Assessment of the Inflamark method as a sensitive and cost-effective measure of oxidative stress in cultured fish.

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2021

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

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- The University of Western Australia (UWA) and
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Abbreviations

ANOVA	Analysis of variation
CTAB	Hexadecyltrimethylammonium bromide
DBS	Dried blood spots
DMSO	Dimethyl sulfoxide
DOW	Days-out-of-water
DPIRD	Department of Primary Industries and Regional Development
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
FCR	Food conversion rate
FM	Fishmeal
IP	Intraperitoneal
LF	Lateral flow
MPO	Myeloperoxidase
PBS	phosphate-buffered saline
POS	Protein oxidative stress
ROS	Reactive oxygen species
SBM	Soybean meal
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
UWA	The University of Western Australia
YTK	Yellowtail Kingfish

Executive Summary

This collaborative project between industry and academia was developed in response to the need for a reliable and simple measurement of fish health status in farmed populations as well as in a research context, to assess the outcomes of trials on health, nutrition and environment. We have developed a tool to measure physiological/oxidative stress in aquaculture species.

Aquaculture species are subject to a variety of stressors because their homeostatic physiology is dependent on their environmental surroundings. Common stressors can include parasitism and associated bathing practices, water pollution, stress from increasing water temperatures, sub-optimal diets, transport, handling and overcrowding. However, there is no practical analytical method of assessing physiological health. Most methods such as haematology, blood biochemistry, flow cytometry and histology have limitations regarding sensitivity, repeatability, cost and simplicity. The lack of a physiological measure of aquatic animal health not only impacts industry operations but also affects research programs designed to improve industry productivity. For example, the Fisheries Research and Development Corporation (FRDC) have invested heavily in aquaculture health and nutrition research, but the lack of a rapid and effective method of assessing physiological health following such trials is still lacking.

An example of measure of stress currently used, is cortisol. It is known to be unreliable due to its sudden increase from handling stressors, inaccuracy in detecting stress in fish that have previously experienced stress, its variability with sex and age, as well as differences between species.

In this project, we proposed that measuring a protein oxidative stress (POS) biomarker in blood could be used to monitor fish health. The objective of the project was to develop a stress test tool which can be used by the aquaculture industry for routine monitoring of fish health and to assess the outcomes of research trials on health, nutrition and environment.

Our goal was to develop an early warning measure of stressors affecting the physiological health of key Australian and global aquaculture species including salmon, kingfish, trout, barramundi and oysters. Such a tool would allow aquaculturists to develop monitoring and management systems to minimise the negative impacts of stressors; develop more sustainable aquafeeds and maximise industry productivity and profitability.

Trials testing the applicability of Inflamark to measure physiological stress were conducted in several available aquaculture species including Yellowtail Kingfish (YTK; *Seriola lalandi*), Barramundi (*Lates calcarifer*), Atlantic Salmon (*Salmo salar*), Rainbow Trout (*Oncorhynchus mykiss*) and Sydney Rock Oysters (*Saccostrea glomerata)*. These trials include looking at stressors which exist within the industry in addition to being involved with some active research trials surrounding aquafeed composition trials.

In human blood, there is one protein biomarker which demonstrates sensitivity to alterations in oxidative stress (Lim et al., 2020). In YTK blood, we identified several key proteins which were modulated from various external and internal stressors through our trials. This discovery provides the Inflamark technique with potentially greater sensitivity as a multi-protein biomarker tool in aquaculture species. This technical advancement added additional complexity to the adaptation of the method from requiring fresh blood collection and the related processing, to the use of dried blood spot (DBS) cards.

Inflamark has been successful in detecting physiological stress in fish blood and oyster haemolymph. Of additional interest, different stressors appear to change different protein biomarkers, which indicates the

possibility of identifying the nature of stress through changes in the proteins modified. This research is very exciting because it has demonstrated that it is possible to measure physiological stress in aquaculture species. Inflamark could help the aquaculture industry monitor stock health and provide an outcome metric in research trials, both long and short duration.

With further validation and development of the Inflamark technology, aquaculture producers would be able to undertake routine testing to monitor the health status of stock; and enable earlier detection and timely intervention strategies which could prevent or mitigate the stressors leading to poor health outcomes.

Keywords: *Oxidative stress, biological stress, biomarker, Yellowtail Kingfish, Barramundi, Atlantic Salmon, Sydney Rock Oysters*

Introduction

The farming of aquatic species for consumption (aquaculture) is an expanding industry. Most of the world's aquatic food is now produced through aquaculture rather than by wild capture. Global seafood consumption per-capita has increased from 9.0 kg in 1961 to 20.5 kg in 2018 which has been attributed to an increase in health consciousness (Food and Agriculture Organization of the United Nations, 2019; Food and Agriculture Organization of the United Nations, 2020). As of 2018, global aquaculture reached 82.1 million tonnes of production with an estimated sales value of USD$ 250 billion (Food and Agriculture Organization of the United Nations, 2020). In Australia, aquaculture has been growing at 4.3% (2015 - 2020) with expected revenue in 2020 of $ 1.9 billion with 59.4% of that from salmonid products (IBISWorld, 2019). Despite the rapid expansion of the global aquaculture industry, the technological advancement to rapidly evaluate the physiological health and welfare status of aquaculture stock is lacking.

In a recent article "*Stress in aquaculture: a rough guide"* Aerts (2019) stated that "Understanding and optimising the stress response by quantification of the stress level at all stages in the production cycle will support disease prevention and improve production performance and efficiency". Researchers and farmers have been increasingly interested in the effects of chronic and acute stress over the last decade. Furthermore, consumers are becoming more aware of, and concerned about, the health and welfare of farmed animals. Stress on fish can have adverse effects on their health and welfare with impacts on growth, reproduction, susceptibility to illness, and increase mortality rates.

Aquaculture species are subject to a variety of stressors because their homeostatic physiology is dependent on their environmental surroundings. Common stressors experienced by aquaculture organisms include changing water temperatures, water contamination and pollution, sub-optimal diets, transport, handling, overcrowding, and parasitism, and associated management practices. In sub-optimal conditions fish become stressed which can increase susceptibility to disease, decrease growth rates and cause mortality. Infectious diseases, both indirectly and directly, account for losses of up to 30% of the total aquaculture production worldwide, as estimated by the Food and Agriculture Organisation of the United Nations (FAO). A sensitive, early warning measure of stressors affecting aquaculture species health would allow aquaculture farmers to react more quickly to remove stressors and improve conditions prior to adverse effects on stock.

Currently, there are no practical and effective analytical methods to rapidly assess physiological health in aquaculture species. Most analytical methods such as haematology, blood biochemistry, flow cytometry and histology have limitations regarding sensitivity, repeatability, cost and simplicity. The lack of a physiological measure of aquatic animal health not only impacts industry operations but also affects research programs designed to improve industry productivity and sustainability. For example, the FRDC have invested heavily in aquaculture health and nutrition research, but an effective method of assessing physiological health following such trials is still lacking.

The oxidative stress research group at the University of Western Australia's School of Molecular Sciences have developed a simple, finger-prick blood oxidative stress test that is sensitive and cost-effective. This test has been named Inflamark for simplicity purposes. The technology is being developed in mammals to test the efficacy of treatments in a chronic childhood disease, Duchenne muscular dystrophy, and to assess damage caused by overtraining in muscles of elite athletes. Inflamark measures the oxidation state of plasma proteins from a drop of finger-prick blood and is a particularly sensitive measure of excess generation of reactive oxygen species (ROS), also known as free radicals. ROS are generated in excess when organisms are under physiological stress, which can occur as result of poor nutrition, inflammation associated with muscle injury, infections caused by viruses or bacteria, and in multiple chronic diseases (e.g., Duchenne

muscular dystrophy, arthritis, cystic fibrosis). Hence, this measurement of oxidative stress can be applied as a biomarker of physiological stress. We aimed to determine if Inflamark could be used as a tool for aquaculture to detect physiological stress in fish and molluscs.

Objectives

The following project objectives were agreed in the contract:

1. Optimise field methods to ensure blood is collected and preserved appropriately for shipment and analysis.

2. Determine the range of baseline oxidative stress values for healthy and moribund fish from four species across the full range of sizes and culture temperatures under commercial grow out conditions.

3. Obtain data from fish in various Fisheries Research and Development Corporation, and industry funded research projects, investigating health and nutrition.

Methods

Samples were obtained from Department of Primary Industries and Regional Development (DPIRD, Western Australian Government) and industry partners involved in this project. Samples were collected as part of small validation studies or as samples a part of larger projects. The specific methods for each of these are defined within the given results sections. Some specifics have not been included due to the project having been a part of larger projects or industry sensitivities. Due to a delay in the commencement of the project, a YTK trial from which we were to acquire samples, had finished. In place of these samples and at the discretion of the partner who provided the funds, we gained an opportunity to evaluate Inflamark in Sydney Rock Oysters.

Various trials were conducted across these available aquaculture species, Yellowtail Kingfish (YTK; *Seriola lalandi*), Barramundi (*Lates calcarifer*), Atlantic Salmon (*Salmo salar*), Rainbow Trout (*Oncorhynchus mykiss*) and Sydney Rock Oysters (*Saccostrea glomerata)*.

Oxidative stress measurement

Oxidative stress was measured through plasma protein thiol oxidation. Due to the biological difference between species tested, including variations in plasma protein abundance and molecular weight of potential protein biomarkers, each species required a tailored approach to the method described below. The method used was modified from Lim et al. (2020), known as the malpeg assay. Briefly, whole blood was collected from the caudal vein (less than 0.5 mL) and added to a preservative buffer (polyethylene glycol maleimide in imidazole buffer, pH 7.4) to trap the oxidative state of the proteins. Blood samples were centrifuged at 3,000 *g* for 10 minutes, supernatant was collected and stored at -80 °C prior to analysis. To perform the assay, samples were thawed and diluted in phosphate-buffered saline (PBS) and sample buffer (125 mM Tris, pH 6.8, 4 % SDS, 30 % (v/v) glycerol, 0.02 % bromophenol blue). Samples were heated for five minutes at 95°C prior to loading on a gel. A molecular weight standard and samples were loaded into separate sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) wells and run at 180V. Analysis was performed as previously described (Lim et al., 2020).

Within Objective 2 and 3, for each species assessed, protein oxidative stress (POS) biomarkers were named based on the protein's appearance in a gel electrophoresis image. Hence, each species has their own labelling system for POS biomarkers, the same number between species, may not reflect the same protein identified. Trials detailed below may have different percentages for similar protein identifications, particularly in YTK trials due to various analytical methods being tested.

Objective 1. Simplification of in-field blood collection

The gold-standard method of Inflamark to measure oxidative stress in blood is shown as a workflow in Figure 1A (also described above). This method requires specialised laboratory equipment, skills and cold-chain logistics. These requirements limit the capacity to take samples on-farm and limit the engagement with aquaculture researchers and industry. Objective one of this project was to develop a simplified method of collecting blood with a device which would not require such technical skill and equipment, and could have samples transported at room temperature (Figure 1B). This would enhance the ability to obtain samples and collaborate with aquaculture researchers and industry, nationally and internationally to validate the technology.

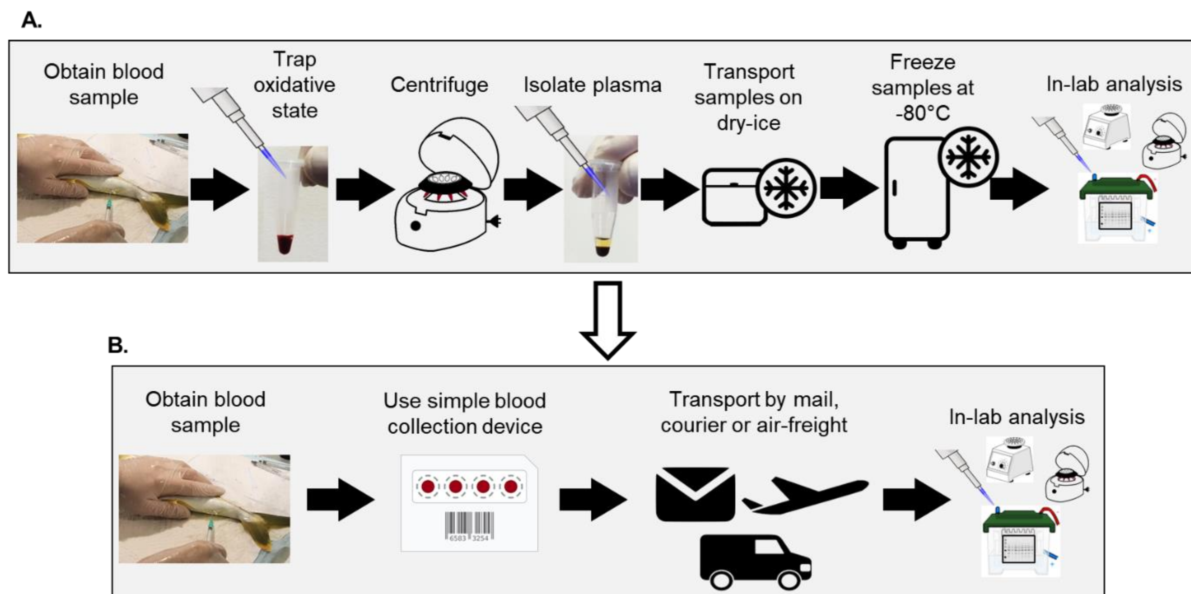

Figure 1. Inflamark gold-standard blood collection method isolating plasma prior to transport to laboratory for analysis (A), compared to the goal of a simple dry-blood collection device that can be easily transported to the laboratory for analysis (B).

To adapt the Inflamark method to one which is simpler and user friendly, we identified the use of dried blood spot (DBS) cards as a potential mechanism for fish blood collection and stable transportation of proteins. These cards are routinely used for human blood collection, particularly in infants for health screening tests. Dr Arthur's laboratory at the University of Western Australia (UWA) has adapted their oxidative stress assay in human blood to successfully work with, and produce repeatable and reliable results from specialised DBS cards. The aim was to adapt this technique for use with finfish blood using YTK. Blood samples were collected via the gold-standard Inflamark blood collection method using plasma isolation (Figure 1A) and on specialised DBS cards for direct comparison of the two methods.

Lateral flow (LF) membranes were also trialled as a potential blood collection medium in comparison to DBS cards. LF membranes work by the migration of a blood sample laterally through a paper-like matrix to result in the red blood cell component being separated from the plasma component. Due to this, LF membranes require less post-processing of the samples to isolate plasma proteins as they are already contained within a particular portion of the membrane.

Flow cytometry

Fluorescence-activated cell sorting (FACS) analysis was conducted in YTK blood after an acute 24-hour temperature trial. YTK blood was collected in heparinised tubes and kept at room temperature until processing. A cell-permeant fluorescent dye 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$) was prepared in ethanol at a stock concentration of 500 µg/ml. Fresh blood (10 µL) was added to Hank's balanced salt solution (1850 µL) and $DiOC_6$ dye solution (40 µL) (Inoue et al., 2002). Hoechst 33342 stock was added to each sample (final concentration of 5 µg/mL). Samples were gently mixed and incubated at room temperature for 30 to a maximum of 90 minutes. Immediately prior to data acquisition, samples were transferred to TruCOUNT tubes (BD Biosciences), allowing for downstream calculation of absolute cell counts.

Flow cytometry was conducted on a FACSCanto II (BD Biosciences) using FACSDiva software. A 30 mW 405 nm solid state laser was used to excite Hoechst 33342 and emission was captured with a 450/50 nm bandpass

filter in linear mode. A 20 mW 488 nm solid state laser was used to excite $DiOC^6$ and TruCOUNT fluorescent beads and emission was captured using a 530/30 nm and 585/42 nm bandpass filter in logarithmic mode. Data was acquired at a slow flow rate to accommodate the high cell concentration in this high-throughput method.

FlowJo (v10) was used to refine data events into cell counts. Briefly, this was achieved by gating single cells (singlets; i.e. no clumps of cells were analysed) using forward scatter for cell size, side scatter for cell complexity and granularity, and fluorescence emitted by the dyes.

