**Department of Environment and Agriculture**

# Investigating the effects of myxozoan parasites on post-harvest quality of Yellowtail Kingfish *Seriola lalandi*

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## PREAMBLE

The purpose of this research is to better understand the effects of infection by the myxozoan parasite *Unicapsula seriolae* on the post-harvest quality of yellowtail kingfish (YTK) *Seriola lalandi* (Valenciennes, 1833). A quantitative assessment of the detrimental effects of the presence of these parasites on the flesh quality of YTK was undertaken through a variety of laboratory techniques. These techniques included histological spore counts, cooking trials, texture analysis and enzyme analysis. Correlations with other factors such as fish morphometrics, post-harvest quality and harvest methods were evaluated to assess if these parameters influenced the severity and/or prevalence of infection.

Chapter 1 is an introduction to aquaculture and its increasing importance in terms of worldwide seafood and food production. This chapter also outlines the farming of yellowtail species on a global and local scale, and the significance of myxozoan parasites in a newly emerging YTK industry in Western Australia. The need, significance, aims and objectives of this research are stated in this chapter.

Chapter 2 reviews the literature of myxozoan parasites, specifically the myxozoans which have a detrimental effect on flesh quality and therefore the marketability of wild and cultured fish species globally. This chapter outlines the significance of these parasites, methods of detection, prevention and treatment (or lack of) and the specific myxozoan parasite, *Unicapsula seriolae (U. seriolae)*, upon which this thesis is based. Flesh quality issues in finfish are also covered, as potential effects by myxozoan parasites may affect the final product quality.

Chapter 3 describes the materials and methods used including the collection and analyses of YTK samples, procedures used to measure infection rates and effects of *U. seriolae* in individual fish, as well as analytical and statistical techniques to measure the severity and prevalence of this parasite.

Chapter 4 reports the results that emerged from the analysis of data collected throughout the course of this project.

Chapter 5 discusses the findings of the project in detail and explains correlations within the tested parameters. This chapter aims to prove and/or disprove the hypotheses stated for this project, including correlations between flesh texture, severity of infection, enzyme analysis and other parameters.

Chapter 6 outlines the conclusions and recommendations for the management and mitigation of the effects of myxozoan parasites in aquacultured YTK in Western Australia.

## ABSTRACT

*Unicapsula seriolae* is a common myxozoan parasite affecting flesh quality in yellowtail kingfish (YTK), *Seriola lalandi*, in temperate waters of Australia. This parasite has been identified as a major threat to the development of a viable commercial YTK industry in WA due to myoliquefaction of infected flesh upon cooking. Cultured and wild YTK were collected from Geraldton, WA to quantify infection rates through spore counting, texture analyses and enzyme activity to measure the prevalence and effects of the parasite.

The prevalence of *U. seriolae* in wild fish from WA was 80%, whereas 97% of all cultured fish sampled were infected. Unacceptable cooked flesh texture in YTK was defined by traits including subjective texture scores of 3 and above, texture analyser scores of less than 100 grams of maximum compression force, histological spore count values greater than 50 and an enzyme assay absorbance greater than 0.25. Other findings included significant ($p<0.0001$) correlations between subjective and objective texture scores, high enzyme activity was a reliable predictor for soft flesh texture, high histological spore counts were correlated with soft flesh texture and a positive relationship between spore counts and enzyme activity were found.

In addition, older fish had firmer textures than younger fish, fish with higher condition factors had firmer flesh texture and condition factor was found to decrease with increasing water temperature. Investigations into simple field tests to enumerate infection rates were undertaken, with one method using a refractometer to measure free protein showing potential. If flesh quality parameters such as pH, temperature and quality index were maintained within optimal levels, it was found that infection from *U. seriolae* had minimal effect on flesh quality.

Although there is no prevention or cure for this parasite, strategies have been identified which could mitigate the impacts *U. seriolae*, in order to facilitate a viable YTK seacage industry.

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## List of Acronyms

| | |
|---|---|
| ACAAR | Australian Centre for Applied Aquaculture Research, Fremantle, WA. |
| ANOVA | Analysis of Variance |
| CESSH | Centre of Excellence for Science, Seafood and Health. Curtin University |
| CV | Coefficient of Variation |
| DAFWA | Department of Agriculture and Food, Western Australia |
| IOFA | Indian Ocean Fresh Australia Pty Ltd, Geraldton |
| MFA | Marine Fishfarmers Association, Western Australia |
| PCA | Principle Component Analysis |
| SCRC | Australian Seafood Co-operative Research Centre |
| SCD | Spinal Cord Destruction |
| SD | Standard Deviation |
| SE | Standard Error |
| TA | Texture Analysis |
| WA | Western Australia |
| WKL | Western Kingfish Limited, Jurien Bay, WA. |
| YTK | Yellowtail kingfish *Seriola lalandi* (Valenciennes, 1833) |

## List of Definitions

| | |
|---|---|
| Myoliquefaction | Otherwise known as ‘soft flesh syndrome’. It is caused by the release of proteolytic enzymes into the muscle tissue of fish by myxozoan parasites. |
| Proteolytic enzyme | Any enzyme that catalyses the splitting of proteins into smaller peptide fractions and amino acids through proteolysis. |

# CHAPTER 1. INTRODUCTION

## 1.1. BACKGROUND

### 1.1.1. Worldwide Aquaculture

The growing demand globally for seafood has resulted in a significant increase in aquaculture activities worldwide. Aquaculture is the fastest growing food sector and has doubled every decade for the last 50 years, increasing 39 fold within this period of time (Samuel-Fitwi et al. 2012). In terms of world production, the total amount of aquacultured product in 1957 was 1.7 million tonnes, compared to 64 million tonnes in 2011 (FAO 2012). The largest volume of cultured aquatic products is from Asia, supplying almost 70% of the world's seafood (FAO 2012). Due to the vulnerability and exploitation of wild fish stocks, aquaculture is viewed as a reliable and potentially sustainable source of protein; however the boom in aquaculture has also led to negative views from society due to disease and mortality issues, escapees and interaction with wild aquatic species, environmental pollution and inferior product in comparison to wild seafood (Aubin et al. 2009, Grigorakis et al. 2011). The sustainability of aquaculture has been questioned in recent times, based on high Fish-In Fish-Out (FIFO) ratios (Tacon et al. 2008). This ratio relates to feed supply and the amount of 'trash fish' utilised to produce a known quantity of farmed fish. Despite aquaculture species experiencing faster growth, the demise of the world's 'trash fish' populations is of growing concern (Tacon et al. 2009).

### 1.1.2. Worldwide Farming of Yellowtail Species

Yellowtails, *Seriola spp.*, are large, predatory, pelagic fish ranging from temperate to sub-tropical and tropical zones of the Indian and Pacific oceans (Nugroho et al. 2001). They are ideal for aquaculture due to attributes such as

rapid growth rates, recent improvements in hatchery technologies and good feed conversion rates (Nakada 2000, Ma et al. 2013).

Regions and countries that are growing *Seriola spp.* include Hawaii, Australia, Korea and Japan. The largest producers of yellowtail are the Japanese who have been intensively farming *Seriola quinqueradiata*, Buri or Japanese yellowtail, for the past 50 years and to a lesser extent other yellowtail species such as *Seriola dumerili*, Kanpachi, and *Seriola lalandi*, Hiramasa (Nakada 2000). Total Japanese production of yellowtail accounts for over 80% of the worldwide total at approximately 200,000 tonnes per annum (Miranda et al. 2008). In terms of species production, Buri accounts for 70% of the tonnage, Kanpachi accounts for 25% and the remaining 5% is Hiramasa. Although Buri production is by far the highest, it is actually Hiramasa that commands the highest price due to the perception of having better quality attributes for sashimi than the other two species. Around 90% of the farmed yellowtail in Japan is collected from the wild as fingerlings, whilst the remaining 10% are cultivated from captive broodstock under controlled conditions (Kleindienst 2011). Another reason Hiramasa commands higher market prices is due to low volumes of seed stock, juveniles collected in the wild, as they do not congregate in as large numbers as the juveniles of Japanese yellowtail and Kanpachi.

Other farmed yellowtail species include *Seriola rivoliana*, also known as almaco jack or highfin amberjack. Aquaculture of this species began in Hawaii in 2005 where farming occurs in state-of-the-art submersible seacages. Marine hatcheries and research facilities in New Zealand and central and south America also have investigated the potential of rearing and growing yellowtail species but have been unable to sustain a commercial grow out industry due to various reasons including unsuitable water quality parameters, lack of supporting services or lack of interest in further development. Europe and the Middle East is showing increasing interest in the commercial farming of yellowtail, as an alternative to culturing slow growing species such as red sea bream and sea bass.

### 1.1.3. Australian Farming of Yellowtail Kingfish

Investigations of the hatchery production of yellowtail kingfish (YTK), *Seriola lalandi,* began in South Australia in the mid-1990s. Upon closure of the lifecycle of YTK in the hatchery environment, commercial farming began in South Australia in the year 2000 (Kolkovski et al. 2004). Since 2006 Clean Seas Tuna Ltd. has been the only commercial producer of YTK in Australia. Production of YTK in South Australia peaked at 3,280 tonnes in 2008 (Cleanseas 2008). The industry faced heavy mortality and poor growth rates from 2010 to 2012, which was attributed to nutritional deficiencies in the diet (D'Antignana 2012). After thorough investigations into the feeds and fish pathology, with assistance from Japanese yellowtail experts, the problem was rectified by adding taurine to the diet. The industry is slowly recovering from this set back and aims to increase production to 3000 tonnes per annum in the next 5 years (Cleanseas 2013a).

Typically, grow out of YTK in Australia is conducted in circular sea cages and the fish are fed exclusively on extruded pelleted feed. The size of cultured YTK, as demanded by the market, is 2 – 4kg. The market price for YTK is high, fetching over $14/kg net farm gate price in June 2013 (Cleanseas 2013a).

Farming of YTK was attempted in Western Australia in 2007 by Western Kingfish Ltd, with the aquaculture site located in Jurien Bay, 220km north of Perth. The company aimed to capitalise on the warmer water of Western Australia to grow YTK considerably faster than fish reared in the cooler waters of South Australia. A commercial YTK hatchery and grow out facility was established and juveniles successfully reared. Within 7 months of stocking, a second batch of YTK was put to sea; however the farm encountered large scale mortalities that forced the business into voluntary administration in late 2009 (http://www.ferrierhodgson.com/au/publications/specialisation-case-studies/rural-and-agribusiness/western-kingfish-limited. Date accessed 06 May 2013). The causes of the mortalities were investigated by the Department of Agriculture and Food Western Australia (DAFWA) Fish Health

Unit and were not conclusively identified despite extensive pathological and histological research (Stephens et al. 2010).

A collaborative project in 2010, between the Marine Fishfarmers Association of WA (MFA), the Australian Centre for Applied Aquaculture Research (ACAAR) Fremantle, Batavia Coast Maritime Institute (BCMI), Indian Ocean Fresh Australia Pty Ltd (IOFA) and DAFWA Fish Health Unit saw the successful completion of a pilot scale YTK farm in 2011. Funding was provided by the state government's MidWest Development Commission and Royalties for Regions scheme. This pilot scale project aimed to monitor and assess various parameters relating to production and fish health, with an aim to establishing the key risk factors for the culture of YTK in WA. The project also aimed to assess the results from the previous WKL project, in Jurien Bay, to add to the body of knowledge of the species potential for growout in WA. At the commencement of this pilot project, myxozoans were identified as a key risk due to the known occurrences of these parasites in wild fish in similar water temperatures in Australia (Lester 1982).

