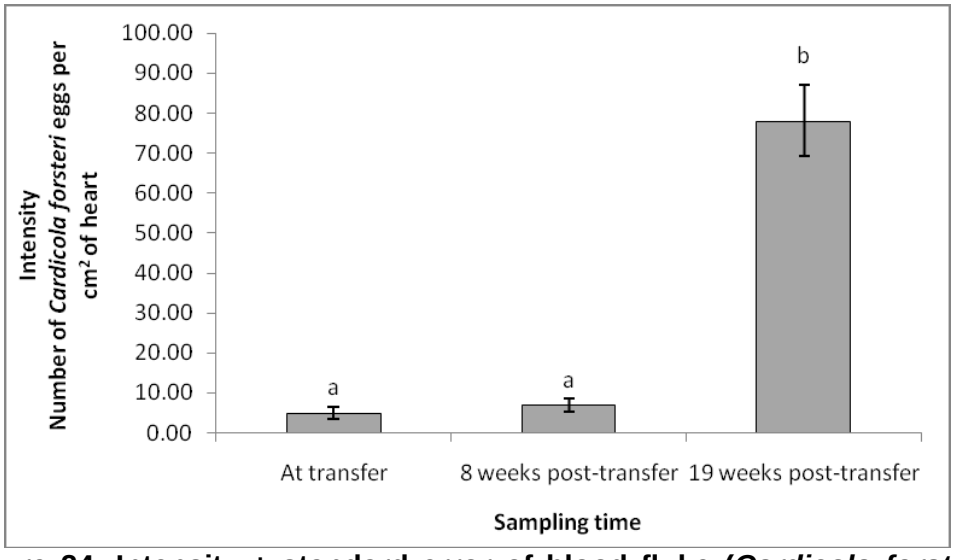

Figure 24. Intensity ± standard error of blood fluke (*Cardicola forsteri*) eggs and associated granulomatous reaction in heart muscle of tuna from immunostimulant trail during three samplings in 2009. Values with different letters within each sampling time were statistically different using one-way ANOVA (F=15.134; df 2,56; P>0.001).

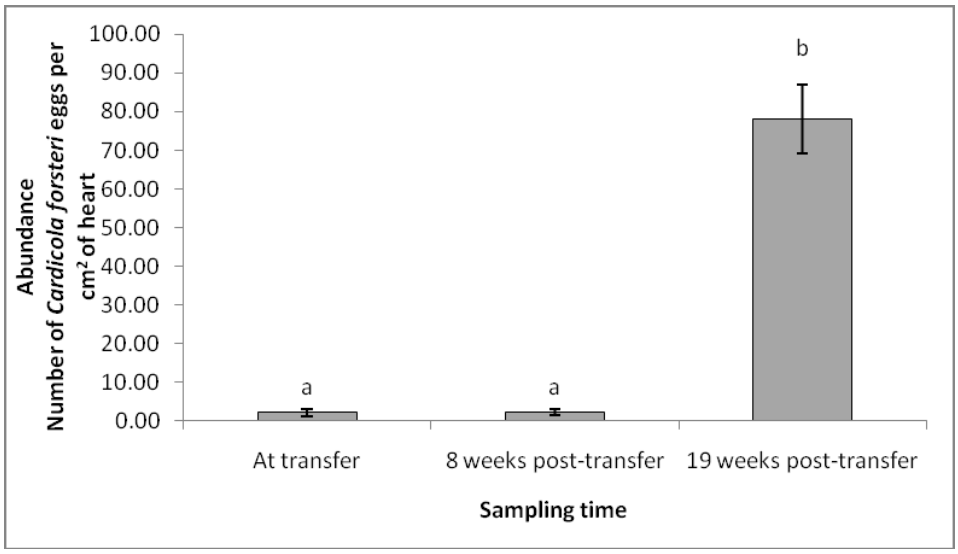

Figure 25. Abundance ± standard error of blood fluke (*Cardicola forsteri*) eggs and associated granulomatous reaction in heart muscle of tuna from immunostimulant trail during three samplings in 2009. Values with different letters within each sampling time were statistically different using one way ANOVA (F=46.846; df 2,89; P>0.001).

Additionally, no correlations were found between the number of eggs and granulomas found in SBT hearts, and the condition index of the fish in any of the three sampling times (At transfer: r=-0.537, P=0.059; 8-weeks post-transfer: r=0.097, P=0.557; 19 weeks post-transfer: r=-0.019, P=0.908), suggesting that the number of eggs in the myocardium does not affect the condition of SBT. There was a statistically significant correlation between the density of eggs in the heart and the gills (r = 0.923, P = 0.025) and number of eggs in the heart and the gills (r = 0.980, P = 0.003) suggesting that the number or density of eggs in the heart can be used as an indicator of the number of eggs in the gills.

Microbiology

Bacteria were only isolated during the first sampling time. A *Vibrio* sp. was isolated from the kidney of fish 39 but could not be further identified. It was suggested that as it was present in very low numbers this finding was probably of little significance. *Enterobacter* sp. (fish 12, mort, spleen) and *Aeromonas* sp. (fish 13, mort, spleen) were also isolated but could not be identified to species level. The significance of their isolation is unknown. *Vibrio sp and V. chagasii* was also isolated from the kidney of fish 13. *Vibrio chagasii* is a member of the *V. splendidus* complex which includes some strains and species that have been implicated as a cause of opportunistic infection in a variety of aquatic animals. *Staphylococcus kloosii*, isolated from the spleen of fish 21 (control) is associated with the normal flora of mammals and has not previously been associated with disease in aquatic animal species, it was most likely a result of sample contamination. All bacteria cultures discussed above were observed to be in occasional quantity (= the lowest level of quantification). No significant findings were observed by either smear or culture for any other fish. No testing has been done for viruses; this

was partly due to the lack of pathology indicating viral disease and the mortality pattern inconsistent with viral disease.

Table 4. Percentage of positive and negative samples for bacterial isolation, obtained from control and moribund/dead (M/D) tuna, during 2009. Negative: no growth on agar plates, no bacteria detected in smears.

	1st Sampling			2nd Sampling			3rd Sampling		
	n	Positive (%)	Negative (%)	n	Positive (%)	Negative (%)	n	Positive (%)	Negative (%)
Controls	10	10	90	8	0	100	10	0	100
M/D	22	12.5	87.5	22	0	100	21	0	100

Blood Samples

Haemoglobin (Hb; g/dL)

Haemoglobin is respiratory pigment present in red blood cells. If haemoglobin concentrations are reduced, for example due to infection with blood feeding parasites, the fish suffers anaemia which can significantly affect oxygen transport within the body of the fish.

During the 1st and 2nd sampling times, there was no difference between the Hb means for control and M/D fish (1st sampling: $F=0.001$, df 1,30, $P=0.977$; 2nd sampling: $F=4.202$, df 1,28, $P=0.050$), and the means for both treatment groups (control and M/D) in both sampling times were below values obtained during 2008 (19.79 and 19.11 g/dL respectively). However, during the third sampling time, control fish not only had a significantly higher haemoglobin concentration than M/D fish ($F=47.825$, df 1,29, $P<0.001$), but the haemoglobin levels were also higher compared to control fish sampled in 2008 (19.79 g/dL), in March 2009 (16.47 g/dL) and early May 2009 (18.45 g/dL) (Figure 26).

Additionally the range of haemoglobin values for controls was considerably less variable in the 3rd sampling time when compared to the 2 previous sampling times. In the case of moribunds, the range of haemoglobin was less variable in the 1st sampling than in the 2 following ones (Table 5).

It was interesting to observe that even though the haemoglobin values of moribund and dead fish appeared to be different from each other in each sampling time, these differences were not statistically significant at any time (1st sampling: $F=0.178$, df 2,29, $P=0.837$, 2nd sampling: $F=2.146$, df 2,27, $P=0.137$; 3rd sampling: $F=2.311$, df 1,19, $P=0.145$). The haemoglobin level means of moribund fish appeared to be higher than those of dead fish during the first (16.7 n=18 and 15.6 n=4 g/dL respectively) and second sampling (15.18 n=3 and 13.6 n=19 g/dL respectively). In contrast, during the third

sampling time, moribund fish (n =3) appeared to have a lower haemoglobin concentration than dead fish (12.64 vs 16.76 g/dL for moribund and dead fish respectively).

During the first sampling time, 6 out of the 10 fish sampled within the control group were below the expected range (17.6-21.5 g/dL, n=110, 2008 data). The two lowest values (6.8 and 14.1 g/dL) were collected from 2 bait-hooked fish from company 3 (this result did not correspond to high gill parasite or blood fluke counts). The four remaining low values were collected from 2 diver caught fish from company 3, 1 fish from company 2 (bait-hooked) and 1 fish from company 4 (bait-hooked).

During the second sampling, three of the five lowest values (3.4, 4.9, and 9.7g/dL) were collected from one company mortalities. All three fish showed low intensities of gills parasites. The blood fluke *C. forsteri* was present in all these fish, and two of them had higher intensities than the mean for the M/D group during this period. The two remaining low values were collected from 2 tuna mortalities from another company (8.3 and 8.9 g/dL). The haemoglobin values for both control and M/D fish did not correlate with the numbers of gill parasites or the number of adult *C. forsteri* found in the heart.

Only during the first sampling, controls were collected using two very different methods: bait-hooked (n=7) and diver caught (n=3). These different capture methods did not appear to influence Hb levels in the control fish (16.3 and 16.9 for bait-hooked and diver caught respectively).