Absolute cell counts for each cell type was determined using the following formula:

$$Cells/\mu L = \frac{cell\ count}{number\ of\ bead\ events\ in\ sample} \times \frac{Number\ of\ beads\ per\ tube}{Volume\ in\ tube}$$

Inflammatory cell presence via myeloperoxidase content

Accumulation of neutrophils as a marker of acute inflammation in YTK hindgut tissue was assessed by measuring the activity of myeloperoxidase (MPO), as previously described (Terrill et al., 2020). Briefly, frozen hindgut was ground using a mortar and pestle cooled in liquid nitrogen and homogenised in 25x 0.5% hexadecyltrimethylammonium bromide (CTAB)/PBS buffer. Samples were centrifuged, supernatant isolated and diluted with CTAB-PBS buffer. Standards were prepared using human MPO (Cayman Chemical) and diluted in CTAB-PBS buffer. Triplicates of experimental samples and MPO standards were loaded into a black 384-well microplate before the addition of a working solution of 10 µM 2-[6-(4-aminophenoxy)-3-oxo-3H-xanthen-9-yl]-benzoic acid (APF; Cayman Chemical) with 10 µM hydrogen peroxide in PBS. The plate was incubated at room temperature in the dark for 30-min with fluorescence measured every minute at excitation and emission wavelengths of 485 nm and 515-530 nm respectively, on a fluorescent plate reader. Samples were quantified with a detergent compatible protein assay (Bio-Rad) and read at 750 nm using a spectrophotometer. The rate of change in fluorescence for each sample was compared to that of the standards and results expressed as $nmole.mg^{-1}$ protein.

Data analysis

The statistical software GraphPad Prism (v7) was used for one-way and two-way analysis of variance (ANOVA), and JMP V15.0 (Statistical Analysis Systems) was used for 4-way ANOVA. Individual statistical analysis is described within each experimental section. Statistical significance was accepted at $p < 0.05$ with data presented as mean ± SEM.

Results and Discussion

Objective 1. Simplification of in-field blood collection

The protocol of human blood protein extraction from DBS cards requires the spots to be punched and incubated in PBS containing 0.05% TWEEN 20 (polyethylene glycol sorbitan monolaurate; detergent for protein extraction) on a shaker at room temperature for 1-hour. Initial experiments with this protocol yielded unsatisfactory results for the extraction of abundant YTK blood proteins. Minimal YTK blood proteins were isolated from the blood cards (Figure 2B) compared to in plasma through the gold-standard blood collection method (Figure 2A). Different concentrations of detergents (TWEEN and SDS) were trialled and it was found that the best elution buffer was 0.1% Tween in 20mM phosphate buffer. However, due to the high number of proteins in the YTK blood with some being less abundant, not all the proteins were successfully being extracted off the blood cards and it appeared protein degradation was occurring during the process (lanes in the gel appear smeared). From this finding, several different strategies were trialled to try and extract all the proteins we were interested in and to reduce degradation of the proteins. Trials included blood card punches being left to elute in buffer on a shaker from 3-hours to 20-hours at room temperature and at 4°C, in addition to having the addition of a protease inhibitor in the elution buffer to prevent protein degradation. It was found that YTK blood card punches eluted in 0.1% TWEEN 20 and 20mM phosphate buffer for 17-hours at 4°C for gave the best level of protein extraction (Figure 2C). Despite this being the best protein extraction method from DBS cards, some key POS biomarkers which are found in the plasma, were not being isolated or retained on the specialised DBS. Based on this result, another approach using lateral flow (LF) membranes was trialled.

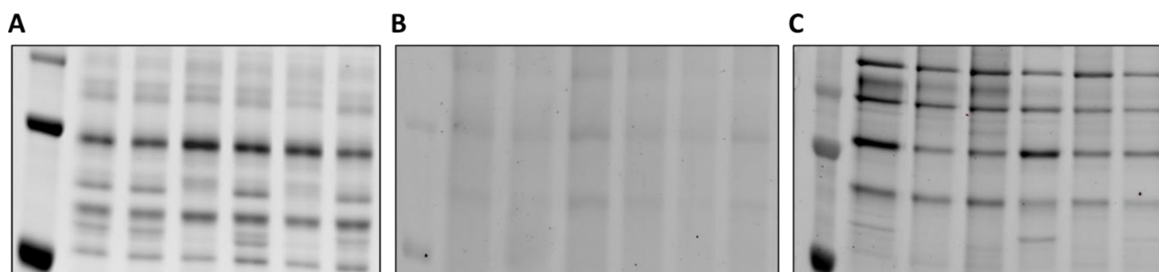

Figure 2. Gel electrophoresis images of Yellowtail Kingfish protein profile in plasma (A) and in dried blood spot (DBS) card samples upon initial testing (B) and after initial optimisation (C).
Molecular weight marker is in the first well of the gels followed by blood/plasma samples. The darker the individual bands, the higher abundance of protein within the electrophoresis gel.

LF membranes were trialled with YTK blood and run through two post-processing protein extraction protocols; a human plasma protein extraction protocol developed in Dr Arthur's laboratory and the extraction protocol developed to work with DBS cards as described above. The human plasma protein extraction protocol involved cutting the plasma protein portion off the membranes and incubating them in PBS containing 0.05% TWEEN 20 on a shaker at room temperature for 1-hour. This protocol gave us similar unsatisfactory results as we had previously seen with the DBS cards (Figure 3B). The DBS extraction protocol (from above) was tested with the membranes and showed a greater yield in protein being extracted for analysis by gel electrophoresis (Figure 3C). However, the less abundant potential biomarkers in YTK blood were absent from the gel electrophoresis with the background in the wells being high. With the high background and little to no signal from certain key proteins, this resulted in unreliable and less accurate levels of oxidative stress in the samples. An increase in the background signal from the samples could come from

the auto-fluorescence of proteins which may have degraded through this blood collection process and show a smearing pattern within the gel electrophoresis wells. LF membrane blood collection requires further optimisation to further explore and reduce this possible protein degradation. A high background signal reduces the sensitivity in the oxidative stress measurements made of the individual protein biomarkers and reduces reproducibility making it a serious issue that needs to be rectified.

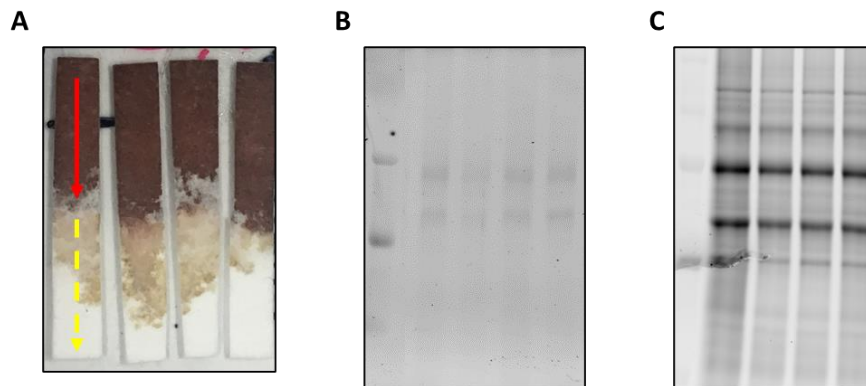

Figure 3. Yellowtail Kingfish blood collected on four individual lateral flow membranes (A) with the plasma portion processed through gel electrophoresis as a preliminary trial (B) and with initial optimisation (C).
Lateral flow membranes separate whole blood (A) into the red blood cell component (indicated by the red arrow showing the direction of flow) and plasma component (indicated by the dashed yellow arrow). Molecular weight makers are present in the first well of the gel electrophoresis images (B and C).

Despite these challenges, we have shown that DBS and lateral flow membranes show initial promise in the possibility of being used for blood collection with the Inflamark technique and could be adapted in the future for use with fish blood.

Prior to commencement of this project objective, risk analysis with threat and contingency plans were outlined. One of the risks associated with Objective 1 was that the simple method of collecting blood (through a blood collection device) in the field does not work for finfish blood. The simple method was trialled and deemed to be inadequate for the complexity of the plasma profile of finfish, without more extensive optimisation which would have taken time out of Objective 2 and 3 of the project. The contingency plan was actioned with the gold standard plasma collection method which we know to work with finfish, used for the remainder of the objectives.

Objectives 2 and 3. Determine baseline oxidative stress values across various species from beneficiaries, and co-current FRDC projects investigating health and nutrition

Trials conducted include:

1. Yellowtail Kingfish temperature trials (including Daniel Soo, University of Western Australia Master's Student Thesis)
2. Yellowtail Kingfish aquafeed trials
3. Barramundi temperature trial
4. Rainbow Trout temperature trial
5. Atlantic Salmon preliminary trial: Hatchery vs. grow-out
6. Sydney Rock Oyster preliminary trial: days-out-of-water and bathing temperature

1. Yellowtail Kingfish temperature trials

An acute 2-hour temperature trial was conducted on 12 juvenile YTK (80 - 100g) at the DPIRD Marine Finfish Commercial Hatchery, Fremantle. Fish were held in two ambient seawater tanks (18.7°C) with six fish stocked per tank. One tank was assigned for warm water bathing and gradually transitioned to 26.6°C. Fish were held for 2-hours at respective temperatures to determine the effect of the acute temperature shock. Fish were placed in an immersion bath to achieve deep anaesthesia with AQUI-S (20 mg/L). Blood was taken from the caudal vertebral vein and blood was immediately placed into ethylenediaminetetraacetic acid (EDTA) tubes for Inflamark and flow cytometry measurements. Warm bathed fish had more blood clotting upon caudal vein blood extraction compared to ambient bathed fish.

The acute 2-hour temperature shock of the warm fish resulted in a significant reduction in the level of protein A oxidation compared to ambient bathed fish (p = 0.0003, Figure 4). There was a trend of the other POS biomarkers being reduced, however none of these were significant (Figure 4).

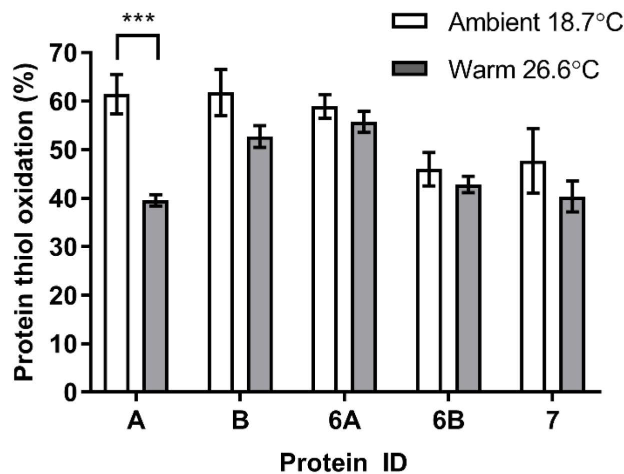

Figure 4. Yellowtail Kingfish had a significant reduction in oxidation of protein A in an acute 2-hour temperature trial at ambient (18.7 °C) and warm (26.6 °C) bathing temperatures.
Significance shown as ***p < 0.001, *n* = ambient, 5 and warm, 6.

Flow cytometry was performed on the same blood samples to assess the level of various blood cell types and if they were affected by the acute 2-hour temperature trial. Results showed a large variation in the number of red blood cells in blood from warm bathed fish compared to ambient held fish (Figure 5A). A significant reduction in the number of monocytes and lymphocytes/thrombocytes was found in blood from warm bathed fish compared to ambient held fish (p = 0.007, Figure 5B and p = 0.013, Figure 5D respectively). A non-significant trend in reduction of the number of granulocytes was seen in warm bathed fish blood compared to ambient held fish (p = 0.099, Figure 5C). The high variability in the number of red blood cells and the significant reduction in thrombocytes could be due to the high tendency of the warm fish blood to clot upon retrieval.

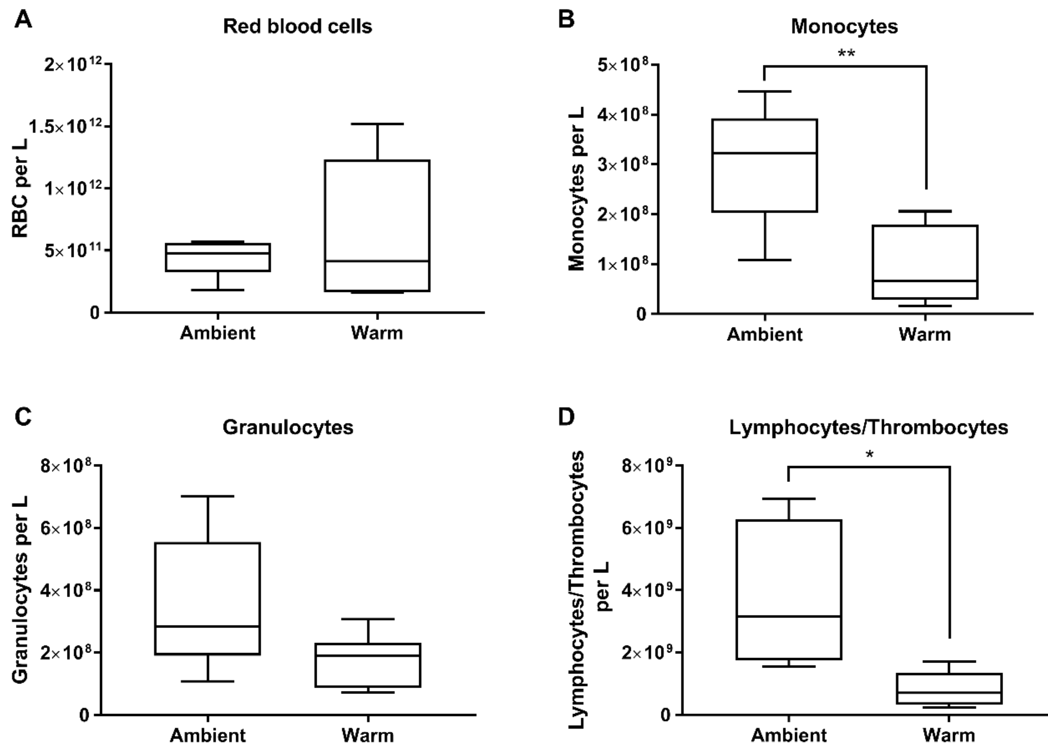

Figure 5. Measurement of red blood cells (A), monocytes (B), granulocytes (C) and lymphocytes/thrombocytes (D) in Yellowtail Kingfish blood after an acute 2-hour temperature trial at ambient (control; 18.7°C) and warm (stressed; 26.6°C) bathing temperatures.

Monocytes (A) and lymphocytes/thrombocytes (D) were significantly reduced in warm bathed fish compared to ambient bathed fish. Significance shown as *$p < 0.05$, *n* = 5 - 6 per group.

A further study on the effect of water temperature and handling on Inflamark in YTK was undertaken by a University of Western Australia Master's student, Daniel Soo. Mr Soo's thesis is presented in Appendix 5. Mr Soo's thesis, showed YTK exposed to the same level of heat stress over 18-days, resulted in an increase in oxidation of the POS biomarkers. This result is distinct from what was found at an acute 2-hour heat shock. We are unsure why there is a differential response between these two trials (acute 2-hour vs 18 days). Perhaps, fish in the first 2-hours of a heat stress event have an acute shock episode where there are alterations in metabolism, white blood cell localisation and high cortisol levels of which collectively, could interfere with the oxidative stress measurements. However, the significant deviation of POS biomarker A from the healthy baseline level does imply a disruption in the health status of the fish. Getting protein identifications of what these POS biomarkers are would enhance our knowledge of the effects which are occurring after an acute shock event, like this in the fish.

Together, these data indicate a differential response in warm bathed fish compared to ambient bathed fish after an acute 2-hour shock of which could be detected by Inflamark.

2. Yellowtail Kingfish aquafeed trials

Trials have been conducted at the DPIRD, Fremantle, on YTK from FRDC project 2017-030. These trials, denoted as Immunotrial 6 and Immunotrial 7, have involved feeding YTK different diets with one of two base dietary protein sources (fishmeal; FM or soybean meal; SBM) with the inclusion of several dietary additives, outlined in each study below.

