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Project Title: Aquafin CRC - SBT Aquaculture Subprogram: improving fish husbandry and performance through better understanding of the relationship of fish stress and health

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Organisation: University of Tasmania

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Final Report

COMMERCIAL IN CONFIDENCE

AQUAFIN CRC – FRDC
SOUTHERN BLUEFIN TUNA AQUACULTURE SUBPROGRAM: IMPROVING HUSBANDRY AND PERFORMANCE OF SOUTHERN BLUEFIN TUNA THROUGH BETTER UNDERSTANDING OF THE RELATIONSHIP BETWEEN FISH STRESS AND HEALTH

Barbara F. Nowak, Daryl Evans, David Ellis, Ryan Wilkinson, Mark Porter, Philip Crosbie, Melanie Leef and Craig Hayward

February 2010

FRDC PROJECT NUMBER: **2006/225**
CRC PROJECT NUMBER: **3.7**

Aquafin CRC – FRDC
Southern Bluefin Tuna Aquaculture Subprogram: Improving husbandry and performance of Southern bluefin tuna through better understanding of the relationship between fish stress and health

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Table of contents

2006/225 Aquafin CRC - SBT Aquaculture Subprogram: Improving husbandry and performance of Southern Bluefin Tuna through better understanding of the relationship of fish stress and health

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OBJECTIVES:
1. To investigate the relationship between husbandry practices and SBT performance (at the level of tow and pontoon)

2. To investigate development of non-lethal indices for SBT health and performance and assess their predictive value

3. To determine the relationship between SBT health, stress and fish performance (individual fish level)

Non-Technical Summary

OUTCOMES ACHIEVED

This project has increased our understanding of the effects of tow conditions on SBT performance. We have developed an industry database including information from four companies from 2003 to 2008, which confirmed the effects of tow condition on mortality. A total of 73 tows and 290 transfers into holding cages is included in this database. New methods were developed for measurement of stress, health and performance of SBT.

Before this project our knowledge of SBT performance and its relationship to stress was based on anecdotal evidence. This project provided scientific evidence showing effects of tow conditions on fish performance. We showed the relationship between husbandry practices and SBT performance during tows. In all six years covered by this study, mortality generally was low during the initial weeks following transfer and began increasing from week 6. In four of the six years (2004, 2006, 2007, 2008) the increased mortality rate at week six was quite marked. By 9-10 weeks mortalities were back to lower levels with the exception of 2008 when mortalities were still elevated after 12 weeks post-transfer. The company can have an effect on the mortality level. This can be due to husbandry practices, the location of the company site or quality of the caught fish. In the initial years (2003 to 2006), the initial mortality (week 1) was positively correlated with biomass of fish on tow, the number of morts on tows was positively correlated with number of days of the tow and negatively correlated with speed of tow (the latter two variables obviously are

highly negatively correlated) and the number of morts later in the period (weeks 8 to 12) was positively correlated to tow length. However, these associations between tow conditions and subsequent mortality appeared to be reduced in more recent years (2007-2008) with the only significant correlation being the positive correlation with tow density and mortality in weeks 5 to 8 after transfer. There was no statistically significant relationship between time taken to transfer fish and mortalities at week 12. The effect of tow on fish was reflected by a peak in cortisol level in SBT sampled at average weight (at transfer to grow-out cages) in comparison to wild fish and fish sampled at mid-season.

We investigated development of non-lethal indices for SBT health and performance and assessed their predictive value. The blood sampler prototype did not perform to a satisfactory level, despite extensive testing and improvements in its design. However, blood samples with low levels of cortisol and good survival of the fish were collected by bleeding fish caught on baited hook from the pontoons. This is currently the preferred non-lethal method of blood sampling from tuna. Cortisol was undetectable in samples of water and faeces. The lack of detectable cortisol concentrations at least partly reflects analytical constraints of this assay. To our knowledge, there have been no reports on the capability of non-invasive cortisol measurement for the assessment of stress in species held in sea cages, but this is not surprising considering the inherent complexities, such as dilution effects in a dynamic open system. Cortisol concentrations extracted from either water and faeces tend to be relatively low compared to their serum counterparts and therefore require both concentration and/or extraction prior to measurement.

The relationship between SBT stress, health and performance was investigated at individual level. There was a high variability for all three stress indicators – cortisol, glucose and lactate. This variability made detection of any seasonal or annual patterns difficult. Individual variability and high and low stress responders most likely contributed to these results. High replication is required to detect patterns in SBT stress variables. SBT showed a high level of individual variability with regard to stress levels and parasite loads. Observed parasite loads did not affect haemoglobin level. No relationship could be detected between parasite loads and stress levels or stress levels and immune response.

KEYWORDS:
SBT, aquaculture, health, stress, performance

Acknowledgments

This study formed part of a project of Aquafin CRC, and received funds from the Australian Government's CRCs Program, the Fisheries R&D Corporation and by other CRC participants. 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 Dr Peter Montague (Aquafin CRC), Dr Patrick Hone (FRDC), Prof Colin Buxton (TAFI), Prof Chris Carter (TAFI) and Brian Jeffriess (ASBTIA) for their continuing support during this project. We would like to thank Dr Richard Morrison, Jen Covello, Victoria Valdenegro, Melanie Andrews, Ryan Longland and Michael Attard for all their contributions to the project, and Adam Watkins and Doug Graske for providing the cover photos.

Background

The effects of stress on farmed fish have been a focus of research for over two decades and are still a key factor in the welfare and performance of stocks (Lymbery 2002). Pickering (1993) defined stress as the reaction observed following a stimulus acting on a biological system and the subsequent effect on that system, which in the case of aquaculture, may be a reduction in immune function (Pickering and Pottinger, 1989, Verburg-van Kemenade et al 1999), an inhibition of reproduction or reduced growth rate (Pottinger 2000, Dyer et al 2004). With the SBT industry relying on wild caught stock these individuals are more likely to experience elevated stress levels when compared to more domesticated species such as rainbow trout (Pottinger 2000; Thomas et al 2003; Rough et al 2005, Thomas et al submitted). It is therefore essential that key points in production stress are identified and methods of reducing these stressors developed if optimal production strategies are to be met. Finfish aquaculture has been a major success of primary industry in Australia in recent years. Driving this success has been the Southern bluefin tuna (SBT) industry in South Australia. Whilst finfish aquaculture is relatively young compared to established livestock industries, aquaculture already makes a significant contribution to the Australian community. For example the tuna aquaculture industry produces about 8500 tonnes of fish worth $AUD250 million (2003/2004). The SBT industry has identified international competitiveness as its main future research priority. This research area is one of their priorities and aims to investigate ways of increasing productivity through stress reduction and the development of integrated databases. This priority resulted in the development of this proposal and the involvement of industry stakeholders in the research team. Stress in SBT is already considered by industry to be a major cost. In the future it is proposed that SBT will be cultured for longer (12-15 months) than the existing industry protocol (3-6 months). If cultured fish are exposed to potentially stressful situations on an ongoing basis, stress may even further compromise production. The physiological effects of stress have been shown to negatively affect the fish immune system *in vitro* and these effects have also been observed *in vivo*. Therefore understanding sources of stress in SBT and health and their links with fish performance may increase productivity and international competitiveness.

The project proposed therefore sets out to answer the following questions:

1. Do variations in the procedures and conditions during capture, tow and transfer significantly influence subsequent health, performance or mortality of ranched SBT?
2. Do variations in on-farm husbandry practices significantly influence health, performance or mortality of ranched SBT?
3. Is “stress” (acute or chronic) a major factor mediating effects of catching and ranching practices upon subsequent health, performance or mortality?
4. If so, is the effect of stress in SBT mediated primarily through effects on the immune system or otherwise?

The answers to these questions are intended to provide information that the industry can use to develop strategies to avoid or minimise adverse effects of catching, tow, transfer and farming practices. Furthermore, the best correlates of SBT performance (either stress measurement for example glucose or health measurement for example lysozyme activity) will be developed as non-lethal predictive indices for future use by the SBT industry.

Husbandry practices within the tuna industry have developed since 1991 through a mixture of 'bush' engineering and, more recently, collaborative R&D supported by the Aquafin CRC. For various reasons, not the least of which are logistical practicalities, formal R&D has focussed on the grow-out and market qualities of tuna, leaving the initial tow, transfer and early grow-out component of the husbandry cycle unstudied. As industry practices have developed, anecdotal evidence suggests that many operators experience fluctuating results (at least in terms of mortality) during these production stages. An initial objective of the project has been to collate industry data from four participating farms to investigate the relationship between tow and transfer conditions and subsequent survival in the first three months of grow-out. Of particular interest was the reported spike in mortality of fish experienced by some operators around 6 to 8 weeks after transfer.

Stress is a physiological response to behavioural, physical or homeostatic threats. This response is characterised by a primary activation of the neuroendocrine system, resulting in a cascade of physiological and metabolic changes that enable an animal to adapt or avoid the potentially hostile environment. Levels of cortisol, glucose and lactate in blood (plasma or serum) have been used to measure stress. Elevated levels of cortisol are an indicator of acute stress, elevated levels of glucose may correspond to acute and chronic stress and increased lactate shows cellular metabolism in response to stress.

The effects of stress on farmed fish have been a focus of research for over two decades and are still a key factor in the welfare and performance of stocks (Lymbery 2002). Pickering (1993) defined stress as the reaction observed following a stimulus acting on a biological system and the subsequent effect on that system, which in the case of aquaculture, may result in a reduction in immune function (Pickering and Pottinger, 1989, Verburg -van Kemenade et al 1999), an inhibition of reproduction or reduced growth rate (Pottinger 2000, Dyer et al 2004). In fish, stress has been shown to impact on growth through the growth hormone/IGF axis. Maintenance of growth in fish is influenced by many environmental and genetic interactions and to a large extent is hormonally controlled by the somatomedin *or* growth hormone/insulin-like growth factor (GH/IGF) axis (Reinecke et al*,* 2005). In response to environmental stimuli and nutritional inputs, the pituitary production and endocrine secretion of GH stimulates the production of insulin-like growth factor-I (IGF-I) in a range of tissues (Moriyama et al*,* 2000). In turn, IGF-I stimulates DNA synthesis, cartilage sulfation and protein synthesis, and enhances osmoregulatory ability (Wood et al*,* 2005). Furthermore, it has been demonstrated, that circulating levels of IGF-I correlate positively with growth rate in a range of species (Beckman et al*,* 2004; Dyer et al*,* 2004a).

Circulating IGF-I levels could therefore be used as an important predictor of growth performance in fish.

With the SBT industry relying on wild caught stock these individuals are more likely to experience elevated stress levels when compared to more domesticated species i.e. rainbow trout (Pottinger 2000; Thomas et al 2003; Rough et al 2005, Thomas et al submitted), therefore it is essential that key points in production stress are identified and methods of reducing these stressors developed, if optimal production strategies are to be met.

Need

The tuna industry in Australia is limited by catch quota. Increased competitiveness through production efficiency is the main way to improve the value of the industry. Improved performance can be achieved through stress reduction and minimising mortalities. 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.

Tuna are hardy under current husbandry practices, and the industry experiences low mortality. The wild capture of immunocompetent 3 – 5 yr old fish is the main reason; however the short growout time and advances in ranching technology have been significant factors. Despite its newness, the industry enjoyed healthy returns for the first 10 years.

Since 2002 there has been a significant fall in revenue (prices down over 50% and strengthening of the Australian dollar). The 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 stress impacts limiting production. Mortality is an obvious area and the current level is no longer acceptable. Possibly even more important, stress may cause economic costs to the tuna industry in lost growth and condition. There is a potential for increased productivity through stress reduction.

The quota places a limit on what biomass can be ranched each year. Rather than merely growing out for 3-6 months, the option of longer term holding is a priority to increase productivity. Then the role of stress and the influence of husbandry practices on fish health and production will be even more important.

As each individual fish has high commercial value, there is a need to develop non-lethal indices for SBT monitoring. Predictive indices will be valuable for planning production and harvesting.

Objectives

1. To investigate the relationship between husbandry practices and SBT performance (at the level of tow and pontoon)

2. To investigate development of non-lethal indices for SBT health and performance and assess their predictive value

3. To determine the relationship between SBT health, stress and fish performance (individual fish level)

Methods

Objective 1. To investigate the relationship between husbandry practices and SBT performance (at the level of tow and pontoon)

Industry database

This study provided the first opportunity to look at various aspects of the period from towing through early grow-out, in five companies over a six year period (2003 to 2008). For confidentiality purposes these companies are referred to as Companies 1 to 5. Unfortunately the sale of one company through the study period precluded continuing this data source and another company was enlisted to fill the void. For this reason subsequent company based comparisons were made from reduced datasets of only five years (x 4 companies) or only 3 companies (x 6 years). Tow and early mortality data were recorded from each of four companies for the last six years (2003-2008). Table 1 provides a list of the variables recorded and derived (*italics*).

Table 1. Principal variables used in historic data review

Tow No.	*Assigned tow identifier*
Protec Tow No	Number of tow cage as recorded by Protec
Cage No (growout)	Number of holding cage fish transferred into
Diameter	Diameter of tow cage
Leadline depth	Depth of tow cage to leadline
Latitude	Latitude of capture area
Longitude	Longitude of capture area
1st fish capture	Date of 1st fish into tow cage
Last fish capture	Date of last fish into tow cage
Average weight	Average of 40 fish sample taken with Protec
Average weight date	Date of 40 fish sample -- taken as end of tow
Tow length (days)	*No days between 1st fish capt and Av wt date*
Tow distance	*Distance between capture lat/long and end of tow pt*
Tow speed	*Tow lgth dist/tow lgth days/24 hours*
Tow density	*Density of fish in tow cage in fish/m^3*
Tow biomass	*Biomass of fish in tow cage in kg/m^3*
No of fish in tow cage	Sum of fish transferred to holding cages plus tow morts
Tow mortalities	No. of morts in tow cage
No of fish	No of fish transferred to holding cage
Transfer date	Date of transfer into holding cage
Diameter H	Diameter of holding cage
Leadline depth H	Depth of holding cage to leadline
Start transfer	Time of starting transfer
Finish transfer	Time of finishing transfer
Transfer time	*Minutes between start and finish of transfer*
Mortalities weekly for 3 mths	Mortality in respective weeks to week 12

Data were received for each individual grow-out cage of the four companies that had fish transferred into them over the review period. Where comparisons were made at a tow cage level, individual grow-out cage numbers and mortalities were summed and expressed as a total for the relevant tow cage.

Due to timing and confidentiality constraints, a geographic point approximately 1.5 nm north east of Davidson Rock in the centre of the farming zone was used as the end of tow position for all tows. Relationships between tows and variables were investigated using graphical, classification (hierarchical cluster analysis) and correlation (Spearman's rho coefficient) techniques.

Objective 2. To investigate development of non-lethal indices for SBT health and performance and assess their predictive value

Underwater blood sampler

Prototype underwater blood sampler was developed, designed and tested.

Non-lethal bleeding

Fish caught on baited hook were transported on board the vessel and blood sample taken using a syringe and needle. Fish were tagged and their survival assessed. Blood samples were analysed.

Measurements of cortisol in water, fish mucus and fish faeces

The potential for cortisol measurements in water, SBT mucus and SBT faeces has been investigated. Faecal samples were collected by divers. Mucus samples were collected from skin of the fish during sampling. Cortisol was assayed as previously described (Pankhurst & Sharples 1992). *Please see Appendix 3 for detailed methods.*

Objective 3. To determine the relationship between SBT health, stress and fish performance (individual fish level)

Survey of stress indices

The pilot survey included three tows, one pontoon/tow. Fish (ten on most occasions) were sampled wild (two tows only), at the end of tow (three tows) and at harvest (three tows). The survey in 2007 and 2008 followed 5 tows (2 from one company). Blood samples from Longer Term Holding (LTH) were also included in the survey. Additionally, the effect of harvest method was investigated in 2006, method 1 was a normal net harvest while method 2 was a harvest with a quicker fixed hook/gaff method. All wild fish and fish at the end of tow were caught with a hand line. Fish at harvest were caught by diver or gaff. The wild fish and the fish sampled at the end of tow were bled either from the lateral artery posterior to pectoral fin (traditional bleed cut position) or from a duct of Cuvier. Blood was collected through normal harvest bleeding for harvest fish. The stress indices measured were blood plasma cortisol, glucose and lactate. Cortisol was determined by radioimmunoassay (RIA) as described by Pankhurst & Sharples (1992). The assay involves a standard ethyl acetate extraction step and has a detection limit of 0.6 ng/mL. Three samples had cortisol concentration below detection limit and a value of 0.6 ng/mL was used for these samples in statistical analyses. Lactate and glucose concentrations were determined using a GM7 Analyser (Series II) developed by Analox as *per* manufacturer's instructions. IGF-I was measured

in blood serum using a commercially available RIA kit (GroPep Ltd., Thebarton, Australia). The kit, developed by Dyer et al, (2004b) uses recombinant tuna IGF-I as the standard, while the antibody used was raised in rabbits against recombinant barramundi IGF-I. This kit has been validated for use with tuna serum and plasma.

The data were analysed using ANOVAs (Cochran's test was used to confirm homogeneity of variance); if the results were significant ($P<0.05$), SNK test was used for comparison of means. Relationships between variables were evaluated using correlation coefficient. Results are shown in graphs as means and standard errors.

Multiple transfer experiment (Hayward *et al* submitted)

In late March 2008, 563 SBT were transferred from a commercial tow cage to a holding pontoon in Port Lincoln (Figure 1). Of these, 474 SBT were measured with callipers and moved into the first of two research pontoons These fish were caught on a baited hook. Between 7 and 9 April 2008, 16 swim through transfers comparable to commercial transfers were conducted between the two research pontoons under variable environmental conditions. These fish were the multiple transfer treatment (cage 2). The remaining fish in the holding pontoon (cage 1) were considered unstressed controls. Blood samples were taken from fish on regular basis. Parasite loads were evaluated from late April. Additionally, fish were sampled from two companies on one occasion. SBT were sampled from two cages at each farm – one fed vitamin injected baitfish and a control cage.

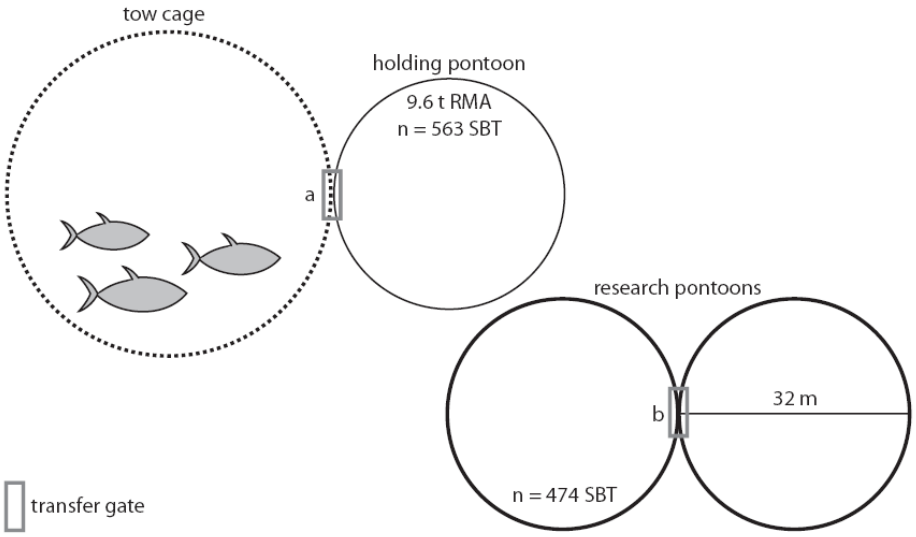


Figure 1. Swim through transfer of SBT from the tow cage to research pontoons. Based on mean weight (17.13 kg) of the 40-fish sample taken from the tow cage on 19 March, 563 SBT were counted through Transfer Gate (a) by conventional underwater video. A subset (n = 474) was then hooked by handline, tagged with conventional spaghetti tags and moved by stainless steel slide into the first of two 32 m diam. research pontoons. SBT were transferred multiple times (n = 16) through Transfer Gate (b) between 7 and 9 April.

Transfer data were subject to a one-way ANOVA with transfer time as the fixed (independent) factor. When the Levene's homogeneity of variance test failed and data could not be transformed the level of ANOVA significance was dropped to ($P < 0.01$). Following a significant ANOVA effect a Tukey's *post hoc* test was used to determine differences among the transfers.

SV 2008 (multiple transfer) data were subject to a two-way ANOVA with time (date) and treatment (control/stressed) as fixed (independent) factors. When the Levene's homogeneity of variance test failed and data could not be transformed the level of ANOVA significance was dropped to ($P < 0.01$). When a significant time effect was found each individual treatment was subject to a one-way ANOVA and, if required, subsequent Tukey's *post hoc* test to determine differences among the time points. When treatment was found to be significant an independent t-test was used to compare the combined data for each treatment irrespective of time. When a significant interaction between time and treatment was found, paired t-tests were used to compare the treatments at the respective time point. Only when the interaction effect was not significant, could significant time and treatment effects be further examined. Commercial data presented as means +/- SEM but not subject to any form of statistical analysis.

Vitamin experiment data were subject to independent t-tests to compare treatment (control and vitamin) within each company.

<u>Relationship between stress indicators and parasitological status of the fish</u>
Coefficient of correlation was calculated for each of the blood variables and parasitological status of the fish (abundance of blood fluke or sea lice) for each cage and each sampling time. If the $P<0.05$ then the results were considered to be statistically significant.

Results/Discussion

Objective 1. To investigate the relationship between husbandry practices and SBT performance (at the level of tow and pontoon)

Fishing for SBT is managed by the CCSBT (Commission for the Conservation of SBT). A quota for Australia has been set at 5265 tonnes since 1989. In late 2009 this was reduced to 4015 tonnes. Summary tow statistics for the farming calendar years of 2001 to 2008 are provided in Table 2 (Australian Southern Bluefin Tuna Industry Association, B.Jeffries pers. comm.). Note actual fishing periods include the December of the year before.

Table 2. Historic tow effort

Year	Catch boats	Quota caught (T)	Morts in catching	Number of tows	Morts pre-transfer (%)
2001	8	5161	71	38	0.42
2002	7	5234	61	40	0.34
2003	7	5375	82	38	0.37
2004	6	4860*	86	36	0.24
2005	7	5215	113	36	0.33
2006	6	5302	128	38	0.25
2007	6	5228	106	33	0.17
2008	6	5212	73	30	0.18

***Reduced due to payback for over catch in 2003**

There has been a small reduction in the number of individual catch boats as a result of industry takeovers and rationalisation. Quota caught has been consistent as would be expected of a quota-restricted sector. Mortality experienced during the capture and transfer of tuna into tow cages on the fishing grounds has also been consistent and low.