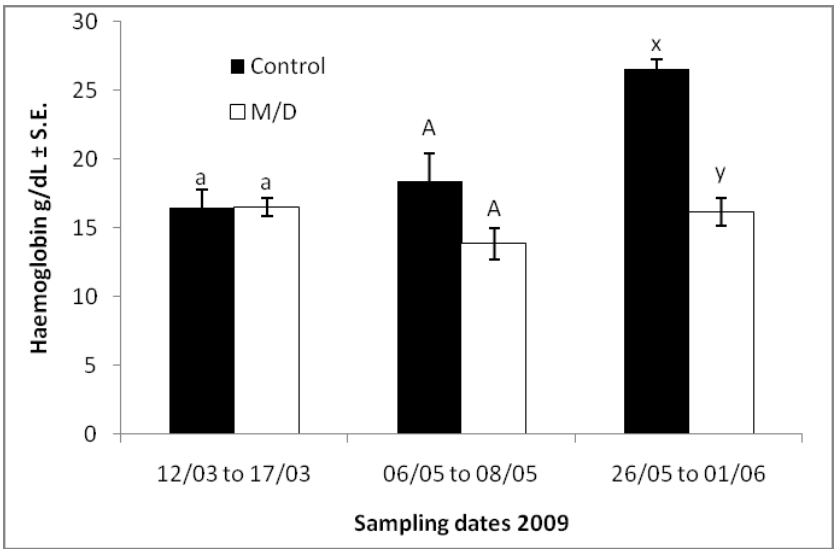

Figure 26. Average haemoglobin values (g/dL) ± standard error (SE) of control and moribund/dead (M/D) tuna sampled during 2009. Average values with different letters within each sampling time were statistically different.

Table 5. Average and range of haemoglobin values (g/dL) for control and moribund/dead (M/D) tuna sampled during 2009. Average values with different letters within each sampling time were statistically different.

	1st Sampling			2nd Sampling			3rd Sampling		
	n	Average	Range	n	Average	Range	n	Average	Range
Control	10	16.47[a]	6.88-23.51	8	18.45[A]	13.52-30.68	10*	26.59[x]	24.15-30.07
M/D	22	16.51[a]	11.69-21.53	22	13.82[A]	3.48-24.21	21	16.18[y]	6.99-25.38

***=** Control fish for the 3rd sampling were all obtained from a single company and were bait-hooked.

Cortisol (ng/mL)

Cortisol is a hormone that can be used as an indicator of stress. Raised levels of blood cortisol suggest that the fish is stressed. Stress activates cortisol secretion and elevates its levels in the blood (primary stress response). This reaction takes minutes in most fish species. Cortisol then activates secondary (metabolic) stress response. Concentration of cortisol in fish blood is used as an indicator of stress.

Average cortisol for M/D was significantly higher compared to control fish in all three sampling times (1st sampling: $F=19.194$, df 1,30, $P<0.001$; 2nd sampling: $F=12.575$, df 1,28, $P=0.001$; 3rd Sampling: $F=80.707$, df 1,29, $P<0.001$), indicating that M/D fish were stressed either during capture (moribund fish) or prior to death. Within the M/D group, no significant differences were observed in the average cortisol value between moribund and dead fish in any of the 3 sampling times (Figure 27).

In all three sampling times, cortisol values for M/D fish this year were higher and also considerably more variable than the values observed during similar months in 2008. Within M/D fish obtained in 2009, cortisol values of fish obtained in May were not significantly different from those observed in M/D fish sampled during March, but cortisol values for M/D fish caught in the third sampling time were slightly higher than those obtained in both March 2009 (377.0 ng/mL) and early May 2009 (414.15 ng/mL).

Mean cortisol values of control fish in 2009 were also higher than values obtained during similar months of 2008, for all three sampling times. Cortisol values obtained in March 2009 were four times higher than the ones obtained in May 2009, but this difference was not statistically significant. The mean cortisol level of control for the third sampling time was twice as high as that reported for early May 2009, but lower than that reported in March 2009 (80.02).

Average cortisol for control fish was higher during the first sampling time, probably due to the fact that some fish were collected by divers. Within the

control group in the first sampling, bait-hooked fish had a mean cortisol concentration of 15.3 ng/mL (n=7) while diver caught controls (all fish from company 5) had a mean of 230.9 ng/mL (n=3). This again potentially indicates that diver caught fish are either already stressed ("sick") or are stressed by capture method. Interestingly, of the 7 bait-hooked control fish, those obtained from company 5 also had the 3 highest cortisol values (9.4, 17.7 and 64.7).

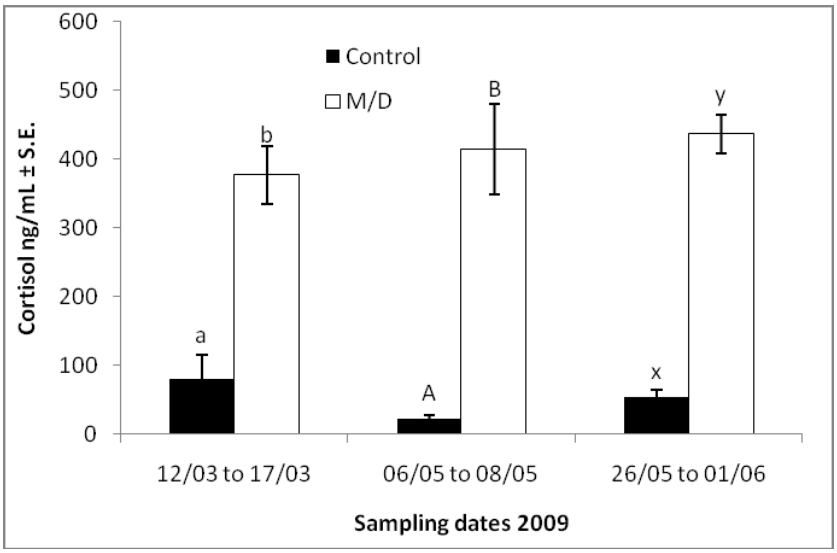

Figure 27. Average cortisol values (ng/mL) ± standard error (SE) of control and moribund/dead (M/D) tuna sampled during 2009. Average values with different letters within each sampling time were statistically different.

Table 6. Average and range of cortisol values (ng/mL) for control and moribund/dead (M/D) tuna sampled during 2009. Average values with different letters within each sampling time were statistically different.

	1st Sampling			2nd Sampling			3rd Sampling		
	n	Average	Range	n	Average	Range	n	Average	Range
Control	10	80.02[a]	1.40-297.20	8	23.37[A]	3.40-40.1	10	54.33[x]	22.0-107.90
M/D	22	377.00[b]	140.7-837.5	22	414.15[B]	107.4-976.6	21	436.57[y]	250.6-692.5

Glucose (mmol/L)
Glucose is a simple sugar, used as a source of energy and metabolic intermediate. Cortisol increases levels of glucose in blood and an increased glucose level in blood is secondary (metabolic) response to stressors, providing energy. However, glucose levels in blood can be also modified by feeding.

While similar to mortalities sampled in Mar 2008 (3.2 mmol/L), mean glucose level for M/D fish in all three sampling times of 2009 was very low compared to other values obtained in 2008. However, the average glucose for M/D fish in 2009 was very similar among all three samplings (Table 7).

For all three sampling times, glucose for control fish was slightly lower than the average obtained from all control fish sampled in 2008 (8.03 mmol/L), but still appeared to be within normal range for tuna.

Compared to dead fish (average 2.29 and 2.1 mmol/L, for first and second sampling respectively), moribund fish had a higher mean glucose value (5.35 and 6.29 mmol/L) during the first and second samplings (Figure 28). These higher values were closer to those obtained for controls, suggesting that low glucose is most likely a post-mortem related change, since the glucose reserves become depleted after death. However, during the third sampling time, moribund fish were found to have a lower average glucose value of 0.99 mmol/L compared to dead fish (average 3.02 mmol/L) (Figure 28). At least 7 dead fish (31%) had higher glucose values than the 3 moribund fish. Plasma glucose values in fish are affected by several factors such as nutritional status and time elapsed between last feeding and dead and/or sampling, which may explain the differences found.

The average for diver caught controls (6.33 mmol/L) was only slightly higher compared to bait-hook controls (5.46 mmol/L) suggesting that diver capture does not adversely affect glucose levels of fish.

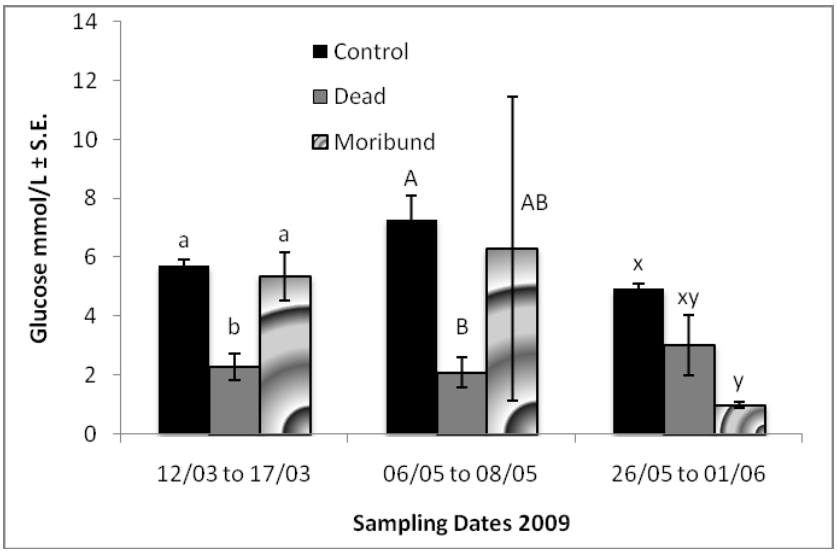

Figure 28. Average glucose values (mmol/L) ± standard error (SE) of control, dead and moribund tuna sampled during 2009. Average values with different letters within each sampling time were statistically different.