Immunotrial 6

Immunotrial 6 feed trial under FRDC 2017-030 was a three-month trial in which YTK were fed on two protein base feeds containing SBM or FM with two different immunostimulants/additives and a control. The SBM diet in this trial contained 20% whole soybean and 10% soybean concentrate. The six combinations of fish feeds were FM/SBM control, FM/SBM with garlic and FM/SBM with 10% w/w hydrolysate. Individually tagged fish within trial tanks were treated as replicates. Bloods from the fish were sampled each month for Inflamark (a part of FRDC 2017-030 project). After the third month the fish were challenged with an intraperitoneal (IP) steroid injection (dexamethasone, an anti-inflammatory and immunosuppression agent, 30 mg/kg dissolved in dimethyl sulfoxide; DMSO at 79 mg/mL) or DMSO as a control. Fish were subsequently sampled at three- and seven-days post-injection for a range of parameters including Inflamark.

Weight gain per fish over the three-month trial was significantly affected by dietary protein source (FM = 744 g/fish, SBM = 656 g/fish; $p = 0.007$), but not by dietary additive ($p = 0.22$). After the three-month trial, there was no evidence of chronic enteritis in the SBM fed fish based on histology and none of the POS biomarkers showed any significant change in blood oxidative stress levels (data not shown; $p > 0.05$). In addition, it appeared the additives made no difference to blood oxidative stress levels within each feed group (data not shown; $p > 0.05$). These data suggest that under these set of tank-based experimental conditions, whilst the SBM-based diets impacted on growth, they had no measurable deleterious effects on the health of the fish and that none of the additives tested had any impact on fish performance. These data allowed us to obtain healthy baseline expected ranges for the POS biomarkers across three months for YTK in a controlled environment.

At three- and seven days post-dexamethasone injection, we found two plasma proteins (YTK protein A and 1) which had completely degraded in all those fish administered with dexamethasone (i.e. across both dietary protein sources and additives) but which were still present in DMSO control fish. This is a result we have not seen before and whilst blood oxidative stress levels could not be quantified because of this degradation, we speculate these two proteins may be involved directly with the immune system and their degradation could be a sign of an immune attack on the fish or, could be an effect of dexamethasone causing immunosuppression, possibly prior to other physiological symptoms. Identification of these plasma proteins and their roles will continue beyond this project and will be of interest as their presence and abundance might be useful as biomarkers of immune dysfunction.

Results from Immunotrial 6 post-dexamethasone challenge were run through a 4-way ANOVA (challenge; days post-challenge, dietary protein and dietary additive) for detecting significances in POS Biomarkers. We found POS biomarker proteins 6B and 7 showed significant changes in oxidative stress in response to the effects of the dietary protein source and when fish were challenged with dexamethasone (Figure 6). Protein B was significantly less oxidised in dexamethasone challenged fish compared to DMSO controls ($p = 0.0001$), in addition to showing SBM fed fish had higher levels of oxidative stress than FM fed fish at day three and seven ($p = 0.003$) (Figure 6A and B).

Results also showed a significant effect of how FM fed fish responded to the immune challenge compared to SBM fed fish. At seven days post-injection FM fed fish had lower levels of oxidative stress compared to SBM fed fish in protein 6B (day, $p < 0.0001$; dietary protein, $p = 0.00002$, Figure 6D) and in protein 7 (day, $p < 0.0001$; base protein, $p = 0.0008$, Figure 6F). These data suggest SBM fed fish were more susceptible to stress compared to FM fed fish which were more resilient. In addition to FM fed fish being more resilient to stress by injection of dexamethasone/DMSO, the FM group with the dietary additive hydrolysate, showed no

change in their levels of oxidative stress. These results suggest that fish fed a diet of FM with added hydrolysate (10% w/w) were protected against a physiological response to dexamethasone. Other additives in the diets did not have any significant effect towards how the fish handled the challenge. Mr Luke Pilmer is analysing the other parameters from this trial as part of FRDC 2017-030, which will enable us to compare the Inflamark results against more traditional measurements of health and fish performance.

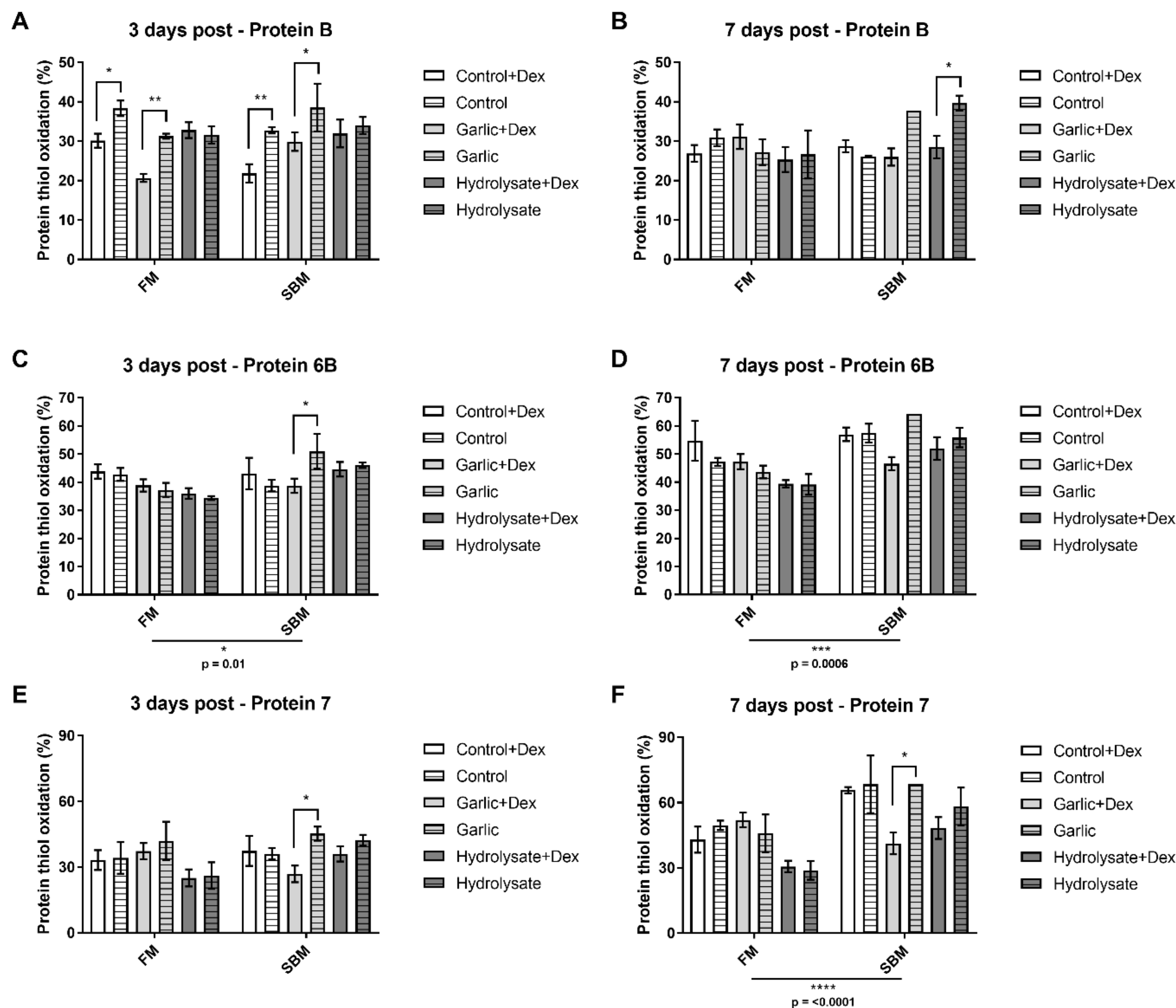

Figure 6. Inflamark found significant differences in blood protein biomarkers of oxidative stress in YTK fed for three months on various diets (Immunotrial 6) and then subjected to an IP injection of dexamethasone (Dex) or carrier control via DMSO (control). Protein B showed FM and SBM control fish at day three, had higher levels of oxidative stress compared to fish injected with dexamethasone. Protein 6B (C and D) and protein 7 (E and F) showed increased levels of oxidative stress across all control and dexamethasone treated fish from 3 to 7 days post injection except in protein 7 for fish fed on FM + Hydrolysate. Each + Dex group contains n = 5 - 6 fish, and DMSO controls contain n = 1 - 3 fish in each group. Data are presented as mean ± SEM. All proteins are shown in Appendix 4. Please note, significant interactions are not shown on the graph and are outlined in the 4-way ANOVA table below.

Table 1. Four-way ANOVA interactions of data from Immunotrial 6 for protein oxidative stress biomarkers B, 6B and 7.

		POS B	POS 6B	POS 7
Before Challenge (end of growth trial)	Dietary protein (FM, SBM)	0.77	0.33	0.095
	Additive (garlic, hydrolysate, no additive)	0.26	0.26	0.065
	Dietary protein x additive	0.96	0.13	0.001
Post Challenge	Challenge	0.0001	0.43	0.29
	Day	0.22	0.00000	0.000
	DietProtein	0.14	0.00002	0.001
	Immuno	0.45	0.06	0.007
	Day*Challenge	0.25	0.78	0.88
	Challenge*Immuno	0.54	0.09	0.16
	Day*Immuno	0.57	0.35	0.36
	DietProtein*Immuno	0.004	0.03	0.04
	Challenge*DietProtein	0.13	0.02	0.10
	Day*DietProtein	0.34	0.10	0.06
	Challenge*DietProtein*Immuno	0.27	0.14	0.30
	Day*Challenge*DietProtein	0.33	0.32	0.40
	Day*Challenge*Immuno	0.04	0.93	0.78
	Day*DietProtein*Immuno	0.10	0.78	0.97
	Day*Challenge*DietProtein*Immuno	0.11	0.80	0.81

Immunotrial 7

Immunotrial 7 feed trial under FRDC 2017-030 was conducted over one-month period (28 days) with fish being sampled for Inflamark and other measures at the conclusion of the trial period. This trial compared FM diets against SBM diets composed of 30% whole (unprocessed and ground) soybeans (i.e., with no soybean concentrate) and a reduction in the amount of choline. This diet was tested on the basis that the previous SBM diet did not induce gross enteritis, even when fed over three months. One of three additives and a control with no additives were trialled in each base diet, eight diets in total. The additives tested were Siberian chaga mushroom extract (2% w/w) and two concentrations of the aforementioned hydrolysate (10 and 20% w/w).

The SBM had a significant negative impact on growth ($p < 0.001$) compared to FM fed fish. Regardless of the base dietary protein source, there was no benefit (or negative impact) of any of the additives on growth ($p = 0.902$). Fish fed FM with the inclusion of 20% hydrolysate, had a significantly higher (worse) food conversion ratio (FCR) than those fed FM alone (control diet). Of the eight plasma proteins analysed, three showed significant differences using the Inflamark assay between fish fed SBM diets and FM diets (Figure 7). Protein 6A and 6B increased significantly in fish fed SBM diets, whist protein B decreased significantly. Protein B is the same protein that was significantly impacted by dexamethasone treatment. Then single POS biomarker in humans only increases in oxidation value in response to stress, however our data are consistently showing that this particular POS biomarker (Protein B) in YTK is reducing in oxidation under stress conditions and this requires further investigation. Both protein 6A and 6B both increased significantly in fish fed the SBM diets. This is in contrast to Immunotrial 6 where no effect of SBM inclusion on POS biomarker oxidation at the end of the growth trial and indicates that this higher inclusion of whole soybeans (30%) was more detrimental to the YTK than the SBM inclusion used in Immunotrial 6 (20% whole soy beans and 10% soy protein concentrate). The inclusion of the feed additives did not affect the oxidative state in most plasma proteins, regardless of base protein. The only

exception was protein 6A which showed an increase in oxidative state with the inclusion of 20% hydrolysate in soybean meal compared SBM control (Figure 7 and Appendix 4).

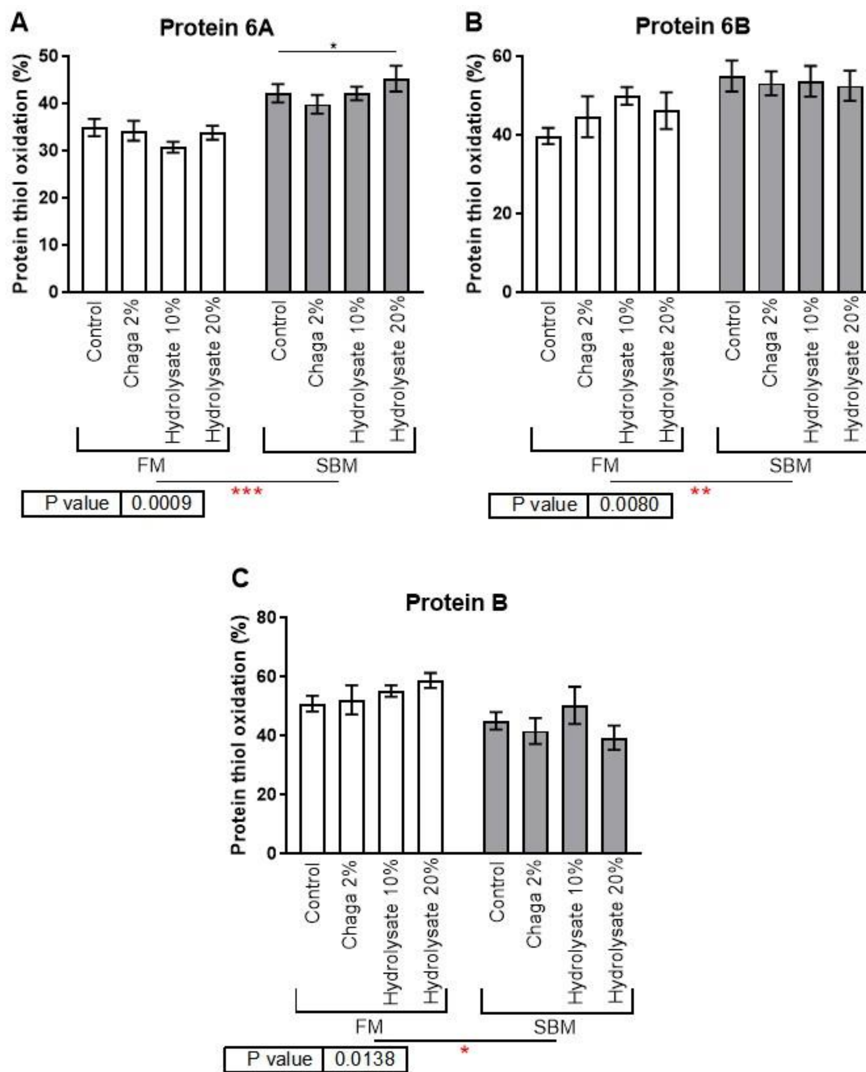

Figure 7. Inflamark found significant differences in blood protein biomarkers for oxidative stress in YTK fed for 28 days on various diets in Immunotrial 7.

Protein 6A (A) and protein 6B (B) showed increased levels of oxidative stress in SBM fed fish compared to FM fish whereas, protein B (C) showed a decrease in oxidative stress in SBM fish compared to FM fed fish. Each group contains *n* = 4 - 6 fish in each group. Data are presented as mean ± SEM with significances determined by two-way ANOVA with Fishers LSD tests. Individual differences between FM and SBM groups are not shown.

Histological examination of the hindgut in the fish from Immunotrial 7, showed no signs of gross enteritis in the SBM diet fed fish, however the laminar propria area was significantly higher in SBM fed fish than FM fed fish ($p = 0.02$), indicating some degree of inflammation. There was no effect of dietary additives on laminar propria area ($p = 0.38$). The POS biomarker data is therefore consistent with the data on laminar propria area.