The number of tow cages used to transport the tuna back to the ranching sites offshore Port Lincoln has decreased in recent years (2007 and 2008). With respect to fish numbers this has resulted in an increase from around 7000 tuna per tow cage in 2001 and 2002 to 10000 to 11000 tuna in 2007 and 2008 (ASBTIA pers. comm.). Some of the increase in fish numbers can be attributed to a general increase in the size and holding capacity of the tow cages over the last several years. These aspects are discussed further in subsequent investigations of the five companies involved in this study. Whether or not there has been a significant increase in towing biomass since 2001, the continued reduction in the combined mortality experienced during catching, on tow and prior to transfer would suggest industry practices during this period have improved. Tuna mortalities from the time of purse seine capture to transfer into ranching cages at Port Lincoln in recent years (0.18% in 2008, 0.17% in 2009) are less than half (42-45%) of those achieved in the first few years (0.42% in 2001 and 0.37% in 2003).

Of interest is whether such a postulated improvement in operating practices is apparent further into the farming cycle. Anecdotal evidence would suggest that not to be the case. In the early 1990's using pole caught fish tuna farmers experienced exceptionally high mortalities of up to 35% (ASBTIA pers com.).

Figure 2 indicates that by 2001 mortalities (industry total) had reduced to below 5% and remained below 4% until 2006. Since then there has been a marked increase in mortality. Because of this marked change in the industry's mortality levels since 2006, subsequent investigations have focussed on pre and post 2006 tow characteristics.

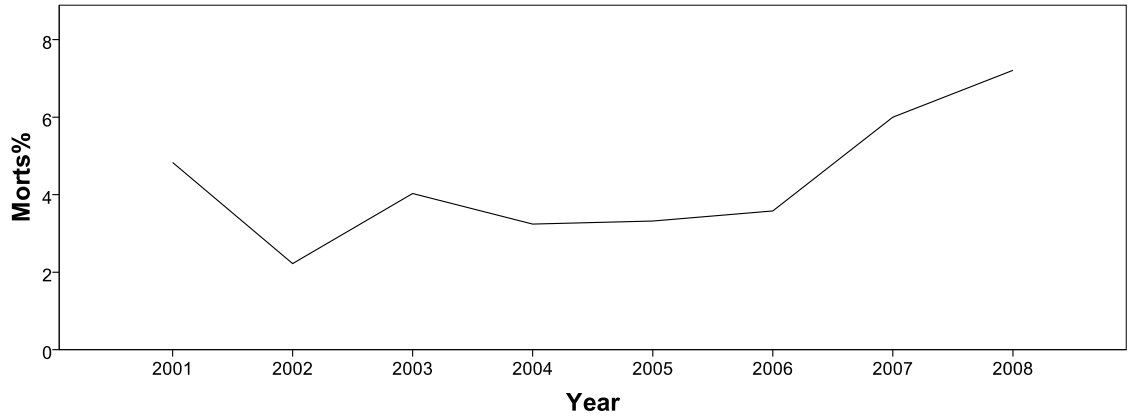


Figure 2. Total mortalities as percentage of tuna caught 2001 to 2008

Historical database

The database consisted of 290 transfers into grow-out cages from a total of 73 tow cages over six years (2003-2006: 204 transfers from 50 tow cages; 2007-2008: 86 transfers from 23 tow cages). Table 3 provides a summary of descriptive statistics for relevant tow variables.

Tow data for the five participating companies do not suggest any reason for the higher mortalities in recent years. Slightly more fish (4%) on average were brought back in 2007 and 2008 although the fish were 0.5 kg smaller. Tow distances were about the same but average tow speeds were reduced by 25% (2003-2006: 0.52 knots, 2007-2008: 0.4 knots) increasing the length of tow by four days. No change in the number of mortalities recorded on the tows is apparent. No obvious reasons can be identified from the tow data as to why subsequent mortalities in grow-out have increased since 2006. Unfortunately no data are available on feeding regimes on tows to assess if lack of food on longer tows may be a contributing factor.

Table 3. Descriptors of relevant tow variables

2003-2006	Mean	Min	Max
Average weight (kgs)	16.55	11.97	23.53
Tow length (days)	22	12	40
Tow distance (nm)	246	186	314
Tow speed (kts)	0.52	0.28	0.87
Tow density (fish/m^3)	0.38	0.16	0.63
Tow biomass (kg/m^3)	6.15	2.82	10.42
No of fish in tow cage	8476	3153	12801
Tow mortalities	14	2	49
Transfer time (min)	67	9	218
2007-2008	Mean	Min	Max
Average weight (kgs)	15.96	13.18	22.75
Tow length (days)	26	15	40
Tow distance (nm)	231	195	307
Tow speed (kts)	0.4	0.23	0.69
Tow density (fish/m^3)	0.38	0.09	0.76
Tow biomass (kg/m^3)	5.90	2.06	12.07
No of fish in tow cage	8828	1741	18155
Tow mortalities	15	2	68
Transfer time (min)	Not recorded	Not recorded	Not recorded

Companies 4 and 5 had slower average tow speeds that the other three and as a result have tows lasting 25-45% longer on average. Company 5 and 2 had higher stocking rates than the other companies. Table 4 summarises the tow parameter means for each company.

Table 4. Descriptors of relevant tow variables for each company

	Company				
	1	2	3	4	5*
Average weight (kgs)	16.51	15.81	16.60	15.93	17.14
Tow length (days)	21	24	22	30	31
Tow distance (nm)	240	246	242	242	230
Tow speed (kts)	0.51	0.46	0.49	0.35	0.31
Tow density (fish/m^3)	0.38	0.45	0.32	0.34	0.61
Tow biomass (kg/m^3)	6.02	7.01	5.40	5.30	10.49
No of fish in tow cage	8210	10370	7680	8710	12654
Tow mortalities	10	24	15	25	2

*Only one tow for this company in data set

Figure 3 shows the percentage of total mortalities for the companies combined for the first 12 weeks after transfer for each of the years 2003 to 2008.

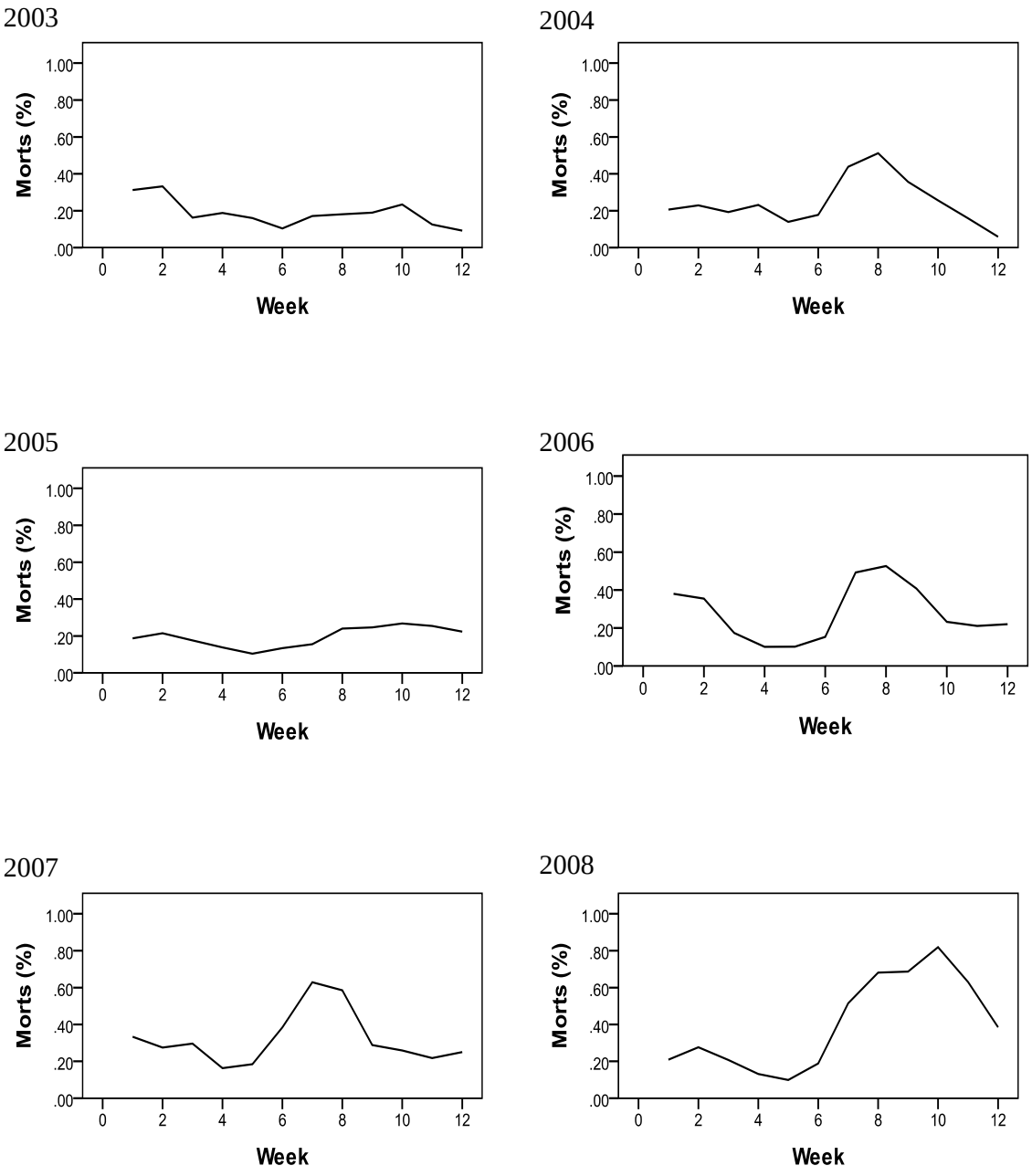


Figure 3. Percentage mortality in the first twelve weeks 2003-2008

In all of the years, mortality generally was low during the initial weeks following transfer and began increasing from week 6. In four of the six years (2004, 2006, 2007, 2008) the increased mortality rate at week six was quite marked. By 9-10 weeks mortalities were back to lower levels with the

exception of 2008 when mortalities were still elevated after 12 weeks post-transfer. Although 2009 records are not part of this report and were not available at the time of writing, anecdotal accounts suggest the mortality pattern in 2009 was similar to 2008. The increase in mortality from 2006 evident for the entire industry (Figure 2) was also evident in the five companies of this study (Figure 3). It is believed this justifies the continued separation of analyses into pre and post 2006.

Figure 4 shows the average percentage of total mortalities for the first 12 weeks after transfer for the four individual companies that provided data for at least 5 of the years of the study.

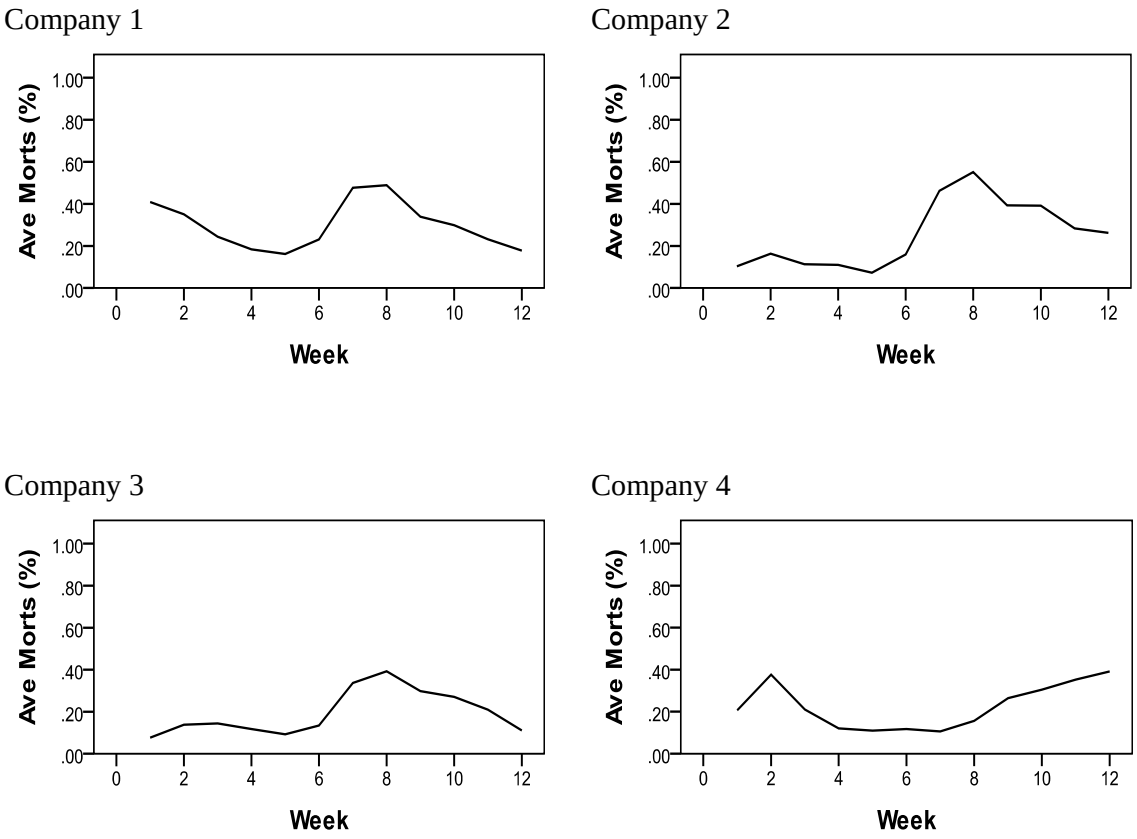


Figure 4. Average percentage mortality in first twelve weeks for each company 2003-2008

Subtle differences exist. Companies 1 and 4 had the highest initial mortalities suggesting possibly sub-optimal tow/transfer procedures. Mortality levels at company 4 reduced to 'industry average' levels for a few weeks but then experienced escalating average weekly mortality through to at least 12 weeks. Company 2 had low initial mortality but experienced the highest mortality of the four over the period of elevated mortality post 6 weeks. Company 3 performed best of the four companies in terms of mortality. This company had the lowest average number of fish in the tow cage and we suggest 9000

fish in a 45 m tow cage as a threshold and 12000 fish as definitely too many for the tow.

Figure 5 shows the percentage of total mortalities for the first 12 weeks after transfer in 2008 for four of the companies.

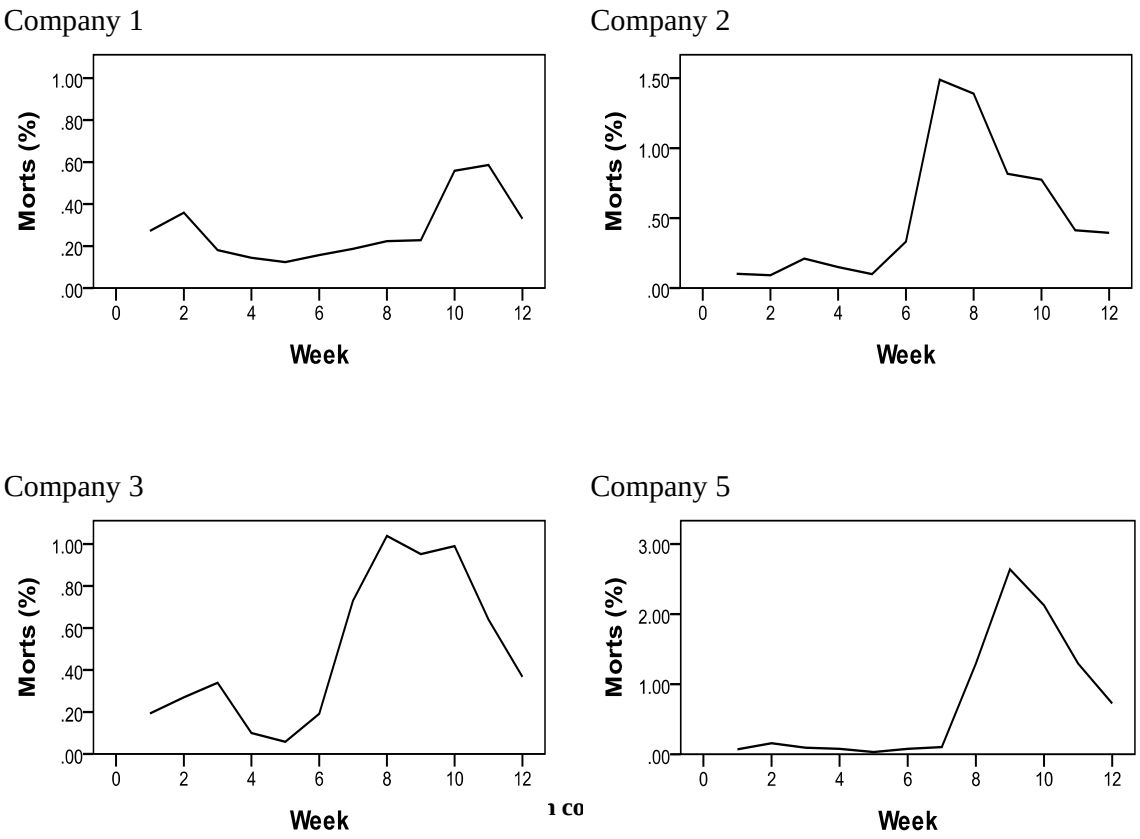


Figure 5 Percentage of total mortalities for the first 12 weeks after transfer in 2008.

As expected mortality levels were consistently higher across all companies in 2008 than average levels in earlier years. Company 5 experienced substantially higher mortalities (up to 2.5% in week 9) than the others, followed by Companies 2 and 3 (Figure 5). Company 1 continued its trend of having higher initial mortalities in weeks 1 to 3 following transfer but then performed better than the other three companies in 2008.

Table 5. Correlation matrix for tow parameters 2003 - 2006

	Fish Total										
%TM	-0.22 0.122	% Tow Morts									
Dens	0.753 0.000	-0.24 0.088	Density								
Biomass	0.584 0.000	-0.16 0.264	0.815 0.000	Biom							
Length	-0.01 0.957	0.351 0.012	0.053 0.714	0.044 0.763	Lgth.						
Distance	-0.04 0.773	0.031 0.830	-0.14 0.348	-0.18 0.224	0.271 0.057	Dist.					
Speed	-0.01 0.948	-0.35 0.012	-0.10 0.480	-0.14 0.346	-0.92 0.000	0.092 0.524	Spd.				
% Morts 1st W	0.090 0.536	-0.03 0.863	0.236 0.099	0.350 0.013	0.169 0.242	0.030 0.834	-0.22 0.131	%W1			
% Morts 1st M	0.043 0.767	-0.01 0.982	0.097 0.502	0.263 0.065	0.200 0.164	0.158 0.273	-0.19 0.193	0.895 0.000	%M1		
% Morts 2nd M	0.115 0.426	-0.18 0.217	0.161 0.263	0.239 0.095	0.001 0.995	0.135 0.350	0.013 0.929	0.457 0.000	0.533 0.000	%M2	
% Morts 3rd M	0.069 0.634	-0.03 0.823	0.155 0.282	0.122 0.399	0.320 0.023	0.261 0.067	-0.25 0.083	0.640 0.000	0.647 0.000	0.498 0.000	%M3
% Morts Tot	0.091 0.531	-0.08 0.569	0.162 0.261	0.272 0.056	0.227 0.114	0.224 0.118	-0.19 0.189	0.784 0.000	0.861 0.000	0.802 0.000	0.820 0.000

Legend:

Top : Spearman's rho coefficient
Bott : Level of probability (2-tail)
Significant correlations

From Table 5 it is evident that:

- Initial mortality (week 1) is positively correlated with biomass of fish on tow
- Number of morts on tows is positively correlated with number of days of the tow and negatively correlated with speed of tow (the latter two variables obviously are highly negatively correlated)
- Number of morts later in the period (weeks 8 to 12) is positively correlated to tow length

Table 6. Correlation matrix for tow parameters in 2007 and 2008

	Fish Total										
%TM	-0.27 0.206	% Tow Morts									
Dens	0.854 0.000	-0.22 0.304	Density								
Biomass	0.809 0.000	-0.14 0.532	0.957 0.000	Biom							
Length	0.114 0.612	-0.16 0.471	0.088 0.698	0.019 0.934	Lgth.						
Distance	-0.37 0.086	0.231 0.301	-0.23 0.306	-0.30 0.175	0.111 0.623	Dist.					
Speed	-0.26 0.244	0.229 0.306	-0.16 0.465	-0.15 0.503	-0.88 0.000	0.308 0.164	Spd.				
% Morts 1st W	-0.05 0.837	-0.01 0.955	0.039 0.861	0.060 0.786	-0.38 0.079	-0.23 0.312	0.288 0.194	%W1			
% Morts 1st M	-0.15 0.497	-0.02 0.939	-0.03 0.911	-0.01 0.950	-0.28 0.203	-0.27 0.221	0.182 0.417	0.865 0.000	%M1		
% Morts 2nd M	0.297 0.168	-0.16 0.455	0.559 0.006	0.498 0.016	0.091 0.688	-0.19 0.391	-0.13 0.566	0.255 0.239	0.413 0.050	%M2	
% Morts 3rd M	-0.18 0.425	0.052 0.812	-0.28 0.198	-0.18 0.414	0.052 0.818	0.007 0.974	-0.14 0.533	-0.11 0.618	-0.05 0.805	-0.24 0.274	%M3
% Morts Tot	0.157 0.474	-0.11 0.628	0.285 0.188	0.315 0.143	-0.04 0.857	-0.19 0.393	-0.07 0.751	0.356 0.096	0.485 0.019	0.657 0.001	0.455 0.029

Legend:

Top : Spearman's rho coefficient
Bott : Level of probability (2-tail)
Significant correlations

The associations between tow conditions and subsequent mortality appear to be reduced in the more recent years with the only significant correlation being the positive correlation with tow density and mortality in weeks 5 to 8 after transfer.

Figure 6 displays a grouping of like tows for 2003 to 2006 using a hierarchical cluster analysis. A summary of mean values for characteristics apparently common to each grouping is provided in Table 7.