### 1.1.4. Significance of *Unicapsula seriolae* in Australian Yellowtail Kingfish Aquaculture

Parasitism is ubiquitous in the marine environment. Understanding the ecology and economic effects of parasites in cultured fish is essential to be able to minimise the impact that they may have on fish stocks and aquaculture (Hutson et al. 2007b).

The main parasites of concern in yellowtail culture worldwide are Monogenean parasites such as skin fluke (*Benedenia seriolae*) and gill fluke (*Zeuxapta seriolae*). These parasites can be managed effectively, although costly and time consuming, through regular hydrogen peroxide baths and/or the use of chemotherapeutants (Sharp et al. 2004). Left untreated, gill flukes can result in heavy mortalities through severe anaemia and asphyxiation of the fish (Grau et al. 2003), whereas outbreaks of skin flukes can cause

interrupted growth, skin lesions and eventual mortality (Chambers et al. 2005). Knowledge gained through studies of parasite assemblages and parasite breeding cycles have assisted in parasite management of aquacultured YTK in Southern Australia (Hutson et al. 2007b).

*Unicapsula seriolae* is a common myxozoan parasite affecting flesh quality in wild and farmed yellowtail kingfish (YTK) in temperate waters of Australia (Pers. Comm. F. Stephens. November 2011). The first published record of this parasite in YTK in Australia was from Lester (1982), who described *U. seriolae* as a new species. There are many knowledge gaps in the understanding of myxozoan lifecycles, especially in the primary host stage, and also the mechanisms of infection of these parasites (Yokoyama 2003). Investigating these complex aspects of the life cycle is beyond the scope of this research, which aims to evaluate and minimise the effects of myxozoan parasites on YTK.

Proteolytic enzymes released by the parasite during cooking can result in flesh myoliquefaction. Marketability of the product is a potential issue that arises from such mushy flesh, as texture is one of the most important quality parameters in fish quality (Rasmussen 2001).

Subsequent to the completion of the pilot scale YTK grow out project in Geraldton from 2010 – 2011, managed by Indian Ocean Fresh Australia Pty Ltd (IOFA), the viability of a large-scale commercial venture in Western Australia is currently undergoing assessment. The opportunities and limitations of farming YTK in the Mid-West region of WA have been identified and the presence of *U. seriolae* has been acknowledged as a significant threat to the viability of a successful industry in WA. The putative risk of myxozoan parasites infecting farmed YTK in WA is based on similar water temperatures and infected wild YTK found in eastern Australia (Hutson et al. 2007a). This project therefore investigates methods to detect, control and minimise the effect of this parasite in the Western Australian YTK aquaculture industry.

### 1.2. Aim

To quantify and develop strategies to mitigate the effects of myxozoan parasite infection on flesh quality of yellowtail kingfish, *Seriola lalandi*, aquacultured in WA.

### 1.3. Objectives

Achieving the aim will be supported by the following objectives:

1. Confirm identification of *Unicapsula seriolae* species seen in cultured WA YTK.
2. Quantify the rate and prevalence of infection by *U. seriolae* in WA farmed and wild YTK.
3. Understand and quantify the relationship between *U. seriolae* infection and product flesh quality in YTK fillets**.**
4. Correlate *U. seriolae* spore count with other parameters including protease activity and texture of cooked YTK fillets.
5. Investigate other parameters such as water temperature, fish size and condition factor that may influence the prevalence of *U. seriolae.*
6. Identify the effects of harvest, handling and stocking strategies to limit the occurrence and economic impacts of myoliquefaction caused by *U. seriolae* in YTK.

### 1.4. Significance

There are no known treatments for fish infected by myxozoan parasites, including *U. seriolae*, and means of control are primarily avoidance of infection (Gómez et al. 2014). *U. seriolae* was cited as a low risk to YTK grow out in South Australia but of high risk if an industry were to develop in north eastern Australia (Hutson et al. 2007a). A risk analysis for the YTK industry in South Australia conducted by Shepphard (2005) stated that *U. seriolae* infection would reduce fish performance and result in reduced product quality or saleability. However, the outcome of this analysis cites the likelihood of *U. seriolae* impacting on farmed YTK in South Australia is low. The warmer

waters of the Australian east coast reflect a similar water temperature profile on the west coast of Australia, which could increase the risk of myxozoan infection.

There is evidence to suggest that wild and cultured YTK in WA could harbour the myxozoan parasite *U. seriolae*. It has been recorded in the Jurien Bay kingfish aquaculture operations in 2008/9 (Stephens et al. 2010), found in wild samples from mid-west WA and has been observed and monitored closely from development in the recent Geraldton YTK aquaculture project from 2010 - 2012. The parasite can cause myoliquefaction of flesh (Lester 1982) in the same species of wild fish in Queensland and its impact on a potential commercial *Seriola lalandi* aquaculture industry in WA needs to be assessed.

Similar species of parasites, most notably *Kudoa spp.*, have caused serious problems to product quality at the point of consumption in wild capture and aquaculture industries in other parts of the world across multiple species (Langdon et al. 1992). The myxozoan spores can also cause unsightly cysts which impact on consumer perception and therefore marketability (Whipps et al. 2003). Such infestations are considered to be a serious impediment to the development of a viable sea cage YTK industry in Western Australia. The occurrence of *Unicapsula seriolae* and its impact on flesh quality has not been thoroughly studied in Western Australia, and its origin and prevalence is virtually unknown. This increased knowledge may assist in the development of management strategies to decrease the prevalence, intensity of infection and post-harvest effects on YTK aquacultured product.

## 1.5. Hypothesis and Research Questions

Research suggests that the primary cause of the myoliquefaction found in YTK is due to enzymes released by the myxozoan spores, which contain a proteolytic enzyme that breaks down muscle tissue (Lester 1982). A similar protease has been characterised from a *Kudoa sp.* infecting Pacific Whiting, *Merluccius productus*, and has been described as a cathepsin-L like protease.

This protease has also been identified in Atlantic salmon causing soft flesh in post-harvested fish (Martinez et al. 2011).

During development of this research, a number of hypotheses around the impact of *U. seriolae* infection on the fillet quality of YTK were formulated based on scientific publications, previous research and anecdotal evidence. This project aimed to prove or disprove the statements below.

The main research questions for this study include:

- Is there a quantifiable relationship between spore counts, enzyme levels and flesh quality in YTK?;
- Do environmental conditions and harvest strategies impact on *U. seriolae* infection in YTK?; and
- Are there methods/strategies to minimise the effects of myxozoan parasites on YTK flesh quality?

Figure 1.1 displays the proposed hypothesis, correlations and effects of the myxozoan parasite, *Unicapsula seriolae*, on post-harvest flesh quality. The subject is complex and many factors are involved in the final flesh quality of the fish. The parameters are broken into four main categories – fish morphometrics, effects and measurement of *Unicapsula* infection, post-harvest flesh quality and harvest methods. This project aims to prove the proposed correlations, as indicated by the arrows linking each of the factors.

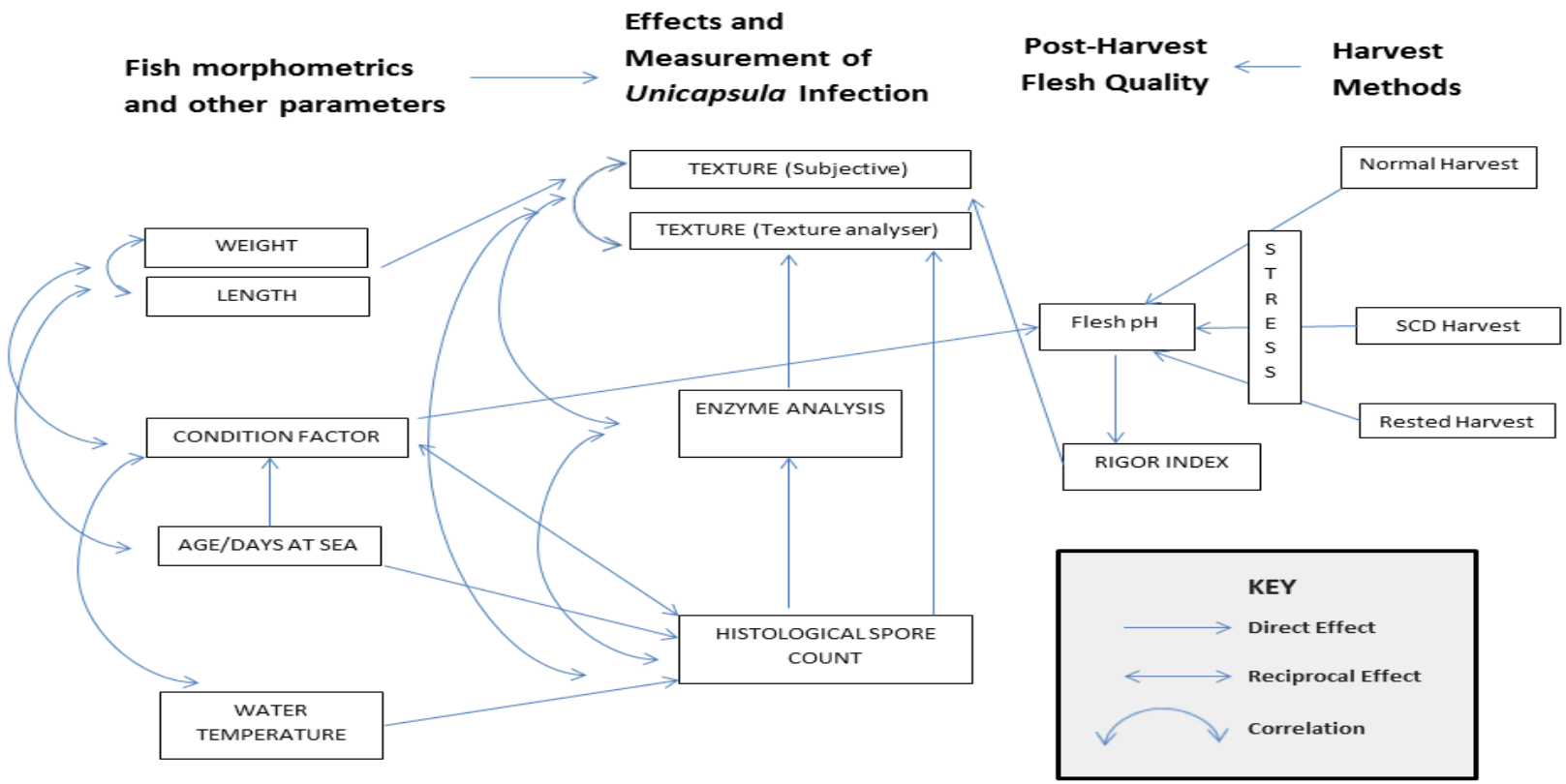



Figure 1.1 Hypotheses of correlations regarding myxozoan infections in yellowtail kingfish, *Seriola lalandi*, in Western Australia.

# CHAPTER 2. LITERATURE REVIEW

## 2.1. Overview of Myxozoan Parasites

### 2.1.1. General Background

Myxozoa are microscopic obligate endoparasites with complex live cycles (Nesnidal et al. 2013). There are over 1350 described species of myxozoa spanning across approximately 52 genera (Kent et al. 2001).

Myxozoan parasites are a common parasite of many fish species found in marine and freshwater environments world-wide (Whipps et al. 2003). Myxozoans can affect any organ within a fish, but it is the parasites found within the flesh that are of highest economic consequence to aquacultured and wild caught product (Moran et al. 1999b).