Table 7. Average and range of glucose values (mmol/L) for control and moribund/dead (M/D) tuna sampled during 2009. Average values with different letters within each sampling time were statistically different.

	1st Sampling			2nd Sampling			3rd Sampling		
	n	Average	Range	n	Average	Range	n	Average	Range
Control	10	5.72^{a}	4.63-7.02	8	7.30^{A}	4.98-12.37	10	4.95^{x}	4.36-5.85
M/D	22	2.84^{b}	0.23-7.30	22	2.67^{B}	0.72-16.60	21	2.73^{y}	0.22-16.88

Lactate (mmol/L)

Lactate is a product of carbohydrate metabolism. High levels of lactate are produced when there is not enough oxygen – for example during vigorous exercise, burst swimming, has been used as indicator of stress in fish. Increased level of lactate in blood will reduce blood pH.

Average lactate for M/D fish was significantly higher than controls in all three sampling times (1st sampling: F=67.800, df 1,30, P<0.001; 2nd sampling: F=38.392, df 1,28, P<0.001; 3rd Sampling: F=67.581, df 1,29, P<0.001) (see graph), again suggesting that M/D fish are considerably more stressed (prior to death) than "healthy" normal fish. No significant difference was observed between the means of moribund or dead fish in any of the three sampling periods (1st sampling: 34.03 and 39.84 mmol/L , 2nd sampling: 44.42 and

44.30 mmol/L and 3rd sampling: 44.73 and 41.87 mmol/L, for moribund and dead fish respectively) (Figure 29).

While higher than 2008 values (<2.0 mmol/L for Feb, Mar, May, June but not July), average lactate of control fish during the 2009 sampling were similar among the three sampling times. Similarly, average lactate from M/D fish was not considerably different among sampling dates of 2009.

Regarding the average lactate values for control fish of the first sampling time, diver caught fish value (9.4 mmol/L; n=3) was more than 2.5 x higher than that of bait-hooked controls (3.67 mmol/L n=7). Only 2 of the bait-hooked control fish (from company 2) had lactate levels above 3.8 mmol/L. These results strongly suggest that capture method can elevate lactate levels in tuna.

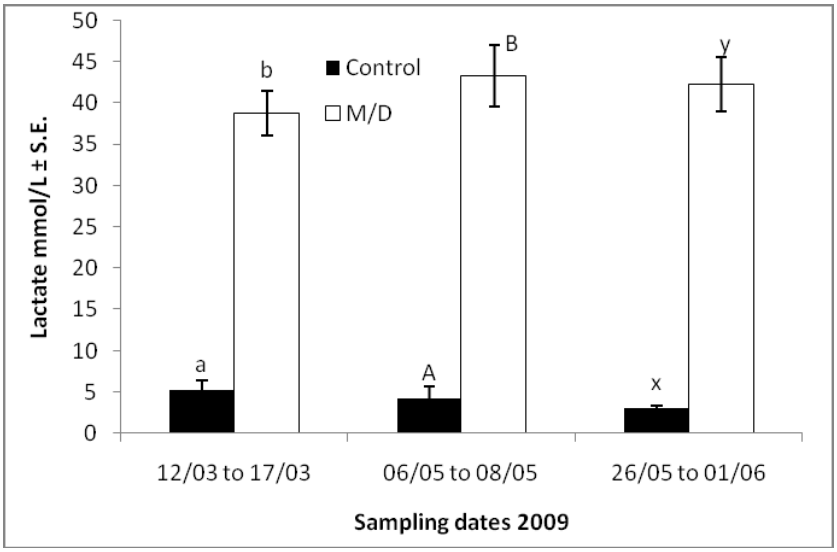

Figure 29. Average lactate values (mmol/L) ± standard error (SE) of control and moribund/dead (M/D) tuna sampled during 2009. Average values with different letters within each sampling time were statistically different.

Table 8. Average and range of lactate values (mmol/L) for control and moribund/dead (M/D) tuna sampled during 2009. Average values with different letters within each sampling time were statistically different.

	1st Sampling			2nd Sampling			3rd Sampling		
	n	Average	Range	n	Average	Range	n	Average	Range
Control	10	5.27[a]	1.38-11.45	8	4.48[A]	0.62-11.20	10	3.11[x]	1.92-4.0
M/D	22	38.78[b]	10.56-59.65	22	44.31[B]	8.6-70.4	21	42.28[y]	13.69-69.6

pH

pH is measured to determine if blood is acidic (low pH), neutral (pH 7) or alkaline (high pH). Acidic blood can be a result of high levels of lactate or carbon dioxide in blood.

Mean pH for control fish was significantly higher than pH from M/D fish in all three sampling times by almost a full pH unit (1st sampling: $F=117.234$, df 1,30, $P<0.001$; 2nd sampling: $F=43.205$, df 1,28, $P<0.001$; 3rd Sampling: $F=110.799$, df 1,29, $P<0.001$), a finding which is consistent with results from 2008. The low pH average for M/D fish was possibly influenced by high lactate. The blood pH of the control group was within expected values and very consistent throughout all the sampling times of 2009. A similar tendency was observed for the pH values of the M/D group, but values were lower than the mean from all 2008 months = 7.39 (Figure 30).

During the first sampling, no major differences were observed in terms of capture method (7.90 n=3, and 7.91 n=7, for diver caught and bait-hooked fish respectively).

Similarly no major difference was observed between the dead and moribund fish during the first and third samplings (6.9 and 7.0 for first sampling; 6.97 and 7.10 for the third sampling, in dead and moribund fish respectively). However, a significant difference was observed between the moribund and dead fish (7.3 and 6.8 respectively) during the second sampling time.

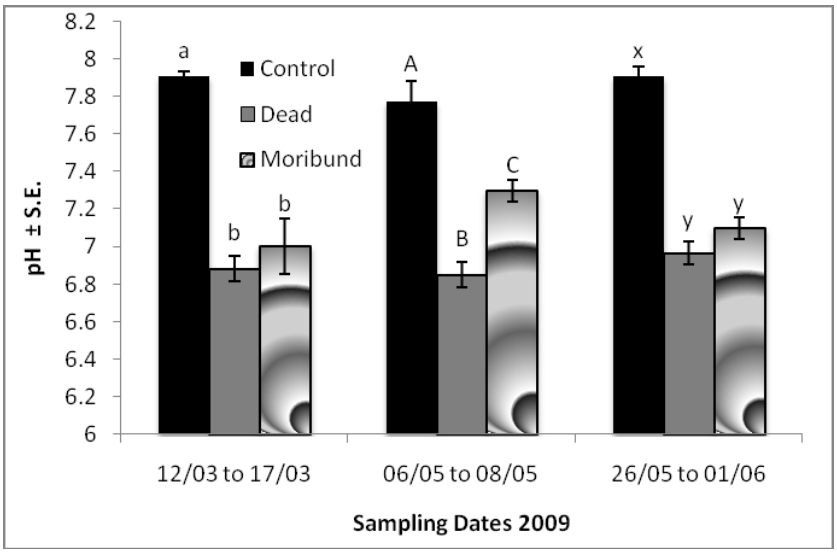

Figure 30. Average pH values ± standard error (SE) of control, dead and moribund tuna sampled during 2009. Average values with different letters within each sampling time were statistically different.

Table 9. Average and range of pH values for control and moribund/dead (M/D) tuna sampled during 2009. Average values with different letters within each sampling time were statistically different.

	1st Sampling			2nd Sampling			3rd Sampling		
	n	Average	Range	n	Average	Range	n	Average	Range
Control	10	7.9^{a}	7.8-8.1	8	7.7^{A}	7.3-8.1	10	7.91^{x}	7.5-8.1
M/D	22	6.9^{b}	6.5-7.5	22	6.9^{B}	6.4-7.5	21	6.99^{y}	6.6-7.7

Osmolality (mmol/kg)

Fish maintain a reasonably constant level of salts in their blood, independent of the level of salts in the external environment. This means that fish are osmoregulators. Osmoregulation is an energy-consuming process. Gills (in particular chloride cells), kidney and intestine are the main osmoregulatory organs in fish. Without osmoregulation marine fish would dehydrate as the passive process of osmosis removes water from their body and the passive process of diffusion results in salts moving to the body of the fish through semi-permeable membranes (gills). Osmolality is a measure of total salt content by determination of freezing point (the higher the salt content the lower the freezing point). If a fish is compromised and cannot maintain the normal level of salt in its blood, the osmolality of blood will be more similar to the external environment (higher in marine environment and lower in freshwater environment). Sea water osmolality is around 1000 mmol/kg and

fresh water osmolality is around 0 mmol/kg. Normal marine fish blood osmolality is around 360-460 mmol/kg.

The average osmolality for M/D fish was significantly higher compared to control fish during all three sampling times (1st sampling: F=60.582, df 1,30, P<0.001; 2nd sampling: F=41.038, df 1,28, P<0.001; 3rd Sampling: F=73.073, df 1,29, P<0.001), indicating osmoregulatory stress. No major differences were observed between dead or moribund fish in any of the three sampling periods (1st sampling: 567.5 and 530.3 mmol/kg; 2nd Sampling: 550 and 539.4mmol/kg; 3rd sampling: 553.9 and 587.0 mmol/kg for dead and moribund fish, respectively) (Figure 31).