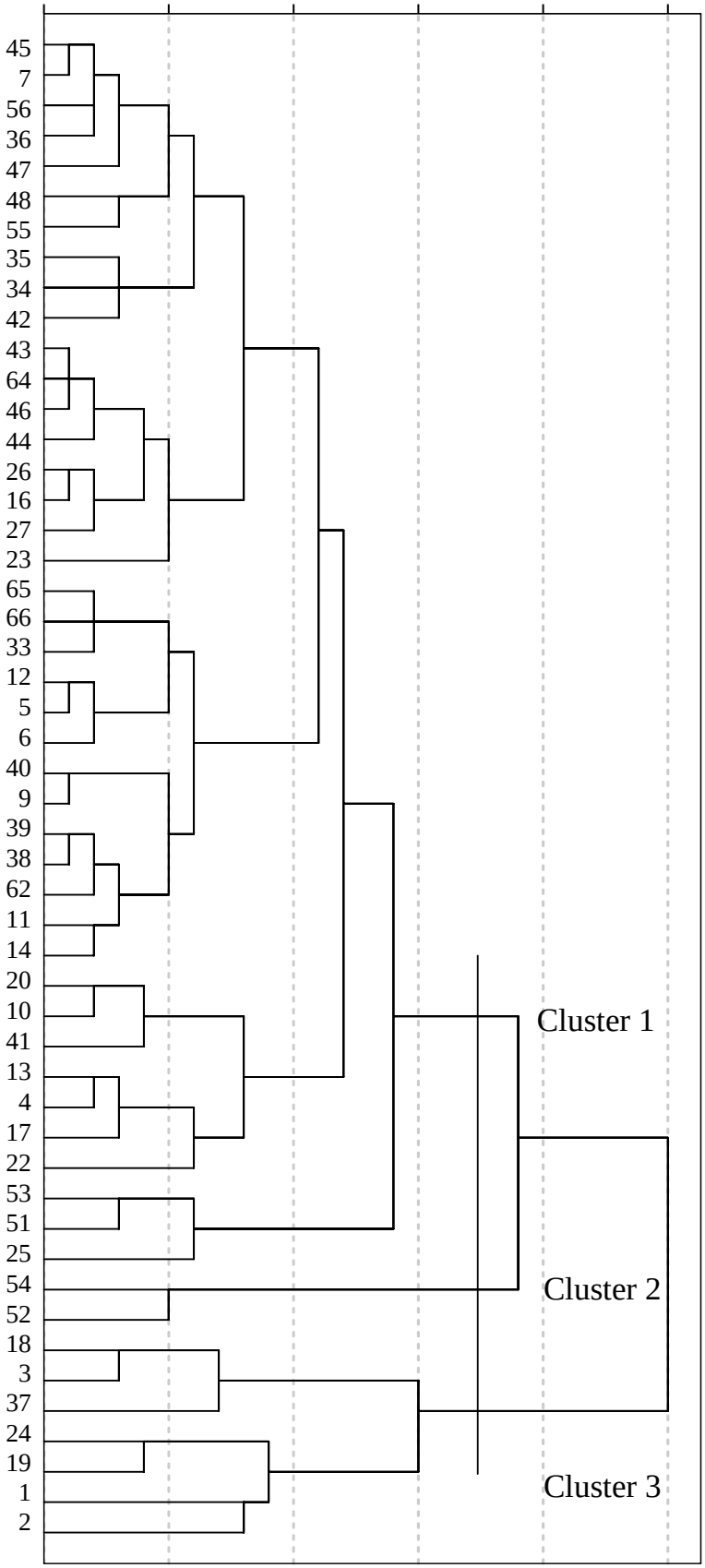


Figure 6. Hierarchical cluster of tow groupings 2003-2006

Table 7. Characteristics of tow groups

	No of tows	No of fish	Tow density (fish/m^3)	Tow biomass (kg/m^3)	Tow speed knots	% Morts after 12 wks
Cluster 1	41	8717	0.39	6.2	0.53	2.1
Cluster 2	2	4200	0.22	3.8	0.35	3.6
Cluster 3	7	8280	0.40	6.8	0.49	6.9

The dominant cluster 1 represents a diverse grouping of tows from mixed companies that experienced reasonable mortalities over the earlier years. It is more instructive to consider the smaller tow clusters. Tows grouped in cluster 2 had comparatively low stocking densities and the lowest tow speed yet had moderate mortalities. All tows in this group were from Company 4. Similarly, all tows grouped in cluster 3 were from the same company, Company 1. This group is characterised by the highest tow biomass and the highest subsequent morality over the 12 week following transfer.

Figure 7 and Table 8 provide the same grouping of tows for the years 2007 and 2008.

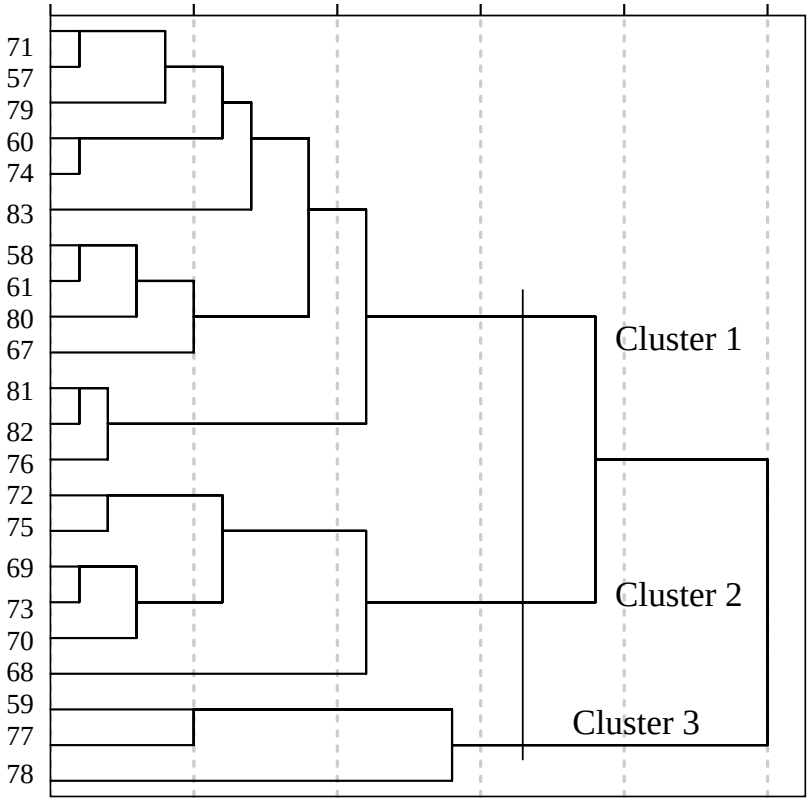


Figure 7. Hierarchical cluster of tow groupings 2007 – 2008

Table 8. Characteristics of tow groups

	No of tows	No of fish	Tow density (fish/m^3)	Tow biomass (kg/m^3)	Tow speed knots	% Morts after 12 wks
Cluster 1	13	6932	0.39	4.4	0.41	2.9
Cluster 2	6	10064	0.47	7.0	0.40	5.4
Cluster 3	3	14950	0.65	10.1	0.35	7.7

Cluster 1 is again the 'norm' with moderate mortalities. Tows grouped in cluster 2 again were all from the same company, Company 1 and the characteristics of this cluster are similar to those of the same company in previous years (cluster 3, Table 7). This suggests this company has made few changes to its towing practices over the years. The biggest change in recent years is represented by cluster 3, characterised by high tow stocking densities and the highest subsequent mortalities. Two of these tows were from one company, Company 2. This possible change in operation to perhaps reduce the cost of bringing in fish would appear to have been counter-productive.

Figure 8 is a scatter plot of time taken to transfer fish against percentage mortality within each grow-out cage after 12 weeks for all cages for which this information was recorded. No apparent relationship exists. This was confirmed with the correlation analysis of these two variables returning a Spearman's rho of –0.112 and a 2 tailed probability of 0.161.

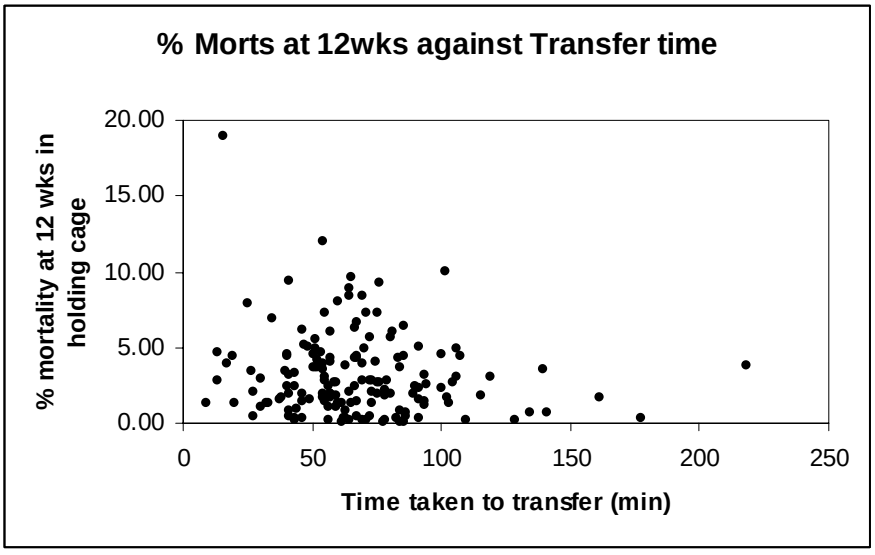


Figure 8. Transfer time against percentage mortality after 12 weeks

Objective 2. To investigate development of non-lethal indices for SBT health and performance and assess their predictive value

Underwater blood sampler

The prototype blood sampler has been developed and manufactured (Figure 9, 10 and 11). A vacuette contained within an aluminium housing was pierced with a needle attached to a collection manifold fed by three 18 gauge needles. The sampler housing was attached to a 1200 mm lance that was pushed by a diver into the side of the free swimming fish in the vicinity of the main lateral blood vessel. The three needles increased the chances of piercing the blood vessel.

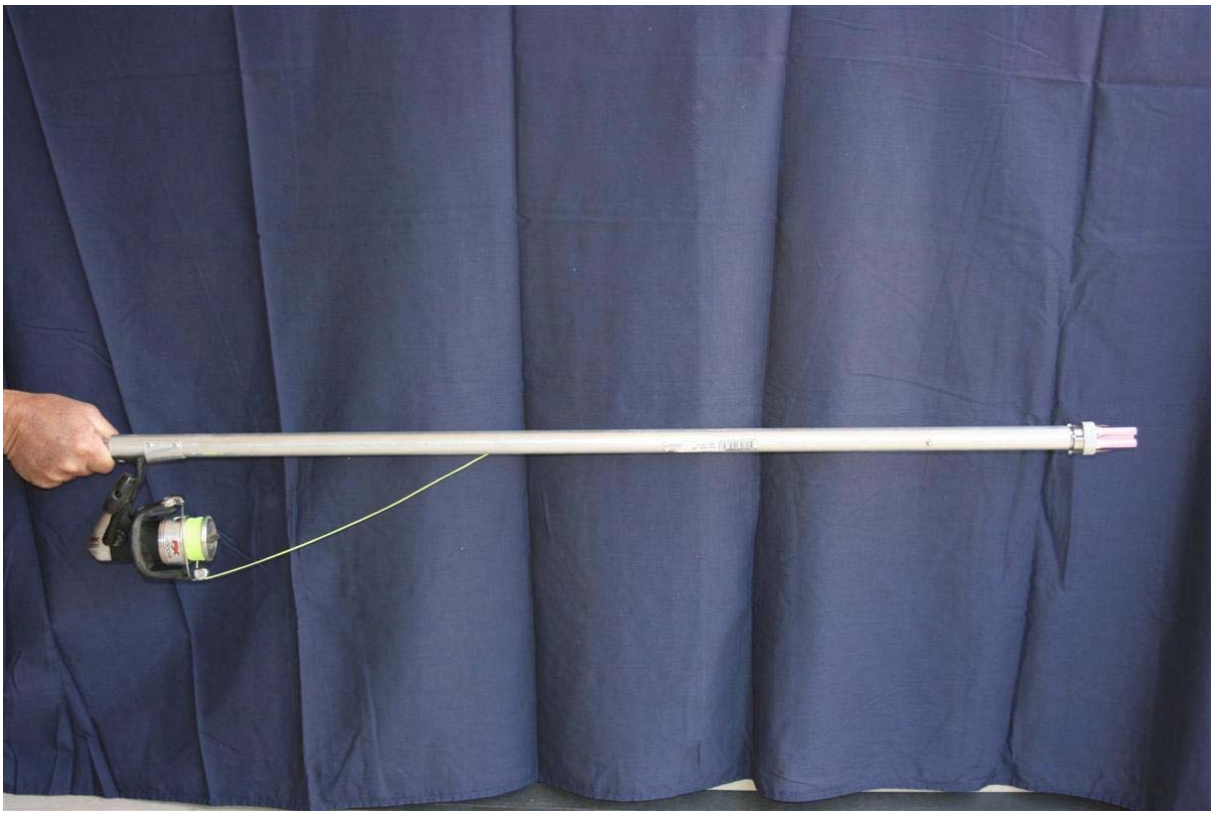


Figure 9. Sampling lance.

The sampler was tried on a fish at the surface. While blood was drawn in the chamber aft of the needles it did not enter vacuette. The vacuette was then pre -seated into holder to make it more effective. The improved blood sampler was further tested throughout harvest period.

To assist the blood collection the housing has three barbed ‘spears’ to hold it in the flesh while the blood is withdrawn. The housing and vacuette are retrieved by a tether to the lance that first removes the vacuette from the collection manifold, then removes the housing from the fish. In controlled trials the sampler successfully collected blood from restrained fish.

Trials on free swimming fish have not been successful. Fish rapidly swim off once struck with the lance by the diver resulting in dislodgement of the housing before blood is removed. A modified housing with larger barbs has also been tried without success. It is considered likely that the size of barb required to hold the sampler in place during the burst of speed following impact, and the subsequent damage upon release, is unlikely to be commercially acceptable.

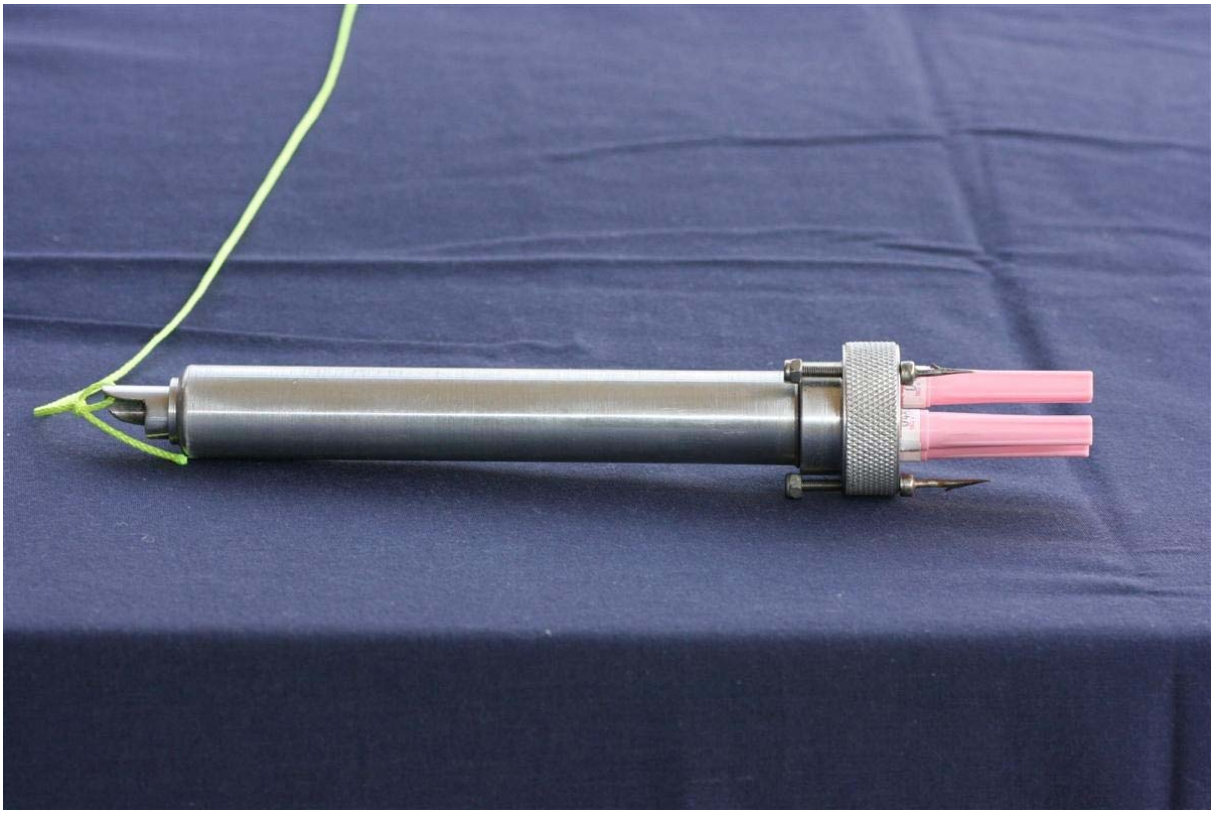


Figure 10. Blood sampler.

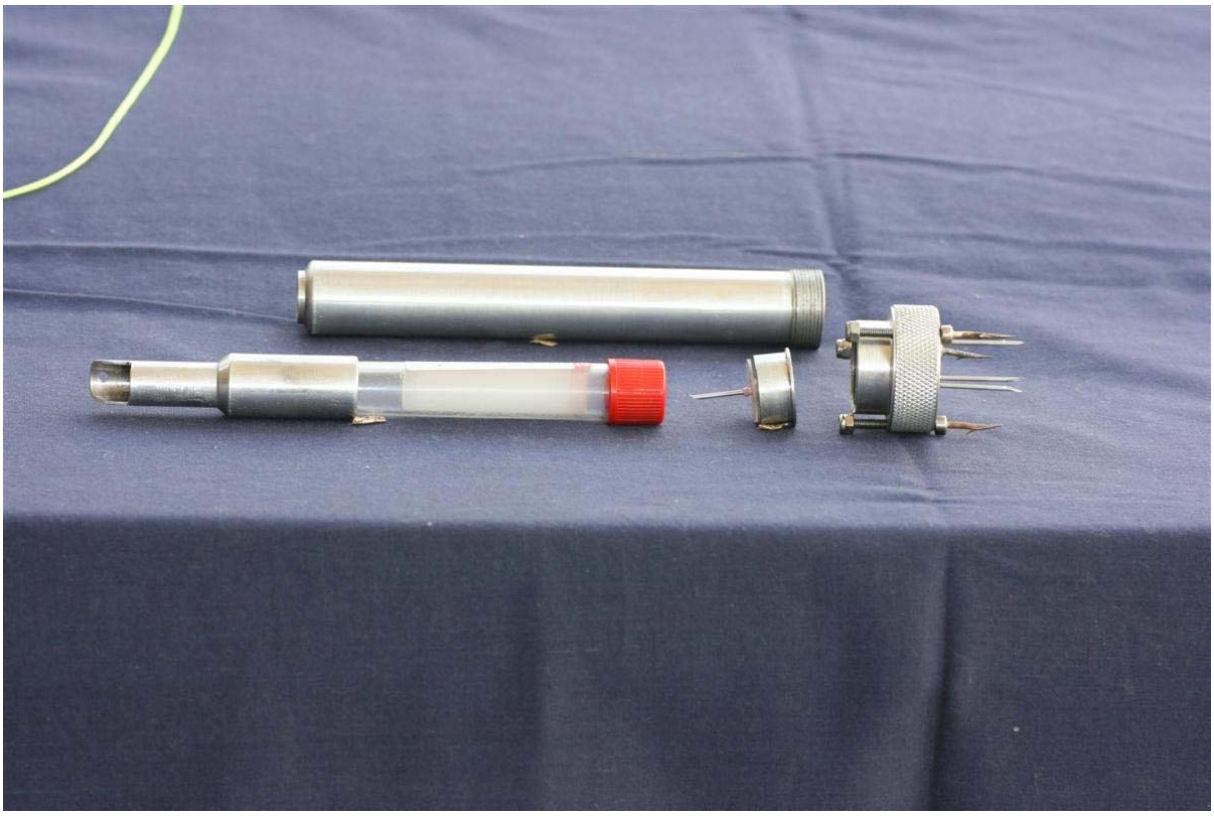


Figure 11. Blood sampler dismantled.

The blood sampler prototype was redesigned to improve its retention in fish during blood sampling. Despite a number of design changes (including adding barbs) and extensive testing, the sampler did not performed at satisfactory level. .

Non-lethal bleeding

We have confirmed that it is possible to collect blood samples from tuna. Sixty five individuals were bled, tagged and returned to the cage. The mortality rate was 4.6% which was normal (the same as unhandled fish) for the company at which the sampling was performed and for the period when it occurred. Blood samples with low levels of cortisol and good survival of the fish have been collected by bleeding fish caught on baited hook from the pontoons. This is currently the preferred non-lethal method of blood sampling from tuna

Measurements of cortisol in water, fish mucus and fish faeces

Cortisol was undetectable in samples of water and faeces (*please see Appendix 3*), both from the field and experimental (salmon faeces). The lack of detectable cortisol concentrations most likely reflect the problems experienced with the low end of the cortisol standard curve. Increased amounts of either freeze dried or wet material for the initial extractions did not appear to improve the sensitivity of the assay. It was also noticed that the slurry samples containing more than 500 mg tended to separate from the ethyl acetate and not remain in suspension even when subject to vigorous shaking. Increased amounts of material (either water or faeces) can

additionally result in matrix effects that can lead to non-specific cross reactivity with assay antibodies or labels, yielding false results. Although the non-invasive measurement of cortisol to assess stress in fish has been shown to be technically possible, the current RIA method employed for these studies requires further investigation for use with Southern bluefin tuna. The use of non-invasive cortisol measurement for the assessment of stress in species held in sea cages has to overcome inherent complexities of the farming systems such as dilution effects in a dynamic open system. Cortisol concentrations extracted from either water or faeces tend to be relatively low compared to their serum counterparts and therefore require both concentration and/or extraction prior to measurement.

Objective 3. To determine the relationship between SBT health, stress and fish performance (individual fish level)

Stress variables for different tows in 2006

Cortisol level ranged from below detection limit (0.6 ng/mL) to 1139.7 ng/mL. Cortisol concentrations were lowest in wild fish (Figure 12). The cortisol in wild fish was significantly lower than at the end of tow or during harvest (ANOVA, $P<0.05$); however, there was no difference in cortisol levels in ranched fish at the end of the tow and at harvest. The levels were highly variable, particularly during the end of tow sampling (two fish had cortisol above 1000 ng/mL, were considered outliers and removed from the analysis, fish with cortisol levels above 100 ng/mL but below 1000 ng/mL were included in the analysis). There was no significant effect of tow or sampling time on glucose level (Figure 13).