As described by Lom et al. (2006) myxozoan parasites are "characterised by spores composed of several cells transformed into one to seven spore shell valves, one to many infective amoeboid infective germs (sporoplasms) and one to several nematocyst-like polar capsules." The size of the individual organisms can range from 5µm to 20µm in overall diameter and can take form in many shapes such as spherical, elongated, pyramidal, ovoid, and symmetrical and asymmetrical (Moran et al. 1999b).

The spores are not commonly found individually within muscle cells but are usually encapsulated as a cluster of spores within a protective sheath called a plasmodium. In severe infections of fish, plasmodia can be visible to the naked eye in the form of unsightly white or black spots or streaks on the muscle (Moran et al. 1999b).

Even mild infections of myxozoan parasites can result in soft flesh in fish at any post-harvest stage. Cooking the flesh of fish with high infection rates can result in myoliquefaction (mushy flesh), as some species of myxozoans, such as *Unicapsula seriolae*, can release proteolytic enzymes upon exposure to heat (Lester 1982). Other species such as *Kudoa thyrsites* can cause muscle

degradation and softness in fish upon storage in ambient temperatures (Patashnik et al. 1982) and can even display excessive softness immediately post-harvest and during chilled storage (Dawson Coates et al. 2003). Such infections may render the fish unpalatable and unsalable which severely affect marketability and in turn influence profitability. Examples of important myxozoan infections significantly affecting aquaculture are *Kudoa thyrsites* in Atlantic salmon, *Salmo salar* (Whitaker et al. 1991), and an example of a wild capture fishery is Pacific whiting, *Merluccius productus*, which suffers from a combination of *K. thyrsites* and *K. paniformis*, resulting in downgrading of flesh to manufacture surimi or animal feeds (Zhou et al. 2009).

### 2.1.2. Distribution of Myxozoan Parasites and Species Affected

Myxozoan parasites occur in a range of fish species that inhabit shallow water habitats to deep oceanic waters. Myxozoa have been recorded from fish species in all ecological niches including demersal and pelagic species, short lived pelagic fish such as pilchards (Langdon et al. 1992) and high longevity species like snappers, swordfish and sharks (Gleeson et al. 2010). The wide range of hosts has led to an extensive distribution which spans across all oceanic environments including tropical, sub-tropical and temperate areas. Some species are endemic to localised areas and only occur in specific species of fish whereas other species can have a cosmopolitan distribution and infect a very wide host range, such as *Kudoa thyrsites* (Moran et al. 1999b). Aquaculture species which have had significant problems with myxozoan parasites include yellowtail (*Seriola spp.*), Atlantic salmon (*Salmo salar*), Atlantic cod (*Gadus morhua*) and halibut. Problems are emerging in relatively new aquaculture species such as flounder, sea bass, sea breams and tuna.

Other than fish hosts, some species of myxozoans have also been found to infect other aquatic organisms such as reptiles, Platyhelminthes, amphibians (Densmore et al. 2007) and waterfowl (Bartholomew et al. 2008). Myxozoan

parasites have also been found in terrestrial moles (Friedrich et al. 2000) and shrews, a small mouse-like terrestrial mammal, proving that infections from myxozoan parasites are not exclusive to cold-blooded and/or aquatic hosts (Prunescu et al. 2007). Early literature has stated that fish infected with the parasites are not known to be harmful to humans, although recent studies have verified that humans have suffered food poisoning as a result of ingesting *Kudoa septumpunctata* infected fish (Grabner et al. 2012). Section 2.2.3 provides more detail on food safety and human health issues regarding myxozoan parasites.

### 2.1.3. Classification of Myxozoan Parasites

Since the discovery of Myxosporea in the musculature of fish almost two centuries ago by a French scientist (Jurine 1825), the classification and phylogeny of Myxozoa has been an ever-changing and highly debated topic between parasitologists.

For the last century myxozoans were categorised as protozoans, which are single-celled eukaryotic organisms (Lom et al. 2006). During the mid-1990s publications demonstrated that myxozoans are of metazoan nature, possessing traits of multicellular eukaryotic organisms (Cavalier-Smith 1998). After this was discovered, molecular studies placed myxozoans within a sub-kingdom of Bilateria, containing animals with bi-lateral symmetry (Smothers et al. 1994). A recent article by Nesnidal et al. (2013) investigated the phylogeny of Myxozoa and concluded that they are undisputedly placed within the Phylum Cnidaria. This was based upon phylogenomic analyses of new genomic sequences of *Myxobolus cerebralis* which is the causative parasite for whirling disease in Atlantic salmon.

The stinging cells of Cnidarians, called nematocysts, have been found through molecular and morphological studies to be the equivalent of polar capsules of myxozoans. The polar capsules of myxozoans possess a coiled polar filament which functions as a point of attachment to the host and possibly assisting the

parasite in entering the host (Holland et al. 2011); whereas nematocyst cells in Cnidarians possess a cylindrical capsule body to which a long tubule is attached that might be armed with spines, which are used for defence, stunning and capturing of prey (Özbek 2011).

The myxozoans with the highest significance and impact to finfish belong to the order Multivalvulida, the focus of this study. The phylogenies of these myxozoan parasites are found in Table 2-1.

Table 2-1: Phylogeny of harmful myxozoan parasites.

| **Classification** | ***Kudoa*** | ***Unicapsula*** |
|---|---|---|
| Phylum | Myxozoa<br>Grassé, 1970 | Myxozoa<br>Grassé, 1970 |
| Class | Myxosporean<br>Bütschli, 1881 | Myxosporean<br>Bütschli, 1881 |
| Order | Multivalvulida<br>Shulman, 1959 | Multivalvulida<br>Shulman, 1959 |
| Family | Kudoidae<br>Meglitsch, 1960 | Trilosporidae<br>Shulman, 1959 |
| Genus | *Kudoa*<br>Meglitsch, 1960 | *Unicapsula*<br>Davis, 1924 |
| Species | *Kudoa spp*. | *Unicapsula seriolae*<br>Lester, 1982 |

Due to the simple morphology and lack of distinct features of myxozoans, it is difficult to characterise different species. Myxozoa from different regions of the world are being compared in DNA banks with more information on distribution and host species becoming available. New species are continually being discovered, classified and re-classified by researchers, especially with advancements in 18s rDNA sequencing resulting in greater efficiency and user-friendly methods (Fiala 2006, Alama-Bermejo et al. 2009).

In a number of publications (Eszterbauer et al. 2006, Fiala 2006, Burger et al. 2011) it has been found that the same species can be found in separate

geographical locations, infect different hosts and have significantly different morphological structure, yet share 100% homology in sequence data. This was the case for the discovery of an unknown *Kudoa* species in Australia which was found to have previously been characterised as *Kudoa yasunagai* from Japan (Burger et al. 2007). Another species, *Kudoa amamiensis,* had only been documented in Japan and was found to infect fish species from Eastern Australia (Whipps et al. 2003).

This thesis is primarily focussed on the Genus *Unicapsula*, more specifically *Unicapsula seriolae,* which is a species of myxozoan known to infect wild and farmed YTK in Western Australia. An overview of the genus *Unicapsula* is found below in section 2.1.4.

### 2.1.4. Overview of Genus *Unicapsula*

To date, 11 species of *Unicapsula* have been described worldwide. A recent paper by Miller et al. (2013) described the 11$^{th}$ species, *U. andersenae*, which was discovered in five different fish species from eastern Australia.

Prior to Lester discovering *Unicapsula seriolae* in YTK off Brisbane, Australia in 1982 (Lester 1982), only two species of *Unicapsula* had previously been described. *U. muscularis* was observed by Davis in 1924 when investigating soft and mushy flesh in halibut on the Pacific coast of the USA (Davis 1924) and *U. pflugfelderi* was found in the musculature of picarel, *Spicara smaris*, formerly known as *Maena smaris*, in France in 1975 (Schubert et al. 1975).

Lester (1982) noted that the differences lay in the morphology of the polar capsule, which contains a filament that varies in the number of turns between species. He observed that *U. muscularis* had 5 turns, *U. pflugfelderi* had 1.25 turns and *U. seriolae* had 2.5 - 3 turns within the polar capsule. These differences and the fact that all three species were found in different geographical areas led to the description of a new species (Lester 1982).

A summary of described *Unicapsula* species in Table 2-2 outlines the fish host, region of discovery and the site of infection and effects on host of the 11 described *Unicapsula* species.

Table 2-2: Summary of the 11 described *Unicapsula* spp., as outlined by Miller et al. (2013).

| Name | Host | Region Discovered | Site of Infection | References |
|---|---|---|---|---|
| ***U. muscularis*** | *Hippoglossus stenolopis*<br>**Pacific Halibut** | NW Pacific coast of USA | Muscle | (Davis 1924) |
| ***U. pyramidata*** | *Nemipterus japonicus*<br>**Japanese Threadfin Bream** | Indian Ocean | Muscle | (Naidjenova et al. 1970) |
| ***U. galeata*** | *Pseudopeneus pleurotaenia*<br>*(*reclassified as *Parupeneus ciliatus)*<br>**Whitesaddle Goatfish** | Indian Ocean | Muscle | (Naidjenova et al. 1970) |
| ***U. pflugfelderi*** | *Maena smaris*<br>(reclassified as *Spicara smaris*) **Picarel**<br>*Lithognathus mormyrus*<br>**Striped Seabream** | France<br><br>Mediterranean | Muscle<br><br>Visible pseudocysts in muscle | (Schubert et al. 1975)<br><br>(Alama-Bermejo et al. 2009) |
| ***U. seriolae*** | *Seriola lalandi*<br>**Yellowtail Kingfish** | Eastern Australia (Brisbane) | Muscles. Causes myoliquefaction | (Lester 1982) |
| ***U. chirocentrusi*** | *Chirocentrus dorab*<br>**Wolf Herring** | Southern India | Muscle | (Sarkar 1984) |
| ***U. marquesi*** | *Polydactylus quadrifilis*<br>**African Threadfin** | Atlantic Ocean off Senegal, Africa | Gills | (Diebakate et al. 1999) |
| ***U. maxima*** | *Pseudosciaena coibor*<br>*(*reclassified as *Nibea coibor)* | Indian Ocean off West Bengal, India | Kidney | (Sarkar 1999) |

| Name | Host | Region Discovered | Site of Infection | References |
|---|---|---|---|---|
| | **Ganges Jewfish** | | | |
| ***U. schulmani*** | *Albatrossia pectoralis*<br>**Giant Grenadier** | Okhotsk Sea, NW Pacific Ocean | Urinary bladder | (Aseeva et al. 2001) |
| ***U. pacifica*** | *Coryphaenoides pectoralis*<br>**Pectoral Rattail** | Okhotsk Sea, NW Pacific Ocean | Muscle | (Aseeva et al. 2001) |
| ***U. andersenae***<br><br><br>***U. andersenae*** | *Argyrosomus japonicas*<br>**Mulloway**<br>*Acanthopagrus australis*<br>**Yellowfin Bream**<br>*Eleutheronema tetradactylum*<br>**Threadfin Salmon**<br>*Lutjanus russellii*<br>**Russell's Snapper**<br>*Sillago ciliata*<br>**Sand Whiting** | SE Qld, Australia<br><br><br><br><br><br>Lizard Island, QLD. Australia | Muscle | (Miller et al. 2013) |

*Unicapsula seriolae* has traditionally been known to infect *Seriola* spp. only, however this has recently been disproven by Miller et al. (2013) who matched the DNA sequence to an unidentified *Unicapsula sp*. from a Japanese grouper, *Epinephalus septemfasciatus*. There has also been an incidence of *U. seriolae* in pink snapper, *Pagrus auratus,* from Carnarvon, WA and it was also found in highfin amberjack, *Seriola dumerili*, from the Abrolhos islands, WA (Pers. Comm. F. Stephens. July 2012). The discovery of *U. seriolae* in multiple species verifies that there is low host specificity and *U. seriolae* is not unique to yellowtail kingfish.