The mean osmolality for control fish was very similar among the three sampling periods of 2009, and they were similar to values obtained for both Feb and Mar 2008 (413.8 and 423.3 mmol/kg). Average osmolality for the M/D treatment group was also consistent for all three sampling times of 2009.

No difference in osmolality was observed between bait-hooked and diver caught (411.4 and 431.0 mmol/kg respectively) tuna during the first sampling time of 2009.

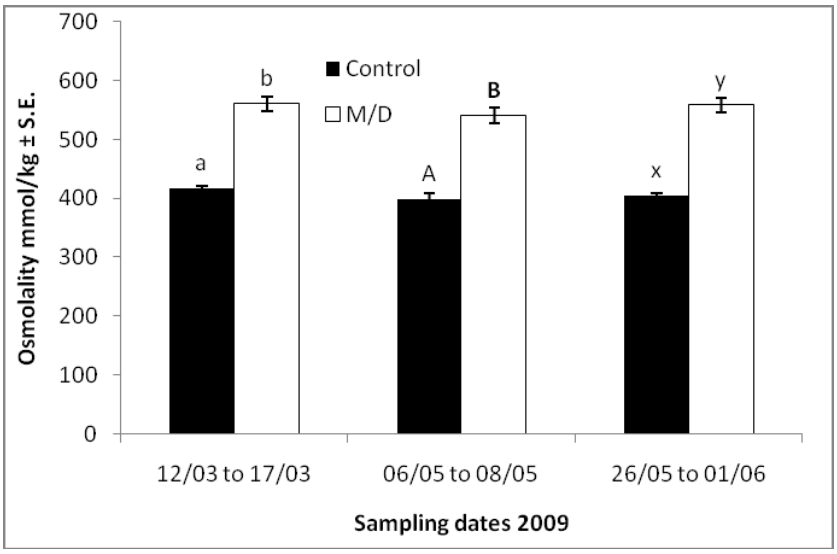

Figure 31. Average osmolality values (mmol/kg) ± standard error (SE) of control and moribund/dead (M/D) tuna sampled during 2009. Average values with different letters within each sampling time were statistically different.

Table 10. Average and range of osmolality values (mmol/kg) for control and moribund/dead (M/D) tuna sampled during 2009. Average values with different letters within each sampling time were statistically different.

	1st Sampling			2nd Sampling			3rd Sampling		
	n	Average	Range	n	Average	Range	n	Average	Range
Control	10	417.3[a]	399.0-439.0	8	399.2[A]	354.0-438.0	10	404[x]	387-419
M/D	22	560.8[b]	444.0-626.0	22	540.9[B]	415.0-630.0	21	558.67[y]	427-623

Lysozyme (µg/mL)

Lysozyme is an enzyme which can lyse bacteria. Lysozyme is one of the components of humoral (soluble) immune response. There are contradictory reports in the literature about the relationship between stress and lysozyme levels or parasitic loads and lysozyme levels in fish.

Although highly variable, lysozyme levels were significantly higher in M/D fish compared to controls for all three sampling times (1st sampling: $F=18.619$, df 1,30, $P<0.001$; 2nd sampling: $F=27.305$, df 1,28, $P<0.001$; 3rd Sampling: $F=36.544$, df 1,29, $P<0.001$). Compared to all 2008 data, both control and M/D fish displayed elevated lysozyme levels throughout 2009. No significant differences were seen between dead and moribund fish in all three sampling periods (1st sampling: 582.5 and 563.3 µg/mL ; 2nd sampling: 521.8 and 452.06 µg/mL; 3rd sampling: 726.80 and 630.79 µg/mL , for dead and moribund fish, respectively) (Figure 32).

The lysozyme mean value obtained during the third sampling time for control fish was higher than control fish sampled during both March 2009 (240.65 µg/mL) and early May 2009 (112.21 µg/mL). Average lysozyme for M/D fish was similarly higher during the third sampling period than both March and May 2009 values (578.99 and 512.33 respectively). It has been previously observed in tuna that the lysozyme activity is greater in fish maintained in captivity for longer periods of time.

For the first sampling time, mean lysozyme for bait-hooked fish (175.3 µg/mL) was considerably lower than diver caught fish (393.2 µg/mL), within the control treatment group. It has been observed that and stressful event, such as that generated by handling, could have an effect on the lysozyme activity in fish.

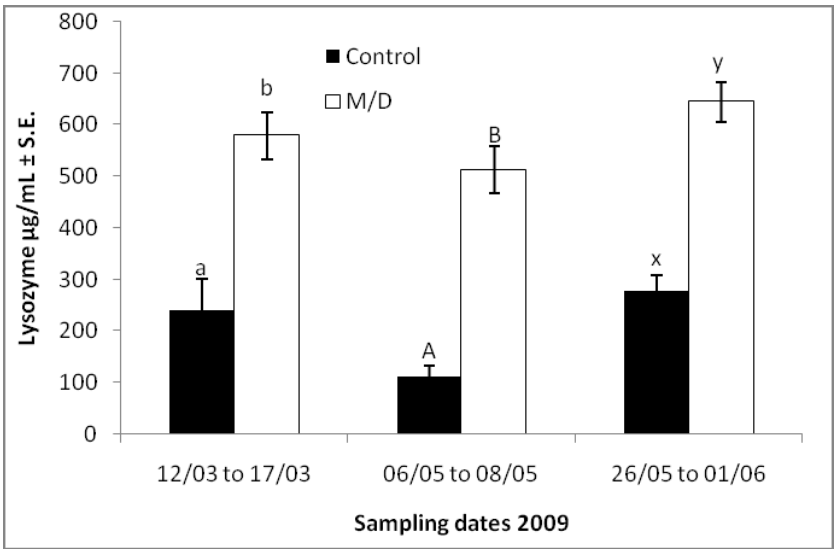

Figure 32. Average lysozyme values (µg/mL) ± standard error (SE) of control and moribund/dead (M/D) tuna sampled during 2009. Average values with different letters within each sampling time were statistically different.

Table 11. Average and range of lysozyme values (µg/mL) for control and moribund/dead (M/D) tuna sampled during 2009. Average values with different letters within each sampling time were statistically different.

	1st Sampling			2nd Sampling			3rd Sampling		
	n	Average	Range	n	Average	Range	n	Average	Range
Control	10	240.65^{a}	18.83-502.89	8	112.21^{A}	43.5-198.15	10	278.06^{x}	122.85-469.40
M/D	22	578.99^{b}	211.80-1162.80	22	512.33^{B}	92.25-935.2	21	644.5^{y}	344.20-1034.00

Cholesterol (mmol/L)

Cholesterol is a fatty substance which is a crucial part of the outer lining (membrane) of animal cells. Cholesterol can be also found in the blood circulation. The cholesterol in blood originates from two major sources; dietary intake and liver production. Cholesterol is carried in the blood as lipoproteins. Low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) cholesterol are the "bad" cholesterol because elevated LDL and VLDL levels are associated with an increased risk of coronary artery (heart) disease in humans. The non-HDL level (which equals to LDL + VLDL) might be a better a good predictor of heart disease in individuals with high triglycerides because as triglycerides increase, so do LDL levels and VLDL (not just LDL) starts to play a larger role in atherosclerosis.

The majority of individuals obtained during the three sampling times presented triglycerides values above 150 mg/dL (Table 12), and therefore non-HDL values were calculated for SBT. Analyses of the triglycerides values showed that these presented no differences between control and moribunds in any of the three sampling times (Table 12).

Table 12. Average triglycerides levels ± standard error in blood plasma of controls and moribund tuna for each sampling time (n: Control March=5, Moribund March=4; Control and moribunds early May and late May-early June=3). One-way ANOVA results for each sampling showed in the table.

	1st Sampling		2nd Sampling		3rd Sampling	
	Control	Moribund	Control	Moribund	Control	Moribund
Ave±SE (mg/dL)	250.97±38.94	153.89±28.28	275.37±42.73	155.78±81.33	434.18±165.02	196.04±42.86
F	3.663		1.699		1.951	
Df	1,7		1,4		1,4	
P	0.097	Not significant	0.262	Not significant	0.235	Not significant

Preliminary results suggested that cholesterol level in moribund fish sampled during the first and second sampling (March, n= 4; early May, n=3) were significantly higher than in control fish (March, n= 5; early May, n=3) sampled at the same time (1^{st} sampling: F=11.684; df 1,7; P=0.011; 2^{nd} sampling: F=9.155; df 1,4; P=0.039) (Figure 33). The data for all three sampling times were pooled, since there was no interaction between the sampling time and group factors (F=1.706; df 2,15; P=0.215), and there were no significant differences between the three sampling time values (F= 2.586; df 2,15; P=0.108). When fish from all three sampling were analysed together, it was observed that the moribund fish had a cholesterol value more than 1 mmol/L higher than that observed for the control fish (F=25.642, df 1,15; P<0.001) (Figure 34).