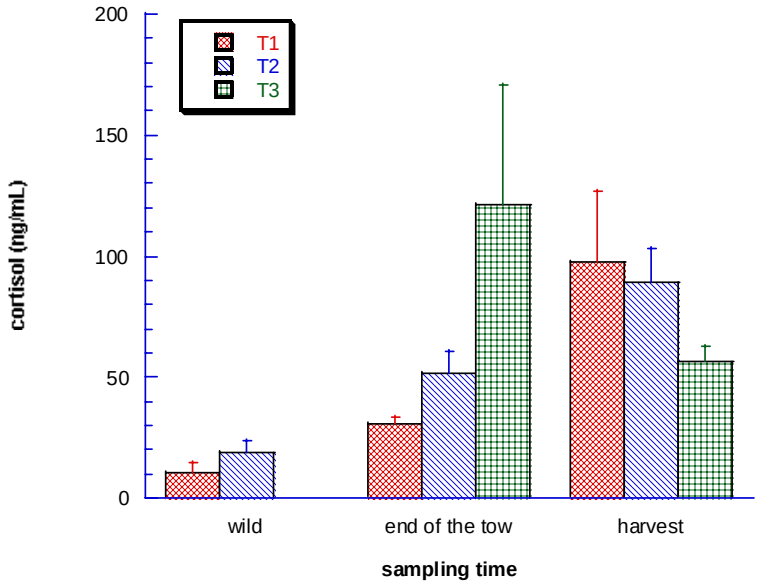


Figure 12. Effect of tow and sampling time on cortisol level (2006).

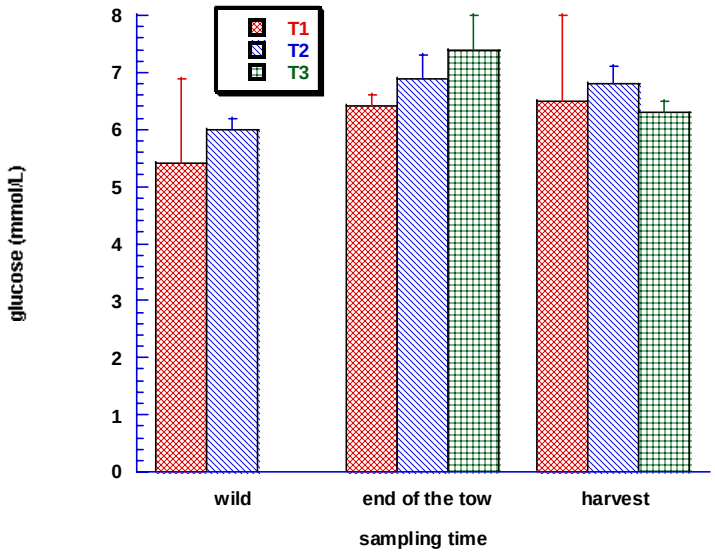


Figure 13. Effect of sampling time and tow on glucose level (2006).

There was a significant difference in the level of lactate in wild fish from the two different tows ($P<0.0005$, Figure 14). Tow had a significant overall effect on lactate level with fish from T3 showing lower levels.

The results of cortisol levels at harvest also demonstrated differences in stress levels between pontoons at harvest time ($P<0.05$). Anaerobic metabolism was most likely a result of fish being chased before they were caught during harvest. The elevation of the mean lactate level at harvest time was most likely due to different capture method used at that time.

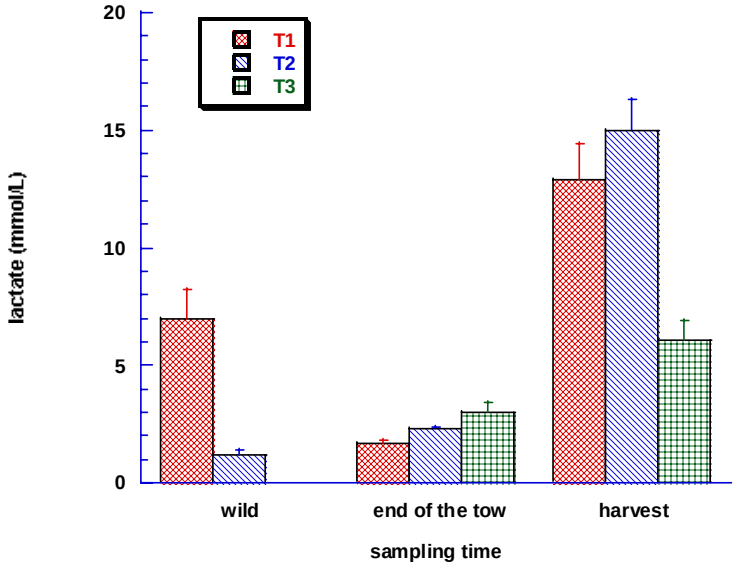


Figure 14. Effects of sampling time and tow on lactate levels.

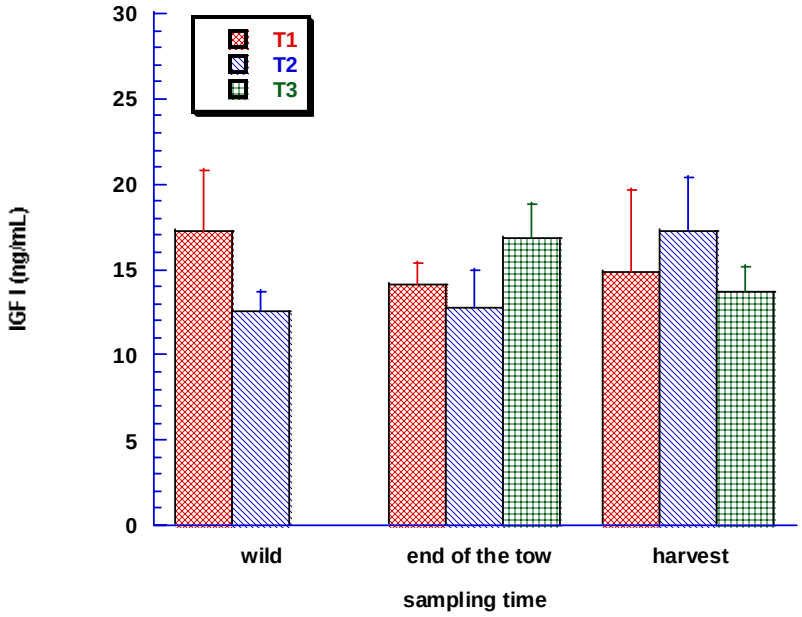


Figure 15. Effects of sampling time and tow on IGF I levels.

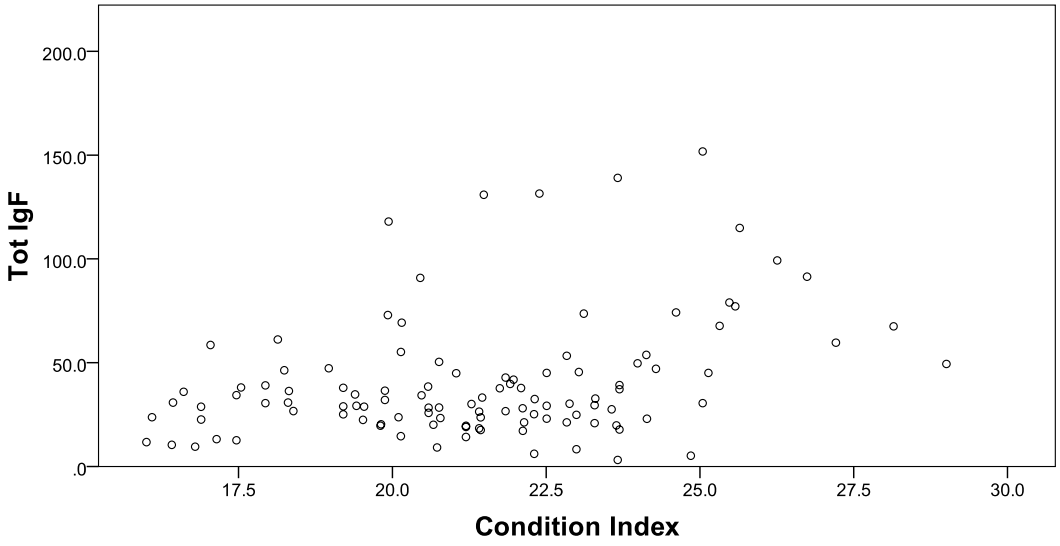


Figure 16. Relationship between the condition index and total IGF

There were no significant effects of sampling time or tow on IGF I concentrations (Figure 15). There was a positive correlation between condition index and total IGF (Figure 16, R= 0.339. k=1, n=108, P=0.000).

Effect of harvest method on stress variables

There was a significant effect of method of harvest on stress indices (Figure 17). Quick harvest resulted in significantly lower levels of glucose ($P<0.05$) and lactate ($P<0.005$). While the mean cortisol level was lower for fish

harvested using quick method, the results were not significantly different, most likely due to high individual variability, particularly in the normally harvested fish.

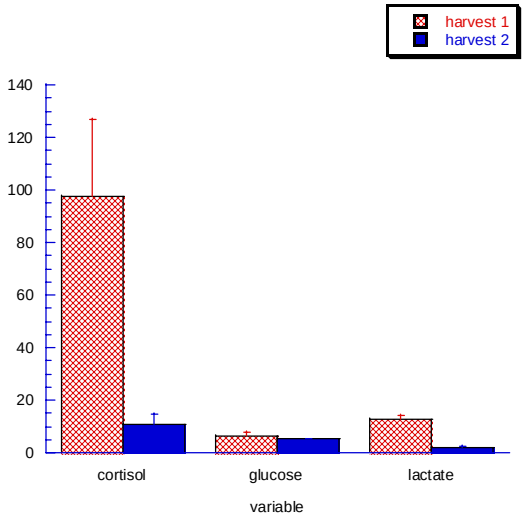


Figure 17. Effects of harvest on cortisol, glucose and lactate levels.

Effect of bleeding method

There was no significant effect of bleeding method (live bleeding or harvest bleeding) on cortisol, glucose or lactate concentrations in wild fish (Figure 18).

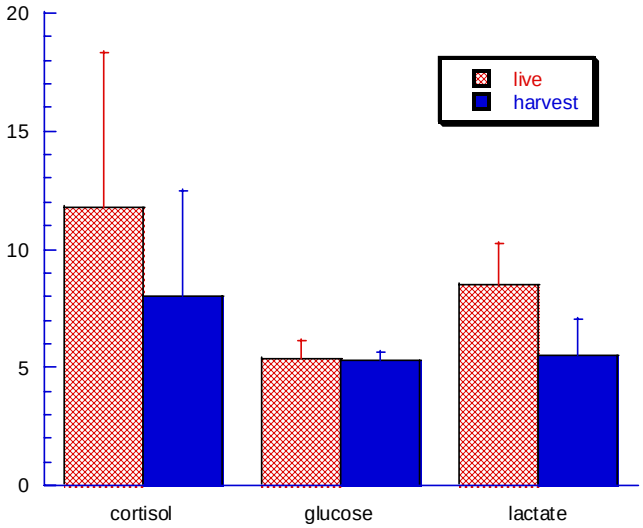


Figure 18. Effect of bleeding method on cortisol, glucose and lactate (wild fish, tow 1).

Ranges for stress indices

Blood plasma cortisol concentration ranged from normal to elevated, particularly in ranched fish sampled at the end of the tow (T2 and T3) and

harvest. Mean blood plasma glucose ranged from 5.2 mmol/L (quick harvest) -7.4 mmol/L (T3 end of tow). Mean blood plasma lactate ranged from 1.2 mmol/L (T2 wild fish) to 15 mmol/L (T2 harvest). Blood haemoglobin ranged from 12.4 g/dL (T2 end of tow) to 18.8 g/dL (T1 end of tow). This suggests that both cortisol and lactate were elevated at times, whereas other variables were within normal ranges reported for southern bluefin tuna (Table 9).

Table 9. Normal ranges for stress indices reported for southern bluefin tuna.

variable	ranched fish normal mean (SE) Munday et al 1997	ranched fish normal mean range Thomas et al 2003	ranched fish normal mean (SD) Rough 1998	wild fish (poled) Thomas et al submitted
cortisol	15.7 (5.48) nmol/L	0.2 - 7.18 ng/mL	50 (94) nmol/L	81.6 ng/mL
glucose	6.94 (0.27) mmol/L	NA	6.9 (0.9) mmol/L	4.6 mmol/L
lactate	NA	0.65 - 2.02 mmol/L	3.5 (1.4) mmol/L	3 mmol/L

Seasonal and annual changes in stress indicators

Cortisol, glucose and lactate were measured in blood plasma of SBT sampled in 2005 (Longer Term Holding - LTH), 2006, 2007 and 2008.

Cortisol values were highly variable and reflected the method by which the fish were caught. Overall, if the SBT were caught on a baited hook then the cortisol value was lower than if a diver caught them from an aggregation. Most harvest fish were collected by divers, which resulted in high cortisol levels in the harvest samples. However, the early samples (wild fish, average weight and midseason fish sampled in May or June) were usually caught on a baited hook so the initial (post-tow) elevation of cortisol level is a real effect and not an artefact of a sampling method.

For LTH fish there was a significant increase in cortisol levels in SBT in May 2005 in comparison to SBT in April 2005 (Figure 19). Cortisol values ranged widely, particularly in 2005. The mean blood plasma cortisol level in SBT in 2005 was 286.81 ng/ml (minimum 0.2 ng/ml, maximum 2227.68 ng/ml). The 2006 cohort showed a mean cortisol of 145.09 ng/ml (minimum 13.89 ng/ml, maximum 861.82 ng/ml). SBT sampled in 2006, 2007 and 2008 also showed the post-tow peak in cortisol level (Figure 20). This difference was statistically significant in 2008. These differences were apparent early during the sampling season so they were not a result of the use of different sampling methods as all fish were caught on a baited hook. Higher cortisol levels later in the season were due to the different methods of catching SBT. Cortisol values appeared to be lower on average in 2008 (mean cortisol in 2008 39.26 ng/ml, in 2006 the mean was 87.18 ng/ml, in 2007 the mean cortisol concentration was 176.01 ng/ml). This was reflected by a similar pattern for

the maxima of cortisol concentrations in blood plasma (maximum for 2008 359.6 ng/ml, in 2006 – 1139.7 ng/ml and in 2007 the maximum was 1246 ng/ml).

Most mean values for cortisol (except for wild fish in 2006 and most fish in 2008) were outside what is considered normal for SBT (<50 ng/ml, Thomas et al 2003, Rough et al 2005). This suggests that either the normal values should be revised or that the sampling methods are more stressful than desirable or that most of captive SBT are stressed. It is also possible that these elevated means are due to a few higher measurements. In previous studies high level outliers were often removed from further analysis (Rough et al 2005), this could be considered as another option for analysing the results.

There were some annual differences in cortisol levels, in particular SBT sampled in 2008 seemed to have lower cortisol concentrations in their blood plasma. There appeared to be seasonal differences, in particular the post-tow cortisol peak. The post-tow increase in cortisol level may result in immunosuppression and have implications for 6-8 week mortalities. However, this hypothesis requires further investigation.

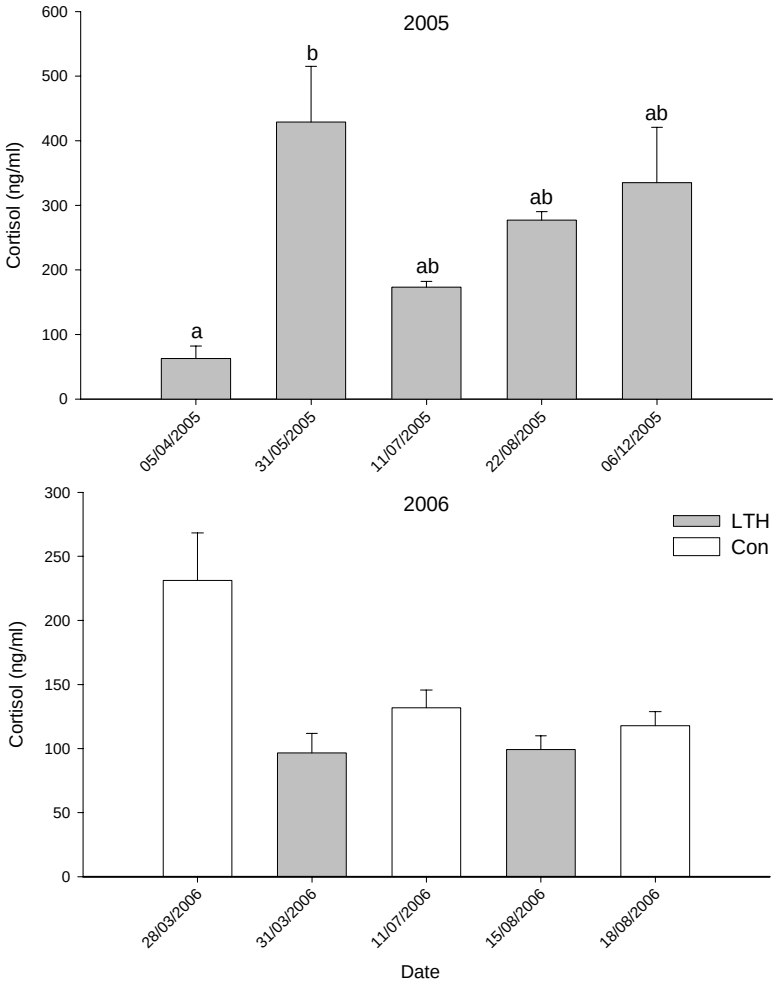


Figure 19. Mean ± SE plasma cortisol levels in SBT sampled during 2005 and 2006. Note long term holding (LTH) held for 361 and 498 days respectively following initial transfer to grow-out in 2005. Control (Con) fish held 37, 116 and 154 days respectively following transfer to grow out in 2006. Different superscripts denote significant differences among time points ($P < 0.05$).

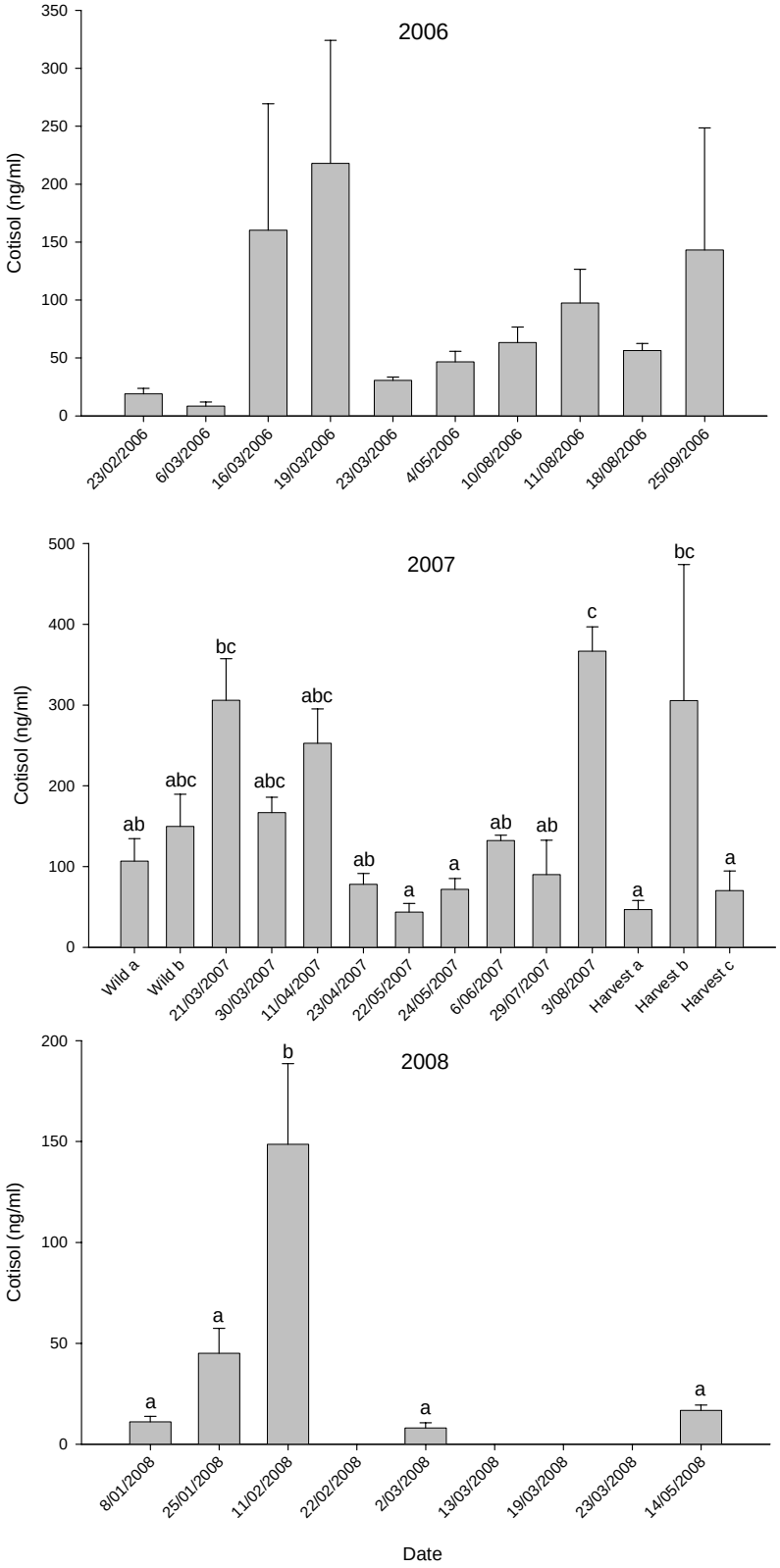


Figure 20. Mean ± SE plasma cortisol levels in SBT sampled during 2006, 2007 and 2008. Different superscripts denote significant differences among time points ($P < 0.05$).