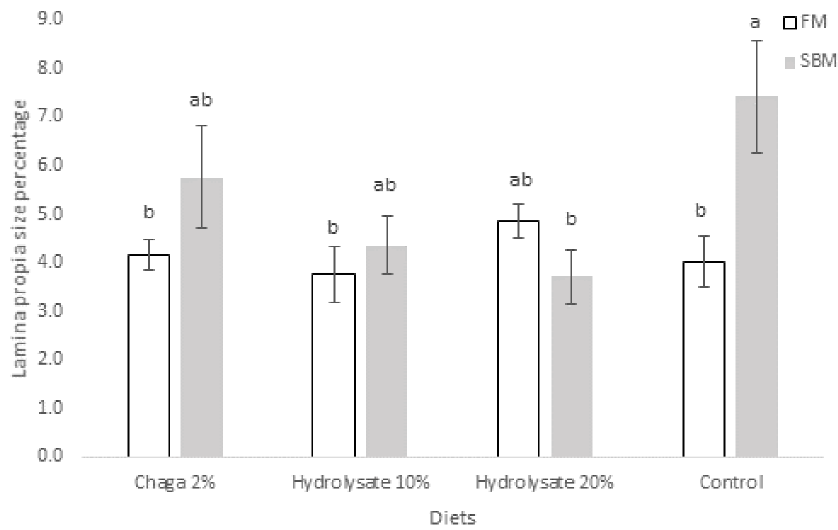

Figure 8. Laminar propria area as percentage of villus area as an indicator of enteritis.

The activity of MPO was used to assess the accumulation of neutrophils as a marker of acute inflammation in the YTK hindgut tissue. Fish fed on SBM diets showed significantly elevated levels of MPO activity in their hindgut compared to FM fed fish (p = 0.009, Figure 9). Increased MPO activity shows higher levels of acute inflammation through the accumulation of neutrophils in SBM fed fish. This data is consistent with the findings in POS biomarkers protein 6A and protein 6B (Figure 7A and B respectively) and the data on laminar propria area.

A correlation analysis was performed between MPO activity and the level of oxidation of protein 6A, 6B and B (Table 2). A weak but significant correlation was found between the level of MPO activity and protein oxidation in protein 6A with a positive correlation (r = 0.364, p = 0.013, n = 46), and protein B with a negative correlation (r = 0.35, p = 0.025, n = 41) (Table 2). These results suggest hindgut inflammation can influence POS biomarkers in YTK plasma.

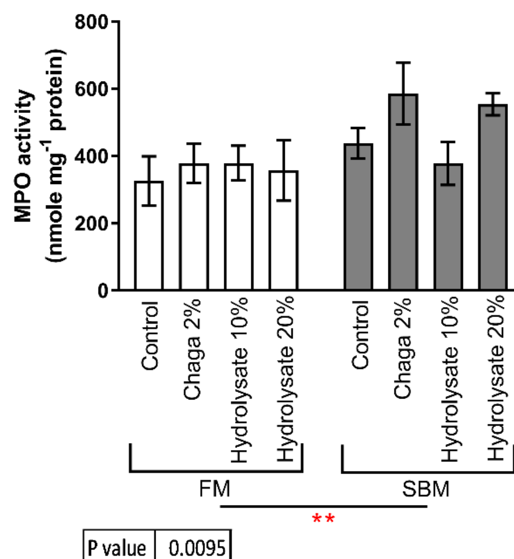

Figure 9. Myeloperoxidase activity in the hindgut of YTK was significantly increased in fish fed on soybean meal (SBM) compared to fishmeal (FM) base protein diets in Immunotrial 7.

Fish fed on SBM showed increased levels of myeloperoxidase activity in the YTK hindgut tissue compared to FM fed fish, indicating higher levels of acute inflammation through the accumulation of neutrophils. Each group contains n = 4 - 6 fish in each group. Data are presented as mean ± SEM with significances determined by two-way ANOVA with Tukey's posthoc comparison.

Table 2. Correlation analysis of myeloperoxidase (MPO) activity in the hindgut tissue of YTK vs POS biomarkers, protein 6A, protein 6B and protein B from Immunotrial 7 analysis.

Correlation coefficient (*r*), p value and the number of pairs in the correlation analysis are shown. A weak association with significant correlation was found between MPO activity and the level of protein 6A oxidation, and MPO activity and the level of protein B oxidation. Significance is shown as *p < 0.05.

	MPO vs.		
	Protein 6A	**Protein 6B**	**Protein B**
r	0.364	0.182	-0.35
p value	0.013*	0.226	0.025*
Number of XY Pairs	46	46	41

3. Barramundi temperature trial

A trial assessing potential proteins susceptible to oxidative stress in Barramundi blood was conducted using water temperature as an external stressor. Twelve Barramundi (50 g average weight) were placed in two holding tanks (six in each tank) and held for seven days either at 27°C (optimal growth temperature) or 19°C (sub-optimal temperature). Fish were anaesthetised with AQUI-S (20 mg/L) before blood was taken from the caudal vertebral vein and immediately placed in EDTA tubes for Inflamark measurements. Six Barramundi blood samples were used for optimisation of trapping and run conditions (three from each temperature), and the others were analysed for Inflamark assessment with the optimised run conditions. Samples involved in the optimisation process are not shown in the results below.

Based on the small preliminary sample size of three fish at each temperature, there were significant increases in POS biomarkers protein 0 ($p = 0.01$) and protein 7 ($p = 0.038$) (Figure 10). In addition, there was a trend for an increase in POS biomarkers protein 1 ($p = 0.106$) and protein 5 ($p = 0.134$) despite not reaching significance (Figure 10). These data show Barramundi blood proteins are sensitive to changes in oxidative stress through an external stress of rearing temperature. These results also suggest the low levels of variability between the Barramundi for the POS biomarkers measured, despite the small sample size.

It will be of interest establishing a time course on how quickly these changes occur and identifying the proteins which are responding to the stressful stimuli. This could help develop industry guidelines as to the length of time which fish are able to be exposed to non-optimal conditions before the effects become detrimental to the health status of the fish.

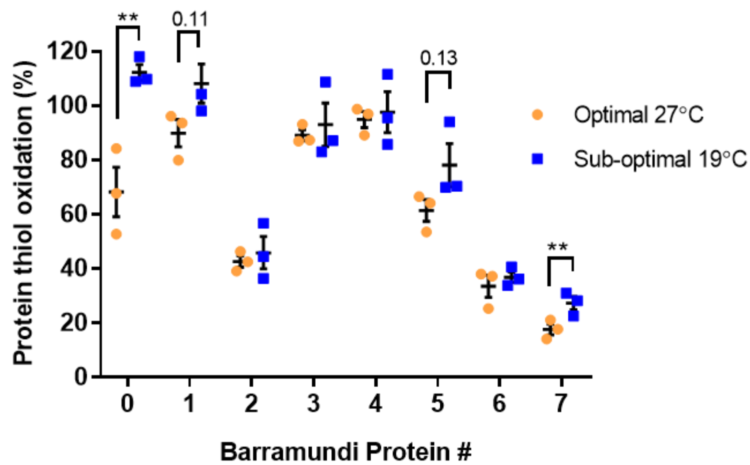

Figure 10. Barramundi have a biological stress response in their blood from exposure to cold water (19°C; sub-optimal) tanks compared to when in their optimum temperature water (27°C) after 7 days.

Several proteins in Barramundi show an increase in oxidative stress under cold-water conditions compared to their optimal temperature. Data are presented as mean ± SEM with significances determined by unpaired *t*-tests *$p < 0.05$, with p values of interest shown, $n = 3$ fish in each group.

4. Rainbow Trout temperature trial

A trial on Rainbow Trout was conducted to evaluate potential blood proteins susceptible to oxidative stress using thermal stress as a modulator. Fish were maintained in 16°C freshwater as a baseline measure for the protein biomarkers and then transitioned to 24°C over a two-week period where they were sampled at 24 h after having reached 24°C.

Fish were maintained at 16°C in their trial tanks for 7 days prior to having blood sampled for Inflamark. On day one, fish were anaesthetised with MS-222, had individual length and weight recorded, blood withdrawn from their caudal tail vein (less than 0.5 mL) for Inflamark assessment and passive integrated transponder (PIT)-tagged in their abdomen for individual identification. On the subsequent sampling day, fish were anaesthetised, weight and length recorded and blood sampled for Inflamark.

Inflamark detected a significant difference in oxidative stress levels of four different plasma proteins in rainbow trout when they were held at 16°C compared to 24°C (Figure 11). Protein A had a significant reduction in oxidation ($p = 0.0002$) under acute stress at 24°C compared to at 16°C (Figure 11). Protein B, C and D all significantly increased in oxidation when under acute stress ($p = 0.029$, $p = 0.0002$, $p = 0.0003$ respectively). This trial demonstrates the ability of Inflamark to detect stress in rainbow trout with multiple POS biomarkers evident.

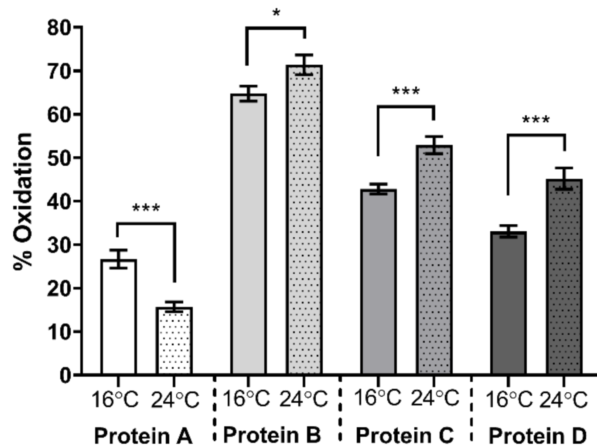

Figure 11. Rainbow Trout held at 16°C and 24°C for 24-hours showed significant differences in oxidative stress levels of several plasma proteins.

Data are presented as mean ± SEM with significances determined by unpaired *t*-tests with *p < 0.05 and ***p < 0.001, n = 10 - 12 fish in each group.

5. Atlantic Salmon preliminary trial: Hatchery vs. grow-out

A trial on Atlantic Salmon was conducted to evaluate potential proteins susceptible to oxidative stress in their blood and to provide baseline POS biomarker levels. Samples were collected from two cohorts of salmon. One of the cohorts were maintained in hatchery conditions (freshwater, recirculating system, 14°C) and were approximately 230 g each in body weight. The other cohort was maintained in grow-out conditions (ocean-cage, approximately 11-11.5°C) and was approximately 1.2 kg in body weight. Fish were anaesthetised prior to their blood being sampled as described above. Hatchery salmon were anaesthetised with benzocaine and ocean-cage salmon with AQUI-S.

Inflamark successfully identified differences in the oxidative stress level of four different plasma proteins between freshwater, hatchery and salt-water, grow-out salmon (Figure 12). This demonstrates that it is possible to analyse samples from salmon and detect differences from four potential POS biomarkers thus this will enable trials to be conducted in salmon to test for various stressors in line with industry needs.

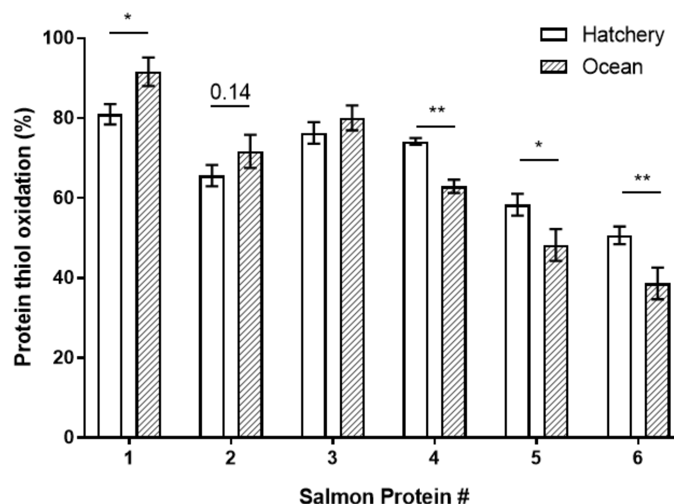

Figure 12. Hatchery and ocean-cage Atlantic Salmon show differences in oxidative stress levels of several plasma proteins.

Data are presented as mean ± SEM with significances determined by unpaired *t*-tests with *p < 0.05 and **p < 0.01, with near significance p value shown on the graph, *n* = 10 - 11 fish in each group.

6. Sydney Rock Oyster preliminary trial: days-out-of-water and bathing temperature

To evaluate if Inflamark could measure physiological stress in Sydney Rock Oysters (*Saccostrea glomerata*), two trials of potential external stressors were evaluated with six oysters in each group; the first trial involved an acute, 24 h temperature change of oysters into 5°C water, compared to a hatchery temperature of 22°C. The second trial involved leaving oysters out to dry at room temperature (days-out-of-water, DOW) for 1 or 5 days. Leaving oysters out of the water to dry is a common strategy used by industry to control fouling organisms.

The two trials were performed on young wild-stock Sydney Rock Oysters (protandrous hermaphrodites) from Port Stephens, New South Wales, Australia. Haemolymph was collected from individual oysters and immediately snap-frozen and stored at -80°C until analysis. Extra haemolymph was also collected on specialised DBS cards to evaluate their potential in being able to dry and transport the samples at room temperature. Analysis for calculation of POS oxidation was conducted on freshly snap-frozen haemolymph samples. Some oysters were excluded from the analysis due to very low protein concentrations, sample numbers for each group are shown in figures. All groups have been presented together on the figures to show the 22°C hatchery control group as a 0 DOW reference, as the DOW oysters came from this temperature. One-way ANOVA with Sidak multiple comparisons was used to determine significances between groups.

A comparison visual between the two methods of collection (Figure 13) shows that oyster haemolymph could be collected and transported on specialised DBS cards with optimisation required for future use.

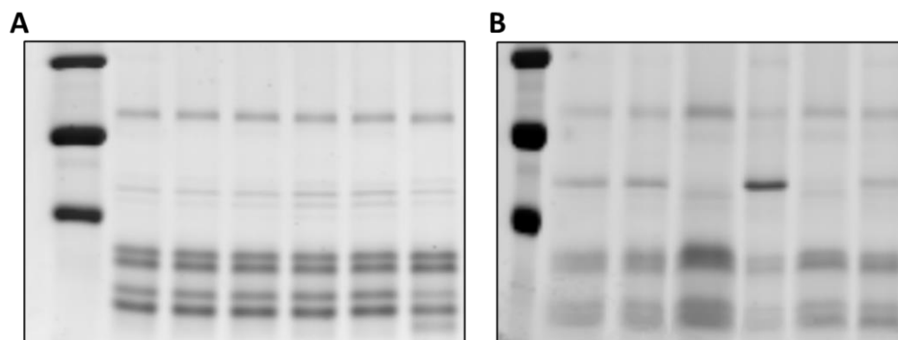

Figure 13. Sydney Rock Oyster haemolymph proteins imaged on SDS-PAGE gel from snap-frozen samples (A) and samples collected and stored on specialised dried blood spot (DBS) cards (B).

In the DOW trial, at day 1, oysters had significantly higher levels of oxidative stress than the 22°C control group (0 DOW) for two potential POS biomarkers, protein 2 ($p = 0.021$, Figure 14A) and protein 5 ($p = 0.035$, Figure 14B). At 5 DOW, protein 2 had a trend of having higher levels of oxidative stress compared to 1 DOW oysters however, there was no significant difference (Figure 14A). Protein 5 did not change in oxidation levels between 1 and 5 DOW (Figure 14B).

The acute temperature stress trial showed a significant increase in oxidative stress when oysters were transferred to 5°C from 22°C based on two potential POS biomarkers; protein 2 ($p = 0.0039$, Figure 14A) and protein 5 ($p = 0.032$, Figure 14B).

Therefore, results in young oysters show they were most stressed at 5 DOW and 5°C, and least stressed at 22°C. These data show two promising POS biomarkers that can detect differences in oxidative stress in Sydney Rock Oyster haemolymph caused by environmental stressors.