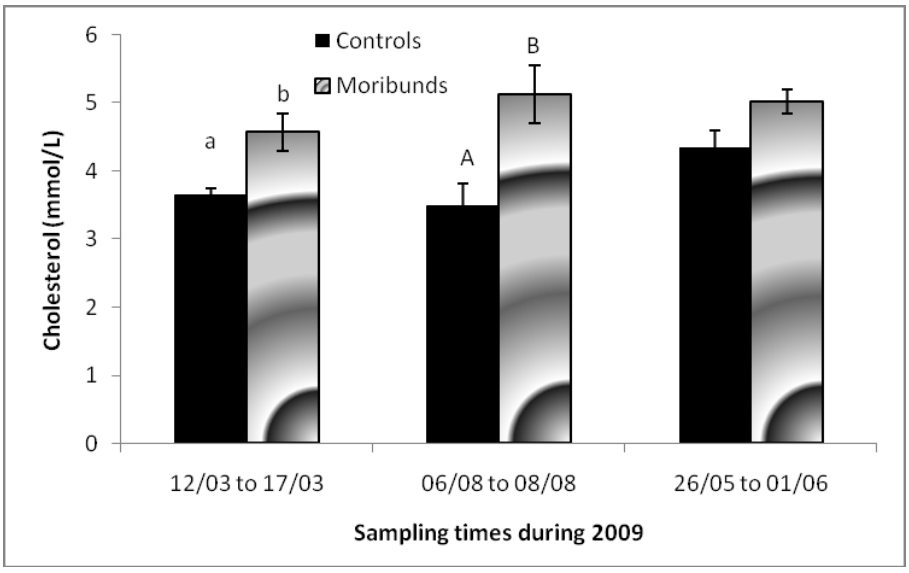

Figure 33. Average cholesterol levels ± standard error in blood plasma of controls and moribund tuna for each sampling time (n: Control March=5, Moribund March=4; Control and moribunds early May and late May-early June=3). Average values with different letters within each sampling time are significantly different from one another by one-way ANOVA.

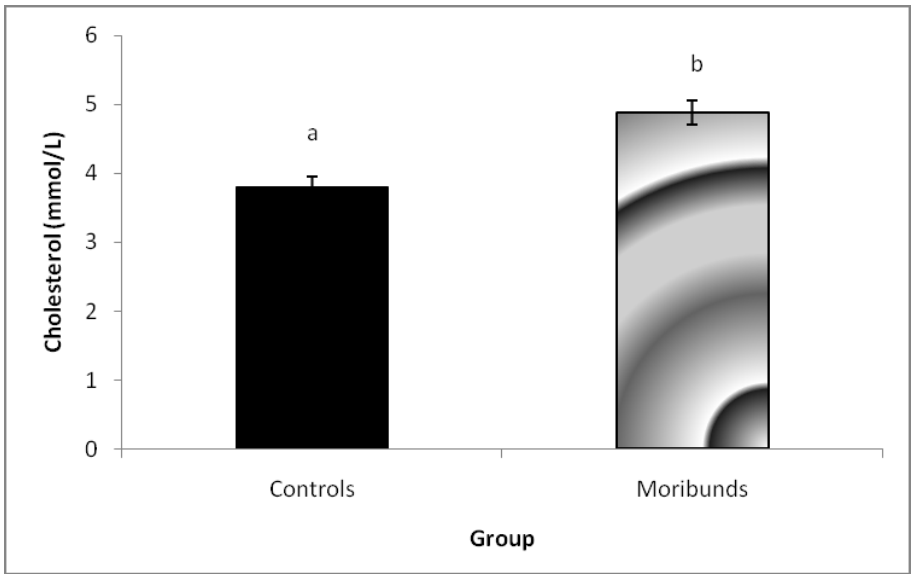

Figure 34. Average cholesterol levels ± standard error in blood plasma of controls and moribund tuna for all three sampling times (n: Control=11, Moribund=10). Average values with different letters are significantly different from one another by two-way ANOVA.

Non-HDL cholesterol (LDL + VLDL) was highly elevated in the moribund fish for all three sampling times (F=24.602; df 1,7; P=0.002; F=45.941; df 1,4; P=0.002; F=22.708; df 1,4; P=0.009 for the 1st, 2nd and 3rd sampling times,

respectively). The means for moribunds were higher than 2.87 mmol/L in all three sampling times, while it was lower than 2.2 mmol/L in controls (Figure 35). Values obtained from all three sampling times were pooled again for non-HDL cholesterol in order to observe differences between the whole control and moribund fish groups. Similarly to what was observed with total cholesterol, the non-HDL cholesterol was higher in moribund fish than in controls ($F=81.257$; df 1,15; $P<0.001$) (Figure 36), and there were no differences in the non-HDL values between sampling times ($F= 3.074$, df 2,15; $P=0.076$). No lesions or changes associated with coronary disease were observed in these hearts that could be related to the increase in cholesterol. The levels of cholesterol were lower than in ranched NBT, but this could be a species-specific difference.

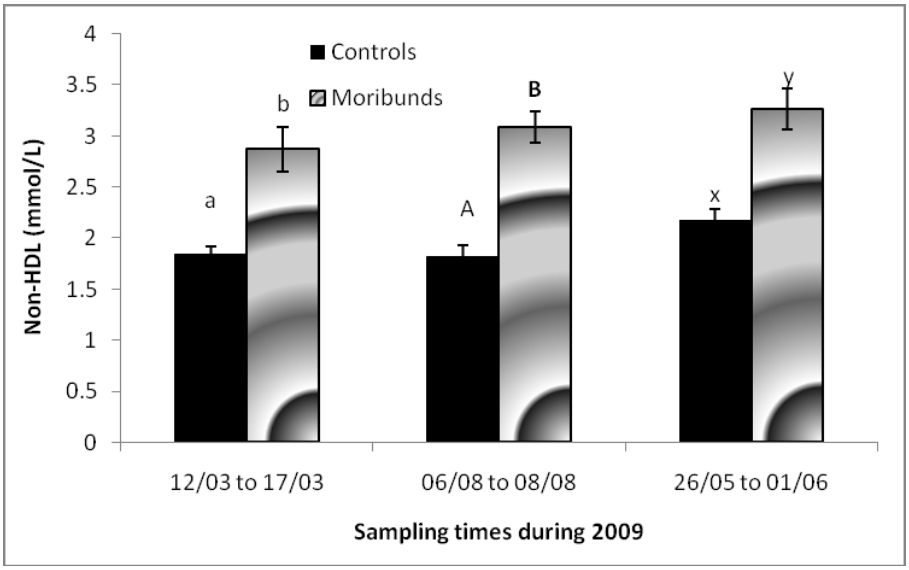

Figure 35. Average non-HDL cholesterol levels ± standard error in blood plasma of controls and moribund tuna for each sampling time (n: Control March=5, Moribund March=4; Control and moribunds early May and late May-early June=3). Average values with different letters within each sampling time are significantly different from one another by one-way ANOVA.26

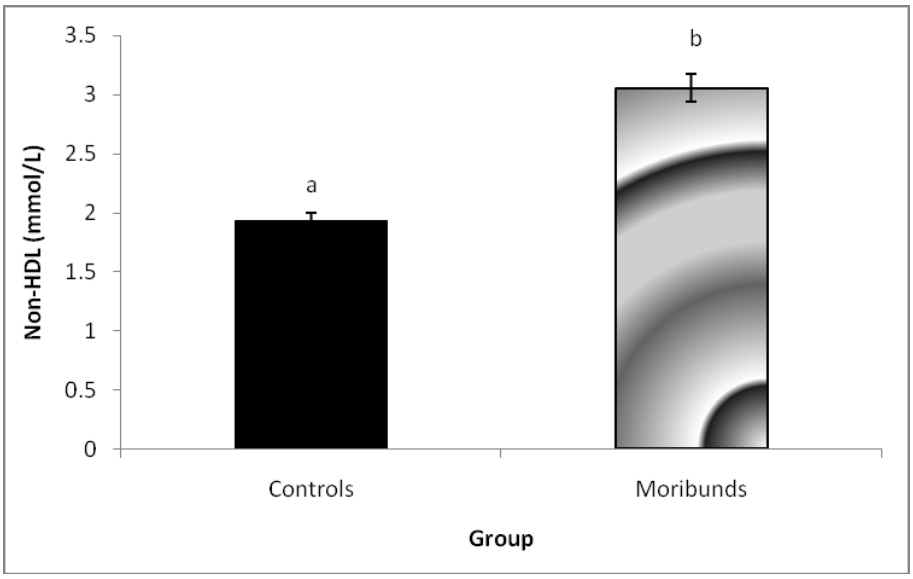

Figure 36. Average non-HDL cholesterol levels ± standard error in blood plasma of controls and moribund tuna for all three sampling times (n: Control March=11, Moribund=10). Average values with different letters are significantly different from one another by two-way ANOVA.

Additional Studies

Gills

Fresh preparations of gills were examined under the microscope in order to look for the presence of blood fluke eggs. It was thought this method may have been faster than histology and could have included a larger gill sample, and therefore provide more accurate answers. A few mortalities were examined during the second sampling time, but the methodology proved to be very time consuming and difficult to carry out. Additionally, no eggs were detected in any of the samples examined.

Gill flushes were performed to recover adult blood flukes. While some flukes were recovered, this was inconsistent and very time consuming. The method, like heart flushes, required analysis of fresh samples.

Hearts

Hearts from a few fish during the second sampling period, were macerated and the presence of adult flukes and eggs were compared with the presence of eggs in histology slides, in order to assess if there was a direct relationship in the numbers obtained with both methods and to decide which method was most accurate and fastest to perform. However, the maceration was time consuming and the preparations obtained were not consistent. A few eggs were found (Figure 37), but the method was time consuming, not really quantitative and gave inconsistent results. Furthermore, the samples had to be analysed fresh, whereas the use of histology for quantification allows for the analysis of fixed samples which is logistically preferable.