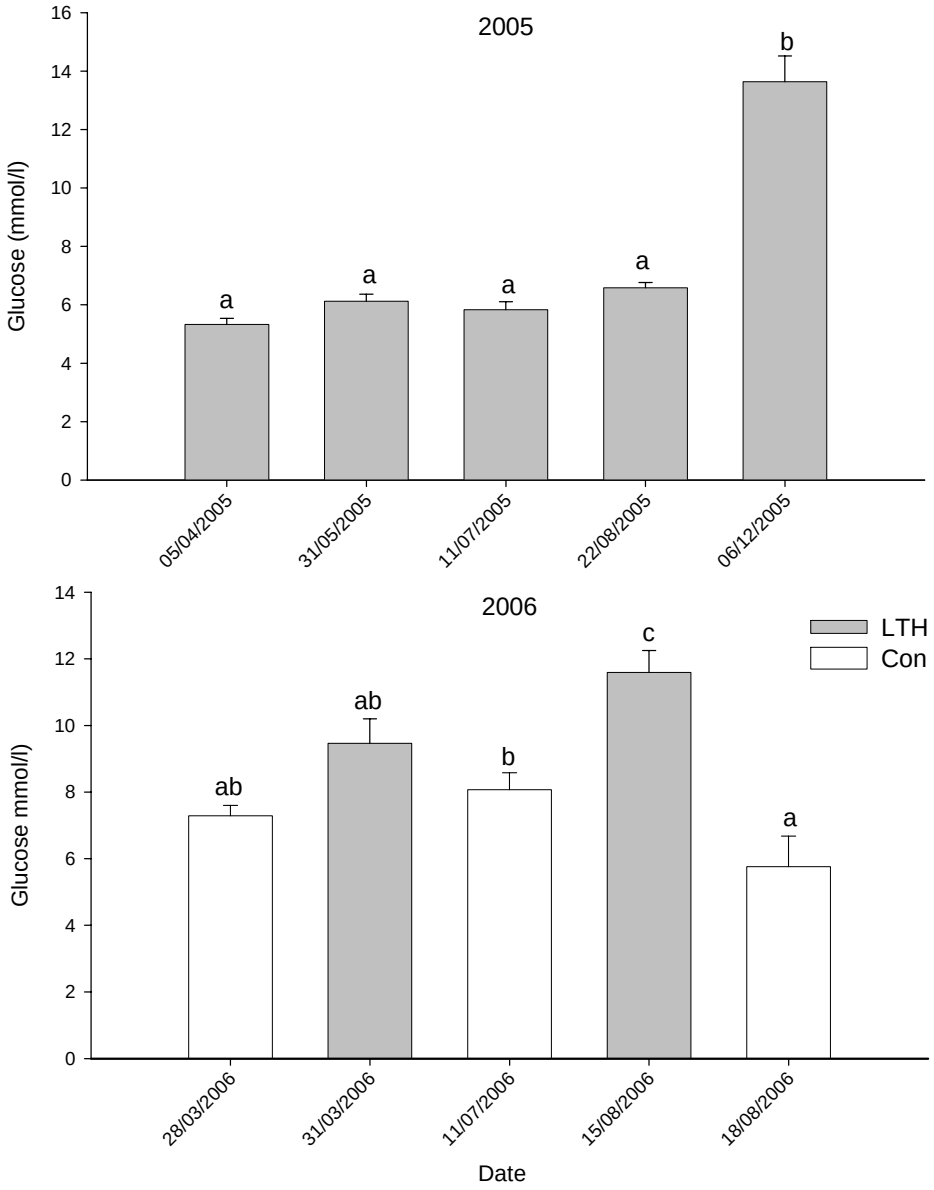


Figure 21. Mean ± SE plasma glucose levels in SBT sampled during 2005 and 2006. Note longer term holding (LTH) held for 361 and 498 days respectively following initial transfer to grow out in 2005. Control (Con) fish held 37, 116 and 154 days respectively following transfer to grow out in 2006. Different superscripts denote significant differences among time points (P < 0.05).

Glucose levels were variable and did not show any seasonal pattern (Figure 21 and 22). The mean was highest for the SBT sampled in 2005 and lowest for SBT sampled in 2007. The greatest range for glucose level was observed in LTH fish – minimum 0.82 mmol/l and the maximum 24.95.mmol/l. Blood plasma glucose level was significantly greater in LTH fish than 2006 cohort. All mean glucose results were within the normal range reported for SBT (<12.5, Thomas et al 2003, Rough et al 2005).

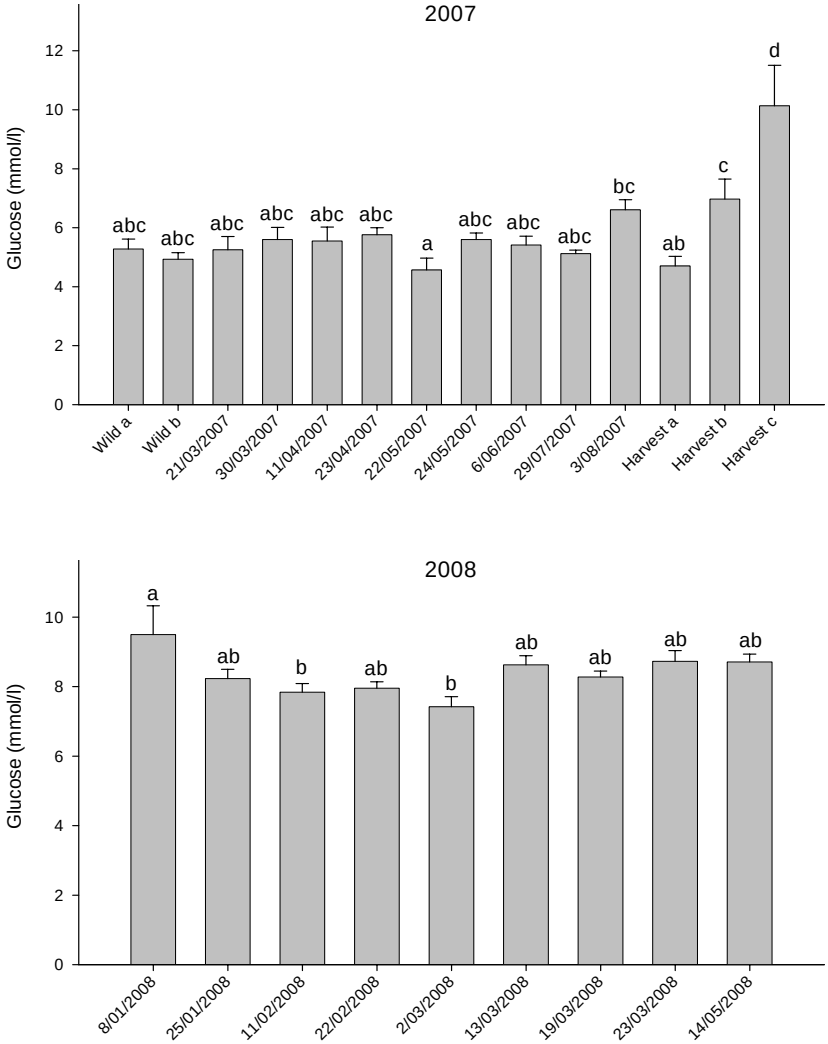


Figure 22. Mean ± SE plasma glucose levels in SBT sampled during 2006, 2007 and 2008. Note dates for 2006 Wild and Harvest points unknown. Different superscripts denote significant differences among time points ($P < 0.05$).

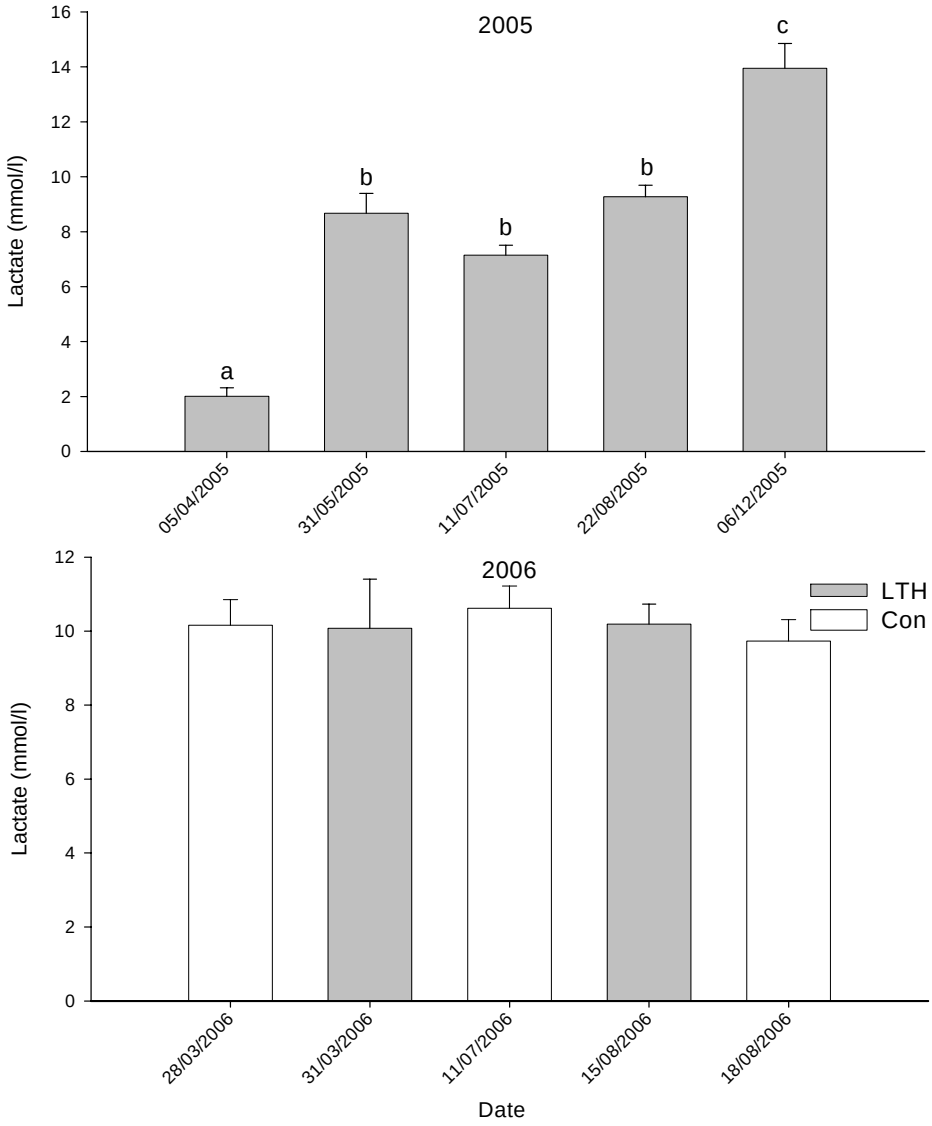


Figure 23. Mean ± SE plasma lactate levels in SBT sampled during 2005 and 2006. Note longer term holding (LTH) held for 361 and 498 days respectively following initial transfer to grow out in 2005. Control (Con) fish held 37, 116 and 154 days respectively following transfer to grow out in 2006. Different superscripts denote significant differences among time points ($P < 0.05$)

With the exception of SBT sampled on 8th January 2008, wild fish had lower lactate levels in their blood plasma (Figure 23 and 24). The elevated lactate levels later in the season were most likely due to the change in the catching method. At the time of the harvest it is often impossible to catch fish on a baited hook and the fish caught by divers have elevated cortisol and lactate levels in their blood plasma. For LTH fish the mean lactate levels were always above the normal range (<3 mmol/l, Thomas et al 2003, Rough et al 2005)) except for wild fish. However, for the 2006, 2007 and 2008 samples most means were within the normal range, with the exception of harvest fish (sampling artefact), post-tow fish in 2006 and wild fish in 2008.

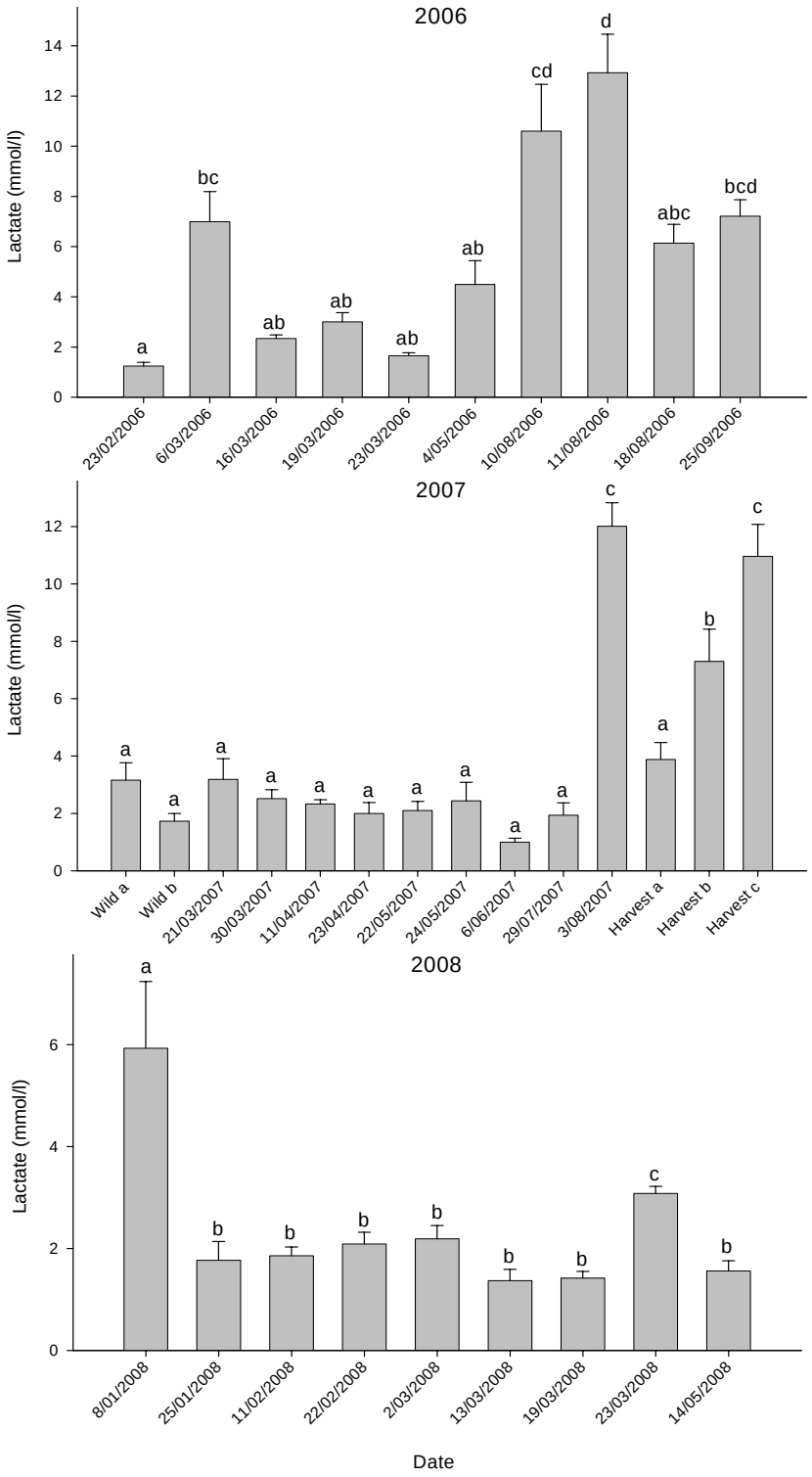


Figure 24. Mean ± SE plasma lactate levels in SBT sampled during 2006, 2007 and 2008. Note dates for 2006 Wild and Harvest points unknown. Different superscripts denote significant differences among time points ($P < 0.05$).

Fish sampled in 2005 had the greatest mean cortisol value and the greatest mean glucose value, however, SBT sampled in 2006 (LTH) had the greatest mean lactate value. SBT sampled in 2008 showed the lowest means for cortisol and lactate but not glucose.

There was a high variability for all three stress indicators. This variability made detection of any seasonal or annual patterns difficult. Individual variability and high and low stress responders most likely contributed to these results. High replication is required to detect patterns in SBT stress variables.

Relationship between stress indicators and parasitological status of the fish in 2005 and 2006 (LTH)

There was no relationship between any species of parasite and stress indicator which was consistent over time. Except for two cases of a significant relationship between the number of adult *Cardicola forsteri* in the heart and glucose level in blood (however, on one occasion 28.03.2006 positive and on another 31.05.2005 negative, each relationship occurred only once during the sampling) Increased cortisol level was positively correlated with the number of skin copepod *Caligus* spp. on 31.03.2006 and negatively correlated with the number of gill copepod *Pseudocycnus appendiculatus*. There was a negative relationship between the number of gill copepod *Euryphorus brachypterus* and glucose on 11.07.2005 and the skin copepod *Caligus* spp. and glucose on 28.03.06. There was a positive relationship for lactate level and the gill copepod *Pseudocycnus appendiculatus* (31.05.2005), the gill copepod *Euryphorus brachypterus* (31.05.2005), as well as the skin copepod *Caligus* spp (15.08.2006). This lack of a consistent relationship over time suggests that parasite loads observed in SBT in 2005 and 2006 did not have any relationship with stress indicators.

This is in agreement with published literature for wild fish. There was no relationship between gnathiid isopod loads and cortisol or glucose level in coral reef fish *Hemigymnus megapterus* (Grutter and Pankhurst 2000). However, it is possible that much higher parasite loads could induce stress or that combination of acute stress and high parasite loads could affect SBT performance. This would require further investigation.

Relationship between stress indicators and immune response in 2005 and 2006

Only on one occasion (22.08.2005) there was a negative correlation between lysozyme and glucose as well as lactate. There were no consistent patterns as there were no other significant relationships between immune variables (lysozyme and total immunoglobulin) and stress indices (cortisol, glucose and lactate).

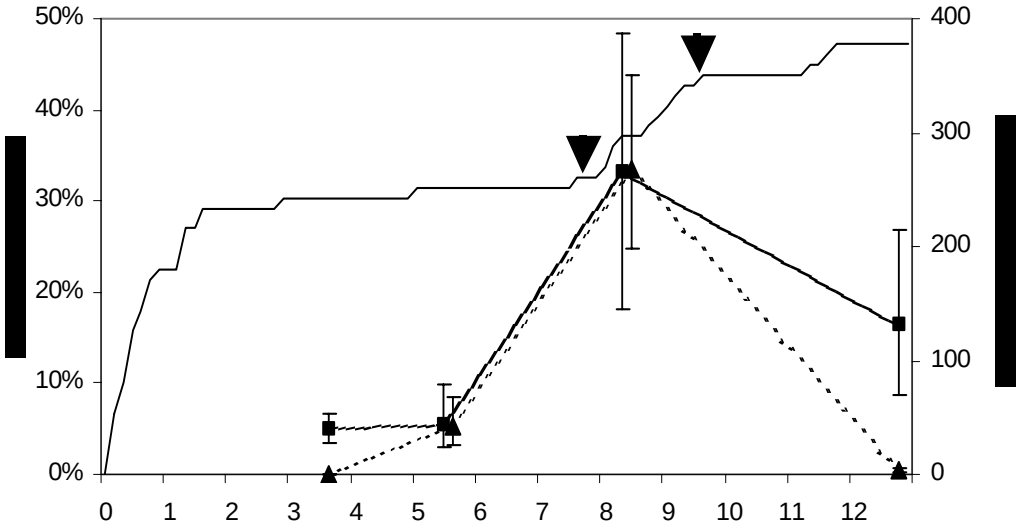
Multiple transfer experiment (Hayward et al, submitted)

Burdens of both parasites in fish in the experimental pontoons reached levels far heavier than previously documented in farmed tuna. For sea lice, the prevalence in most pontoons was 100%; the highest intensity reached 495 individuals, and mean burdens at the peak of the infection exceeded 265 lice

per fish. Almost all of the 5,407 individual lice counted were identified as adult *Caligus chiastos* (89.44% female, 10.14% male); adult females of two other species were also present, *C. amblygenitalis* (0.13%), in addition to an undescribed species, *Caligus* sp. (0.04%). Lice counts were correlated positively with gross eye pathology scores ($r_{s,151df}$=0.3394, p=0.0000), negatively correlated with condition index ($r_{s,151df}$=-0.5396, p=0.0000) – also reported previously, and positively correlated with plasma cortisol ($r_{s,131df}$=0.3906, p=0.0000) and glucose ($r_{s,131df}$=0.2240, p=0.0096). For the blood fluke, prevalences were less uniform than those of sea lice, with lower rates of infection at the beginning (ranging from 10 to 40%), reaching 100% mid-study, and declining again (40% in one pontoon). The highest intensity reached 441 individual flukes. Fluke counts were positively correlated with sea lice burdens ($r_{s,150df}$=0.3143, p=0.0000). Fluke counts were also correlated with plasma haemoglobin ($r_{s,151df}$=-0.2436, p=0.0051) and lysozyme ($r_{s,151df}$=0.3013, p=0.0019). Peaks in these epizootics occurred immediately before the onset of elevated mortalities, which started after 6 weeks of ranching.

Sea lice and blood flukes were present in every cage and on every date sampled. The highest prevalence and the highest mean abundance for both parasites were reached in the two experimental cages, cages 1 and 2; the highest blood fluke prevalence (100%) was also reached in two commercial cages on May 12. At the first sampling period, four weeks after transfer into cages, the prevalence of sea lice was 100% in the two experimental cages and 80% in the commercial cage; prevalence remained more or less stable for the rest of the trial. The highest intensity of sea lice on any tuna reached 495 individuals, and mean burdens in one sample (19 May 2008, cage 1) exceeded 265 lice per fish. Prevalence of the blood fluke were less uniform than those of sea lice, with lower rates of infection at the start of the study on 16 April (ranging from 10-40%), peaking at 100% mid-study (from April 29 to 19 June in cage 1, from 8 May to 2 June in cage 2, and on 12 May in cage 3), and in cage 2 then declining to a lower level (40%) at the end of sampling, on 19 June. The total number of flukes counted was 5,664; at the peak of infection (on May 19 2008), mean burdens in cage 1 exceeded 268 flukes per host tuna, and the highest intensity reached 441 individuals.

A



B

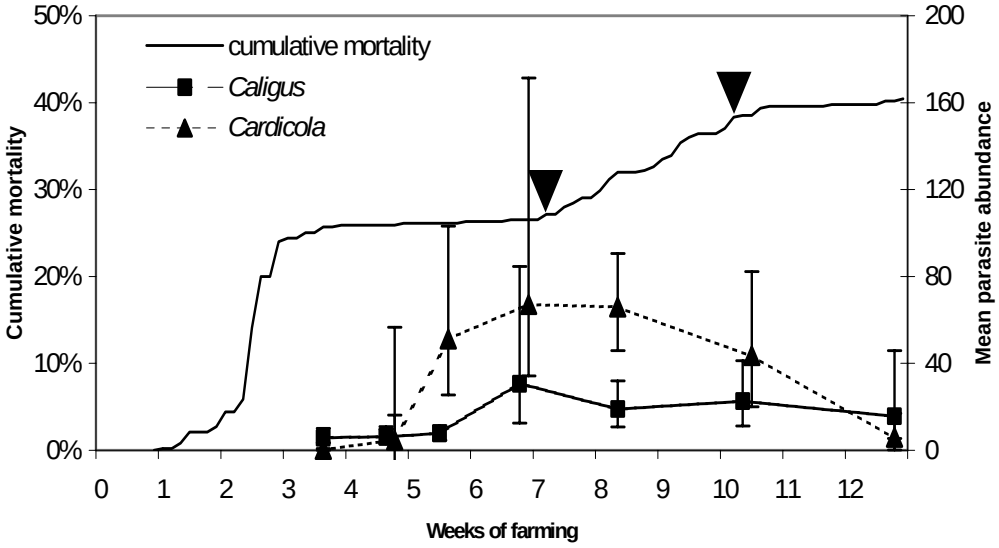


Figure 25. Cumulative mortalities in two experimental cages (A - cage 1, 'control' and B – cage 2, 'multiple transfer') for southern bluefin tuna, *Thunnus maccoyii* early in the 2008 ranching season off Port Lincoln, with a comparison of mean abundances of sea lice (predominantly *Caligus chiastos*) and blood flukes (*Cardicola forsteri*). The mortality syndrome occurs in the period marked by arrows.