Of interest, when assessing oxidation levels of protein 2, an oyster within the 5°C group had an oxidation level of 27.7% compared to the group mean of 74.99 ± 10.3%, and an oyster in the 1 DOW had an oxidation value of 43.9% compared to the group mean of 67.31 ± 6.38%. When assessing protein 5 oxidation levels, the same oyster at 5°C which showed low oxidation levels in protein 2, showed a similar pattern in protein 5 with an oxidation level of 31.3% compared to the group mean of 85.65 ± 11.58%. In addition, the 1 DOW oyster had a lower level of oxidation in protein 5 of 39.13% compared to the group mean 77.7 ± 10.4%. As these oysters showed the similar pattern in protein 2 and protein 5 with lower than group mean oxidation levels, these oysters are considered biological outliers. These results raise the possibility that these two oysters may have a protective advantage over the environmental stressors inflicted within this trial and respond differently compared to other oysters when exposed to similar stressors. With this, these findings suggest that we could potentially be able to provide advice on potential selective breeding strategies where oysters that show remarkable resistance to stress could be bred to create stronger, more resilient breeding lines.

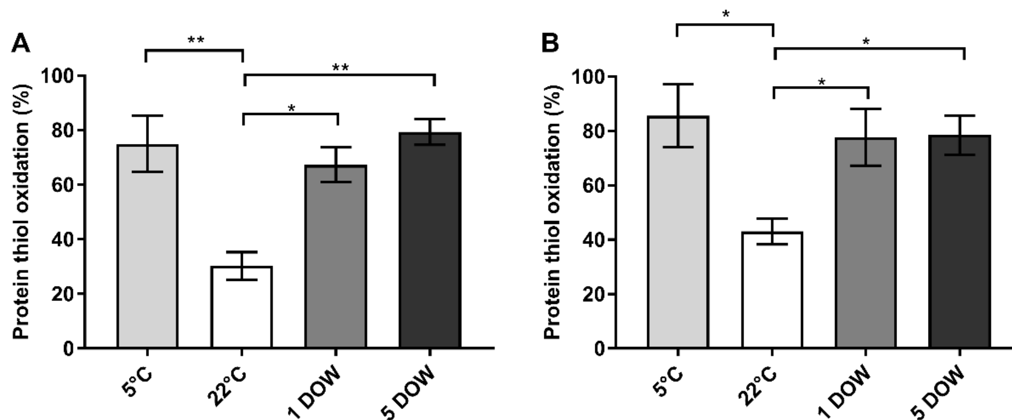

Figure 14. Two potential protein biomarkers of oxidative stress in Sydney Rock Oyster haemolymph show significant differences in protein thiol oxidation in trials performed including days-out of water (DOW) and sub-optimal temperature stress.
Protein 2 (A) and protein 5 (B) show differences between trial groups with the oysters held at 22°C showing the lowest levels of oxidative stress compared to other groups. Significance was determined by one-way ANOVA with significance shown on figure. Data are shown as mean ± SEM, *n* = 6, 4, 5 and 5 respectively.

Conclusions and Implications

This project has successfully demonstrated that Inflamark can detect changes in protein oxidative stress biomarkers in the blood of fish and haemolymph of oysters. This project showed water temperature stress causes a change in the oxidative state of protein biomarkers in blood of YTK and Barramundi, and that high levels of whole soybeans in feed causes increased protein oxidation in YTK. In salmon, we have identified differences in oxidation levels of several proteins between hatchery and ocean-cage salmon. In Sydney Rock Oysters, we have detected oxidative stress caused by acute water temperature change and through extended emersion. In addition, in Sydney Rock Oysters, we identified biological outliers that appeared to have a protective advantage to the environmental stress. This biological protective advantage could be exploited in the future to conduct selective breeding strategies where oysters that show remarkable resistance to stress could be bred to create stronger, more resilient breeding lines. These data suggest that Inflamark will be able to monitor metabolic stress in a variety of finfish and mollusc species and shows much promise.

The ability to accurately measure physiological stress in aquaculture species would provide industry and research groups with an analytical tool to monitor and assess the general health status of stocks. The measurement of protein biomarkers in the blood of finfish and haemolymph of oysters through the Inflamark technology described, could provide a reliable metric to measure physiological stress across different species.

Recommendations

Further development

Currently this novel oxidative stress biomarker tool is being utilised as a research tool. However, there is huge potential that this tool could translate directly into commercial research programs and practices to monitor the welfare and health of finfish. Due to the complexity of this project with identifying multiple protein biomarkers, the development of a simple blood collection device was not achieved under this project. The current blood collection method requires specialised equipment and cold-chain logistics which are not practical on remote fish farms, and sample processing is limited in its throughput ability. We foresee the further development of this technology that will enhance the usability of the test for on-field collection of blood samples and enhancement of sample throughput for quicker result turn-around. This will be the next stage of development.

Identification of the POS biomarkers found in each species will be conducted which may provide insight to the physiological mechanisms behind the stressors eliciting changes. A multi-POS biomarker approach will further enhance the strength of the measurement to detect stressors.

Extension and Adoption

Inflamark is considered as a standard analytical test to be run during research trials on marine finfish at the Department of Primary Industries and Regional Development (DPIRD) where the evaluation of physiological stress is to be determined. The technology is undergoing further proof-of-principal trials to help better understand the capabilities and extension of this technology, along with the limits.

Project coverage

The Inflamark project was written up as an interest piece to be featured in a Western Australia Government distributed article for the Research and Industry Innovation Directorate of Department of Primary Industries and Regional Development.

The leads in this project are currently working toward peer-reviewed publication with describing the oxidative stress protein biomarkers found in Yellowtail Kingfish.

With this research project being undertaken within a University of Western Australia (UWA) research group, the group has presented this exciting technology at the November 2020, UWA Innovation Showcase.

Appendices

Appendix 1. Researchers and Project Staff

Dr Gavin Partridge (Principal Investigator) – Principal Research Scientist, Primary Industries Development, Department of Primary Industries and Regional Development, Government of Western Australia.

Dr Peter Arthur (Co-investigator) – Biochemistry Academic, School of Molecular Sciences, University of Western Australia.

Dr Catherine Wingate (Co-investigator) - Research Scientist, Primary Industries Development, Department of Primary Industries and Regional Development, Government of Western Australia.

Mr Daniel Soo – Masters Student, School of Molecular Sciences, University of Western Australia.

Dr Lindsey Woolley – Research Scientist, Primary Industries Development, Department of Primary Industries and Regional Development, Government of Western Australia.

Mr Luke Pilmer - Research Technician and PhD Candidate, Primary Industries Development, Department of Primary Industries and Regional Development, Government of Western Australia.

Dr Marisa Duong - Technical training of Masters student on mass spectrometry, School of Molecular Sciences, University of Western Australia.

Appendix 2. Intellectual Property

Some methodology and content are protected from publishing in this project. The technology underlying the Inflamark method is patented.

Appendix 3. References

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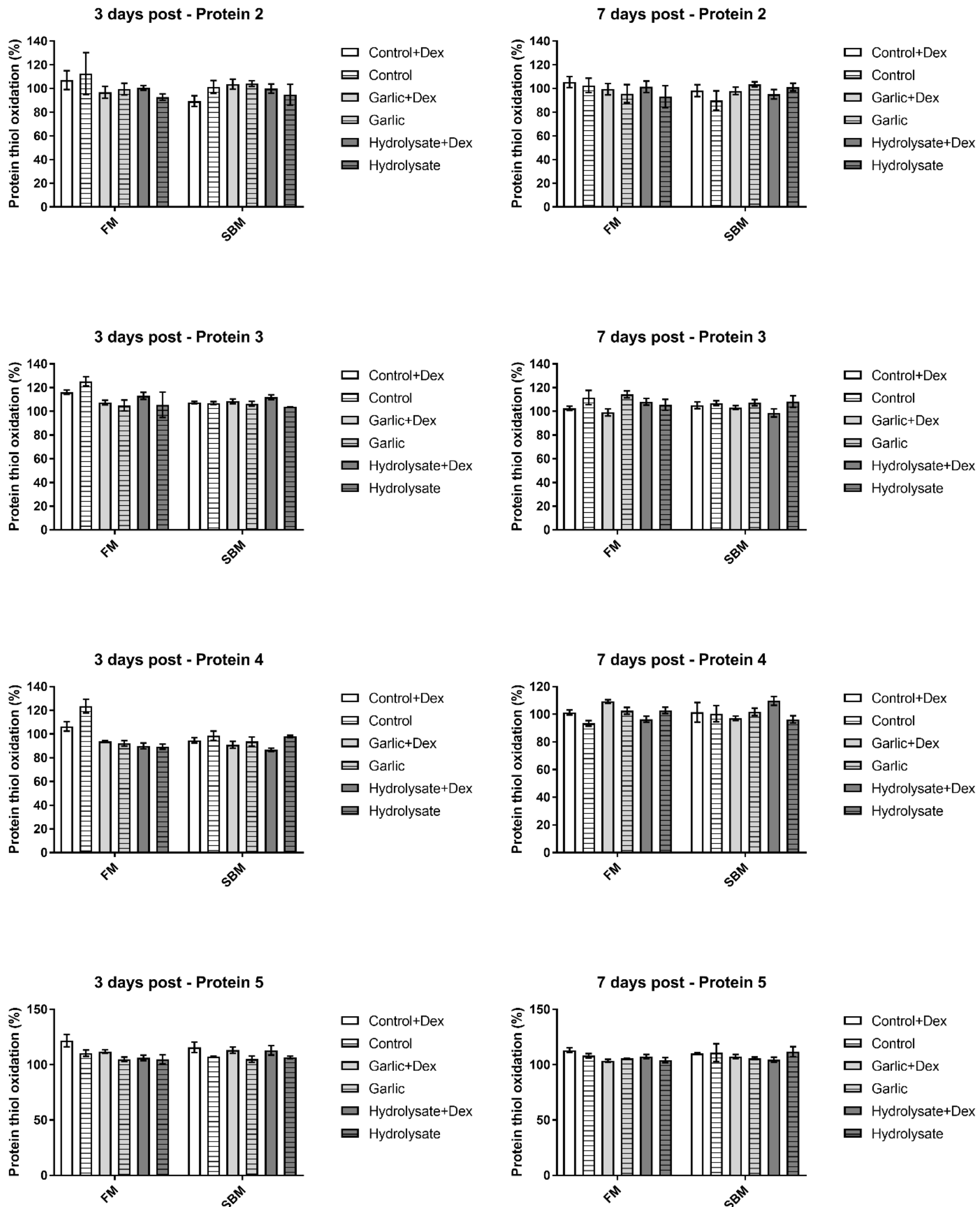
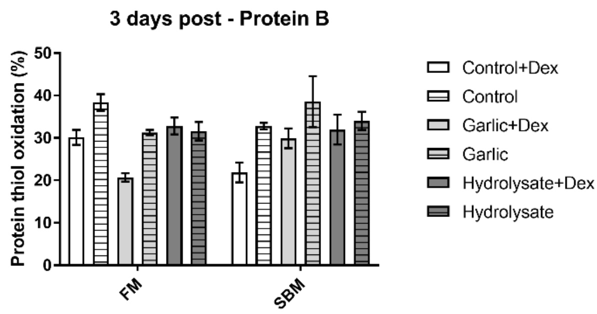
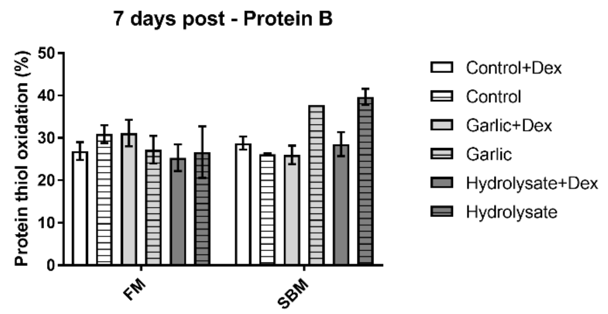
Appendix 4. Supplementary figures

Immunotrial 6

Immunotrial 6 post three-month feed trial challenge by dexamethasone injection results. Graphs show proteins analysed three- and seven-days post-dexamethasone (+dex; *n* = 5 - 6 per group) with controls (*n* = 2 - 3 per group). Note: Two proteins are missing due to not being present in dexamethasone fish and significance differences between groups are not shown on graphs. Protein B, 6B and 7 are of particular interest in this immunotrial (Figure 6).




3 days post - Protein B
Protein thiol oxidation (%)
0
10
20
30
40
50
FM
SBM
Control+Dex
Control
Garlic+Dex
Garlic
Hydrolysate+Dex
Hydrolysate


7 days post - Protein B
Protein thiol oxidation (%)
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30
40
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SBM
Control+Dex
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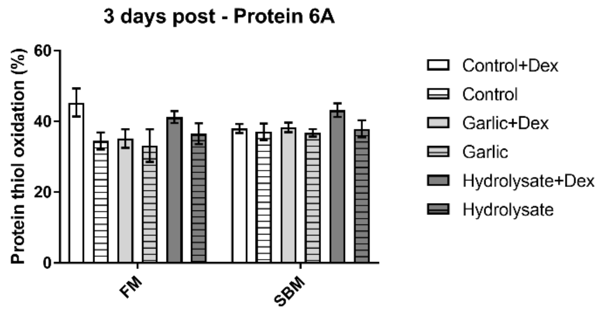
Hydrolysate+Dex
Hydrolysate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Protein thiol oxidation (%)
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SBM
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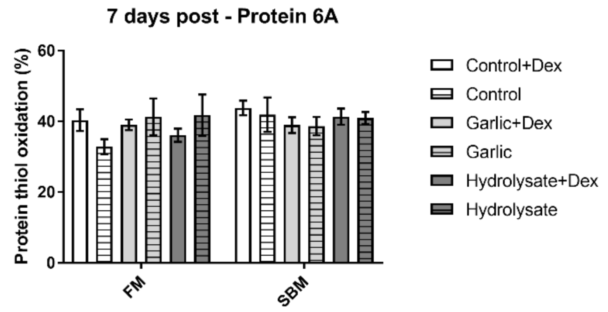
Hydrolysate


7 days post - Protein 6A
Protein thiol oxidation (%)
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40
60
FM
SBM
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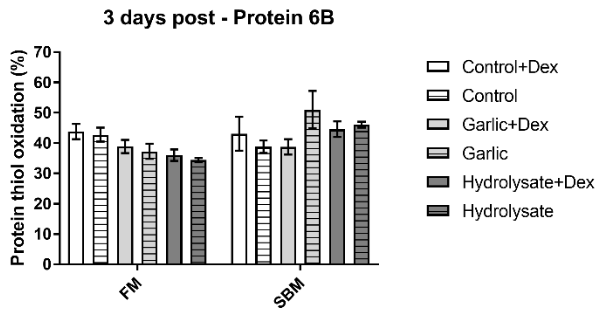
Control
Garlic+Dex
Garlic
Hydrolysate+Dex
Hydrolysate


3 days post - Protein 6B
Protein thiol oxidation (%)
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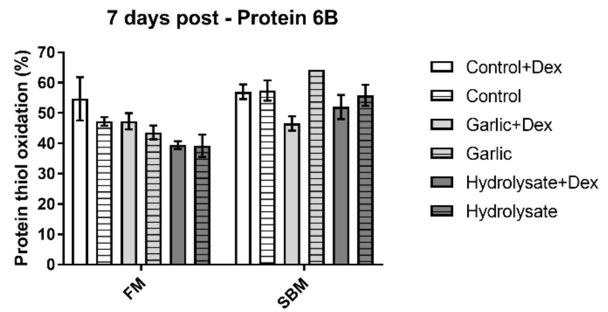
SBM
Control+Dex
Control
Garlic+Dex
Garlic
Hydrolysate+Dex
Hydrolysate


7 days post - Protein 6B
Protein thiol oxidation (%)
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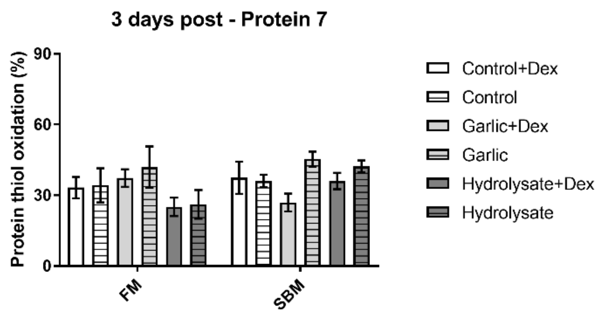
30
40
50
60
70
FM
SBM
Control+Dex
Control
Garlic+Dex
Garlic
Hydrolysate+Dex
Hydrolysate