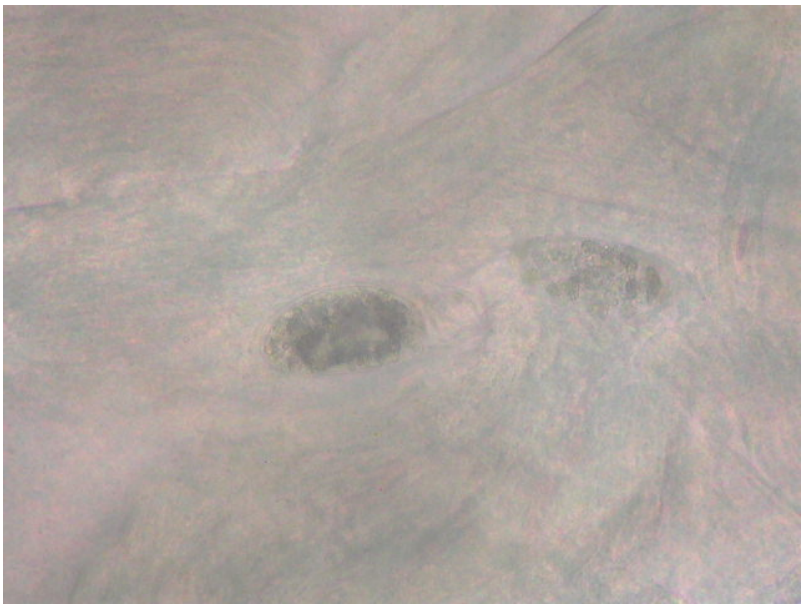

Figure 37. Eggs of the blood fluke (*Cardicola forsteri*) found in macerated heart preparations.

Summary of Results

In 2009 mortality sampling took place during three different periods: March (12/03-17/03), early May (6/05-8/05) and late May-Early June (26/05-01/06). During each sampling time, SBT were delivered to a vessel based laboratory by divers, where they were immediately sampled. The total number of fish was 105, including 12 fish sampled only for swimmer syndrome and 28 controls (Table 13).

Table 13. Number of control and moribund/dead (M/D) tuna sampled during three periods in 2009.

		1st Sampling	2nd Sampling	3rd Sampling	Total
Nº of companies providing fish		3	4	4	
Control	**Diver collected**	3	0	0	3
	Bait-hooked	7	5	10*	22
	Gaffed	0	3	0	3
M/D	**Moribund**	4	3	3	10
	Fresh mortalities	18	19	18	55
Additional M/D	**Swimmer syndrome only examination**				12
Total		32	30	31	105

Control Fish

- Diver collected - from the pontoons
- Bait-hooked - hook harvested during feeding
- Gaffed during feeding
- * - control fish from the third sampling were hook-harvested

Mortalities (M/D) – moribund or dead fish

- moribund - near death
- fresh mortalities – assessed as dead for less than 2 hours

The number of available moribund fish was very low when compared to the total number of M/D fish sampled at all sampling times. The fact that the majority of the M/D fish sampled were fresh mortalities could be interfering with obtaining accurate results and make it more difficult to determine the cause of mortality. This is because some of the differences between morts and controls could be due to the pre-mortem or post-mortem changes (caused by the death not the cause of the death).

Cumulative mortalities of SBT–between weeks 6 and 12 of culture, grouped by grow-out pontoon, were correlated with different parameters related to

conditions during the tow and grow-out periods to investigate any possible associations between these variables. Cumulative mortalities were significantly correlated with: number of fish present initially in each tow pontoon (second sampling), condition index of the fish when initially put in tow pontoon (second sampling), length of transfer (in minutes) from tow pontoon to grow-out pontoon (second sampling), number of fish in each grow out pontoon (second sampling) and number of mortalities in the tow was not correlated with mortalities between weeks 6 and 12 for neither of the sampling times. There were no correlations for the third sampling or for all pooled data. This could be due to either different factors affecting mortalities at different times or different companies or the datasets being too small and at times too variable to allow detection of any relationships. This could be overcome by using all SBT industry data.

SBT were sampled for a broad range of tests including:

1. Gross Pathology – gross changes to external and internal organs
2. Parasitology – the number of key species of grossly visible parasites on the external surfaces (skin and gills – copepods and gill flukes) and in heart of the fish (blood flukes), presence of ciliates in the cerebrospinal fluid
3. Microbiology – presence of culturable bacteria in internal organs (kidney and spleen)
4. Histology –organ structure and changes
5. Haematology – changes in the blood variables

Prevalence and intensity of parasitic infections appeared to be consistent with those reported in the literature for SBT at similar period of time post-transfer for control and M/D fish during all sampling periods. The sea lice, *Caligus* spp., were found in similar percentages of both control and M/D fish. However, for all three samplings, the mean intensity of infection (average number of parasites on infected SBT) with sea lice was greater in M/D group. Presence of blood fluke, *Cardicola forsteri* was examined by heart flushes and counts of adults found in the flushes. Prevalence (percentage of SBT infected) of blood fluke infection in M/D fish was very consistent throughout 2009, but variable in control fish. The intensity of infection of the blood fluke was lower in the M/D group when compared to the controls in all three sampling times. *Uronema nigricans* was the confirmed cause of mortality of 6.5% of the total number of M/D fish.

The method of capture of the control fish did appear to confound some of the results. The 'harvest' control fish caught by divers had higher cortisol, lactate and lysozyme compared to the bait-hooked control fish. This is more than likely related to the harvest process as opposed to the hooked fish that are actively feeding in a relaxed state. Control fish used during the second and third sampling were all bait-hooked, which provided a more standard control sample and base parameters. Additionally, sampled control fish were known to be feeding, and therefore in good condition, as confirmed by condition index and health status (see following sections).

With the exception of haemoglobin, blood parameters for control fish were found to be within the expected range for normal “healthy” tuna. There were a number of control fish during the first sampling, which had lower than expected haemoglobin levels. Results of blood analyses suggest that the fish were stressed prior to death. The elevated blood osmolality indicates that the M/D fish were unable to balance the blood ion or salt concentration. M/D fish had low blood pH (higher acidity). These changes may be related to the organ function being affected by the process of dying, which includes organs failure.

Bacteria were only isolated from SBT during the first sampling time and they belonged to the genera *Enterobacter*, *Vibrio*, *Aeromonas* and *Staphylococcus*. There were no consistent isolations and pathology did not suggest that bacterial infections were a problem.

In general, all SBT appeared ‘normal’ with no visible signs indicating cause of death. No eye damage was observed. There were some grossly visible changes in gills. Some of the gills sampled were striated and banded which warrants further investigation.

The macroscopic dark banding observed during sampling was associated with hyperperfusion of gills with erythrocytes. While some fish had blood fluke eggs in their heart and gills (as detected by histology), the numbers were mostly low (with the exception of one company) and it is unlikely that the levels detected were affecting SBT based on the previous research findings. No differences in egg numbers were found among different areas of the heart.

While we know that 6.5% of moribund/dead (M/D) fish died due to swimmer syndrome, the cause of death of the remaining fish is unknown. Blood changes and the pathology results suggested metabolic problems. It is possible that the cause of fish mortality is multifactorial - that the fish are affected by a range of factors which cause the mortality only in combination, and that there is unlikely to be a 'primary' cause of death. This would make identification of a single cause of death difficult. For example parasites found in SBT during this investigation (with the exception of *Uronema* which is known to cause death) occurred at varying levels in the 'normal' situation, in the years when the SBT mortality was low. That means that either there are synergistic effects of a number of factors that make these parasites more pathogenic, or that there are other as yet unidentified reasons for the mortalities. The findings were inconclusive and did not identify a specific cause of morbidity/mortality, except for the 6.5% of fish which died of swimmer syndrome.

Table 14. Summary of results for M/D tuna in relation to pathogens potentially responsible for SBT mortality (excludes confirmed 6.5% of *U. nigricans* – swimmer syndrome related mortality). Unless specified all summaries relate to M/D fish only.

Potential cause	Summary of results for M/D fish
Caligus spp (sea lice)	Prevalence of this parasite decreased over the sampling period from ~56 to 20% and intensity remained low (~2-3 parasites/affected fish). In general, prevalence, intensity and abundance values were all consistently higher in M/D SBT compared to controls. It is possible that sea lice contributed to SBT mortality but unlikely to have been the primary cause.
C. forsteri (blood fluke) Adults in heart	Prevalence of this parasite was consistently seen in ~56-64 % of fish at all sampling time points. Intensity however decreased from ~5-2 parasites in each affect ed fish over the sampling period. In general, prevalence, intensity and abundance were all consistently lower in M/D fish compared to controls. There is no evidence that the presence of adult blood flukes in heart caused SBT mortality.
C. forsteri (blood fluke) Eggs in heart	There is an increase in the number of eggs during holding time, however the significant difference (peak) occurs around harvest (19 weeks post-transfer) and not at the time of increased mortality. There is no evidence that the presence of blood fluke eggs in heart caused SBT mortality.
H. thynni (gill fluke)	Prevalence of this parasite was less than 10% at both the 1st and 2nd sampling time points however by the 3rd sampling period ~20 of fish were affected. Intensity was also low across all time points with less than 5 parasites/affected fish. While not observed in control fish at the 2nd sampling time point, prevalence, intensity and abundance were similar to control fish at both the 1st and 3rd sampling periods. Unlikely to contribute to SBT mortalities.
P. appendiculatus (gill copepod)	In general prevalence (~14-18%) and intensity (1-2) of this parasite were relatively constant over the 3 sampling periods and values were all similar to that of controls. Unlikely to contribute to SBT mortalities.
E. brachypterus (gill copepod)	Prevalence, intensity and abundance of this parasite all initially increased at the 2nd sampling time point (compared to the 1st) then decreased at the 3rd sampling period. Prevalence and intensity were consistently higher than control fish at all sampling periods. However, due to small numbers unlikely to contribute to mortalities.
Bacteria	No significant findings were observed for either M/D fish or controls – culturable bacteria did not cause SBT mortalities
Metabolic problems	M/D SBT had lower pH, greater osmolality and greater cholesterol level than control fish. This could either mean that metabolic problems contributed to SBT mortality or that these are changes related to the process of dying.
Overall stress	M/D SBT had elevated levels of cortisol and lactate and reduced blood pH. This could either mean that stress contributed to SBT mortality or that these are changes related to the process of dying.