Multiple transfers resulted in a significant increase in glucose level and significant decrease in lactate level (Figure 26). There was a significant increase in cortisol level after 2nd transfer followed by a statistically significant reduction (Figure 26). Cortisol levels were characterised by high individual variability and ranged from 2.18 ng/ml to 163.2 ng/ml (both individuals

sampled after second transfer). Haemoglobin concentration increased significantly after 1st transfer but then decreased again (Figure 5). pH was significantly reduced in transferred fish (Figure 27). Blood osmolality was significantly increased after the 2nd transfer (Figure 27).

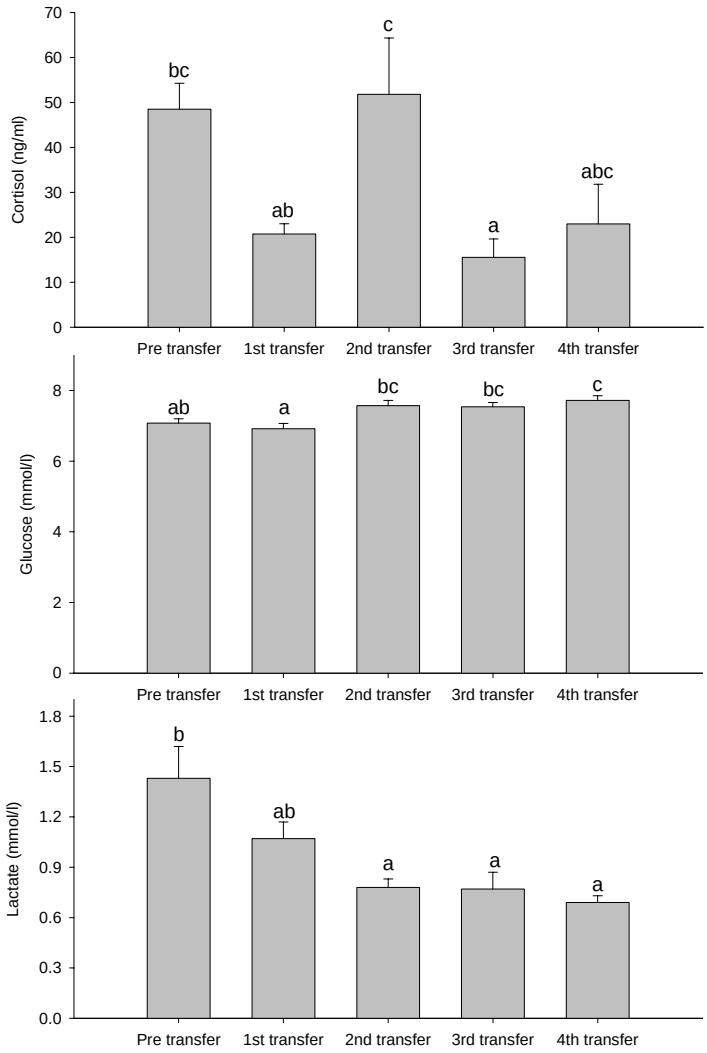


Figure 26. Mean ± S.E.M of various stress parameters in SBT following transfers of wild caught SBT (pre-transfer) to cages. Different superscripts denote significant differences among transfers.

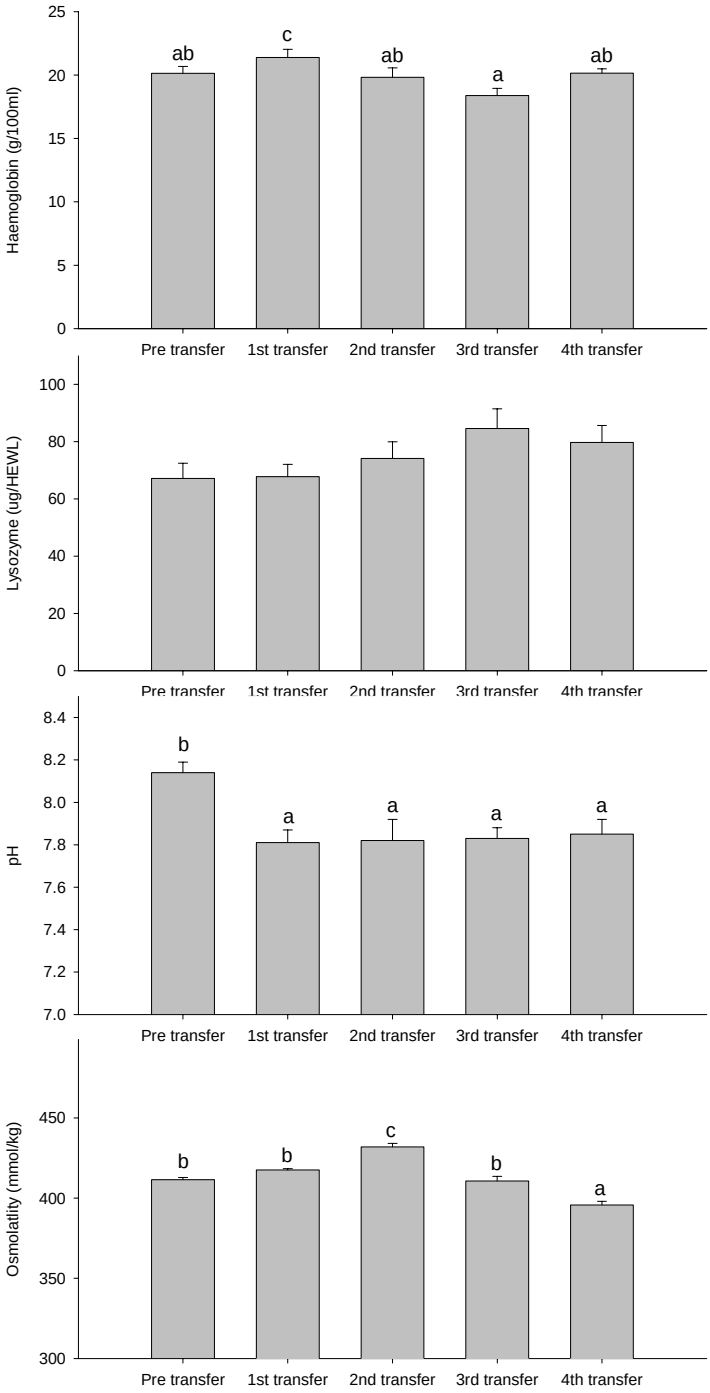


Figure 27. Mean ± S.E.M of various blood and serum parameters in SBT following transfers of wild caught SBT (pre-transfer) to cages. Different superscripts denote significant differences among transfers ($P < 0.05$).

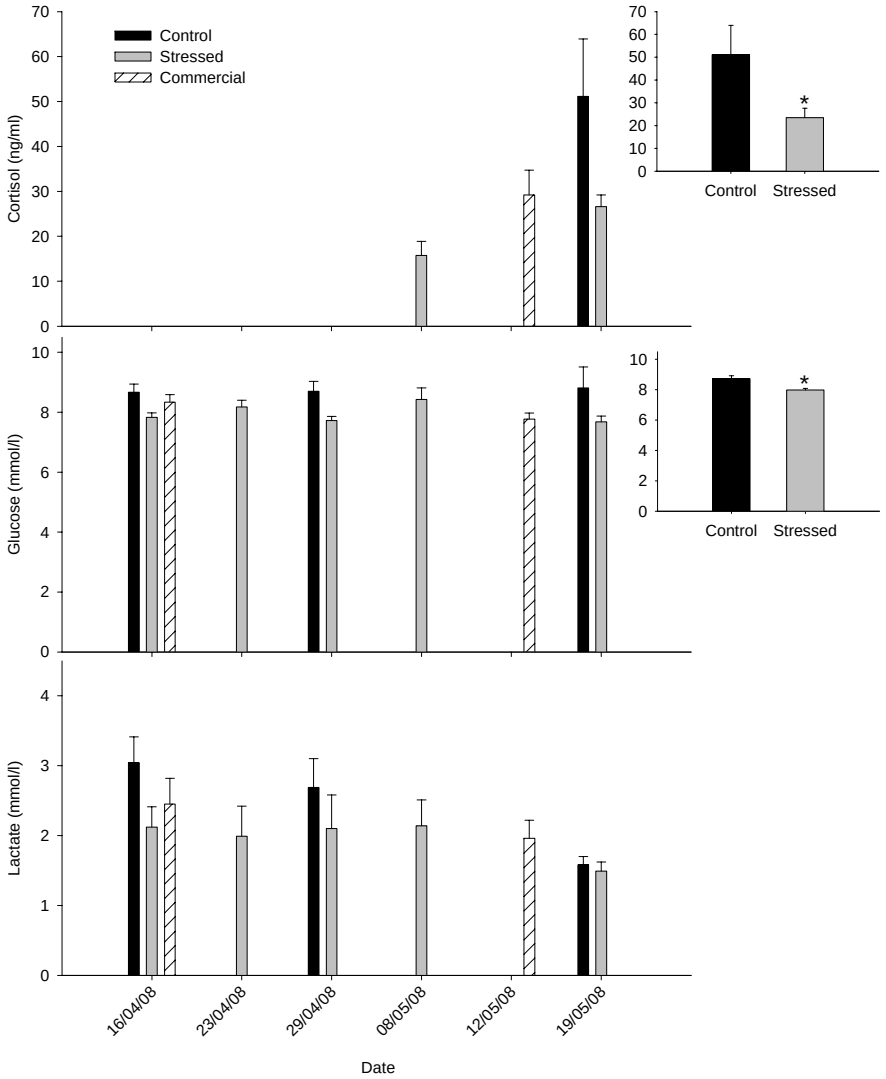


Figure 28. Mean ± S.E.M of various stress parameters in SBT (control) or following stress (stressed). Graph inserts denote a significant effect of treatment (* $P < 0.05$). Commercial cage data (commercial) also presented but not included in statistical analyses.

Cortisol and glucose levels were significantly elevated in the SBT from the control cage in comparison to the multiple transfer fish (Figure 28). Haemoglobin concentrations were significantly greater in the fish from the control cage (Figure 29).

The selection of fish for the two different cages (multiple transfer and control) was biased. The fish that remained in the control cage were those that were not caught on the baited hook. Furthermore, the stocking density was different between the two cages. Thus, any comparison must be treated with caution.

The fish in the control cage performed poorly. However, SBT in both cages had very high parasite loads relatively to commercial SBT. The control fish

had greater parasite loads than SBT held at the same time in commercial pontoons and they had significantly greater blood plasma cortisol level (mean almost double the multiple transfer fish). This is despite high individual variability in cortisol levels.

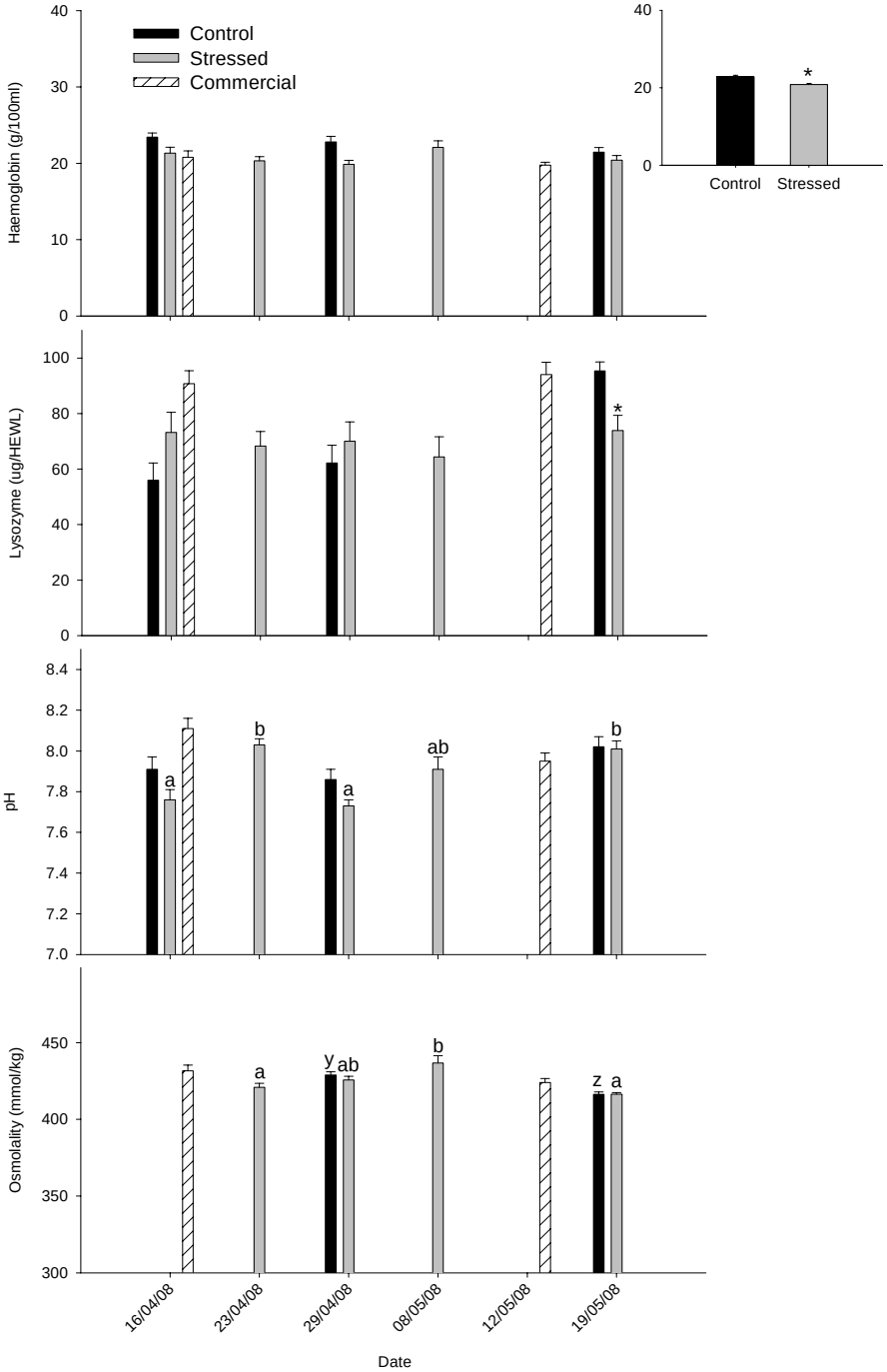


Figure 29. Mean ± S.E.M of various blood and serum parameters in SBT (control) or following stress (stressed). Different superscripts denote significant differences among time points within each treatment group ($P < 0.05$). Graph inserts denote a significant effect of treatment (* $P < 0.05$). Commercial cage data (commercial) also presented but not included in statistical analyses.

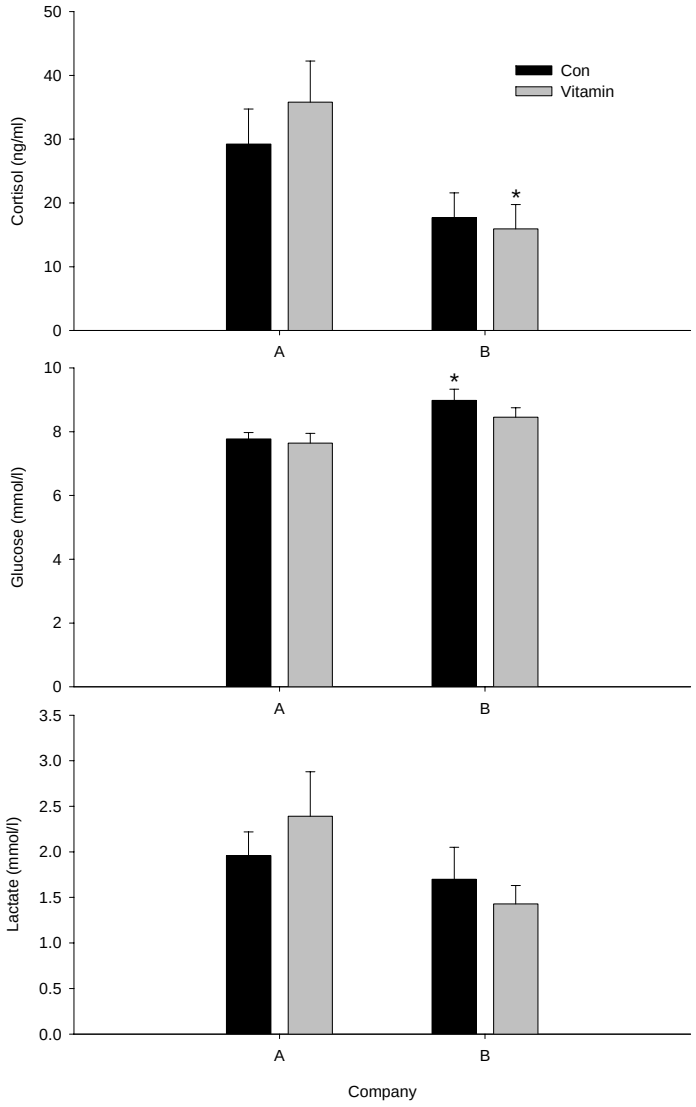


Figure 30. Mean ± S.E.M of various stress parameters in SBT fed a vitamin supplemented (vitamin) or control (Con) diet. Within a treatment an * denotes a significant differences between the companies ($P < 0.05$). No significant differences between the treatments were found for each of the companies.

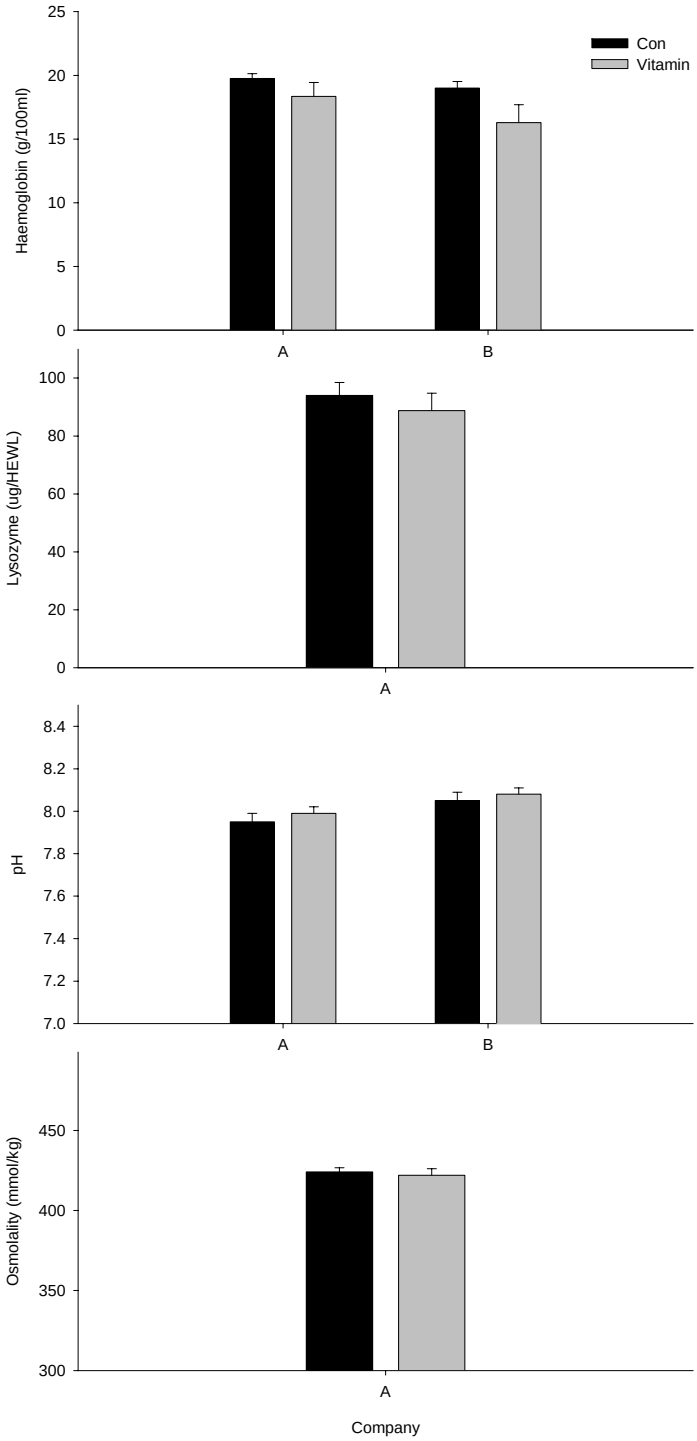


Figure 31. Mean ± S.E.M of various blood and serum parameters in SBT fed a vitamin supplemented (vitamin) or control (Con) diet. Significant differences between treatments and companies were not found. Note lysozyme and osmolality data for company B were not available.

No significant effects of vitamin administration could be detected (Figure 30 and 31). Independent t-tests were also used to compare the respective treatments between the companies. Only glucose data for the control diet were found to be significantly different between the companies.

Benefits and Adoptions

This project directly benefits the SBT industry by contributing to our understanding of the effects of stress and health on SBT performance. The development of a SBT industry database is one of the most significant benefits to the industry. Broader industry contribution to this database will result in increased understanding of the effects of various husbandry and environmental factors on performance of ranched SBT.

The tow history database developed in 2006/225 has already provided tangible benefits to industry. Elucidation of the influence of stocking density and duration/speed of tow on subsequent mortalities has supported the adoption of a reduction in numbers of fish transported in tow cages and an increased attention on the location and timing of fish capture to reduce tow durations.

More importantly, the limited tow database developed through the efforts of five companies in this project has demonstrated the benefits of collaboration to wider industry participants. The example of this database has been the catalyst for developing a more extensive husbandry focused database that the majority of industry operators have expressed a desire to contribute to. Prior to 2006/225 this type of collaboration was virtually non-existent in the industry.

Through the close involvement of industry and in particular the intimate involvement of one farm General Manager, 2006/225 has encouraged industry adoption throughout its duration. As indicated above, early stress indicator results indicated a focus on certain tow aspects likely would bring immediate gains and that grow-out methods were not unreasonable.

The mortality data in the first three months of grow-out collated over five years from five companies confirmed to industry the anecdotal evidence for a post transfer period of low mortality followed by a spike in numbers of deaths in the 6-8 week period. As such 2006/225 was instrumental in focusing attention on research into farm mortality that project 2008/232 was developed to address. Furthermore, the increase and temporal change in mortality pattern in recent years demonstrated in this project added weight to at least one hypothesized cause of increasing mortality: increased parasitic burden. Industry has strongly adopted the consequential remedial action of potentially increased environmental parasite loads by relocating sites. In some cases relocations have been up to 20 to 30 km away and out into water of 35 m depth. The results disseminated from 2006/225 in milestone reports and industry workshops had a significant role in facilitating such industry options.