### 2.1.5. Hosts/Lifecycle

The lifecycles of over 25 species of freshwater myxozoans have been unearthed (Yokoyama 2003) since the discovery of the lifecycle of *Myxobolus cerebralis*, the causative myxozoan of whirling disease (Wolf et al. 1984). Based on freshwater species of myxozoans it has been found that benthic oligochaetes are the primary invertebrate hosts and fish are secondary hosts in a bi-phasic life cycle (Lom et al. 2006).

However, in the marine environment only 4 lifecycles have been described, demonstrating that polychaete worms are the definitive hosts for finfish (Rangel et al. 2011). The difficulty in studying the lifecycle of marine myxozoans is due to locating and identifying primary hosts, the complexity of the lifecycle and lack of understanding of the actinospore stage (Burger et al. 2011). The rapid progression of the applications of Polymerase Chain Reaction (PCR) probes may assist investigations of life cycle and host organisms. Refer to section 2.4.3 for further information on PCR.

Actinospores are the alternative life stages of myxozoan parasites and are morphologically different to the spores found in the secondary hosts, despite being the same species (Wolf et al. 1984). Actinospores represent the infective stage of the parasite and are predominantly found in the definitive host, proven in most cases to be an invertebrate.

The route of infection will vary with the different species of myxozoan, although in fresh water species the mode of infection is typically as follows. Benthic oligochaete worms release actinospores into the water column. The release is likely to be dependent on warmer water temperature through seasonal fluctuations (Xiao et al. 1998). The free actinospores make contact with the surface of a fish, discharging the polar capsule for attachment to the fish and release the sporoplasm (infective germ cells) to enter the fish through the gills, skin or fins. The actinospores then migrate to the tissue that the parasite colonises. In the case of whirling disease, the actinospores travel from the tail through the central nervous system to the brain of the fish

(Potera 1997). After colonisation of a host, asexual replication occurs until maturation of the spores (Canning et al. 2003). Myxozoan spores are either released from the fish body via the renal, reproductive or digestive system or upon death of the fish, if located in the flesh or other deep tissue sites such as the brain or heart. Spores are returned to the sediment where the primary host ingests the spores. In the case of whirling disease, spores can remain dormant for up to 30 years in the benthos of an aquatic environment (Potera 1997). Myxozoan spores are very hardy and have also proven to be freeze resistant and can pass through the guts of birds intact (Yokoyama 2003).

Diamant (1997) demonstrated that infection can be transferred to healthy fish without the definitive host being present. It was established that *Myxidium leei* can be passed onto healthy fish that have ingested waste or fed flesh from infected fish. This research was initiated in response to the outbreaks of myxidiosis in red sea bream, *Sparus aurata*, in the Mediterranean. Heavy mortalities resulted from this disease and it spread rapidly through densely populated sea cage farms.

Langdon et al. (1992) observed that planktivorous bait fish hosts may be reservoirs for infection of large predatory pelagic fish. Cultured mahi-mahi, *Corvphaena hippurus*, were found to be infected with *Kudoa thyrsites,* which was also found to be prevalent and severe in clupeoids such as the Australian pilchard, *Sardinops neopilchardus*, a common fish in southern Australia. Baitfish, such as pilchards, were frequently found within the vicinity of the seacages containing mahi-mahi.

Moran et al. (1999b) showed that seawater contaminated with *Kudoa thyrsites* spores can infect Atlantic salmon, *Salmo salar*. Within six weeks of exposure to seawater containing infective myxospores, 70% of the salmon tested positive for *K. thyrsites* infection. Moran et al. (1999b) also proved that *K. thyrsites* cannot be directly transmitted to fish with fresh myxospores, disproving Langdon's suggestion that ingestion of infected fish may lead to infection. Although, it was discovered in Japan that yellowtail, *Seriola*

*quinqueradiata*, introduced into southern Japan were to develop severe infections of *Kudoa amamiensis*. Fish were grown out in warmer waters to capitalise on the increased growth and were not endemic to that area. It is believed that various species of Damsel fish, of family Pomacentridae, residing in the shallow reefs near the net pens were the reservoir hosts of the parasite. The yellowtails were thought to be 'accidental' hosts after possible ingestion of these reef fish (Egusa et al. 1980). Although some myxozoans have specific hosts, it is apparent that they are opportunistic if the right conditions allow the parasite to establish within the host (Lom et al. 2006).

In a sea cage farm environment it is unlikely that knowledge of lifecycles will facilitate control strategies because it is difficult to avoid the infective stages or manage the immediate cage environment to restrict or interrupt the lifecycle of the parasite.

## 2.2. Impacts of Myxozoan Parasites

This section outlines the effects of myxozoan parasites on finfish as living organisms as well as effects post-harvest, impacts on wild and aquaculture fisheries and the effects on human health.

### 2.2.1. Effects of Myxozoan Parasites on Finfish

***Effects on Live Fish***

Moran et al. (1999b) stated that the main effects of *Kudoa amamiensis* and *K. thyrsites* cause damage to the musculature of fish and do not cause mortalities. Other species of myxozoans can cause obvious changes in physiology and behaviour of the fish it inhabits, such as whirling disease. Whirling disease is a devastating disease caused by *Myxobolus cerebralis* and affects Salmonids in the juvenile stages in a fresh water environment. Fish under 5 months of age are more susceptible due to the lack of ossification of the skeleton and the abundance of cartilage for the parasites to colonise (Halliday 1976). This disease destroys cartilage, induces spinal deformities and causes disrupted swimming patterns in the form of erratic movements and whirling behaviour. Mass mortalities have been caused as a result of this disease, which devastated numerous salmon farms and wild salmonid fisheries in Canada, northern USA and Europe during the early to late twentieth century (Hoffman 1990).

Another species of *Myxobolus*, *M. spirosulcatus*, was found to be the causative pathogen of mass mortalities in yellowtail, *Seriola quinqueradiata*, in Japan. This species of myxozoan causes similar symptoms as whirling disease including reduced feeding, erratic and abnormal swimming, skin ulceration from the jaw to the anterior abdomen and redness of the brain. The cumulative effect of these symptoms results in chronic mortality and the disease affects all size ranges of fish, from juveniles to harvestable 3kg+ fish (Yokoyama et al. 2010).

Almost any organ can be infected with myxozoan parasites including the gills, heart, kidneys, digestive tract, reproductive system and gall bladder with effects varying from reduced fitness and performance to heavy mortality - all of which are summarised in Table 2-3. The effects of myxozoans are dependent on the species of parasite as well as the species of the host fish.

Table 2-3: Myxozoan parasites have adapted to infect different species of fish and can affect almost any organ within a fish host.

| Organ Affected | Myxozoan Species | Fish Species Affected | Location | Effect on Fish | References |
|---|---|---|---|---|---|
| Muscle | *Kudoa thyrsites*<br>*K. amamiensis*<br>*K. septempunctata*<br>*Unicapsula seriolae* | Atlantic salmon,<br>Yellowtail, flounder,<br>Olive flounder,<br>Yellowtail kingfish | Canada,<br>Japan,<br>Australia | Post–harvest myoliquefaction<br>"Jelly meat"<br>Macroscopic white cysts | (St Hilaire et al. 1997a)<br>(Egusa et al. 1980)<br>(Grabner et al. 2012)<br>(Lester 1982) |
| Brain/central nervous system | *Kudoa neurophila*<br><br>*Myxobolus cerebralis*<br><br>*Myxobolus spirosulcatus* | Tasmanian trumpeter,<br>Yellowtail kingfish,<br>Fresh water salmonids<br><br>Yellowtail | Tasmania,<br>Western Australia,<br>North America,<br>Canada,<br>Japan | Whirling behaviour, scoliosis, loss of equilibrium.<br>Whirling disease.<br>Reduced feeding, erratic and abnormal swimming.<br>Chronic mortality | (Grossel et al. 2003)<br>(Stephens et al. 2010)<br>(Potera 1997)<br><br>(Yokoyama et al. 2010) |
| Heart | *K. thyrsites* | Atlantic salmon | British Columbia, Canada | Decreases chance of survival. Reduced cardiac output. | (Kabata et al. 1989) |
| Renal System | *Polysporoplasma sparis* | Gilthead seabream | Atlantic and Med Coasts of Spain | Swelling and hyperplasia of the epithelium in renal tubuli, which can lead to necrosis and kidney failure. | (Palenzuela et al. 1999) |

| Organ Affected | Myxozoan Species | Fish Species Affected | Location | Effect on Fish | References |
|---|---|---|---|---|---|
| Digestive Tract | *Enteromyxum leei, Ceratomyxa shasta* | Sparidae<br>Salmonids in fresh water | Mediterranean, Pacific NW coast of USA. | Anorexia, lethargy, darkening, wasting and eventual mortality. | (Estensoro et al. 2011)<br>(Fiala 2006) |
| Gall Bladder | *Ceratomyxa sparusaurati* | Gilthead seabream | Atlantic and Med Coasts of Spain | Enlarged gall bladders, abdominal distention and high mortalities in heavy infection. | (Palenzuela et al. 1997) |
| Reproductive Tract | *Kudoa ovivora,*<br>*Sphaerospora testicularis,*<br>*Henneguya testicularis* | Wrasse<br>Sea bass<br><br>Glass Tetra | Panama, Caribbean, Spanish Med<br>Amazon R., Brazil | Reduced growth, fecundity and spawning activity. | (Swearer et al. 1999)<br>(Sitja-Bobadilla et al. 1990)<br>(Azevedo et al. 1997) |
| Gills | *Henneguya exilis*<br>*H. psorospermica,*<br>*Thelohanellus pyriformis,*<br>*Myxobolus muelleri* | Channel catfish FW<br><br><br><br>Cyprinids (Carps) | USA and Mexico<br><br><br><br>Eurasia | Causes cysts in gills leading to suffocation and death. | (Rábago-Castro et al. 2013)<br><br><br>(Lom et al. 2006) |

Despite the destructive nature of the myxozoan species mentioned above, myxospores infecting the musculature of live fish do not always have the same dire consequences. From existing literature, it appears that the parasites initial penetration and migration into the fish body does not invoke an immune response. Further to this, it seems that the parasites also go undetected during the development and colonisation of the musculature and they can even fill muscle fibres with multiple plasmodia without an immune response (Morado et al. 1986). Langdon et al. (1992) noted that there was no host inflammatory response in small bait fish such as pilchard, *Sardinops neopilchardus*, and other small bait fish found in Southern Australia.

The parasites may remain undetected by the immune system until the plasmodia reach a critical size or mass, at which time they excrete metabolic wastes. It is these wastes which are thought to invoke an inflammatory response and can lead to the development of black and white pseudocysts in the flesh. Patashnik et al. (1982) observed that in Pacific whiting, *Merluccitis productus,* white pseudocysts are more proteolytically active than black pseudocysts and have a greater myoliquefaction rate post-harvest. In the same species, Morado et al. (1986) found that plasmodium created by two different species of *Kudoa* can form along the entire length of muscle fibre. The infected muscle cells exhibited no fibre enlargement, no inflammation, and were not detected by the immune system of the fish until the fibre was dying or being replaced. Growth of the plasmodia is only limited by the confines of the host muscle cells and eventual immune response.

Patashnik et al. (1982) noted that plasmodium of *Kudoa thyrsites* can vary in size from barely being visible to completely filling up and destroying the muscle fibre. The rupture of the cell membrane of a muscle cell (Sarcolemma) will trigger an immune response in which the pseudocysts will be encapsulated by phagocytes to form a granuloma, followed by the formation of a capsule, plasmodia or fibroblast (Morado et al. 1986, Stehr et al. 1986).