3 days post - Protein 7
Protein thiol oxidation (%)
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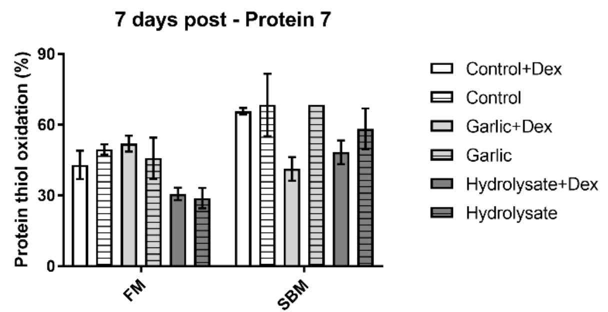
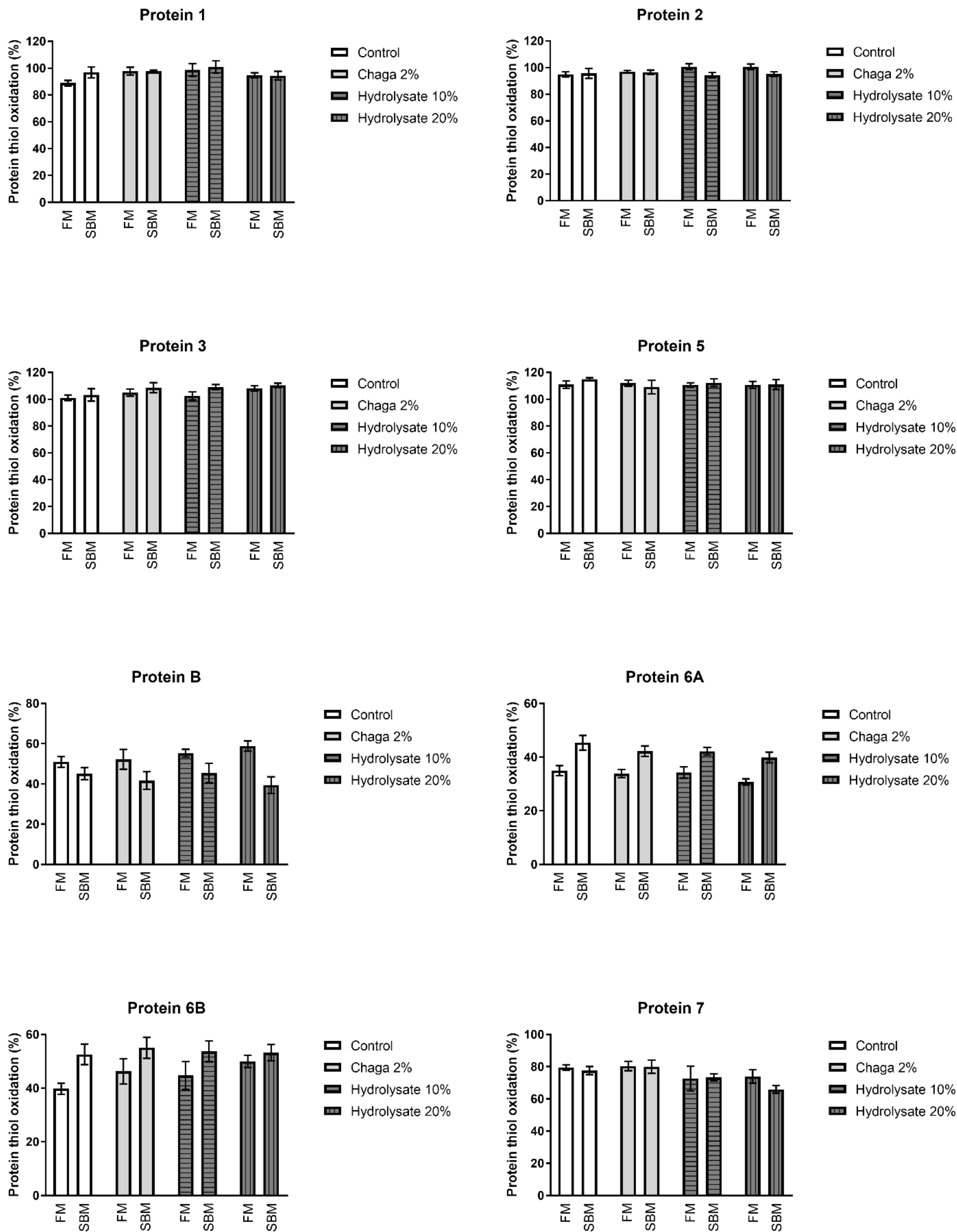
60
90
FM
SBM
Control+Dex
Control
Garlic+Dex
Garlic
Hydrolysate+Dex
Hydrolysate


7 days post - Protein 7
Protein thiol oxidation (%)
0
30
60
90
FM
SBM
Control+Dex
Control
Garlic+Dex
Garlic
Hydrolysate+Dex
Hydrolysate

Immunotrial 7

Immunotrial 7 results showing graphs for proteins analysed. Note: significances are not shown on graphs. Proteins of interest with significances are shown in Figure 7.



Appendix 5. UWA Master Student Thesis – Daniel Soo 2020

*Please note this thesis has been amended to remove confidential information which has yet to secure IP rights. These aspects were funded by The University of Western Australia and not FRDC project funds.

Identifying novel biomarkers of physiological stress in farmed fish

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Abstract

With declining fish populations and overfishing still prevalent today, growing seafood demands must be met with the development and rise of aquaculture practices globally. Preventing and monitoring stress associated with common aquaculture handling practices has become an integral part in ensuring aquaculture farming in open ocean cages are profitable, efficient and sustainable. As climate change is increasingly more prevalent and unpredictable, fish are challenged to adapt to sub-optimal temperatures and temperature stress impairs the growth of fish. Current measures of physiological stress have individual limitations in terms of accuracy, sensitivity, cost, and simplicity. To date, an indicator of oxidative stress in fish as a means of providing a simple, sensitive and cost-effective measure of health for industry applications has not yet been available. Using Yellowtail Kingfish (*Seriola lalandi*) as a model, heat and handling stress were employed to evaluate the potential of protein thiol biomarkers as reliable signatures of stress in fish. A key conclusion from this study was the finding of two potential protein biomarkers: Protein 3 which exhibited the greatest sensitivity to handling and temperature stress and Protein A which showed a later oxidative onset on day eighteen in response to chronic heat stress.

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1. Introduction

Global consumption and demand for seafood has increased dramatically over the past decade. Part of this increase in seafood demand has been attributed to the growing awareness of the various health benefits of certain types of seafoods as a healthy alternative source of protein (1). To match the demands of worldwide fish consumption, current practices employed in captured (wild-caught) fisheries have resorted to unsustainable levels of overfishing and as a consequence, threatens the future viability of consumer seafoods and diversity of wildlife in the oceans (2). Despite these measures, captured fisheries have experienced stagnant levels of production since the 2000's (Figure 1), due to declining wild-fish stocks, restrictions on catching quotas and higher operating costs (3). This dilemma has accelerated the expansion and adoption of aquaculture (farmed) in many parts of the world to ease some of the burden of captured fisheries (Figure 1) (1). In 2016, total fisheries production reached 171 million tonnes of which 80 million tonnes was from aquaculture, and from 1990 to 2016, aquaculture production has significantly increased by six-fold whereas, captured production has stabilised at 90 million tonnes (Figure 1) (1).

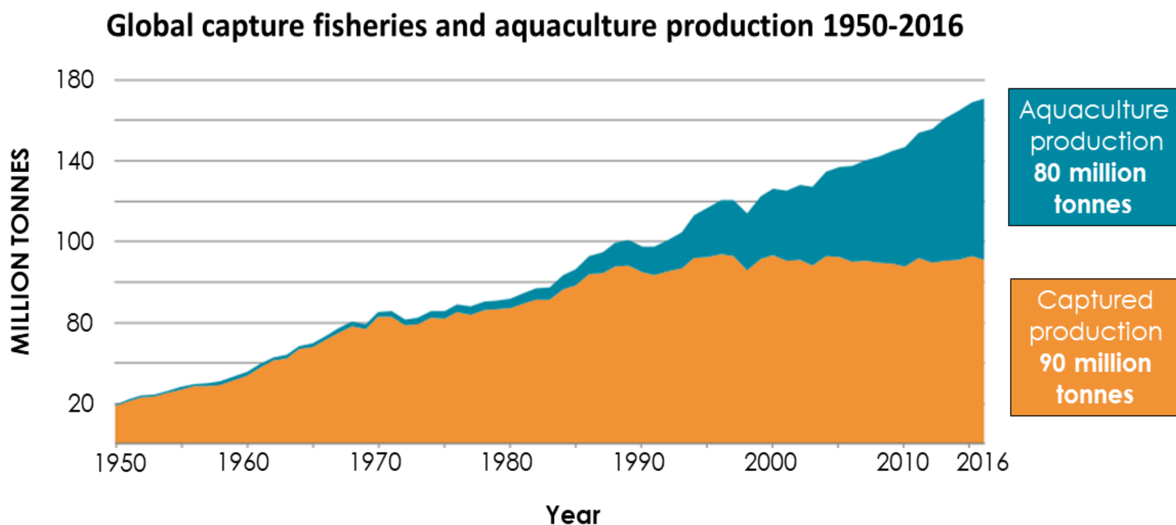


Figure 1: Estimated global fish production by captured (wild-caught) commercial fisheries and aquaculture production over the past 66 years, 1950 to 2016. Captured production is shown by the orange area and aquaculture production is shown in by the blue area. Figure modified from (3).

Current practices employed in commercial aquaculture facilities have been focused on maximising production rates and economic returns. Aquaculture farming in open ocean cages enables farmers to increase their production per unit area and allows for the growth of larger pelagic fish such as Atlantic Salmon, rainbow trout and Yellowtail Kingfish (3). The specific growth rate (percentage increase in size per day, SGR) in fish is widely used as a measure of productivity as rapid growth rate minimises the time required to reach market size (4). A shorter turnaround time is desirable, as it increases yield

whilst reducing the cost of feeds, labour and overheads. As such, various husbandry practices such as increasing stocking densities, changing dietary feeds and patterns, and modulating abiotic factors such as temperature, pH and salinity are currently employed to enhance growth (5-8). However, abiotic factors cannot be regulated in open ocean cages and thus there is little evidence to support what practices are optimal for growth or are stressful and compromise growth of the fish (9).

In Australia, finfish aquaculture is centred around farming of salmonids, southern bluefin tuna, and in recent years, Yellowtail Kingfish (3). Diversification of the aquaculture industry to support new high-value finfish species for international markets, is part of the reason why the culture of Yellowtail Kingfish is the most rapidly growing sector in Australia (3). However, the rapid growth is met with a paucity of information surrounding optimal husbandry, harvesting and welfare practices of new finfish species (9).

Fish are important ectothermic organisms that rely on their surrounding temperature to regulate physiological systems such as metabolism, respiration, locomotion and growth (10). As a result of increasing ocean temperatures due to climate change, aquatic organisms are challenged to adapt to sub-optimal temperatures. Stress inducing factors, known as stressors, can be defined as any change in the environment that disrupts homeostasis in a body (7). Current knowledge about the physiological stress response in fish has been elucidated by studying their neuroendocrine response, in particular, the activation of the hypothalamic-pituitary-inter renal (HPI) axis (11-14). Stimulation of the HPI axis results in the release of catecholamines and cortisol into the bloodstream that propagates and mediates downstream alterations towards the adaptation and redistribution of metabolic energy to respiration, locomotion and tissue repair as opposed to growth and reproduction (13,15). This adaptation is observed through increased liver gluconeogenesis and subsequent glycogenolysis in the liver and muscles by circulating catecholamines and cortisol (9). Consequently, when adaptive mechanisms fail to maintain homeostasis, physiological stress impedes on their fitness and growth (7,10).

Stress induced by temperature change has also been associated with increased generation of reactive oxygen species (ROS) due to elevated metabolic respiration and hypoxia (8,10,16). Superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (•OH) are amongst the suite of ROS that are highly reactive agents of oxidative damage to cellular constituents and in signalling cascades such as cell apoptosis and necrosis (16-18). Under homeostatic conditions, intrinsic sources of ROS arise from the reduction of molecular oxygen from the mitochondria electron transport chain, oxidative burst from inflammatory cells and oxidases such as NAD(P)H and xanthine oxidase (8,16). The levels of ROS are balanced through the interplay with antioxidant defence systems that functions in a dynamic equilibrium to quench oxidants and mitigate their deleterious effects (8,16). In fish,

enzymatic antioxidants such as superoxide dismutase, glutathione peroxidase, catalases and non-enzymatic antioxidants such as glutathione, vitamin C, E and A work to scavenge ROS (16,17).

Stressors such as climate changes and episodic heat waves can exacerbate excessive ROS generation and lead to a state of oxidative stress that attacks cellular components such as nucleic acids, lipids and proteins (8,16,19,20). Lipid peroxidation is one of the most deleterious and prevalent consequence of oxidative stress (21) and has been shown in fish to be very susceptible to temperature stress outside of a species optimal range (20). Fish contains large amounts of polyunsaturated fatty acids, which is a major constituent of membrane phospholipids and highly susceptible to free radical attack (16). The reaction of one lipid molecule with ROS, sparks a self-propagating chain reaction with adjacent lipid molecules and consequently damages membrane fluidity and structural integrity (18,19).

Proteins are also highly susceptible to ROS induced damage and this has been studied extensively through measuring the generation of protein carbonyls (16). Carbonyl derivatives may arise from direct ROS attack on proteins, or indirectly from secondary reactions with oxidised carbohydrates and lipids (reactive carbonyl species) (22). Carbonylation is an irreversible modification that leads to one of two fates, proteolysis or protein aggregates; both of which are detrimental to the function of receptors, enzymes and transport proteins (16,23).

Another readily oxidised protein target for ROS are sulfhydryl groups on protein thiol side chains (-SH in cysteine residues) (24). These sulfhydryl groups are highly sensitive to reversible oxidation, forming disulphide bonds (S-S) in the presence of oxidants and reduce to sulfhydryl groups (-SH) under certain antioxidant enzymes (25). Reversible oxidative thiol modifications as opposed to irreversible protein carbonylation, are important determinants of protein structure and function that influences enzyme catalysis, protein-protein interaction, cell signalling cascades and transcriptional activation (25,26). Measurements of protein thiol oxidation have yet to be studied in fish, but elevated levels have been reported in dystrophic muscles of the *mdx* mouse model (25) and in post-exercise induced stress in humans (27).

As climate change is increasingly more prevalent and erratic in nature, monitoring stress and welfare of fish becomes a priority. Current physiological measures of stress in fish on site, are limited to crude measures of growth rate, mortality, and various other phenotypic indexes such as swimming performance, behaviour and feeding patterns (11,14,28). The measure of growth rate is still by far the most widely used metric in aquaculture, but a disadvantage of such phenotypic indicator is that it takes many weeks to manifest into observable changes compared to physiological changes in blood biochemistry (11). This was especially apparent in a study conducted by Handeland et al (2008),

where the optimal temperature for growth in Atlantic Salmon was not evident until eight weeks into the study. Growth was significantly impaired in the 6ºC temperature group, but differentiation between a tighter range of temperatures (10, 14 and 18ºC) was only apparent in their weights after eight weeks (29).

Other physiological measures of stress in fish, rely on measurements in plasma but are limited to cortisol, glucose and lactose measurements (9), mainly because of the lack of antibody specific tests in fish. Numerous studies have confirmed that plasma cortisol levels sharply rise following acute physical stressors such as overcrowding (30), temperature (29) and net handling stress (31), but under chronic periods of stress, continual stimulation of the HPI axis leads to the saturation of cortisol and subsequently downregulation and attenuation of secondary and tertiary responses through negative feedback inhibition (9). Furthermore, basal and stressed cortisol levels vary considerably by an order of ten times between inter and intra-species (11,15,32) and elevated cortisol can be caused by 'non-stressors' such as age, sexual maturity and nutritional level (9). In this sense, measuring plasma cortisol only provides a snapshot of the physiological state and thus, current measures of physiological stress have their own limitations in terms of sensitivity, reproducibility, cost and simplicity.