Aquatic Animal Health research (human capital development) in Australia benefits from this project through training provided through industry workshop and the involvement of postgraduate students 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 farmed in marine pens.

Further Development

An 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. Inclusion of broader farm data would significantly extend the database and provide the industry with valuable information.

Further investigations of non-invasive cortisol measurement as a potential method of stress assessment should be performed in a model species amenable to tank culture. Correlations of plasma cortisol levels with these future results would be valuable and necessary to assess the value of this non-invasive approach. The use of ELISA for free cortisol measurement in fish has now also been validated. It is therefore also recommended that any further investigations include the potential use of antibodies specific for free cortisol as well as the development of a cortisol ELISA specific for Southern bluefin tuna.

There appeared to be seasonal differences, in particular in the post-tow cortisol peak. The post-tow increase in cortisol level may result in immunosuppression and have implications for 6-8 week mortalities. However, this hypothesis requires further investigation. Only a few immune variables can be currently measured for SBT. The best correlates of SBT performance (either stress measurement for example glucose or health measurement for example lysozyme activity) were to be developed as non-lethal predictive indices. However, there was no opportunity to study non-lethal predictive indices (sampling blood from tagged individuals and then collecting performance data for the same individuals at harvest time to correlate it with blood variables) due to the change in industry priorities.

Current extensive research into high mortalities and the associated survey for the intermediate host of blood flukes in the farming environment had their genesis in part in 2006/225. The demonstrated variation in fish stress and immune response characteristics has resulted in changes to concurrent projects so that more detailed baseline measurements of wild fish characteristics can be made.

Projects have been developed that expand on the immune response investigation that formed a subset of the research undertaken in 2006/225. In vitro and in vivo studies are being undertaken using immunostimulants.

Results of this project have been widely disseminated throughout the SBT industry through industry meetings (at least one/year), workshops for SBT industry (one/year), tunabriefs and through the Aquafin CRC and FRDC SBT Aquaculture Subprogram conferences. The results of this project have been discussed on a one to one basis with each company by David Ellis and Brian Jeffriess. Individual companies can exploit the results commercially if they choose to adopt any of the suggested strategies.

Planned Outcomes

The establishment of the industry database (tow conditions and subsequent mortality) has provided information on the effect of tow conditions on the fish mortalities in the first 12 weeks. Results of this project form the baseline for stress and performance indices. This project added to the existing SBT health database and established SBT industry database. Through showing the benefits of sharing information in the tow database, this project initiated a new husbandry based database, which included most companies.

Conclusions

Objective 1. To investigate the relationship between husbandry practices and SBT performance (at the level of tow and pontoon)

Tow conditions, in particular duration of the tow but also in 2003-2006 tow biomass, had a significant effect on fish performance, including week 6-10 mortalities. In particular:

- Lower tow densities (suggested fish number in a 45 m tow cage is under 9000, 12000 are considered too many on the basis of this project) and shorter duration tows reduce subsequent mortality after transfer
- Duration of single transfers was not shown to be important in influencing subsequent mortality (although no reliable measure of transfer 'intensity' was tested)
- Increased stress levels at end of tow possibly contribute to a spike in mortality over the 6 to 8 week period that prior to 2007 may be considered as 'normal' operational losses of up to 2-5%.
- Post 2007 mortalities have increased beyond the previously established operational levels suggesting a 'deterioration' in farming environment/practices. Historic tow data analysis suggests an environmental influence.

Conventional harvesting increases cortisol level in SBT and any harvest samples or samples collected through crowding and capture by divers should be interpreted with caution. Variations in the procedures and conditions during capture, tow and transfer as well as variations in on-farm husbandry practices have the potential to influence subsequent health, performance or mortality of ranched SBT, however it is difficult to prove in observational studies due to the presence of confounding variables.

Objective 2. To investigate development of non-lethal indices for SBT health and performance and assess their predictive value

The results of this project showed that the best method of non-lethal blood sampling was by bleeding fish caught on a baited hook without anaesthesia. The predictive value of blood analysis is currently under investigation. Levels of cortisol and its metabolites can be measured in water, mucus and faeces samples. The relationship between these variables and cortisol level in blood plasma is being determined. Non-lethal sampling of tuna blood was possible, although handling remains an obvious problem.

Objective 3. To determine the relationship between SBT health, stress and fish performance (individual fish level)

Grow-out techniques appear not to increase stress levels or reduce immune response. SBT showed a high level of individual variability with regard to stress levels and parasite loads. No relationship could be detected between parasite loads and stress levels. Observed parasite loads did not affect haemoglobin level. There was no consistent association between stress and subsequent performance, at least partly due to high individual variability.

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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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Co-investigators:
Daryl Evans

David Ellis

Mark Porter

Ryan Wilkinson

Appendix 3

CORTISOL MEASUREMENT IN SOUTHERN BLUEFIN TUNA (*THUNNUS MACOYII*) FAECES AND MUCUS: NON-INVASIVE MEASUREMENT OF STRESS

Melanie Leef and Ryan Wilkinson

INTRODUCTION

It is a universal fact that stressful events reliably result in an increase of glucocorticoids (cortisol and/or corticosterone) for every vertebrate genus (Klein 2000). Cortisol, the classic stress hormone, is considered to the best quantitative indicator of physiological stress (Ellis et al., 2007) and has been shown to respond to a variety of both acute and chronic stressors associated with aquaculture (Pickering, 1992; Barton, 2000; Fridell et al., 2007). Under normal conditions cortisol is vital for general body function and can have both beneficial and protective effects (Lane 2006). Chronically elevated levels however are more often associated with more adverse consequences such as reduced growth rate (Jentoft et al., 2005) and immunosuppresion (Watanuki et al., 2002). Despite the large body of knowledge documenting the consequences of stress in a range of fish species, assessment of the stress response can be difficult. Ultimately the very act of blood sampling from fish is a stressor and may result in a rapid (i.e. <30sec) elevation of blood cortisol potentially confounding results (Gerwick et al., 1999; Ellis et al., 2004; Lower et al., 2005). Researchers typically attempt to address this problem by using anaesthetics to suppress the cortisol stress response and by employing rapid bleeding protocols during fish sampling (Pottinger et al., 1992). These approaches are suitable for small and easily handled fish species (i.e. Atlantic salmon) however are not feasible for expensive, larger, harder to handle species such as southern bluefin tuna (*Thunnus macoyii*). Additionally the disturbance associated with removing fish for sampling can elevate cortisol concentrations in the remaining fish population (Laidley and Leatherland, 1988). As a consequence there has been a recent shift towards using non-invasive measures of stress in farmed and wild fish populations.

Previously the assessment of stress in fish has typically been quantified in blood-based assays using either serum or plasma; however, various non-invasive sample collection methods are now increasingly being utilised. The measurement of cortisol in water as a non-invasive method for the assessment of stress in fish was first proposed by Scott et al., (2001). The release of cortisol across the gills is thought to represent passive leakage (Ellis et al., 2005; Ellis et al., 2007; Scott and Ellis 2007) however cortisol concentrations in water are extremely low and in most circumstances direct measurement is difficult. As a result cortisol measurement in culture water relies heavily on the concentration of the steroid using solid-phase extraction cartridges and pre-filters followed by extraction using organic solvents (Ellis et al., 2004; Scott and Ellis 2007). Because the rate of cortisol release into the water is directly related to the concentration in the plasma, measurement of free cortisol in water has been validated as a means to assess potential stress

for various fish species (Ruane and Komen, 2003; Ellis et al., 2004; Lower et al., 2005; Wysocki et al, 2006; Earley et al., 2006; Bender et al., 2006; Ellis et al., 2007).

Measurement of faecal glucocorticoid metabolites has been previously used to monitor stress in dangerous or rare species such as rhinoceros (Turner et al., 2002), hyenas (Goymann et al., 1999) and cheetahs (Jurke et al., 1997). More recently methods for faecal glucocorticoid measurement have also been applied to fish (Turner et al., 2003 and Lupica and Turner, 2009). Glucocorticoids are metabolised and excreted with both intact (free) hormones and their associated metabolites present in faeces (Touma et al., 2003). Free glucocorticoids are almost insoluble in water and are readily extractable using organic solvents (Lupica and Turner 2009). Information regarding the measurement of cortisol in fish mucus is scant however the clinical use of salivary cortisol has been commonplace since the early 1980s for humans (Guechot et al., 1982).

Investigation of stress using non-invasive methods such as the measurement of free cortisol in faeces and/or from water offers a variety of advantages including minimal or zero disturbance to the animal itself (i.e. no handling, anaesthetic or bleeding stress etc) (Scott and Ellis 2007). These methods are therefore particularly attractive for the assessment of stress in Southern bluefin tuna. Considering the inherent difficulties (access to a limited number of animals, handling and sampling stress) associated with tuna research, non-invasive methods for the measurement of cortisol in water, faeces as well as mucus were investigated.

METHODS AND MATERIALS

Faecal, mucus and seawater samples

Southern bluefin tuna faecal samples were collected by diver's in situ using funnels and collection jars from fish held in pontoons (Port Lincoln, South Australia) immediately following excretion. Excess seawater was decanted from the sample on board the dive boat. Mucus samples were collected from the posterior dorsal region of harvested fish using a plastic blunt edged spatula. Both faecal and mucus samples were placed on ice prior to storage at -20°C. Samples were later transported to the University of Tasmania where they were similarly stored at -20°C to await analysis. Seawater samples were taken from the incoming seawater supply at the University of Tasmanania's Aquaculture Centre.

Cortisol Measurement (RIA)

Cortisol concentrations were determined using a tritiated radioimmunoassay (RIA) using a commercially available antibody (Sirosera™ supplied by Krius Pty. Ltd.) as described by Pankhurst and Sharples (1992). This RIA was initially developed and validated for the measurement of cortisol in fish plasma samples. For that application, frozen plasma is thawed, extracted with ethyl acetate and assayed in duplicate with overnight incubation. Following separation with ice cold charcoal solution, supernatants are decanted, mixed with scintillation cocktail, and counted in a beta counter. Cortisol extraction efficiency for plasma is typically >95% and minimum detection limit is 6 ng/ml.

This RIA was subsequently adapted for the attempted measurement of cortisol in seawater, faecal and mucus samples described below.

1. Cortisol extraction from seawater
Experiment 1.1
The aim of this experiment was to test the suitability of solid-phase hydrophilic-lipophilic balance (HLB) cartridges for the extraction of free cortisol from seawater samples. Free cortisol was added to seawater at concentrations of 1, 0.5, 0.25, 0.125 and 0 ng/ml. Samples (10 ml) from each of the seawater concentrations were passed through a Waters Oasis HLB 3cc column (Part # WAT094226 / Lot # 066A36 – containing 60 mg sorbent equilibrated with 2 ml methanol and 3 ml MQH_2O). Bound cortisol was subsequently eluted from the column in 1 ml ethyl acetate. The eluate was then transferred to a 5ml RIA assay tube and the ethyl acetate evaporated to dryness (30°C for 48 hrs). Following evaporation the residue was re-suspended in 1 ml phosgel buffer. Aliquots of the re-suspension (100 µl) were transferred into duplicate 5 ml RIA assay tubes and assayed as described in Pankhurst and Scharples (1992).

Experiment 1.2
The aim of this experiment was to determine the sensitivity of the cortisol extraction procedure from seawater. Free cortisol was added to seawater at concentrations of 100, 20, 4, 0.8, 0.16, 0.032 and 0 ng/l. One hundred milliliters of each seawater sample was passed through duplicate Waters Oasis HLB 3cc columns (Part # WAT094226 / Lot # 072A37073A) as previously described. Following loading of seawater samples each column received a 3 mL MQH_2O wash. Bound cortisol was subsequently eluted from the column in 1 ml ethyl acetate. Aliquots of the eluate (200 µl) were transferred into duplicate 5 ml RIA assay tubes, evaporated overnight at room temperature (RT) and assayed.

Experiment 1.3
This experiment was a repeat of the previous experiment which aimed to determine the sensitivity of the cortisol extraction procedure from seawater. Free cortisol was added to filtered (0.2 µm) seawater at concentrations of 250, 83.3, 27.8, 9.3, 3.1 and 0 ng/l. Samples (100 ml) from each seawater concentration were passed through duplicate Waters Oasis HLB 3cc columns (Part # WAT094226 / Lot # 072A37073A) as previously described. Following loading of seawater samples each column received a 3 ml MQH_2O wash. Bound cortisol was subsequently eluted from the column in 1 ml ethyl acetate. Four hundred and fifty microliters of the eluate was transferred into duplicate 5ml RIA assay tubes, evaporated at RT (48 hrs) and assayed.

2. Cortisol extraction from faeces
Experiment 2.1
The aim of this experiment was to determine if free cortisol could be extracted from tuna faecal samples. Frozen faecal slurry samples from two tuna (8T21 – 3 and 4) were thawed at RT and weighted out into 0.5, 0.25 and 0.125 g samples in 1.5 ml plastic tubes. Filtered seawater (250 µl) was added to each sample and vortexed. Ethyl acetate (1 ml) was then added and each tube

was shaken vigorously for 1 hr at RT. Samples were then centrifuged at 15,000 rpm for 10 min at 4°C. The ethyl acetate phase was removed and transferred into 5ml RIA tubes and evaporated to dryness (30°C for 48 hr). Following evaporation the residue was re-suspended in 1 ml phosgel buffer. Aliquots of the re-suspension (100 µl) were transferred into duplicate 5mL RIA assay tubes and assayed.

Experiment 2.2
The aim of this experiment was to continue investigations into optimizing the extraction of free cortisol from tuna faecal samples. Frozen faecal slurry samples from two tuna (8T21 – 3 and 4) were thawed at RT and subjected to the following treatments;

- Freeze dried and extracted with dichloro-methane
- Freeze dried and extracted with ethyl acetate (as described in experiment 1)
- Thawed faecal slurry centrifuged, water removed and extracted with ethyl acetate (as described)
- Water from centrifuged thawed faecal slurry extracted with ethyl acetate

Experiment 2.3
Because cortisol extractions from tuna faeces have not been thoroughly investigated, a number of experiments were designed with a view to investigate the potential limits of cortisol detection and extraction by using differing amounts of sample masses and also different extract volumes. These experiments were as follows:

2.3.1 Cortisol extraction and replication at the level of the eluate.
For most RIA assays, hormone from a single sample is extracted in 1 ml of ethyl acetate. From this 1 ml eluate, replicate extract volumes (100-200 µl) are used for the assay. To assess intra assay variation triplicate samples of 25, 50, 100 and 200 mg were eluted with 1 ml of ethyl acetate. From one of the replicates a 200 µl (n =3) was used as the extract volume. From the remaining 2 replicates the ethyl acetate eluate was pooled and a 500 µl (n=3) extract volume was assessed.

2.3.2 Cortisol extraction and replication at the level of the original sample.
For this experiment triplicate samples of 25, 50, 100, 200 and 500 mg were similarly eluted with 1 ml of ethyl acetate. From each replicate both a 200 and 500 µl extract volume was assessed.

2.3.3 Cortisol extraction and parallelism of the curve
Because faecal material not only contains whole cortisol but also glucocorticoid metabolites a parallelism curve was made. This curve would aid in the determination of whether or not the RIA detects free cortisol or cross reacts to non specific components. To do this the elulates from triplicate 100 mg samples eluted with 1 ml of ethyl acetate were pooled. Samples of 200, 160, 120, 100, 80, 40, 20, 10 and 0 µl of this pooled eluate was then used as the extract volume to simulate a 0–100 % dilution.

2.3.4 Steroid yield determination (spiking the sample)

To determine if cortisol could be extracted from faeces a triplicate 100 mg samples were spiked with 50 µl of assay strength cortisol label. The cortisol was then extracted with 1 ml of ethyl acetate and compared to the control (no faeces sample).

Faeces samples were freeze dried until weights had stabilised. The samples were then pooled together and ground into a fine powder using a mortar and pestle. This pooled material was then placed into a new sealed container and stored at -80 until extractions were begun (~ 1 week). Mucus samples were similarly freeze dried, pooled, ground together and stored at -80 until required for extraction experiments.

Experiment 2.4 Collection of faecal material experiment

In light of the results from experiments 2.1, 2.2 and 2.3, it was thought that perhaps excess seawater contamination in the samples possibly aided the breakdown of cortisol. A collection experiment using seawater acclimated Atlantic salmon (*Salmo salar*) as a model was therefore proposed. A small scale preliminary experiment was conducted in order to determine whether cortisol could be reliably measured in salmon faeces.

Fish

A single pooled faecal sample was collected from approximately 45 Atlantic salmon each weighing around 100 g held across 15 separate tanks. Fish were held at 19°C in full strength seawater (pH 8.0, 35ppt) and fed to satiation twice daily using four different experimental diets. After removing any uneaten pellets, as well as any excess water, the collected faeces were ground to form a consistent bolus.

Experimental design

Following collection the faeces were evenly divided into 4 containers, 2 containing an equal volume of 95% ethanol and 2 without. The samples stored in ethanol were inverted 3 x to ensure faecal contact with the alcohol. The containers were then stored at -80 (n = 2) and -20 (n = 2) for 3 days prior to extraction.

Extraction

Samples were thawed, weighed to approximately 2 g, then extracted using 2 ml ethyl acetate. Samples stored in ethanol were firstly centrifuged and the supernatant was removed. All samples were then placed on a shaker at RT for 1 hour prior to being centrifuged 1500 xg for 5 min. To assess if any cortisol had leeched from the faeces into the ethanol, the ethanol supernatant was also subject to extraction procedures using 2 ml (v/v). Extract volumes of both 500 and 200 µl were taken in triplicate for all samples and allowed to evaporate in a fume hood for 48 hours prior to RIA.

3. Cortisol extractions from mucus

Experiment 3.1

The aim of this experiment was to determine if free cortisol could be extracted from tuna mucus samples. Mucus samples from 3 tuna (9T40 – 2, 3 and 7) were freeze dried and hand ground to a fine powder (see Table 1). Each sample was subsequently split between two new 1.5 ml plastic tubes and weighed.

Table 1. Freeze dried mucus weights used for extraction and measurement of cortisol.

	Fish #2	Fish #3	Fish #7	Extraction method
1	18.8 mg	24.3 mg	17.1 mg	Ethyl acetate
2	15.0 mg	37.4 mg	27.8 mg	Schultz et al., 2005

Mucus extractions were performed as follows;

1. Ethyl acetate extraction method

Ethyl acetate (1 ml) was added to each tube and mixed. Tubes were subsequently centrifuged for 10 min at 10,000 rpm. Either 100 µl or 200 µl of the extract was then transferred into triplicate 5 ml RIA assay tubes, evaporated overnight at RT and assayed.

2. Schultz et al (2005) extraction method (used previously for sex steroid extractions from carp mucus)

Freeze dried mucus samples were re-suspended in 0.5 ml MQH_2O followed by 1 ml Tris (20 mM, pH 7.8) and vortexed. Samples were then transferred into 5 ml RIA tubes and diluted with a further 3ml Tris. Samples were vortexed before being centrifuged at 3000 rpm for 10 min. The supernatant (1 ml) was collected and transferred to a new tube before being diluted with 3 ml diethyl ether. Samples were then vortexed and centrifuged as previously described. The top 2 ml of the diethyl ether layer was removed into a new tube (fraction 1). An additional 2 ml of diethyl ether was added to the original sample tube, vortexed and centrifuged as previously described. A further 2 ml of diethyl ether was removed and added to fraction 1. Either 200 µl or 1000 µl of the extract was transferred into triplicate 5 ml RIA assay tubes, evaporated overnight at RT and assayed.

Experiment 3.2

Based on the results of experiment 8, only the ethyl acetate method was reassessed.

Mucus samples were freeze dried until weights had stabilised. The samples were then pooled together and ground into a fine powder using a mortar and pestle. This pooled material was then placed into a new sealed container and stored at -80 until extractions were begun (~ 1 week).

All mucus experiments were identical to those conducted for the faecal samples and included:

3.2.1 Cortisol extraction and replication at the level of the eluate.
3.2.2 Cortisol extraction and replication at the level of the original sample.
3.2.3 Cortisol extraction and Parallelism of the curve
3.2.4 Steroid yield determination (spiking the sample)

RESULTS
1. Cortisol extraction from seawater
Experiment 1.1.
Recovery of free cortisol from spiked seawater samples ranged from 67 to 129% (Table 2). This experiment confirmed the ability of HLB solid-phase extraction cartridges to remove cortisol from seawater samples. Ethyl acetate also appeared to be suitable for column elutions.

Table 2. Recovery of free cortisol added to seawater.

Cortisol in seawater (ng/ml)	*Expected recovery (ng)	Rep.	Observed recovery (ng)	% recovery	Mean % recovery
0	0	1	BDL	0	0
		2	BDL	0	
0.125	0.125	1	0.098	78.4	76.0
		2	0.092	73.6	
0.25	0.25	1	0.182	72.8	72.0
		2	0.178	71.2	
0.5	0.5	1	0.357	71.4	67.2
		2	0.315	63.0	
1.0	1.0	1	1.31	131	128.5
		2	1.26	126	

* = expected recovery if 100% of available cortisol binds and is subsequently eluted from column.
BDL = below detection limit.
NB – detection limit of RIA = 0.003 ng/tube

Experiment 1.2
As a consequence of the standard curve which failed at the lower concentrations, no sensible concentration determinations could be made for the seawater samples spiked with 0.032, 0.16, 0.8 and 4 ng/l cortisol (Table 3). Using a truncated form of the cortisol standard curve (cortisol standards from 800 – 80 pg instead of down to the normal 3 pg detection limit) the following values were derived for the 20 and 100 ng/l cortisol spiked seawater samples. In this case recovery of cortisol from spiked seawater samples was 48 % and 23 % for 20 ng/l and 100 ng/l respectively. Due to the failure of the assay standard curve a repeat of this assay was warranted. Furthermore, in this instance 100 ml of seawater was passed through the column however the final 20-30 ml of each load was extremely difficult to pass and may have impacted on column binding efficiency. More stringent pre-filtration of the seawater samples is recommended.

Table 3. Recovery of free cortisol added to seawater.

Cortisol in seawater (ng/l)	*Expected recovery (ng)	Extraction cartridge	Assay rep.	Observed recovery (ng)	% recovery	Assay rep. mean % recovery	Mean % recovery
0	0	1	1	BDL	0	0	0
			2	BDL	0		
		2	1	BDL	0	0	
			2	BDL	0		
20	0.4	1	1	0.21	52.5	47.5	48.2
			2	0.17	42.5		
		2	1	0.16	40.0	48.8	
			2	0.23	57.5		
100	2.0	1	1	0.60	30.0	25.3	22.8
			2	0.41	20.5		
		2	1	0.49	24.5	20.3	
			2	0.32	16.0		

* = expected recovery if 100% of available cortisol binds and is subsequently eluted from column.
BDL = below detection limit.
NB – detection limit of RIA = 0.08 ng/tube due to failure of assay standard curve.