Moran et al. (1999a) experimentally infected Atlantic salmon, *Salmo salar*, with *K. thyrsites* spores and observed the progression of the infection. Once the infection was fully developed within the muscle cells, host response was only triggered when the plasmodia/muscle fibre ruptured. The myxospores released from the muscle cells were actively engulfed by macrophages which destroyed them. At 87 weeks post-exposure, the myxozoan infections subsided and were eradicated to the point that there was no sign of infection.

***Post-Harvest Effects***

The major problem of fish infected by myxozoan parasites, in a post-harvest sense, is that there are no external signs or indicators that the product may be unsuitable for consumption. The effect on the muscle tissue only becomes obvious after the death of the fish usually after processing and/or cooking and is revealed as mushy flesh or myoliquefaction (Dawson Coates et al. 2003). This poses a major problem in wild fisheries and cultured product, as the enzymes contained within the myxozoans are responsible for post-mortem muscle deterioration. The order Multivalvulida is of highest concern due to the economic ramifications of unsightly cysts and/or myoliquefaction of the muscle (Moran et al. 1999b).

Proteolysis, the breakdown of protein, in fish flesh occurs as the myxospores rupture. The damage to the spores can either be through mechanical means (rough handling when harvesting) and/or temperature (inadequately chilling post-harvest) or through exposure to temperatures at which the proteolytic enzymes are found to be most active; usually between 52° – 55°C (Samaranayaka et al. 2007). The release of these proteases has an immediate effect on the muscle tissue of fish and can result in gradual or immediate loss of texture, depending on post-harvest handling, storage conditions and processing. The soft flesh is often accompanied by a bitter taste when cooked (Lester 1982). Myoliquefaction of fish flesh can vary in severity, with highly infected fish containing myxozoans such as *Kudoa thyrsites* or *Unicapsula*

*seriolae* literally liquefying the flesh. Fish of the same species with low levels of infection or no infection will remain firm.

### 2.2.2. Myxozoan Impacts on Aquaculture and Wild Fisheries

The effects of myxozoan parasites, both directly and indirectly, on aquaculture and wild capture fisheries are significant. Direct impacts include economic consequences through product loss and degradation. Highly parasitised fish are either downgraded into low value product such as bait, pet food, stock feeds or fertilisers. These fish can also be utilised for manufacturing of seafood analogues or hydrolysates which can be made into food additives for human consumption.

Some fish species such as Pacific whiting, *Merluccius productus*, are considered 'under-exploited' due to susceptibility to parasites. Large quantities of these fish stocks are readily available but capabilities do not exist to be able to cope with large scale, high technology processing (Mazorra-Manzano et al. 2008). Sales and marketing of these inferior fish is a problem due to a reputation as soft-fleshed product which will not be readily accepted by consumers.

In Japan, the high prevalence and severity of *Kudoa amamiensis* in yellowtail, *Seriola quinqueradiata*, led to the closure of several farms in the 1970s. The farms shut down due to the lack of commercial value of infected fish (Egusa et al. 1980).

Investigations into the culture of a commercially important fish species in Tasmania, Australia were severely hindered by *Kudoa neurophila* (previously *Pentacapsula neurophila*). Striped trumpeter, *Latris lineata*, displayed nervous abnormalities from this particular myxozoan which attacks the central nervous system. This condition prevented commercial quantities of fingerlings needed for the project to be viable (Grossel et al. 2003).

The decline in fish stocks from devastating diseases such as whirling disease have been felt in central and northern USA due to economic effects. Some states such as Montana and Wyoming rely heavily upon income generated from nature-based tourism activities such as fly fishing. Popular salmonid species such as rainbow trout*, Oncorhynchus mykiss*, have experienced significant declines in populations; seriously damaging these high value fisheries (Neudecker et al. 2012). Whirling disease originated in Europe and endemic fish have developed a natural immunity to the disease. During the 1970s and 1980s in the USA, whirling disease spread rapidly in natural waterways and aquaculture facilities due to lack of immunity of local salmonids; however in recent times, in farmed salmonids, it is considered less of a risk due to improved culture practices (Gómez et al. 2014).

### 2.2.3. Food Safety and Human Health Issues

Due to the difficulty in screening fish and fish products for myxozoan parasites, it is likely that consumers will regularly ingest infected flesh from a variety of common fish species. End users may not notice macroscopic cysts in the muscle or the effects of myoliquefaction may not be severe enough to notice.

The majority of published literature suggests that myxozoan parasites are not harmful to human health if ingested (Lester 1982, Langdon et al. 1992, Alvarez-Pellitero et al. 1993, Mazorra-Manzano et al. 2008). Andreu-Ballester et al. (2008) describes the recent increase of imported fish into Spain which were infected with *Kudoa sp*., and no associations between consuming fish containing myxozoan parasites and human health issues had arisen. It is possible that the enzymes of myxozoan spores may be denatured by stomach acid when ingested or the amount of enzyme may not be sufficient to invoke an immune response in humans. Fish with obvious myxozoan infections, such as fillets exhibiting severe myoliquefaction or macroscopic cysts would most likely be discarded by the consumer.

In Queensland, Australia, *Myxobolus* sp. spores were found in the stools of humans suffering from abdominal pain and diarrhoea. The patients had all recently consumed freshwater golden perch, *Plectroplites ambiguous,* prior to becoming ill. The spores had remained undigested and intact after travelling through the digestive system, which led to the conclusion that they were incidental findings and not the cause of the symptoms. It was noted that in two of the three cases the fish consumed was noted as having an ‘awful’ or ‘funny’ taste (Boreham et al. 1998).

A study by Velasco et al. (2003) showed that there was some immunological response in mice after *Kudoa* extracted from fish was introduced orally and subcutaneously. The aim of their study was to demonstrate the potential for hypersensitivity and allergic reactions in humans. The same authors performed an experiment involving a skin prick test of *Kudoa* extracts to 15

patients. Results showed that 26% of patients showed signs of reaction to the *Kudoa* extract (Martinez de Velasco et al. 2008). Patashnik et al. (1982) fed heavily infected fish flesh to mice over a six week period, with no colonisation of the parasite found in the flesh or any organs. The spores passed through the digestive tract of the mouse intact.

Despite the numerous claims that myxozoan parasites are harmless to human health, a recent study in Japan has proven that people experienced food poisoning as a result of ingesting fish flesh containing *Kudoa septempunctata.* The flesh of olive flounder, *Paralichthys olivaceus,* eaten as sashimi has been linked to diarrhoea and vomiting in patients within 2-20 hours after consumption. The food poisoning symptoms usually abate within 24 hours and patients usually make a full recovery. *K. septempunctata* has been detected in Korean olive flounder, raising concerns that this parasite could spread globally due to the rapid increase in the aquaculture trade and the translocation of culture species (Kawai et al. 2012).

## 2.3. Flesh Quality in Finfish

This section outlines the basic factors involved in the flesh quality in finfish and demonstrates the complex and multifactorial nature of optimising finfish quality.

### 2.3.1. What is Optimum Flesh Quality?

It has been suggested that optimal flesh quality in finfish products should include parameters such as colour and appearance, flavour, texture, odour, nutrition and food safety (Haard 1992). According to Chéret et al. (2007), texture is the most important quality attribute in fish in terms of consumer acceptance and marketability. Concerns about soft texture of seafood contrasts greatly with mammalian meats, in which toughness and firmness is the major concern (Ladrat et al. 2003).

Seafood and fish products are very susceptible to spoilage and have historically demonstrated a short shelf life, especially if optimal storage and handling conditions are not met. Spoilage, as defined by Ashie et al. (1996), is 'any change in the condition of food in which the latter becomes less palatable, or even toxic; these changes may be accompanied by alterations in taste, smell, appearance or texture.' Spoilage can be caused by enzymatic, chemical and microbial activity within fish flesh. There are many methods for measuring spoilage in seafood, including sensorial, microbial and biochemical, which allow enumeration of freshness. Correlating these three parameters will give a reliable measurement of fish freshness and overall quality (Ravn Jørgensen et al. 1988).

### 2.3.2. Parameters Influencing Flesh Quality in Aquacultured Finfish

***Spoilage by Microorganisms***

Upon the death of a fish normal metabolic activities cease to function, including the body's defences against pathogens. As bacteria in the gills, viscera and skin begin to multiply rapidly upon death, certain compounds are metabolised resulting in off flavours and smell, loss of texture and changes in colour. The mechanisms of microbial deterioration work in conjunction with endogenous enzyme activity, resulting in the rapid spoilage of seafood products if not stored and chilled in optimal conditions. Chilling does not stop enzymatic deterioration or microbial growth; it only inhibits and slows down growth (Ashie et al. 1996).

Changes in pH to neutral or slightly acid when under aerobic conditions can facilitate the growth of Gram negative aerobic bacteria like *Photobacterium*, *Shewanella*, *Aeromonas*, and *Pseudomonas spp.* (Ghaly et al. 2010). For this reason it is important to optimise harvest techniques, handling and post-harvest storage conditions in order to maintain high quality in finfish by minimising microbial growth.

***Spoilage by Proteolytic Enzymes***

Post-mortem fish changes are also due to the activity of enzymes, which breakdown peptides, nucleotides and proteins. Enzymes include cathepsins, peptidase and proteases. The denaturation of these proteins may also provide a favourable setting for microbial growth and subsequent spoilage (Pedrosa-Menabrito et al. 1988). It has been documented that storing fish at low temperatures may act to inhibit the activity of these proteolytic enzymes, thus prolonging shelf life and quality as mentioned in the above text.

Hypoxanthine is a by-product of the breakdown of adenosine triphosphate (ATP), an important component in the storage of energy in cells. The presence of hypoxanthine in fish flesh is related to softening of muscle tissue and bitter off flavours (Jones et al. 1964). Concentrations of hypoxanthine are positively

correlated with storage time, as ATP in the muscle cells continually degrades over time. As well as enzymatic activity, microbial loads on fish products may be another cause of high levels of hypoxanthine, a result of metabolic activity (Ravn Jørgensen et al. 1988).

Naturally occurring proteolytic enzymes such as cathepsins are another cause of spoilage in finfish. High levels in fish have been linked with the spawning cycles (Yamashita et al. 1990) and also parasitic infestation of the flesh by myxozoan parasites (Seymour et al. 1994) but are always present in fish muscle despite these factors.

Activity of cathepsins, in fish unaffected by parasites, is related to physical abuse or temperature abuse in the form of freeze/thaw cycles during storage The most active enzymes involved in the breakdown of fish muscle during chilled and frozen storage have been identified as cathepsin L, and to a lesser extent cathepsin D, whereas the most active enzyme in mammalian meat is cathepsin D (Chéret et al. 2007).

### *Storage Conditions and Temperature*

Quality of fish flesh can also be influenced by storage conditions and temperature post-harvest, especially in terms of inhibiting microbial growth as mentioned previously. Other benefits of storing harvested fish at low temperatures include slowing the rate of protein denaturation and phospholipid degradation and the overall reduction in biochemical activities within the flesh (Ashie et al. 1996).

Optimal storage conditions at certain temperatures have also been demonstrated to suppress the development of soft meat in fish. Ando et al. (2007) showed that yellowtail, *Seriola quinqueradiata*, flesh quality benefitted from storage at -1.5°C or 10°C degrees post-harvest. The mechanism responsible was hypothesised to be the continual function of the sarcoplasmic reticulum and the retention of $Ca^{2+}$ ions post-mortem. The sarcoplasmic reticulum is an organelle found in muscle fibres which is responsible for

controlling and storing calcium ions within the muscle cell. Once the sarcoplasmic reticulum has suffered damage from temperature shock, such as placement directly into an ice slurry, $Ca^{2+}$ ions will leak into the cytoplasm and activate $Ca^{2+}$ dependent proteases which cause soft flesh. As fish cannot be stored for long periods of time at 10°C, due to microbial restrictions, a combination of 10°C and -1.5°C would significantly improve flesh quality.