Approaches to quantifying physiological stress experimentally are still underdeveloped, with current biochemical assays requiring fish to be culled for enzyme activity assays and transcriptional studies (33-35). Fresh liver extracts are commonly used for enzyme assays while transcriptome and genomic tests requires fish to be fixed in ethanol to extract DNA (33,36,37). Consequently, this cross-sectional design only provides a snapshot of their physiological state and nullifies the ability to repeatedly sample and track individual stress responses over chronic periods of stress. The significance of tracking stress responses in longitudinal studies stems from the ability to establish causality and a baseline level of physiological indicators which can then be used to compare subsequent changes to quantify individual stress responses (14). Even though studies have shown the activity of key antioxidant biomarkers such as superoxide dismutase and lipid peroxides to significantly increase after temperature stress (8,19,34), it is debatable whether these enzyme assays can pick up low-moderate levels of stress.

A novel method to measure the redox state of protein thiols in human plasma has recently been published that showed greater sensitivity than conventional oxidative stress assays, whilst utilising less than 10 µl of blood from fingerpicks (27). This has granted an opportunity to apply a longitudinal experimental design to study protein thiol oxidation in the plasma of fish, as a potential simple, sensitive, and cost-effective measure of physiological stress. Using Yellowtail Kingfish as a model, acute and chronic temperature stress were employed to evaluate the applicability of protein

biomarkers as a sensitive indicator of physiological stress in fish. The effects of repeated handling and sampling was assessed to determine its influence on the stress response on the potential protein thiol biomarkers.

2. Method

2.1 Stocking procedure

Yellowtail Kingfish (*Seriola lalandi*) were maintained at the Australian Centre for Applied Aquaculture Research, Department of Primary Industries and Regional Development, Fremantle, Western Australia. On day zero of the trial period (Table 1), 48 Yellowtail Kingfish (340.58 ± 68.41 g) were removed from ambient temperature water (20°C) holding tanks, anaesthetised (Tricaine Methanesulfonate (MS222), Western Chemical Inc, USA), individual body weight recorded and each fish tagged with an identification tag (RFID chip) before being transferred into trial tanks.

Fish were placed in six 500 L trial tanks, stocking eight fish per tank (Table 1). Three tanks received ambient temperature water (20°C), whilst the other three tanks were supplied with warm temperature water (25.36 ± 0.26°C). Warm water was continuously heated, and temperature was measured every

five minutes using a temperature logger (HOBO). Fish were maintained in their respective ambient or warm water temperature trial tanks for 18 days (Table 1). The tanks received a constant supply of oxygen, and dissolved oxygen was measured daily with a multimeter (Dissolved Oxygen Instrument, YSI). Fish were fed to satiety once per day on a commercial diet.

2.2 Blood collection and storage

Fish from ambient and warm temperature water conditions were sampled periodically over an 18-day period (Table 1). On the sampling days, fish were anaesthetised through dosing their bathing water with MS222, while maintaining respective bathing temperatures. Once anaesthetised, fish were identified through their RFID chip, body weight recorded and blood was taken from the caudal vein using a heparinised syringe and subsequently placed in heparin tubes (MiniCollect Lithium Heparin, Greiner Bio-One). Fish were then returned to their respective trial tanks to recover.

Table 1:
Experimental set-up of trial tanks with tank sampling days shown. Trial tanks 1, 2 and 3 contained ambient temperature water (20°C), and 4, 5 and 6 contained warm temperature water (25.36 ± 0.26°C). Day 0, the set-up day, consisted of weighing, tagging, and transferring fish to the temperature trial tanks, n=8 per trial tank. Greyed out boxes indicate that the tank was not sampled on that day. On day 8 and subsequent days, only 7 fish were sampled from tank 2 and tank 4 (shown by annotation*), as two fish died (one euthanised due to poor health and other died from abnormal growth condition).

	Ambient	**Ambient**	**Ambient**	**Warm**	**Warm**	**Warm**
	Tank 1	**Tank 2**	**Tank 3**	**Tank 4**	**Tank 5**	**Tank 6**
Day 0	Set-up day					
Day 1	Sample	Sample	Sample	Sample	Sample	Sample
Day 4	Sample			Sample		
Day 8	Sample	Sample*		Sample*	Sample	
Day 12		Sample*			Sample	
Day 18	Sample	Sample*	Sample	Sample*	Sample	Sample

2.3 Trapping of protein thiols in blood

The malpeg assay was used to measure protein thiol oxidation in blood as described previously (27). In summary, blood obtained from heparin tubes was immediately aliquoted into two microcentrifuge tubes. One tube, control sample and the other, trapped sample, contained the thiol oxidative state trapping solution (62 mM polyethylene glycol maleimide in 40 mM imidazole buffer). Control and trapped tubes were briefly mixed and centrifuged at 3,000 *g* for two minutes at room temperature and plasma collected. Plasma from control tubes was snap-frozen on dry-ice, and plasma trapped with the

trapping solution was incubated at room temperature in the dark for 10 minutes prior to being snap-frozen on dry-ice. Samples were stored at -80ºC until use.

2.4 Protein separation by 1D-gel electrophoresis

Frozen plasma was thawed by vortexing and subsequently diluted 1:20 in phosphate-buffered saline (PBS). All samples were further diluted with equal amounts of sample buffer (125 mM Tris, pH 6.8, 4 % SDS, 30 % (v/v) glycerol, 0.02 % bromophenol blue) and heated for five minutes at 95ºC. Control and trapped samples were loaded alongside a molecular weight marker (All Blue Precision Plus Protein standards, Bio-Rad) on a hand-cast polyacrylamide gel. Gel electrophoresis was performed using the Bio-Rad Mini Protean III system and each gel ran at 180V in a cold room. The gel was scanned using the ChemiDoc MP Imaging System (Biorad) with the stain-free setting.

2.5 Quantification of protein thiol oxidation

Reduced and total thiols were measured using the malpeg method as described previously (27). Incubation of the blood with the trapping solution (malpeg) labelled reduced thiols (Band B, Figure 1), while the unlabelled samples contained total untagged thiols (Band A, Figure 1). Reduced thiols bound with malpeg migrate less than unlabelled oxidised thiols on an SDS-PAGE, allowing them to be separated. Quantification of protein thiol oxidation was measured by first quantifying the oxidised and control bands by densitometry using ImageJ (v.1.52a) with the mean gray value, after first removing the background signal. Then, the percentage of thiol oxidation was calculated ratiometrically using the formula below:

Fluorescence from oxidised signal: B

Fluorescence from total signal: A

$$\% \text{ thiol oxidation} = \frac{fluorescence\ from\ oxidised\ signal}{flourescence\ from\ total\ signal} \times 100$$

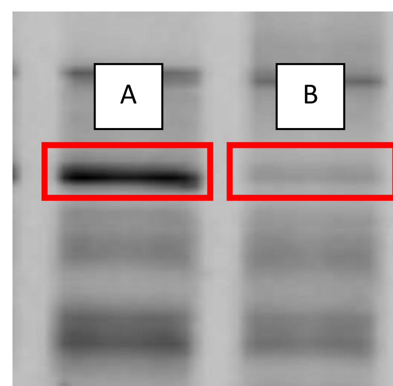


Figure 1: (A) Left lane contained total thiols and (B) right lane contained oxidised thiols.

2.6 Statistical analysis

All analyses were performed using GraphPad Prism (v8.4.2) for Windows. Statistical differences in candidate proteins between temperature groups were analysed using a two-way analysis of variance

(two-way ANOVA). Multiple comparisons were carried out by Tukey's post-hoc. Differences in weight and percentage of dissolved O_2 between tanks and temperature was measured using a two-way ANOVA and multiple comparisons were carried out by Tukey's post-hoc. Sample sizes varied depending on the sampling day, and outliers were removed before analysis.

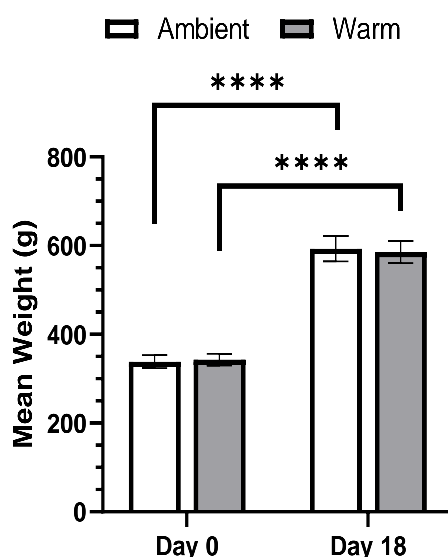
3. Results

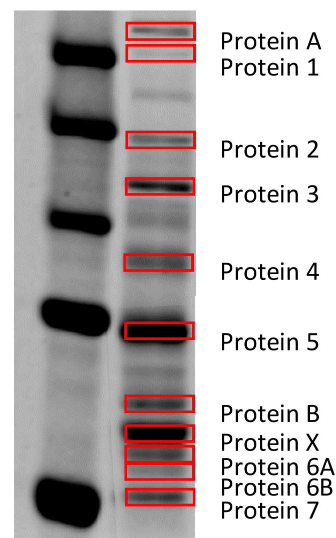
To determine if protein thiol oxidation could be used as a potential biomarker of physiological stress in Yellowtail Kingfish, handling (net catching of fish, anaesthesia) and heat stress were evaluated. The effects of handing stressors on protein oxidation were assessed to determine its potential influence over protein thiol oxidation measurements independent of physiological stress caused by heat stress.

Fish in ambient temperature water saw a 77 % increase in body weight at the end of the 18-day study period as compared to a 71 % increase in warm water (Figure 2). There was no significant effect of water temperature on body weight of the fish at 18-days (p value = 0.644) and the SGR of both cohorts was 4 %. There were no significant differences in dissolved O_2 content between temperatures, or between days (Figure 8, Appendix 1). Fish survival rate was 96 % at the end of the 18-day trial period.

3.1 Yellowtail Kingfish plasma protein profile and candidate protein bands

Eleven protein bands were selected for protein identification, based on observing the bands that were conserved across plasma control samples of fish (Figure 3). The same eleven protein bands represented our pool of candidate protein thiols that were assessed for oxidation changes. Bands were numbered based on their position in the gel (Figure 3).





3.2 Acute 1-day and chronic 18-day heat stress on thiol oxidation of candidate proteins

Most of the proteins identified did not show changes in thiol oxidation after one day of acute heat stress. Protein 3 was the only protein identified to show an acute change in oxidation (p value = 0.0322, Figure 4A). While the differences between temperature conditions were not significantly different in most proteins even after 18-days of chronic heat stress, significant changes were observed in protein A, 3 and, 4 at the end of the chronic period (Figure 4B). Protein 3 was the stand-out protein which had significantly higher levels of protein thiol oxidation in warm temperature fish on day one and day eighteen compared to ambient temperature fish (1.13-, 1.54-fold respectively; Figure 4). In addition, protein A in warm temperature fish showed a significant increase in protein thiol oxidation on day eighteen compared to ambient temperature fish (p value < 0.0001) (Figure 4B).

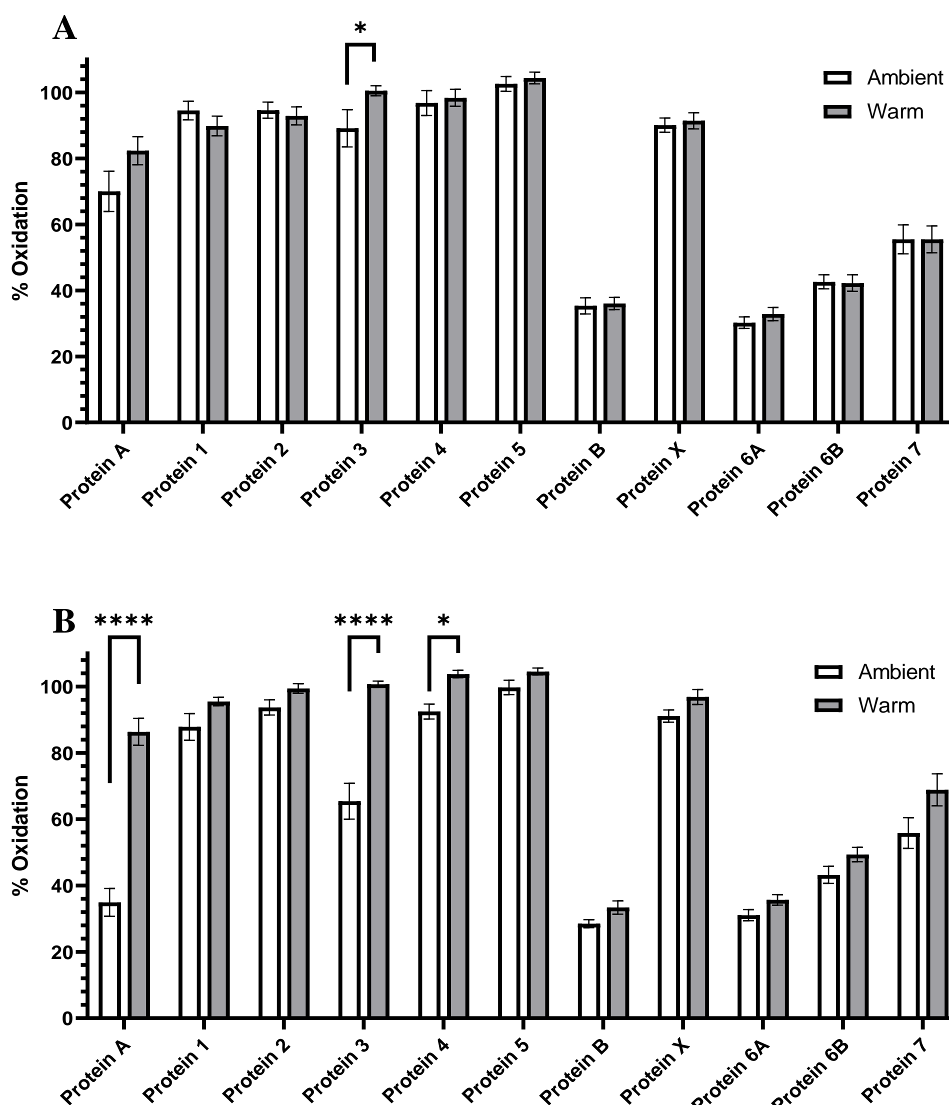


Figure 4: Percentage oxidation of eleven candidate proteins after (A) acute one-day temperature stress and (B) chronic 18-day temperature stress compared with respective day ambient control. Data is shown as mean ± SEM. Significance is shown as *p<0.05, ****p<0.0001. *n* = day 1: 8-24, day 18: 11-23.

3.3 Effect of handling stressors on thiol oxidation

Handling stress (net chasing, anaesthesia treatment) influenced the levels of protein thiol oxidation in protein 3 similarly between the two water temperature conditions on day one of sampling (p value = 0.419). The protein oxidation level on day one of the trial period, was significantly higher in ambient (2.25-fold; Figure 5A) and in warm (2.5-fold; Figure 5B) temperature fish, compared to their respective levels on day four. Comparatively, fish at day one were exposed to more stress stimuli from the initial setup day (day zero), which included relocating fish from ambient water temperature holding tanks to trial tanks, anaesthetising, weighing and tagging individuals with an RFID chip. This was opposed to subsequent days of sampling (day four, eight, twelve, eighteen), where fish were only exposed to a short window of net chasing and anaesthesia stressors.

To gain a better understanding of how frequently fish could be sampled and whether repeated sampling would affect protein thiol oxidation, a three- and seven-day window in between sampling days were assessed. Tanks one and four were sampled every three days, while fish from tank two and five, were given seven days to rest before sampling again on day eight (Figure 5). Comparing the oxidation levels on day eight, no significant difference was observed between fish sampled on day four (tank 1, 4) from fish sampled after eight days (tank 2, 5) (Figure 5A and 5B). This observation followed through to day twelve, where fish from tank two and five were sampled after three days, and no difference in oxidation was observed relative to day eight. However, after a period of steady-state levels between day four and day twelve, a significant increase in oxidation occurred on day eighteen in tank two, four, five, which indicated that the fish were again stressed.