Experiment 1.3
Filtering of seawater samples (prior to cortisol addition) down to 0.2 µm resulted in much easier loading of 100 ml samples. It now appears possible to load more than 100 ml if required. Recovery of cortisol from spiked seawater samples ranged from 3% (250 ng/l) to 64% (3.1 ng/l) (see Table 4). Significant assay cross-reactivity was detected from un-spiked seawater samples. The nature of this cross-reactivity (specific or non-specific cross-reactivity) will require further investigation.

Table 4. Recovery of cortisol added to seawater.

Cortisol in seawater (ng/l)	*Expected recovery (ng)	Extraction cartridge	Assay rep.	Observed recovery (ng)	% recovery	Assay rep. mean % recovery	Mean % recovery
0	0	1	1	0.014			
			2	0.013			
		2	1	0.024			
			2	0.021			
3.1	0.14	1	1	0.078	55.7	58.6	64.3
			2	0.086	61.4		
		2	1	0.107	76.4	70.0	
			2	0.089	63.6		
9.3	0.42	1	1	0.131	31.2	30.9	33.5
			2	0.128	30.5		
		2	1	0.156	37.1	36.0	
			2	0.146	34.8		
27.8	1.25	1	1	0.179	14.3	14.4	13.9
			2	0.180	14.4		
		2	1	0.181	14.5	13.4	
			2	0.153	12.2		
83.3	3.75	1	1	0.268	7.1	7.7	6.6
			2	0.311	8.3		
		2	1	0.244	6.5	6.2	
			2	0.223	5.9		
250	11.25	1	1	0.231	2.1	2.3	2.9
			2	0.286	2.5		
		2	1	0.412	3.7	3.5	
			2	0.368	3.3		

* = expected recovery if 100% of available cortisol binds and is subsequently eluted from column.
NB – detection limit of RIA = 0.003 ng/tube

2. Cortisol extraction from tuna faeces

Experiment 2.1

This experiment indicated that significant cortisol assay cross-reactivity can be obtained from tuna faecal samples (Table 5). Interestingly the observed recovery did not occur in a manner reflective of the amount of original faecal slurry used. Freeze drying of faecal samples is suggested for further work to allow more accurate determination of initial faecal weight used for extractions.

Table 5. Cortisol recovery from faecal samples.

Fish	Faecal slurry weight (g)	Rep.	Observed recovery (pg)	Mean recovery (pg /tube ± SE)
8T21 – 3	0	1	BDL	0
		2	BDL	
	0.125	1	15.1	15.2 ± 0.05
		2	15.2	
	0.25	1	15.9	14.7 ± 1.2
		2	13.5	
	0.5	1	BDL	0
		2	BDL	
8T21 – 4	0	1	BDL	0
		2	BDL	
	0.125	1	15.9	15.4 ± 0.5
		2	14.9	
	0.25	1	20.2	23.0 ± 2.8
		2	25.7	
	0.5	1	17.3	15.5 ± 1.8
		2	13.7	

BDL = below detection limit
NB – detection limit of RIA = 0.003 ng/tube

Experiment 2.2

The results for the faecal slurries were inconsistent with the original amount of material used (Table 6). The results for the centrifuged water samples were very high compared to both freeze dried and slurry material suggesting leaching of cortisol from the original slurry. Cortisol determination of freeze dried material therefore appears to be the most accurate method for measurement. Significant assay cross-reactivity was observed from a range of extractions for fish 8T31-3. Interestingly a significant amount of assay cross-reactivity was also observed in the seawater removed from the thawed faecal slurry for this same fish. This result indicates potential leaching of cortisol from the stored faecal samples. As observed previously (see experiment 2.1) recovery did not occur in a manner reflective of the amount of original faecal slurry used. The inconsistency of these results warrants further investigation.

Table 6. Recovery of cortisol from faeces using different extraction methods.

				Extraction method				
Fish	Sample	Freeze dried (FD), Thawed slurry (T) or Water spun from thawed sample (W)	Amount used (mg)	MQ H_2O (µL)	Filtered seawater added (µL)	Dichlroro-methane (µL)	Ethyl acetate (µL)	Mean cortisol (pg) ± SE
8T31-3	1	FD	44.8	50	-	1000	-	11.2 ± 0
	2	FD	50.8	-	250	-	1000	BDL
	3	FD	25.3	-	250	-	1000	15.3 ± 1.87
	4	T	203.7	-	250	-	1000	11.5 ± 0.5
	5	T	104.8	-	250	-	1000	22.6 ± 6.6
	6	T	52.7	-	250	-	1000	25.6 ± 22.6
	7	W	200 µL	-	250	-	1000	46.2 ± 4.6
	8	W	100 µL	-	250	-	1000	64.9 ± 13.5
8T21-4	10	FD	51.8	50	-	1000	-	29.7
	11	FD	49.3	-	250	-	1000	BDL
	12	FD	22.8	-	250	-	1000	BDL
	13	T	201.5	-	250	-	1000	BDL
	14	T	104.0	-	250	-	1000	BDL
	15	T	22.8	-	250	-	1000	29.8 ± 22.6
	16	W	200 µL	-	250	-	1000	BDL
	17	W	100 µL	-	250	-	1000	BDL

* Assay performed with both 100 and 200 µL of extract. Presented 200 µL results only
BDL = below detection limit
NB – detection limit of RIA = 0.003 ng/tube

Experiment 2.3

With the exception of the steroid yield determination experiment, most of the measured cortisol concentrations assessed from the freeze dried material were below detectable limits. Recently a new cortisol antibody has been employed for cortisol RIAs and consequently the standard curve which previously covered 800 – 6.25 pg/ml now only reliably reaches 800 – 25 pg/ml (Fig. 1). Despite this, concentrations for the 10 and 20 % dilution (experiment 2.3.3) were detectable possibly indicating a potential inhibitory effect by a substance contained within the faeces. With the steroid yield determination (experiment 2.3.4) approximately 50% of the steroid label was extracted compared to the control; however, it is difficult to know the relevance of this result as we do not know if the label was taken up by the faecal matrix or was situated around it.

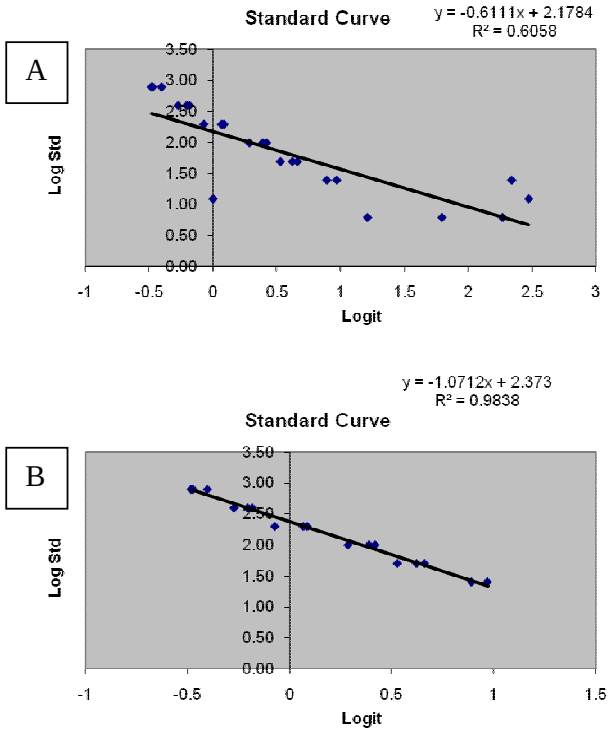

Fig 1. Standard curves obtained from the mucus/collection methods RIA. Curve A includes all points from 800- 6.25 pg/ml. Curve B only includes points from 800-25 pg/ml.

Experiment 2.4
Cortisol levels in all faecal slurries, regardless of collection or storage methods, were below detectable limits.

3. Cortisol extractions from tuna mucus
Experiment 3.1
This preliminary experiment indicated that significant cortisol assay cross-reactivity can be obtained from tuna mucus samples (Table 7). Use of 1000 µl extract from the method described by Schultz et al., (2005) provided detectable cross-reactivity for all 3 tested fish. Use of increased amounts of freeze-dried mucus material per extraction is suggested for future experiments.

Table 7. Cortisol recovery from freeze dried mucus samples

Extraction method	Fish	Extract volume (µl)	*Mean cortisol detected (pg/tube ± SE)*
Ethyl acetate	2	100	*BDL*
		200	*5.7 ± 2.9*
	3	100	*BDL*
		200	*BDL*
	7	100	*3.6 ± 0.1*
		200	*5.4 ± 0.8*
Schultz et al., 2005	2	200	*BDL*
		1000	*4.4 ± 1.1*
	3	200	*BDL*
		1000	*7.7 ± 0.4*
	7	200	*BDL*
		1000	*3.8 ± 1.1*

BDL = below detection limit
NB – detection limit of RIA = 0.003 ng/tube

Experiment 3.2
Cortisol levels in freeze dried mucus were below detectable limits in all experiments. Again the standard curve was only reliable above 25 pg/ml (see Fig 1).

DISCUSSION
1. Cortisol extractions from seawater
The above experiments outlined within the present report have clearly shown that cortisol can be extracted from 'spiked' seawater samples using solid-phase hydrophilic-lipophilic balance (HLB) extraction cartridges. However, although cortisol was detectable, the lack of optimum recovery suggests that not all the cortisol added to the sample was indeed extracted. Additionally, extraction efficiencies were not consistent among spiked samples which suggest that this method cannot reliably be used as a direct measure of cortisol concentrations in fish and will require further investigation. In general the recommended flow rates of water through solid-phase cartridges are low 2-15 ml/min depending on the manufacturer. The manufacturer recommendation for the cartridges used in these studies suggested that flow rates not exceed 2 ml/min when loading the cartridges. Although the flow rate was not actually determined it was likely to have exceeded this 2 ml/min recommendation. A reduction in flow rate may therefore have improved the observed extraction efficiencies.

The extraction and concentration of cortisol in water using expensive single use solid phase extraction cartridges and pre filters has limited the use of this method of stress assessment to species in tank culture studies (see Ruane and Komen, 2003; Lower et al., 2005; Ellis et al., 2007). The use of this method for species cultured in open water has not been reported, most likely due to inherent difficulties relating to dilution and dissipation of cortisol as well as steroid degradation. Cortisol extractions from water taken from sea cages

would be costly and require considerable validation. The development of this particular method for the assessment of stress in Southern bluefin tuna therefore represents both logistical and technological challenges that will be both time consuming and economically expensive.

2. Cortisol extractions from tuna faeces

The results for above experiments demonstrated considerable variability in the measurement of cortisol from tuna faeces. Particular sources of error and variability for faecal glucocorticoid measurements have been linked to dietary effects on the gastrointestinal system (von der Ohe and Servheen, 2002), faecal collection methods, storage of samples and time held prior to measurement (Millspaugh and Washburn, 2004). Faecal glucocorticoid metabolites are considered to be relatively inert and stable compounds (Lane, 2006); however, water can result in significant fluctuations as its presence in a sample can allow metabolic processes to occur (Terio et al., 2002; Tempel and Gutierrez, 2004). Faecal samples used for cortisol measurement in experiments for parrotfish (*Sparisoma viride and Scarus spp*) were stored at -40°C (Turner et al., 2003; Lupica and Turner, 2009). For the above studies samples were collected in Port Lincoln, transported to the University of Tasmania and stored at -20°C in the presence of saltwater. Storage temperature and time may have therefore contributed to the observed variability. Results from the faecal samples experiments have also demonstrated considerable cross-reactivity for the various extractions. Attempts to investigate parallelism were not successful however and it appears that this cross-reactivity is related to a non-specific component of the sample matrix. In general RIA techniques measure both free and protein bound cortisol (cortisol metabolites); however, the cross reactivity of both cortisol and cortisone metabolites with this assay is at present unknown. Additionally, it is possible that the salt content of the samples acted as an inhibitory factor. This however will require further investigation. Recent investigations into the decrease in cortisol RIA sensitivity now suggest that the problem is related to the antibody stability and storage time.

3. Cortisol extractions from tuna mucus

Although variable, measureable amounts of cortisol were detected in tuna mucus samples. There is little to no literature regarding the measurement of cortisol in fish mucus however, a number of studies for other animals have reported that salivary glucocorticoids are not consistent with plasma levels (Dorn and Susman, 1993; Anderson et al., 1999; Wong et al., 2004). In fish, cortisol in the circulation exists principally bound to cortisol-binding proteins and only approximately 12-40% of plasma cortisol exists in the ‘free’ state (the only state where cortisol has any biological activity) (Caldwell et al., 1991; Barry et al., 2001). Free cortisol in animals enters the saliva mainly by passive diffusion. The bound fraction is unable to cross the blood-saliva interface due to its large size (Lane, 2006). Therefore in contrast to both blood and faecal samples, that contain both free and bound cortisol, salivary cortisol is directly and accurately correlated with the free fraction of cortisol (Aardal and Holm, 1995). The clinical use of salivary cortisol has been commonplace since the early 1980s for humans (Guechot et al., 1982) and highly sensitive antibodies that only recognise and measure free cortisol have been produced

(Lewis et al., 2003). As the majority of free cortisol is excreted across the gills, cortisol measurement in gill mucus seems could be an attractive method for the investigation of stress. Considering the difficulties associated with trying to collect gill mucus samples however this method most likely has limited application for Southern bluefin tuna.

Conclusion

The lack of detectable cortisol concentrations in the above experiments most likely reflect the problems experienced with the low end of the cortisol standard curve. Increased amounts of either freeze dried or wet material for the initial extractions did not appear to improve the sensitivity of the assay by shifting the assay signal into the linear part of the standard curve. It was also noticed that the slurries samples containing more than 500 mg tended to separate from the ethyl acetate and not remain in suspension even when subject to vigorous shaking. Increased amounts of material (either water or faeces) can additionally result in matrix effects that can lead to non-specific cross reactivity with assay antibodies or labels yielding false results (Scott and Ellis, 2007). Although the non-invasive measurement of cortisol to assess stress in fish has been shown to be technically possible the current RIA method employed for these studies requires further investigation for use with Southern bluefin tuna. To our knowledge, the capability of non-invasive cortisol measurement for the assessment of stress in species held in sea cages has not been reported but this is not surprising considering the inherent complexities such as dilution effects in a dynamic open system. Cortisol concentrations extracted from either water and faeces tend to be relatively low compared to its serum counterpart and therefore require both concentration and/or extraction prior to measurement.

In order to refine the current collection, extraction and measurement techniques outlined within this report, it is suggested that further investigations of non-invasive cortisol measurement as a potential method of stress assessment be performed in a model species amenable to tank culture. Correlations of plasma cortisol levels with these future results would be valuable and necessary to assess the value of this non-invasive approach. The use of ELISA for free cortisol measurement in fish has now also been validated (Lupica and Turner, 2009). It is therefore also recommended that any further investigations include the potential use of antibodies specific for free cortisol as well as the development of a cortisol ELISA specific for Southern bluefin tuna.

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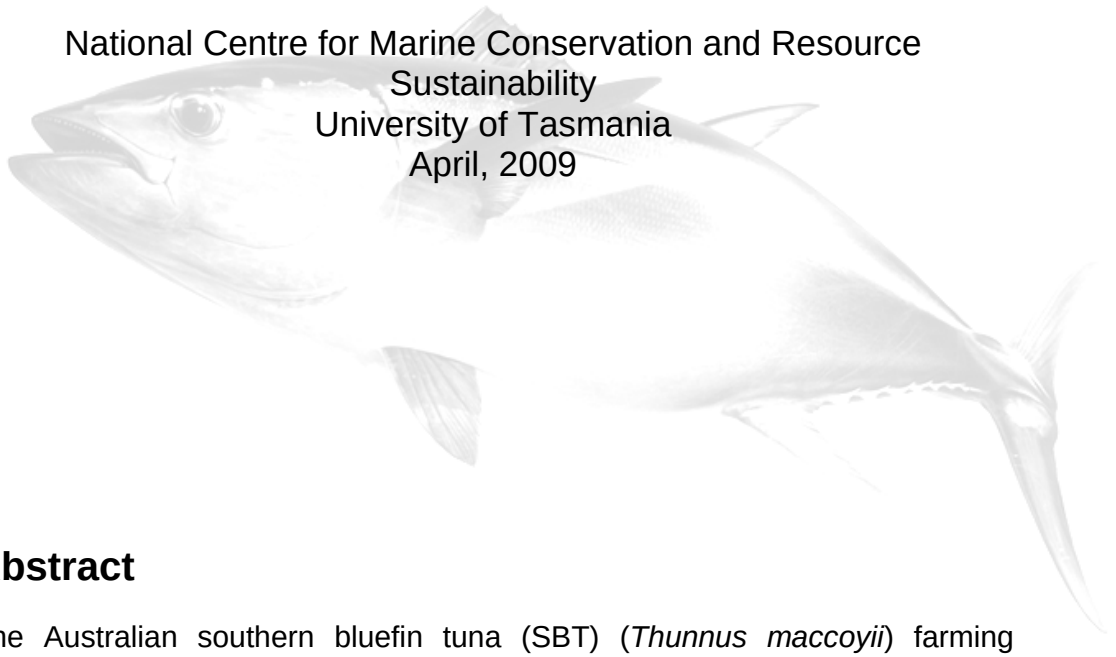
Appendix 4

Investigation of disease and mortality causes in farmed southern bluefin tuna (*Thunnus maccoyii*)

Victoria A. Valdenegro

This thesis is submitted in partial fulfilment
of the requirements for the degree
of Masters of Applied Science in Aquaculture
by coursework and dissertation

National Centre for Marine Conservation and Resource Sustainability
University of Tasmania
April, 2009



Abstract

The Australian southern bluefin tuna (SBT) (*Thunnus maccoyii*) farming operation is currently considered the largest of this type in the world. The health status of SBT had been good until recent times. During the last few years an increase in mortalities of SBT a few weeks post-transfer to the Port Lincoln area has concerned members of this industry. This project aimed to identify possible causes of mortalities in SBT by comparing normal, moribund fish and fresh mortalities.

Sampling of fish was carried out in the 2008 SBT farming season from commercial cages from 5 different companies. Fish were allocated into two groups: normal and moribund/dead, which were compared using a range of analyses. Intensity and prevalence were calculated for the counts of ectoparasites; cerebrospinal fluid (CSF) was analysed for the presence of *Uronema nigricans*; blood samples were analysed for levels of haemoglobin and plasma used for cortisol, lactate, pH, osmolality and lysozyme estimation; microbiological samples from spleen and kidney were obtained in TCBS/BA

plates and isolated bacteria were identified. Histological samples from diverse organs were also collected and slides were examined at different magnifications for the presence of any histopathological changes or pathogens. Granulomatous reactions caused by *Cardicola forsteri* in the heart were categorized. Hearts were flushed in order to remove and count any adult *C. forsteri* present and to calculate prevalence and intensity of infection.

Histopathology generated by the eggs of *C. forsteri* was significant. However, similar severity of pathology was observed between normal and moribund/dead fish indicating that this pathology is probably not the solely cause of mortality in SBT. The severity of the granulomatous reaction observed in the SBT heart increased over time, probably in relation with the typical development of the reaction and with increasing numbers of adult parasites in the heart. Intensities of *Hexostoma thynni* and *Euryphorus brachypterus* on gills of SBT did not differ between fish sampled in different months or between the groups, and they were lower than previously reported for *H. thynni* but not for *E. brachypterus*. *Pseudocycnus appendiculatus* presented similar intensities as previously observed, but its prevalence was higher during the winter epizootic than that reported in the past. Under the current SBT culture conditions these parasites have not been reported to affect the condition index and therefore, a small increase in intensity of the copepod should not be a cause of concern. *Caligus* spp. presented higher intensities than in the past; this parasite has the potential of affecting SBT health and hence should be surveyed. *U. nigricans* was not found in CSF of SBT or in histology of olfactory rosettes during the present study.

Mean plasma cortisol and lactate showed trends in agreement with stress events affecting SBT. Plasma glucose levels were very variable and they might have been affected by other factors. pH values did not correlate directly with lactate values as expected. Increases in plasma lysozyme activity seemed to have been associated with bacterial isolations; however, some high values could have been related with individual variability and stress. Bacterial isolations were made from both normal and dead/moribund fish, but in a greater number in the second group. These isolates all belonged to genera *Photobacterium* and *Vibrio*, and were consistent with previous findings from SBT.

Cardicola forsteri was a suspected mortality causing agent but mortalities generated by parasite infestation are hard to identify. No evidence was found that linked *Caligus* spp. directly to mortalities. Bacterial isolations presented suspected environmental contaminants and potential pathogens, but no evidence of organ damage due to bacterial infection or presence of toxins was found in the histological analysis. Without further information and a more complete range of sampled fish, it would be very difficult to determine a definite cause of mortalities in SBT.

Appendix 5

Presentations (other than at the SBT industry meetings)

Hayward, C.J., Ellis, D., Foote, D., Wilkinson, R., Crosbie, P. and Nowak, B.F. Annual elevated mortality in ranched southern bluefin tuna was immediately preceded by concurrent epizootic hyperinfections of sealice (predominantly *Caligus chiastos*) and blood flukes (*Cardicola forsteri*). Fourth National FRDC Aquatic Animal Health Conference, 22-24 July, Cairns.

Valdenegro, V. And Nowak B. Investigation of mortality causes in Southern Bluefin Tuna (*Thunnus maccoyii*), Fourth National FRDC Aquatic Animal Health Conference, 22-24 July, Cairns.

Appendix 6

Publications

Evans, D. (in prep) Tow history and subsequent mortality in ranched SBT, Aquaculture
Evans, D. (in prep) Seasonal and annual stress responses in SBT, Journal of Fish Biology
Evans, D. (in prep) Stress responses during longer term holding of SBT, Aquaculture
Hayward, C.J., Ellis, D., Foote, D., Wilkinson, R., Crosbie, P., Bott, N., Nowak, B.F (2010) Concurrent epizootic hyperinfections of sea lice (predominantly *Caligus chiastos*) and blood flukes (*Cardicola forsteri*) in ranched Southern Bluefin tuna Veterinary Parasitology, submitted
Valdenegro, V., Naeem, S., Carson, J., Bowman, J., Tejador de Real, J.L., Nowak, B.F.. (2010) Microbiology of ranched southern bluefin tuna (*Thunnus maccoyii* Castelnau), Veterinary Microbiology, submitted