Myxozoan parasites may be able to be managed through freezing, storing and handling regimes post-harvest (Langdon et al. 1992). When combined with temperature abuse, a decrease in flesh pH may also trigger the rupture of myxospores, resulting in poor flesh quality and texture. This highlights the importance of maintaining high standards when handling fish post-harvest.

***Harvesting, Stress, pH and Rigor Mortis***

After an extended period of growout, one of the final stages for aquacultured product is harvesting. The greatest care should be taken at this stage to minimise stress in fish, but it is usually overlooked by farmers (Wilkinson 2012).

The common theme for harvesting finfish is the initial crowding of the fish, which allows ease of access when extracting fish from the culture environment, whether it is tank, pond or sea cage. Crowding in tanks involves draining water out of the tank to concentrate fish into a smaller volume of water, whereas in larger culture systems such as seacages and ponds a seine net is used. A seine net, when positioned by fish farm staff, cuts off a proportion of fish from the main population and is then constricted to ‘crowd’ fish into a smaller area. The concentration of fish makes it easier for removal by manual or mechanical means such as netting by hand, fish pump, or the use of a crane with net attached.

Depending on the species best industry practice for slaughtering finfish after crowding, as described by (Robb et al. 2002), may include:

- Sedation, followed by any of the below methods
- Asphyxiation
- Asphyxiation in ice
- Exsanguination (cutting the gills and allowing to bleed to death)
- Placing fish through a percussive stunning machine
- Placing fish through an electrical stunning machine
- Iki jime/nerve destruction/severing the spinal cord

Once fish are subdued, some fish species are bled (exsanguination) prior to being placed into ice slurries for rapid chilling. It has been found that immediate bleeding upon harvest will increase the flesh quality in some species of fish, although bleeding may accelerate the post-harvest deterioration of some fish species (Ashie et al. 1996). Bleeding is especially beneficial in fish with high amounts of red muscle, such as yellowtail and tuna, to increase quality and shelf life, delay fishy smell and prevent dark colouration of the meat post-harvest (Sohn et al. 2007). Rested harvests, with the use of anaesthetic, have been shown to reduce stress in fish at harvest and improve flesh quality significantly. If fish struggle less, using less energy overall then rigor mortis will be delayed and quality parameters will be improved overall (Wilkinson et al. 2008).

Each harvest method has advantages and disadvantages, and despite the knowledge gained over the decades of intensive fish farming, continuous research is still being undertaken by well-established aquaculture industries such as Atlantic salmon in Norway (Hultmann et al. 2004a, Hultmann et al. 2004b, Hultmann et al. 2012). Changes in aquaculture harvest technology, cultivation of new species and consumer preferences will continue to fuel research into improving flesh quality.

Stress during harvesting may be worsened by prolonged crowding and using mechanical pumps for transportation of fish from seacages to harvest platforms. The increase in activity has been demonstrated to decrease time to

rigor mortis and flesh pH (Roth et al. 2012). Low oxygen availability during harvest can also lead to an increase in muscle glycogen and therefore decrease flesh pH (Haard 1992).

A simple overview of stress at harvest is as follows: increased muscular activity depletes ATP levels in muscle, causing a higher production of lactic acid through anaerobic glycolysis, results in a reduced muscle pH. Lowered muscle pH will exacerbate other factors such as rapid onset of rigor mortis, gaping, soft texture, higher muscle tension, and a higher drip and colour loss (Hultmann et al. 2012). This process is depicted in Figure 2.1.

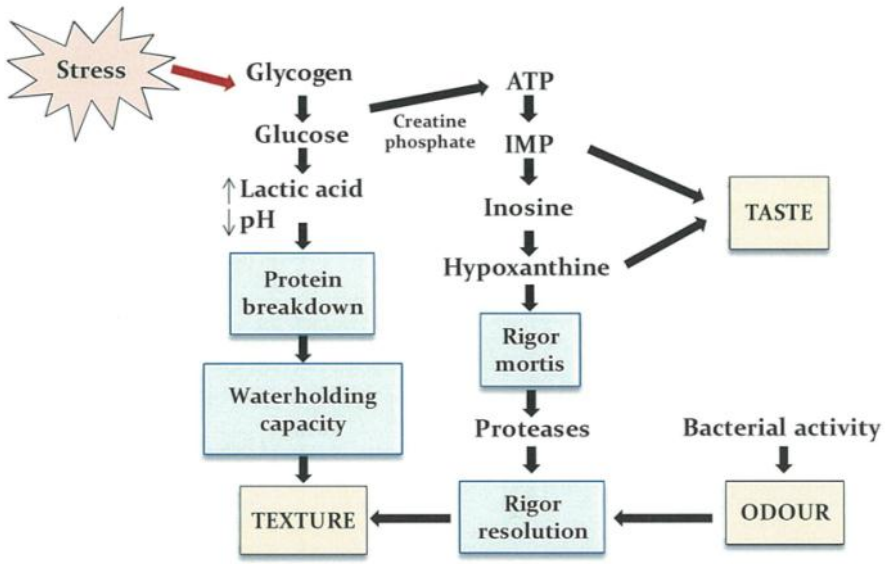



Figure 2.1: Harvest stress from crowding and harvest techniques may impact on the final flesh quality parameters such as taste, texture and odour, through numerous post-mortem biochemical processes (Wilkinson 2012).

Prolonging the time for fish to reach rigor mortis will likely improve flesh quality traits. If the muscles of the fish contract rapidly consequences may include excessive drip loss or gaping of the flesh, appearing as tears or slits. Gaping has led to reductions in market value of fish fillets, with approximately 40% of Atlantic salmon product being downgraded to inferior products in

secondary processing. Gaping is a complex subject and has been linked to factors such as genetics, farming conditions, slaughter methods and processing procedures (Ashton et al. 2010). An index for rigor mortis can be used to measure the onset and resolution in whole fish post-harvest and is a useful tool for determining the best method of harvest for particular fish species (Iwamoto et al. 1987).

Drip loss is another effect from the early onset of rigor mortis, as water holding capacity within fish muscle is greatly reduced. It has been found that texture in raw Atlantic salmon, *Salmo salar*, was softer in stressed fish and experienced drip losses of three times higher than unstressed fish (Roth et al. 2006).

### 2.3.3. Measuring Flesh Quality

Due to the many mechanisms involved in the degradation of finfish after harvest, many parameters are utilised to evaluate seafood and fish quality. Methods may include sensorial, microbial and biochemical analyses.

Sensorial analyses may include the use of a quality index method (QIM) or a quantitative descriptive analysis (QDA) for analysing whole fish such as yellowtail kingfish, *Seriola lalandi*, (Boulter et al. 2009) or Atlantic salmon, *Salmo salar* (Sveinsdottir et al. 2002). Fillets can also be subjected to QIMs, as is the case of Atlantic cod fillets in a study by Bonilla et al. (2007). These systems are demerit based, whereby parameters such as skin, eyes, abdomen, gills or flesh are observed for signs of degradation. A final score is obtained and the lower the final score, the fresher the product. Assessments can be made at set intervals over time to measure the onset and rate of loss of quality. Sensory analysis is a reliable tool for assessing freshness of seafood products and is widely used in the aquaculture and wild catch fisheries sector globally (Cheng et al. 2013). Other methods of sensory analysis may be through a trained sensory panel to objectively measure odour, flavour and texture using an existing criterion such as the Torry scheme (Alasalvar et al. 2001). Texture of raw or cooked fish flesh can also be measured mechanically, which is covered in section 2.3.4. Texture in fish and seafood products is a highly variable parameter and is dependent on factors such as the species of fish, size, maturity, sex, physical activity, environmental conditions, diet, harvest methods, post-harvest handling, processing, cooking – all of which influence biochemical processes within the muscle (Coppes et al. 2002).

Microbial media may be used to enumerate the bacterial load carried by seafood. Analysis of fish flesh may be undertaken using the traditional methods of total viable count (TVC) which determines the total number of aerobic mesophilic organisms present on food (Ravn Jørgensen et al. 1988). The units used in TVCs are CFU/g, which stands for colony forming units per

gram of flesh. Iron agar is used to detect and measure hydrogen sulphide producing bacteria, which are responsible for the off smells generated in seafood products when freshness has been severely compromised (Ravn Jørgensen et al. 1988). Long and hammer agar contains a high salt content which isolates bacteria which thrive in high saline environments such as in vacuum packed marine finfish. These psychrotolerant bacteria are also $CO_2$ resistant and include species such as *Photobacterium phosphoreum* (NMKL 2006).

Biochemical analyses of flesh may include measuring concentrations of ATP and the breakdown product hypoxanthine in the muscle which decrease and increase in concentrations respectively over time. ATP levels, and other related compounds, are measured using chromatography (Alasalvar et al. 2001). Volatile compounds caused by bacterial reduction of trimethylamine oxide to trimethylamine can cause fishy odour in seafood products, which can also be measured using specialised instruments (Rasmussen 2001).

### 2.3.4. Measuring Texture

Investigating the texture of infected fish can help determine the effects of the myxozoan parasites on the quality of the flesh. A consumer's expectation of cooked fish flesh is for the texture to be firm and elastic (Rasmussen 2001). Fish muscle cells (myotomes) are divided by thin membranes of connective tissue (mycommata). The muscles fibres are very short compared to terrestrial mammals and fish contain more uniformly distributed connective tissue. The orientation of the muscle cells runs almost parallel to the longitudinal axis of the fish to allow locomotion in the aquatic environment (Dunajski 1980). Mycommata are primarily composed of collagen, which readily dissolve upon heating. The dissolution of the connective tissue upon cooking results in the distinct characteristic of 'flaking' in fish. Heating flesh denatures and coagulates the proteins within the muscle cells and turns the flesh from translucent in appearance to opaque (Hall 1997).

When fish flesh is infected by myxozoan parasites, effects on fish flesh can vary from myoliquefaction immediately post-harvest at ambient temperatures (Patashnik et al. 1982), during the chilling stage (Dawson Coates et al. 2003) or most commonly during cooking (Lester 1982). The texture of fish flesh has been correlated with the severity of the myxozoan infection in species such as *Kudoa thyrsites* in sardines, *Sardina pilchardus,* (Cruz et al. 2011) and Pacific hake, *Merluccius productus*, (Zhou et al. 2009), i.e. the softer the flesh, the higher the infection rate.

The methods of texture analysis depends on the post-harvest utilisation of the species; for instance Pacific hake, *Merluccius productus,* is usually minced and cooked prior to analysis as it is used as the base ingredient for the production of surimi, a processed seafood analogue (Mazorra-Manzano et al. 2008). Atlantic salmon, *Salmo salar*, will be either tested raw or cooked, as salmon is a popular fish for the Japanese restaurant trade as sashimi. Cooked salmon is also subjected to texture analysis, as the smoking process exposes salmon to

prolonged periods at moderate temperatures, which is optimal for myxozoan parasite enzyme activity (Hultmann et al. 2004a).