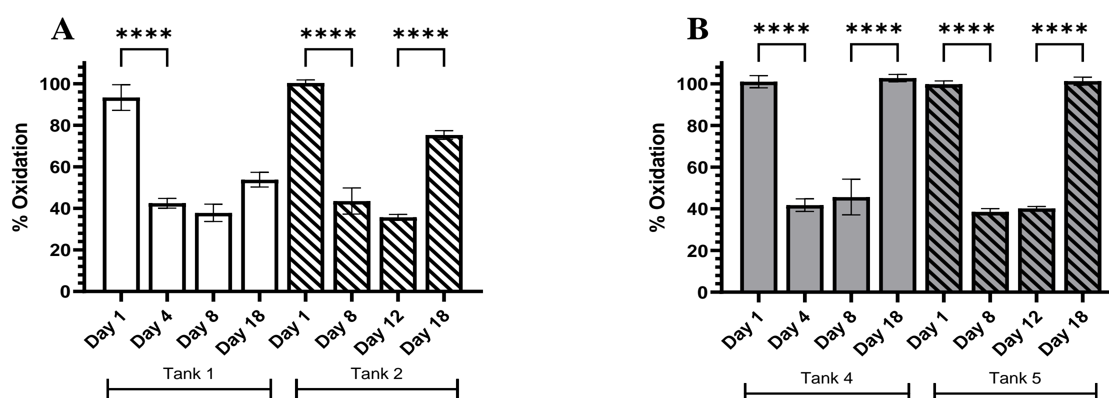


Figure 5: Effect of repeated handling stressors on percentage of oxidation in protein 3 at different water temperatures (A) Ambient tank 1 and 2 protein 3 percentage oxidation values (B) Warm tank 4 and 5 protein 3 percentage oxidation values. Fish from tank 1,4 was sampled on day 1, 4, 8 while fish from tank 2, 5 was sampled on day 1, 8, 12. Data is presented as mean ± SEM. Significance is shown as ****$p<0.0001$. *n* = ambient (5A): 5-8, warm (5B): 3-8.

3.4 Oxidation levels of protein 3 after acute and chronic heat stress

The pattern of oxidation in protein 3, presents an unusual biphasic pattern. After an acute 24-hour temperature shock, oxidation levels of protein 3 were significantly higher in warm temperature tanks independent of handling stress (p value = 0.0322; Figure 6). Levels on day four, eight and twelve were significantly reduced compared to day one, and no differences were observed between the two temperature conditions. A significant increase in protein thiol oxidation occurred in both temperature tanks on day eighteen but interestingly, the levels in warm fish were 1.82-folds higher compared to the day eighteen ambient values (Figure 6).

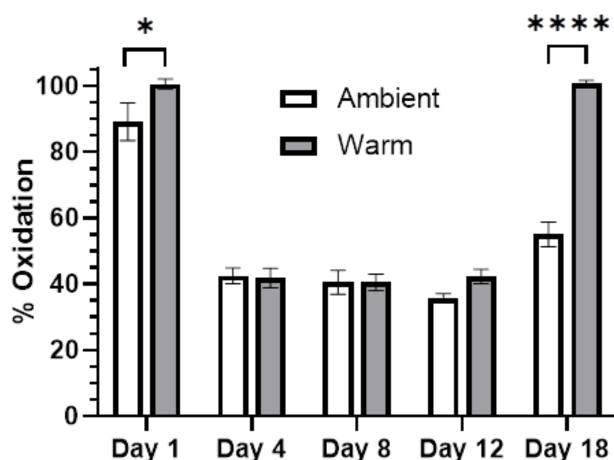


Figure 6: Percentage protein oxidation of protein 3 between ambient and warm temperature conditions over 18 days. Data is presented as mean ± SEM. Significance is shown as *p<0.05, ****p<0.0001. n = 8, 15, 7, 5, 15, 11, 6, 8, 11, 16 respectively.

4. Discussion

To date, an indicator of oxidative stress in fish as a means of providing a simple, sensitive, and cost-effective measure of health for industry aquaculture applications has not yet been available. In this novel study, temperature and handling stress was used as a model of physiological stress in Yellowtail

Kingfish to evaluate the applicability of using longitudinal measures of protein thiol oxidation, as a potential biomarker. A key conclusion from this study is that the redox state of Protein 3 exhibited the greatest sensitivity to handling and temperature stress. Another potential protein biomarker was Protein A, which showed a later oxidative onset on day eighteen in response to chronic heat stress.

Stress caused by the initial day zero handling procedures, which involved relocating fish to a new tank environment, net chasing, anaesthetising, weighing and RFID tagging triggered an acute increase in the oxidation levels of protein 3 as reflected by the elevated levels 24-hours later on day one. Subsequent day handling stressors returned a lower oxidation value that was similar in all days leading up to day eighteen, indicating that the levels observed on day one was result of a stressed state, while subsequent day handling stressors were not stressful for fish. Hence, we can conclude that while the initial day zero handling practices did cause stress in fish, they were able to recover from the initial stressors by day four and repeated sampling every four days did not affect the levels of thiol oxidation in protein 3. Interestingly, even though the sampling procedure was consistent across all five timepoints and temperature levels were not significantly different between days twelve and eighteen (Figure 9, Appendix 1), a significant increase in protein thiol oxidation occurred on day eighteen in warm temperature fish. It is uncertain if this observation is of an actual physiological response of protein 3 in fish, or from new stressors that occurred down at the fish hatchery before the day eighteen sampling. Nevertheless, if fish were exposed to a new unexpected external stress, ambient fish handled it better, while it exacerbated a greater physiological response in the stressed warm fish.

Despite the effect of handling stress, protein 3 on sampling day one had significantly higher levels of oxidation in the warm temperature water fish compared to fish in ambient water. This result is significant because changes in body weights were not detected between the two temperature conditions, but changes in protein 3 oxidation was immediately evident after an acute 24-hour temperature shock. Thus, there is a potential that we can pick up biological stress in fish at its inception using protein thiol biomarkers, before current industry measures can. Further studies are warranted to understand the biphasic response observed in the oxidation levels of protein 3, and currently it is unclear whether measures of thiol oxidation in protein 3 are reliable in chronic stress monitoring.

This study is the first to illustrate that the pattern of protein oxidation of important plasma proteins was modulated by environmental (water temperature manipulation) and routine handling stressors, suggesting that the immune system of fish may have been affected. Currently, it is unclear by which the mechanism of ROS generated by temperature stress causes changes in oxidation levels of key plasma proteins however, a proposed pathway is through myeloperoxidase (MPO) mediated

oxidative damage (38). MPO is a heme enzyme, secreted primarily by activated neutrophils and to smaller extents by monocytes and macrophages at sites of tissue damage and inflammation (39,40). MPO catalyses the production of potent oxidants like hypochlorous acid (HOCl) in the presence of chloride and hydrogen peroxide, that has been shown to target plasma proteins thiols and causing oxidative damage (38,41,42).

In summary, this study demonstrates changes in protein thiol oxidation of proteins in Yellowtail Kingfish in response to temperature and handling stress. The redox state of Protein 3 exhibited the greatest sensitivity to stress followed by Protein A, which showed a later oxidative onset on day eighteen in response to chronic 18-day heat stress. The identification of other immunological proteins associated with the acute-phase response in fish will be useful in developing future studies to test their potential on different environmental and physical stressors.

This study has showcased the potential of measuring protein thiol oxidation in Protein 3 as an indicator of temperature stress in Yellowtail Kingfish, but there is still a paucity of information on the mechanism and consequence of reversibly oxidised thiols present in Protein 3 and other proteins in fish. Validation of the exact thiol groups in Protein 3 where oxidation occurs will enhance our understanding of its role. Understanding the correlation between plasma levels, and oxidation of Protein 3 will allow us to be better informed when developing novel biochemical assays to measure the oxidation levels of Protein 3. Furthermore, studies on other aquaculture species will be important work towards a more reliable assessment of fish welfare.

This study has also demonstrated that a three-day window after the initial setup stress is sufficient time to allow fish to recover before re-sampling. Future temperature trial studies will be tasked to incorporate this three-day acclimatisation period prior to the first sampling to allow for an accurate depiction of the temperature induced oxidative stress response. In addition, further studies will be tasked to bring forward the sampling window and optimise the frequency of sampling to ultimately raise the commercialisation prospects for industry.

This body of work is the first step, towards a more robust welfare and stress indicator in fish, that might help secure the future of aquaculture.

5. References

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Appendix 1:

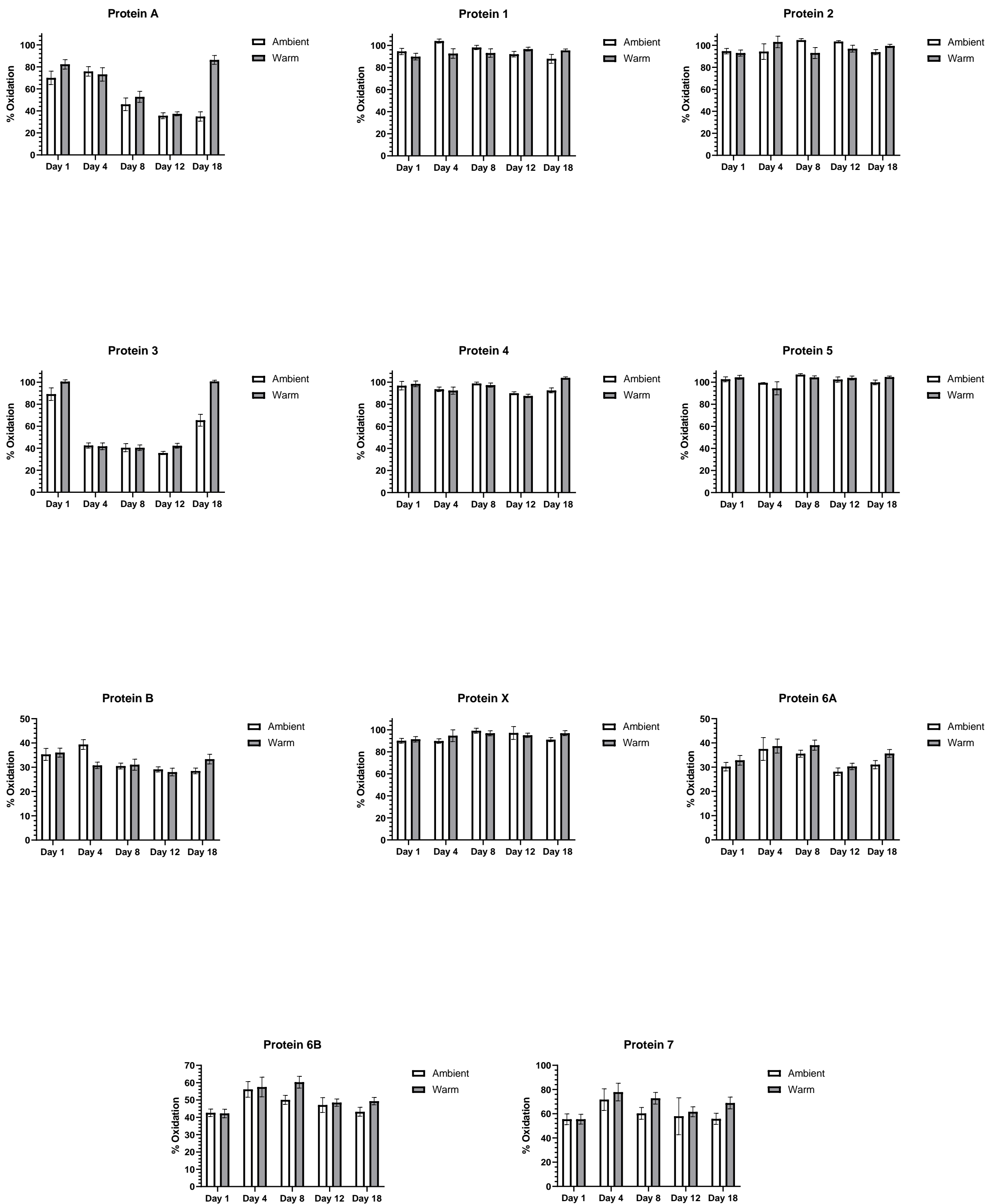

Figure 7: Temperature trail protein oxidation levels of all 11 candidate proteins from day 1 to day 18. Note: Significances are not shown on graphs. See Figure 4 for proteins of interest with significances.

Appendix 2:

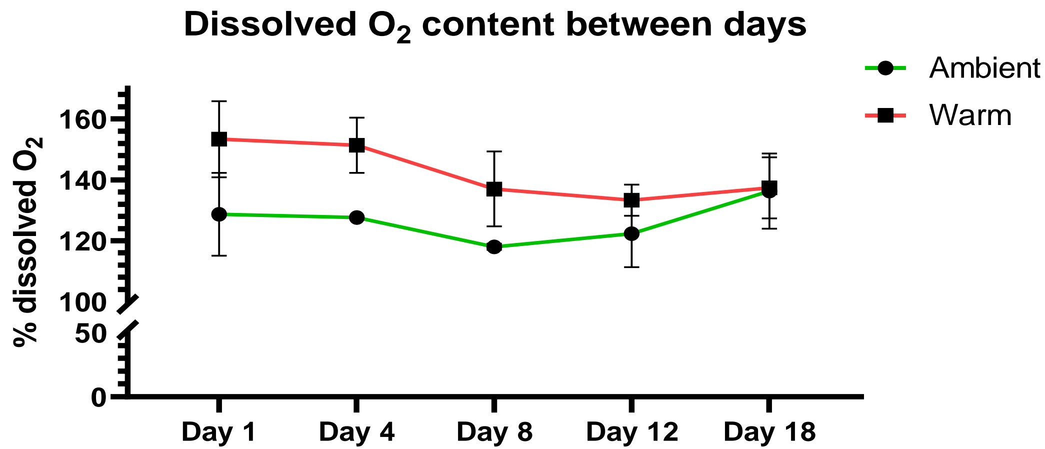

Figure 8: Mean percentage of dissolved O2 in ambient (n=3) and warm (n=3) trial tanks ± SEM. Error bars on day 4 and day 8 ambient, were too small to be displayed.

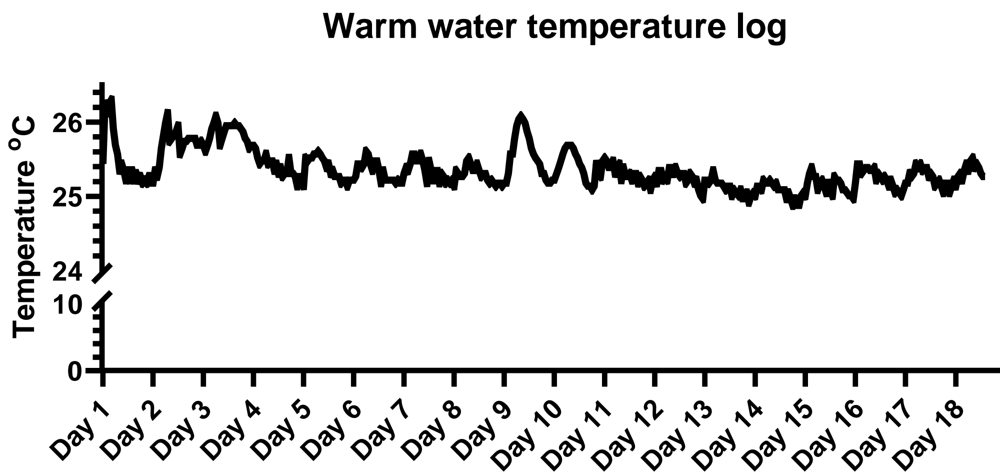

Figure 9: Warm water temperature log. Data is presented as individual values at one-hour intervals. n = 423 timepoints.