Note: Correlation statistics were conducted for all M/D fish irrespective of sampling time point. No significant correlations were found for any parasites and either condition factor or fish weight.
During all sampling periods prevalence and intensity of parasitic infections in both control and M/D fish were consistent with those reported in the literature for SBT at similar period of time post-transfer.

Benefits and Adoption

This project directly benefits the SBT industry by contributing to the understanding of causes of mortalities of ranched SBT.

Aquatic animal health research (human capital development) in Australia benefits from this project through involvement of postgraduate students and ASBTIA staff in this project.

This project has indirect benefit for other aquaculture sectors in Australia. It provides research methods and knowledge that can be applied to other fish ranched in marine pens.

Further Development

Industry database involving four SBT companies was developed during this project. The continuation of the database and inclusion of more companies would be beneficial for the industry.

Results of this project have been widely disseminated throughout the SBT industry through industry meetings, and workshops.

Future research:

- Experimental manipulation to test if blood fluke causes mortalities (antihelminthic)
- Case definition for increased mortality at the commercial pontoon level (investigate at the level of pontoon and not an individual fish)
- Comparison of SBT from pontoons with normal and high mortality (160 SBT) sampled over 2-3 sampling periods
- Focus on sampling affected live fish instead of mortalities
- Analyses of SBT industry data to identify risk factors for mortalities, common patterns and differences between tows, companies and locations
- Define tow and annual variation for health of wild fish and determine their effect on SBT performance

Planned Outcomes

This project is proactive and directly benefited SBT industry. This extension contributed to the planned outcomes as outlined for project 2008/228. The project significantly contributed to our understanding of the factors that underpin high health status of SBT. It has increased our understanding of the causes of mortalities in farmed SBT. Swimmer syndrome caused 6.5% of the investigated mortalities. Mortalities were highest during the second sampling period in early May. Mortalities and moribund SBT had a lower condition index than control fish at all sampling times, suggesting that either the cause of death is chronic, or it has a lethal effect only on compromised fish. Analysis of all industry data would be highly beneficial for the SBT industry as it would allow a much more robust statistical analysis. This project provided training to ASBTIA staff, SBT industry and research students. Future research priorities were proposed.

Conclusions

- *Uronema nigricans* was the confirmed cause of mortality of 6.5% of the total number of moribund and dead fish

- Cumulative mortalities were significantly correlated with: number of fish present initially in each tow pontoon (second sampling), condition index of the fish when initially put in tow pontoon (second sampling), length of transfer (in minutes) from tow pontoon to grow-out pontoon (second sampling), number of fish in each grow out pontoon (second sampling) and number of mortalities in the tow was not correlated with mortalities between weeks 6 and 12 for either of the sampling times

- Mortalities and moribund SBT had a lower condition index than control fish at all sampling times, suggesting that either the cause of death is chronic, or it has a lethal effect only on compromised fish

- The intensity of infection of the blood fluke (or number of parasites in the infected SBT) was lower in the moribund and mortalities group than in the controls in all three sampling times

- The number and density of blood fluke eggs in the heart were correlated with the number and density of blood fluke eggs in the gills and the number of blood fluke eggs per square cm of histological section of heart significantly increased by harvest

- For all three samplings, the number of sea lice on infected SBT (intensity of infection) was greater in moribund and mortalities then in control fish

- The higher blood acidity of mortalities and moribunds may be related to stress and the subsequent increase in lactate level

- Mortalities and moribund fish did not appear to be able to osmoregulate and maintain the balance of salt in their blood, however it is unknown if this contributed to the death or if it was a part of the death process

- Moribund SBT had elevated total cholesterol and in particular nonHDL cholesterol in comparison to the control fish

- Blood changes (including low pH and increased non-HDL cholesterol) and the pathology results indicate potential metabolic problems

- There was a lot of variation in pathology of wild SBT, including degree of inflammatory changes

Appendix 1

INTELLECTUAL PROPERTY

The intellectual property and valuable information arising from this report are:

1. Copyright in this report

Appendix 2

STAFF

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Appendix 3

METHODS

Sample collection in the field

Lengths and weights were recorded for all fish at the time of sampling. Gills and viscera were excised and stored on ice until processed. The olfactory rosette (OR) was dissected and preserved in jars with 10% neutral buffered formalin (10% NBF). In addition, tuna gills and skin surface were examined for external metazoan parasites with the naked eye and their numbers were recorded. Blood samples from healthy and moribund fish were collected from the severed lateral vein behind the pectoral recess using a syringe; samples were aliquoted in heparinised Vacutainer® tubes (BD, USA) and 1 mL plastic tubes. Cerebrospinal fluid (CSF) from all fish was obtained from the cranial orifice left by the "Taniguchi tool" using a transfer pipette. Samples were immediately placed on ice.

Sample processing in the laboratory

Blood samples

Whole blood in the 1 mL plastic tubes was frozen at -20^{o}C. Heparinised blood was aliquoted in 1 mL plastic tubes and centrifuged at 10,000 rpm for 5 min. Plasma was removed and transferred into fresh 1 mL plastic tubes and frozen at -20^{o}C. Samples were then transported on dry ice to the University of Tasmania (UTas) Fish health laboratory in Launceston and stored at -80^{o}C until analysed.

Microbiological samples

Within 2 to 4 h after collection, microbiological samples were obtained aseptically from spleen and kidney from all fish. The surface of the organ was seared with a hot blade and an incision was made (approximately 1 cm deep) with a sterile scalpel on the seared surface. Cotton swabs were inserted in the incision and rotated gently to saturate the swab with blood and tissue. The swabs were then used to produce a smear from each organ on glass slides and to inoculate thiosulphate citrate bile salts sucrose and blood agar (TCBS/BA) plates. These were then streaked out using sterile loops. TCBS is a medium selective for *Vibrio* spp., while BA is a non selective medium for the isolation of a wide range of bacteria (Atlas, 2004). In addition to the routinely sampled organs, an eye lesion was sampled from a moribund fish in April and a muscle lesion from a moribund tuna in June, using the same technique described above.

All the smears and plates were sent to the Mount Pleasant Microbiology Laboratory, Department of Primary Industries and Water (DPIPWE) in Launceston for identification of microorganisms.

Histological samples

Gills, OR, heart (caudal tip of ventricle), liver, spleen, kidney and pyloric caecae were collected from all fish. Firstly, organs were observed for the presence of any macroscopic lesions, which were recorded if present.

Secondly, small samples of each organ were preserved in 10% NBF for 24-48 h. Subsequently, 3 mm thick sections were cut using a scalpel and placed into a “Tissue Tek” (Sakura Finetek, Japan) histology cassette. Then, cassettes were transported in sealed containers to the UTas Fish Health laboratory in Launceston. The samples were processed using standard histological techniques. Gills inside the cassettes were decalcified for 1 hour using Rapid decalcifying fluid (Australian Biostain, Australia) and then washed with water for an hour. After this initial step, all samples were processed in a Tissue Tek II (Sakura Finetek, Japan) tissue processor and embedded into paraffin blocks on a Shandon Histocentre^{TM} 3 (Thermo Scientific, USA). Blocks were then faced at approximately 30 µm to remove the excess wax and 5µm sections were cut using a Microm HM340 microtome (Thermo Scientific, USA). Blocks were cooled on ice before facing and microtoming. Sections were immediately placed in a water bath at 40^{o}C and recovered using glass slides, which were marked and dried for 24 h at 37^{o}C. Slides were then stained with Haematoxylin-Eosin (H&E) using a Shandon Listain^{TM} GLX (USA).

Heart flushes for *C. forsteri*

The hearts were cut opened longitudinally and flushed with 30-50 mL of diluted seawater (1:2 seawater to regular water) in order to dislodge any adults of *C. forsteri*. Flushes were poured into petri dishes (3-4 per heart) and allowed to settle for 20 min. Then, the flushes were observed under a dissecting microscope (Olympus SZX12 stereomicroscope, Olympus, Japan) for the presence of adult *C. forsteri.* Parasite numbers were noted for each fish.

Sample analyses

Lengths and weights

In order to have the largest possible sample number, lengths and weights from fish sampled in this study and the available measurements of fish used in the blood sampling study were combined.

Measurements were used for the calculation of the condition index (C.I.). This index was estimated based on the state of the animal when examined. For whole animals the South Australian tuna industry formula (Aiken et al., 2006) was used:

$$\text{C.I.} = \frac{\text{Whole animal weight (kg)}}{\text{length}^3\ \text{(m)}}$$

A corrected formula (Hayward et al., 2008b) was used to estimate the C.I. in gilled and gutted animals:

$$\text{Gilled and Gutted C.I.} = \frac{\text{Gilled and gutted weight (kg) / 0.87}}{\text{length}^3\ \text{(m)}}$$

Parasite burdens

Parasites counts were obtained from the fish either on boat at the sampling time (always for skin parasites, most of the time for gill parasites) or in the lab immediately after return (sometimes for gill parasites, always for blood flukes). The investigated parasites included copepods from the genus *Caligus* on the skin surface, metazoan parasites on the gills (copepod *P. appendiculatus*, monogenean *H. thynni* and copepod *E. brachypterus*) and a blood fluke in the heart (*C. forsteri*). Prevalence (the percentage of infected individuals within the population) and intensity (the average number of parasites per infected fish) (Bush et al., 1997) were calculated for each parasite species observed in both groups (normal and moribund/dead fish) for each month when data were available.