Texture analysis of raw or cooked flesh can be either instrumental or subjective. The use of texture analysers for instrumental analysis is a simple, cheap and effective method to obtain a measurement (Ashton et al. 2010). A piece of flesh, cooked or raw, is placed onto a platform where a probe is programmed at a set speed to make contact with the flesh to measure the resistance or maximum compression force. Different types of attachments can be used including probes that measure the forces when shearing, puncturing, and compressing or tensile strength (Sigurgisladottir et al. 1999). The parameters that are important in measuring texture are force, distance and time. The parameter of highest importance in raw flesh is elasticity, whereas cooked fish flesh is measured in force or resistance (Coppes et al. 2002).

Subjective assessment involves the use of a scale to assess the texture of either cooked or raw fish flesh which uses the judgement of a person. Subjective measures can either be a visual and touching assessment or organoleptic assessment through a taste testing panel. An example of a subjective texture scale is found in Patashnik et al. (1982), which uses a scale of 1-5 to assess texture of cooked Pacific hake with 1 being mushy/pasty and 5 being firm to normal. Due to health concerns of myxozoan parasites and the obtaining ethics approval, tasting panels for heavily infected fish are not recommended in spite of the perceived low risk of adverse effects on humans. Refer to 2.2.3 for more information on human health concerns.

With certain myxozoan parasites such as *Unicapsula seriolae*, cooked flesh will reveal the presence of parasites and texture will vary with the severity of the infection. Slow cooking is the best method to showcase the effects of these parasites on flesh texture. Cooking at temperatures in line with the optimal temperature of the myxozoan enzymes, approximately 50° – 55°C, is adequate to rupture the polar capsule of the spores to release proteolytic enzymes into surrounding flesh (Samaranayaka et al. 2008). Standard

methods for cooking fish to analyse texture can include water baths, baking, frying and microwave oven (Coppes et al. 2002).

Overcooking fish flesh with no infection will become tough as a result of the muscle cells shrinking and subsequently expelling moisture (Hatae et al. 1990). Flesh with severe infections will degrade to the point of becoming viscous and pasty in texture, unable to hold shape or form (Patashnik et al. 1982).

Lester (1982) noted that the predominant impact on Yellowtail Kingfish infected with *U. seriolae* is the myoliquefaction of the flesh upon heating. This compromises product quality and texture at the point of consumption, being most noticeable after slow cooking. Lester (1982) used the Warner-Bratzler shear test to determine the texture of cooked Yellowtail Kingfish.

Overall, cooking is an excellent way to assess the texture of fish that is suspected to contain myxozoan parasites. Cooking requires no expensive equipment and subjective analysis is simple and accurate as long as the method remains consistent. The texture results can be correlated with other parameters such as histological counts and enzyme analysis to improve accuracy. Cooking trials are ideal for fish farms to sample stock pre-harvest and although the sampling is lethal, it can coincide with regular stock health checks which usually require a number of freshly slaughtered fish.

## 2.4. Methods for Detecting Myxozoan Parasites

To screen fish for myxozoan parasites prior to harvest and sales has proven to be a time consuming and laborious exercise (Taylor et al. 2005). In many cases feedback of the quality of the fish is provided by the end customer to inform the producer of inferior product. There is a need to determine the best method/s for the detection of flesh parasites, as there is still a lack of non-destructive, quick and simple methods to measure the prevalence and severity of infection (St Hilaire et al. 1997b, Taylor et al. 2005, Samaranayaka et al. 2007). This section outlines the methods to detect the presence of parasites infecting the muscle tissue of fish hosts.

### 2.4.1. Spore Counting

***Squash Technique on Raw Flesh***

One of the simplest ways to detect the presence of myxozoan spores is using the squash method. This entails manually dicing a sample of fresh or thawed raw fish flesh (or any organ of interest) on a glass plate, with a small amount of saline solution. Another glass plate is used to squeeze out the resulting fluid into a microfuge tube (Burger 2010a). The supernatant is left to settle, as spores will sink to the bottom of the tube; a small portion of settled matter is extracted from the tube and placed onto a microscope slide with a coverslip, then observed under a compound microscope at 400x magnification. The sample is scanned for anything resembling myxospores and if they are present, the sample can be frozen for further analysis. The microscope operator will need to be familiar with the appearance and external morphology of myxospores, as they can be difficult to spot due to their small size. The lipid globules released from the flesh upon dicing can also be of similar size and shape to myxospores, which may lead to confusion in inexperienced researchers.

***Macroscopic and Microscopic Observation of Raw Flesh***

In cases of severe myxozoan infections the flesh will have obvious macroscopic cysts in the muscle tissue, which can be seen with the naked eye. Filleted fish flesh may be embedded with pseudocysts that appear as black or white streaks running along the muscle segments (Patashnik et al. 1982), or resemble spherical white cysts which may be situated sporadically or focally throughout the flesh of the fish (Egusa et al. 1980). Milder cases of infection may not display any macroscopic signs, so other methods besides visual assessment are advised. Weighed strips of thinly sliced muscle can be squashed onto a microscope slide for observation and the number of plasmodium (pseudocysts) can be counted and expressed as the number of pseudocysts per gram of flesh as demonstrated in Kabata et al. (1986).

St Hilaire et al. (1997b) developed a method to detect the presence of *Kudoa thyrsites* infections in the skeletal muscle of Atlantic salmon by analysing the opercular muscle. The results of the study showed a strong correlation for the presence/absence of the parasite between the two types of muscle. The method is non-destructive and does not damage the appearance of the fish, as only small pieces of unseen muscle are extracted. The downsides include lack of ability to detect low levels of infection accurately and the time required to process each fish is approximately 20 minutes. The application of this method for screening fish post-harvest is impractical; however, it may be very useful to sample fish whilst still in the water due to its non-lethal nature. An insight can be gained into the prevalence of *Kudoa thyrsites* during the growout period by testing a small sample of the population.

***Muscle Digest***

An accurate process to enumerate myxozoan spores is the muscle digest method. This involves breaking down a known weight of muscle tissue with proteolytic enzymes such as pepsin or trypsin at their optimal temperature. The muscle undergoes proteolysis, leaving behind the hardy myxospores,

suspended in solution. The fluid is centrifuged, forcing spores to collect at the bottom of the tube. A sample of the pellet is placed onto a haemocytometer and the total number of spores counted is extrapolated to obtain a value expressed as spores per gram of flesh (Samaranayaka et al. 2007). The flaws in this method includes the need for specialised laboratory equipment such as a water bath, microscope and centrifuge and the process of preparing and counting the spores is very time consuming. If there is more than one researcher involved, counts of spores are prone to error which can lead to discrepancies in results. Also the required dosage of trypsin/pepsin to muscle is imperative; otherwise large pieces of muscle may not completely digest. If undigested muscle is present, then myxospores may be locked up in the tissue which can obscure accurate counting.

### 2.4.2. Histology

Histology is the study of the structure of tissues at a microscopic level and is a very useful tool in the detection of myxozoan parasites. The standard histological methodology for detecting these parasites in fish muscle include cutting a section of fish flesh post-mortem, fixing the flesh in formalin, encasing the piece of muscle within wax and slicing the muscle to approximately 5µm. The finely sliced muscle piece can then be stained using standard hematoxylin and eosin (H&E) stain, or for greater contrast and visibility a stain such as Giemsa can be used to highlight the polar capsules of the parasites. The piece of tissue is then attached to a microscope slide and cover slip is attached. Organs and tissues of fish can be prepared in this manner for detecting parasite infections or other pathological anomalies. The final histology slides are viewed under a compound microscope for analysis, and the original preserved tissue blocks are kept for further histological analyses.

In a study by Moran et al. (1999a), myxospores of *Kudoa thyrsites* were detected, by histology and light microscopy, in Atlantic salmon four months post-exposure to natural waters containing the parasites. Histology cannot detect when fish become infected by myxozoans, as the contagion only becomes obvious and visible when myxospores establish in the tissue and reach a certain size. Other methods such as PCR, as outlined in section 2.4.3, are able to detect parasites at an early stage.

Another disadvantage of histological analysis is the difficulty in accurately counting the spores due to the small amount of flesh, which is not representative of the entire fish. The approximate volume of a histology sample may amount to approximately 0.0005cm$^3$ (based on 1cm x 1cm x 0.0005cm), within a volume of around 640cm$^3$ in one fish fillet (based on a 40cm x 8cm x 2cm). This can be overcome by taking samples from numerous sampling points across the fish, however, this may be a time consuming and

costly exercise if numerous fish samples are to be analysed. Consistency in the sample location on each fish is necessary, as the myxozoan infection may be distributed unevenly within the flesh of the fish, with infections being concentrated towards the head of the fish (Kabata et al. 1981, Patashnik et al. 1982, Kabata et al. 1986), although Samaranayaka et al. (2007) found that infections were uniformly distributed throughout the flesh. A common site to extract flesh is from the left anterior 'shoulder' of the fish from each sample obtained, although flesh collection methods depend on the type of research.

A number of research papers have used this method to ascertain the presence or absence of myxozoan infections (St Hilaire et al. 1997a, Dawson Coates et al. 2003, Whipps et al. 2003, Burger et al. 2010b), highlighting the efficacy of histology. The constraints of histology include time needed for fixation, sample preparation and analysis - if an immediate diagnosis is required. However, the accuracy of detecting the presence of established myxozoan infections cannot be surpassed. Turnover of samples may be slow for fish farms operating in regional areas that rely upon outsourced expertise to process submitted samples. This issue is counteracted by the fact that myxozoan infections on their own are rarely a cause for mortality and a rapid response from pathologists is usually unnecessary.

### 2.4.3. Polymerase Chain Reaction

Screening tests can be undertaken using polymerase chain reaction (PCR) assays to detect the presence of myxozoan parasites within muscle tissue of fish (Hervio et al. 1997). This technology can be useful for fish farms and hatcheries to detect early levels of infection and to gain an insight into the prevalence of infection by specific species of myxozoa (Taylor et al. 2005). PCR can pick up the presence/absence of myxozoan parasites before it is detectable through others methods listed in this section. Since the DNA sequences differ for every organism, PCR can discriminate between DNA of the parasites and the hosts DNA. This useful tool has led to the discovery of many new species and has re-arranged and/or confirmed the evolutionary relationships of numerous groups of organisms, including the phylum Myxozoa (Kent et al. 2001, Canning et al. 2003, Lom et al. 2006, Morris 2010, Grabner et al. 2012). GenBank is a comprehensive database that contains nucleotide sequences for more than 380000 organisms named at the genus level or lower, available to the public (Benson et al. 2011). Researchers can submit genetic sequences to Genbank to compare with existing records held in the database and if the species is not present already, they can contribute to the pool of knowledge through the addition of a new species.

Specific primers can be designed to detect different species of myxozoan parasites, as is described by Grabner et al. (2012). The researchers created an assay to differentiate between three species of Kudoa which infect olive flounder, *Paralichthys olivaceus*, a popular food fish in Japan and Korea. The importance in this test lies in the fact that one of the *Kudoa* species (*K. septumpunctata*) is responsible for a number of food poisoning outbreaks in Japan, whereas the other two species, *K. thyrsites and K. lateolabracis*, is not known to have any adverse effects if ingested by humans. Early detection is critical in managing food safety, especially when a known agent in a common food fish is known to be harmful to consumers. Potera (1997) states that *Myxobolus cerebralis*, causative agent for whirling disease, can be picked up in rainbow trout at 2 hours post-infection in a research setting using PCR.

### 2.4.4. Enzyme Assays and ELISA Tests

***Protease Enzyme Assays***

Enzyme assays that quantify the levels of protease activity can be used to determine the severity of myxozoan infections within individual fish. A positive correlation involving enzyme activity and parasite severity has been demonstrated (St Hilaire et al. 1997a, Dawson Coates et al. 2003, Samaranayaka et al. 2007, Zhou et al. 2009). It is useful to confirm the presence of myxozoan parasites via alternative methods listed in this section prior to undertaking enzyme analyses.