Blood and plasma samples

Haemoglobin concentrations were obtained from whole blood samples without anticoagulant using the cyanometahaemoglobin assay. Samples were thawed for 1 h then mixed for 10 s using a vortex mixer. Blood was diluted 1:50 with Drabkin's reagent (Sigma-Aldrich, USA) (DR) in 1.5 mL plastic tubes and left to stand protected from light for 15 min. Haemoglobin standards were set by preparing a 1:100 stock solution of dried haemoglobin from bovine blood (Sigma-Aldrich, USA) in DR and then performing dilutions to obtain, 0, 5, 10, 15, 20, 25 and 50 $g.dL^{-1}$ standards. Standards and samples were vortexed again for 10 sec and 40 µL of each were placed in triplicate wells of a 96-well microtiter plate along with 160 µL of DR. Their absorbance was measured using a Rainbow Thermo plate reader (Tecan Trading AG, Switzerland) at a wavelength of 540 nm, shaking the plates for 120 s before reading. A standard curve was constructed using the absorbance levels of the standards and sample values were converted into haemoglobin concentrations from this curve. Serum total cholesterol, HDL cholesterol, triglycerides and glucose were analysed on a Konelab clinical chemistry analyser (Thermo Fisher Scientific Inc.,Waltham, MA) using Thermo-trace reagents (Thermo Fisher Scientific Inc.,Waltham, MA).

Blood plasma samples were analysed in duplicates for glucose, lactate, pH and osmolality and in triplicates for cortisol and lysozyme activity (L.A) as follows. Blood plasma glucose and lactate were measured using a GM7 Microstats reader (Analox instruments, United Kingdom), using 10 µL and 5 µL of blood plasma sample, respectively. Results for both variables were obtained as $mmol.L^{-1}$. Additionally, the pH of blood plasma samples was measured by placing a drop (approx. 40 µL) of plasma in a Minilab Isfet pH meter Model IQ125 (IQ Scientific, USA). Blood plasma osmolality was obtained using a Vapro© Model 5520 vapour pressure osmometer (Wescor Inc., Logan, Utah, USA). Results were expressed as $mmol.kg^{-1}$.

Blood plasma cortisol was extracted from the plasma sample by placing 50 µL of plasma in a plastic test tube and adding 1 mL ethyl acetate. Plasma and ethyl acetate were mixed thoroughly and left to stand for 5 min. Then 50 µL of this mixture were removed and placed in fresh assay tubes (three per sample). Assay tubes were left overnight for the solvent to evaporate. Cortisol levels were then measured using a radioimmunoassay (RIA) performed as

follows: 200 μL of assay buffer were added to each of the sample tubes. Then each tube received further 200 μL of ^{3}H-cortisol (label)(about 3500 cpm) and 200 μL of antibody to cortisol and left to stand overnight. A series of dilutions of "cold hormone" (non-radiologicaly marked) (0, 6, 12, 25, 50, 100, 200, 400, and 800 pg standard) were prepared in duplicates as standards by adding 200 μL of label and 200 μL of antibody to cortisol to the hormone. The "zero hormone" standard contained 200 μL of buffer in place of "cold" hormone. Replicate "total count" tubes (200 μL label) and "non-specific binding" tubes (200 μL label plus 400 μL buffer) were also prepared. The following day all the tubes were placed on ice for 10 min and 200 μL of dextran-coated charcoal solution was added, the tubes were vortexed and then centrifuged for 10 min at 3000 rpm at 4^{o}C. The supernatant was decanted in scintillation vials, 4 mL of scintillation cocktail added and the radioactivity in the vials was then counted. Using the standards prepared, a curve was constructed using the following equation:

$$\text{Percent relative binding (\%BO)} = \frac{X - NSB}{H - NSB} \times 100$$

X = mean of replicate for standards or sample.
NSB = mean of counts for non-specific binding tubes.
OH = mean of counts for zero-hormone tubes.
These values indicated the displacement of labelled cortisol from the antibody by increasing amounts of "cold hormone". The amount of cortisol in the samples (ng.mL^{-1}) was then calculated by comparing the values obtained with the standards.

Blood plasma L.A. was measured using a method similar to that of Carrington and Secombes (2007). In brief, a series of dilutions of hen egg white lysozyme (HEWL) (80, 40, 20, 10, 5 and 0 μg.mL^{-1}) were prepared as standards using phosphate/citrate (0.1M) buffer pH 5.8, containing 0.09% NaCl. Using the same buffer, a 1 mg.mL^{-1} suspension of *Micrococcus lysodeikticus* was made. 25 μL of each standard and each serum sample were placed in triplicates in a 96-well plate. Then 175 μL of the bacterial suspension was added to each well. The plate was shaken and the reduction in turbidity was immediately measured in a Rainbow Thermo plate reader (Tecan Trading AG, Switzerland) at 450 nm over 2.5 minutes at 50 second intervals in negative kinetics mode at 25^{o}C. The data were assessed to identify the time at which maximum lysis (T_4) had occurred. The mean difference in absorbance between T_0 and T_4 of HEWL dilutions was used to create a standard curve for L.A. Using this curve and the mean absorbance value from the serum samples, the L.A. of serum was obtained in μg.mL^{-1}.

Microbiological samples

Bacteria were identified using standard procedures (DPIPWE, Animal Health Laboratory, Fish Health Unit). Smears from all the microbiological samples were Gram stained and observed under a light microscope in order to identify the presence of bacteria according to guidelines provided in Table 1.

Table 1 Bacteria presence levels in glass slides for microbiology microscopy analyses.

Presence level	*Description*
-	No bacteria observed
±	1-10 bacteria per 25 fields of view
+	10-50 bacteria per 25 fields of view
++	5-20 bacteria per single field of view
+++	>30 bacteria per field of view

TCBS/BA plates with the primary cultures were examined to determine growth. Any distinct colonies that were present in each media were isolated into a fresh ZoBell Marine Agar (ZMA) media and incubated at 25^{o}C. Biochemical characterisation of microorganisms cultured in this media was performed using the MicroSys® V36 test (DPIW, Launceston). In brief, cultures of the unknown strains less than 24-48 h were used. Bacteria were suspended in 3 mL of 2% NaCl in order to obtain a density equal to 0.5 McFarland standards. 1 mL of this suspension was diluted into 4 mL of 2% NaCl and mixed gently to produce the inoculum. 96-well microtitre plates were set up with the reagents outlined in the manual for MicroSys® V36. 50 µL of the inoculum were placed in wells A1 to D6 of a 96-well microtitre plate. Wells A1 - 6 and D1 - 4 were covered with sterile paraffin oil. The plate was sealed and incubated at 25^{o}C for 48 h, monitoring regularly the change in colouration of the reactions in the wells. After 48 h additional reagents were added to wells A9 - 12 for further analyses as indicated in Appendix 1. In parallel to the microplate, an antibiotic sensitivity test was carried out in Mueller-Hinton agar + 2% NaCl plates. Sensitivity to five antibiotics was tested: Carbenicillin 100 µg (Oxoid, Australia), Novobiocin 5 µg (Oxoid, Australia), Ampicillin 10 µg (Oxoid, Australia), O/129 10 µg (Oxoid, Australia) and O/129 150 µg (Oxoid, Australia). Lawn plates of the unknown microorganism were prepared by swabbing the agar plates with the McFarland 0.5 suspension. Antibiotic discs were placed at regular intervals within each plate and these were incubated at 25^{o}C for 24 h. Then the diameter of the inhibition zone (where no bacterial growth occurred) was measured for each disc and results interpreted according to the guidelines. Results obtained from the biochemical tests were assessed using the Probabilistic Identification of Bacteria (PIBWin) (Bryant, 2004) software and the data matrix for *Vibrio* sp (VibEX7_DB). An unknown isolate was identified as a species when it presented an ID score of 0.99 and a modal score of 0.001.

CSF samples

Samples obtained from the fish were transferred from ice and refrigerated at 4^{o}C until analysed. A drop of fluid was placed on a glass slide and observed

under a dissecting microscope (Olympus SZX12 stereomicroscope, Olympus, Japan) for movement indicative of the presence of the ciliate *U. nigricans*. Some samples were further investigated by compressing them with a cover slide and observing under a light microscope (Eclipse E400, Nikon Corporation, Japan) at 100x and 400x magnification for the presence of the parasite. A sample was considered positive if at least one *U. nigricans* was found and identified (Deveney et al., 2005). Some samples were confirmed using species specific PCR.

Histological samples

Slides were examined under light microscope (Leitz Diaplan microscope, Leica Microsystems, Germany) at 100x magnification and details at 400x or 1000x under oil immersion. Any histopathological changes or presence of pathogens was recorded and photographed using a digital camera attached to the microscope (Progress C14, Jenoptik, Germany).

Statistical analyses

Whenever possible, data were analysed using SPSS 16.0 statistical software (SPSS Inc, USA). A *P*-value < 0.05 was considered a significant difference for all cases.

ANOVA test was performed to estimate effects of different factors. A Levene's test for differences in variances between groups was carried out before the test. If variances differed significantly, data were log-transformed in order to reduce these differences.