The standard enzymes assay used by a number of researchers for measuring protease activity in fish is based on the research by An et al. (1994). The TCA-AZO assay was found to be the most sensitive, fast and simple based compared with other assays.

In simple terms, proteins are made of chains of amino acids held together by peptide bonds. Proteases act by breaking peptide bonds, therefore denaturing and breaking down proteins such as those found in fish muscle. Proteases are fast acting upon activation, and can degrade as many as one million peptide bonds per second (Sajid et al. 2002). The TCA-AZO assay can accurately measure the level of protease activity and subsequent breakdown of protein in fish flesh.

***Cathepsin-L***

Cathepsin-L is one of many enzymes found within vertebrate muscle. Cathepsins are acid proteases located in the lysosomes of cells and are inactive in living tissue (Chéret et al. 2007). They may be released into both the cytoplasm and the intracellular spaces as a result of lysosomal disruption, which can occur after cell death due to a drop in pH (Dutson 1983). Lysosomes are known to harbour about 13 types of cathepsins (Goll et al. 1983). Due to the number of different cathepsins found within fish flesh, it is possible that a

combination of a number of cathepsins cause soft flesh post mortem, although Cathepsin-L has been identified as the major contributor to the total post-mortem proteolytic activities in fish (Yamashita et al. 1990, Chéret et al. 2007). Cathepsin-B, another contributor to soft flesh in fish can be differentiated from cathepsin-L in a method formulated by Godiksen et al. (2007). This method will assist in identifying and differentiating activity between these two important cathepsins when studying myoliquefaction in fish flesh.

It has been found that myxozoan parasites, such as *Kudoa thyrsites* and *K. paniformis*, also harbour cathepsin-L like proteases within their cells (An et al. 1994, Zhou et al. 2009). Muscle pH, lactate content and temperature were also found to affect the activity of protease enzymes in parasitised fish muscle. Fish flesh with an initial pH of 6.5 had negligible protease activity, but a drop in pH to 5.5 was adequate to stimulate the enzymes into high activity (Funk et al. 2008). Sajid et al. (2002) found that endoparasites secrete cathepsin-L proteases in order to invade tissues and cells of hosts, as well as to assist in tissue migration. Funk et al. (2008) also made the conclusion that cathepsin-L is derived from the myxozoan parasite and not the fishes response to the parasite.

***ELISA Tests***

Enzyme-Linked Immunosorbant Assays (ELISA) are plate based assays used to detect the presence of antigens or antibodies to specific proteins, peptide, antibodies or hormones (Thermo-Scientific 2013)

Chase et al. (2001) immunised mice with purified *Kudoa* spores to collect *Kudoa*-specific monoclonal antibodies, created as a result of the mice's immune response. The spores were found to be highly immunogenic, meaning that they incited a rapid immune response post-exposure to the pathogen. It was also discovered that the antibodies may also be species

specific, as the developed antibodies were not attracted to other species of *Kudoa* including *K. paniformis* and *K. crumena*. The previous research was a prelude to a study by Taylor et al. (2005) which developed a quantitative and sensitive diagnostic test to detect *Kudoa thyrsites* in Atlantic salmon. This ELISA test has a similar sensitivity to PCR and is a good early diagnostic tool for use in pre-harvest screening of salmon.

*Kudoa thyrsites* antibodies can be purchased online from a biotechnological supplier in the USA for approximately US$380 (October 2013). The kits are comprised of 100 tests for applications such as ELISA, western blot, flow cytometry, immunocytochemistry and immunofluorescence. (Novus Biologicals 2013, accessed 5 October 2013, http://www.novusbio.com/).

ELISA assays can be designed to be chromogenic, chemifluorescent, or chemiluminescent when displaying detection of the antibodies. This means that an instant colour change can be observed in the test upon contact with the substrate, using instruments such as spectrophotometers, fluorometers or luminometers to measure the colour changes (Thermo Scientific 2013: How to choose your ELISA detection system. Accessed 5 October 2013 http://www.thermoscientific.com/ecomm/servlet/newsdetail_11152_55153_-1).

The usefulness of such tests has limited applications for detecting *Unicapsula seriolae* in yellowtail kingfish, as the tests will need to be customised to individual myxozoans. The production of experimental and commercial antibodies is not feasible at this stage and is beyond the scope of this project.

***Conclusion***

Overall, to detect myxozoan parasites in fish flesh depends on the type of species, the technology available and the skills of the investigator. From published literature, it is recommended that a variety of methods should be utilised to determine the extent of severity and prevalence of myxozoan parasites in fish populations. Most methods require complex laboratory

techniques that are time consuming and expensive, requiring specialised training and knowledge. This is impractical for most aquaculture applications that are located in regional areas away from major centres. Each method for detecting myxozoan parasites is listed below in Table 2-4.

Table 2-4**:** Summary table of the common methods used for detecting Myxozoan parasites. When investigating the prevalence and effects of myxozoan parasites on the host, it is recommended to undertake a number of different methods and not rely solely on one testing parameter.

| **Method** | **Presence/ Absence** | **Count?** | **Counting Accuracy** | **Positive Aspects** | **Constraints** |
|---|---|---|---|---|---|
| Histology | Yes (only with mature spores) | Yes | Average/poor | - Excellent for confirmation of infection.<br>- Good 'snapshot' of the severity of the infection.<br>- Can enumerate spores if counting methods kept consistent between samples.<br>- Slides can be kept indefinitely for future reference. | - Can only pick up infection in tissue when established and mature spores present, therefore newly infected tissue may not be detected.<br>- Need specialised laboratories to process and analyse the samples.<br>- Counting spores from one sampling point of one fish is not always representative of the whole fish.<br>- Sampling is always lethal. |

| Method | Presence/ Absence | Count? | Counting Accuracy | Positive Aspects | Constraints |
|---|---|---|---|---|---|
| Wet mount/ Squash mount | Yes | No | Poor | - Quick and easy method to detect the presence of myxozoans using limited equipment.<br>- Excellent for collecting spores for morphological and DNA analysis, as they can be separated from flesh and frozen.<br>- (St Hilaire et al. 1997b) found a non-lethal method to detect infection in Atlantic salmon using the operculum muscle – counts correlated with muscle infection. | - Not able to detect early infection or low levels of infection as the spores may be too small or sparsely in the flesh.<br>- Counts may not be accurate due to limited release of spores from plasmodia during physical dicing of flesh.<br>- Difficult to count smaller myxozoan species such as *U. seriolae*, due to similar size to lipid globules released from flesh.<br>- Sampling is lethal |
| Spore Counting | Yes | Yes | Average/ Good | - Can give an accurate count of spores<br>- Can be correlated with objective texture scale and enzyme analysis to estimate level of infection. | - Very time consuming.<br>- Prone to error in counting.<br>- Specialised laboratory equipment<br>- Discrepancies between researchers<br>- Sampling is usually lethal |
| Cooking and Texture Analysis | Yes (Indirectly) | No* | Good indicator of severity of infection | - Cooking is simple, fast and effective.<br>- Does not require complex equipment.<br>- Subjective texture scale of cooked flesh easy to use. Can be correlated with objective texture scale, enzyme and spore count to estimate level of infection. | - Objective texture analysis requires specialised equipment and training.<br>- Requires a significant piece of flesh, which may be needed for other analyses, posing a problem for small fish and small pieces of flesh.<br>- Sampling is always lethal. |

| Method | Presence/ Absence | Count? | Counting Accuracy | Positive Aspects | Constraints |
|---|---|---|---|---|---|
| | | | | - Obvious results in severely infected flesh.<br>- Can be used on fish farms. | |
| Polymerase Chain Reaction (PCR) | Yes | No | Excellent at determining presence/ absence | - Can detect infection at very early stages.<br>- Very high accuracy of determining presence/absence of infection. | - Needs specialised laboratory equipment and training.<br>- No indication of infection level.<br>- Sampling is always lethal. |
| Enzyme Analysis & ELISA tests | Yes (Indirectly) | Yes* | Excellent, when correlated with other parameters. | - Very accurate if correct methods used.<br>- Can correlate with methods such as cooking/texture and spore counting to give accurate insight into level of infection. | - Time consuming and good laboratory skills and equipment required.<br>- Unable to use in the field<br>- Time, money and expertise required for ELISA set-up for specific myxozoans.<br>- Sampling is always lethal. |

*Can be correlated with other parameters such as texture and spore counting to give a good indication of infection level.

## 2.5. Factors Influencing Prevalence and Solutions for Myxozoan Infections in Finfish

There are no known treatments for eliminating myxozoan infections in finfish, although in aquaculture studies it has been found that there are some preventative measures which may help to mitigate the effects of these parasites. Further, there are however some seasonal or cultural factors that may influence likelihood of infection.

The factors reported in the literature to influence prevalence and possible preventative measures for myxozoan parasite infection in aquacultured fish are summarised in Table 2-5. These factors are discussed in more detail below.

### 2.5.1. Fish Health and Stress

It is generally accepted that cultured fish exposed to prolonged stress or frequent stressful events are more susceptible to pathogens (Fevolden et al. 1993). Stressful physical events for fish may include transferring, grading, harvesting, net changing or the presence of predators or parasites. Physiological stress can arise from environmental conditions such as water temperature fluctuations, changes in water chemistry or pollution (Iwama 1998). Also, prolonged stress in cultured stock can also be linked to inadequate nutrition in the long term – refer to section 2.5.2.

It was found in two independent studies that stress is a factor in fish becoming more susceptible to myxozoan infections, especially in the juvenile stages (St Hilaire et al. 1997a, Moran et al. 1999a). These examples arise from the Atlantic salmon industry where fish undergo physiological and morphological changes to cope with the differences in salinity when transferred from freshwater hatcheries to sea cage farming. These stressful events expose Atlantic salmon smolt to a higher prevalence of *Kudoa thyrsites*

infections, although it was found in these studies that the fish are able to overcome significant infections to the point where it is undetectable within 18 months.

Condition factors are used to assess the overall health of a fish, based on calculations using length and weight. Condition factor, K, is useful to quantitatively evaluate fish within a population or between populations of the same species (Barnham et al. 1998). The traditional condition factor equation used over the last century, formulated by Fulton in 1911, has been superseded to include the weight of the fish. (Richter et al. 2000).

In this study, hence it will be hypothesised that condition factor may be a useful predictor for fish that are affected by myxozoan parasites. Fish in poor condition may have an increased propensity to myxozoan infection than fish with higher condition factors. It may be that fish with low condition factors are already immunocompromised, leading to increased myxozoan infection, or have become emaciated as a result of myxozoan infection.

### 2.5.2. Feeding

Moran et al. (1999a) suggested that sub-optimal growing and environmental conditions may leave fish more susceptible to myxozoan parasites, and demonstrated that a reduction in feeding led to a higher incidence of infection. This may be due to a depressed immune system due to rationed feed, allowing pathogens to more readily enter the body of a fish. The complex relationship between nutrition, immune status and parasitic infection is well recognised and is described thoroughly for domesticated animals and fish in Fekete et al. (2007) and Oliva-Teles (2012).

In recent times, aquaculture has come under scrutiny for the utilisation of ‘trash fish’ for the manufacture of feeds in order to grow carnivorous fish such as Atlantic salmon, yellowtail, cod, halibut, sea bass, sea bream and tuna

(Tacon et al. 2008). Fish meal and oil, due to its increasing price and limited supply, is now being interchanged in the aquaculture industry with plant based protein and oils. Other sources of protein and oils currently being used in aquaculture include abattoir waste of land animals including bone, feather and blood meal (Powell 2003).

Cost cutting has compromised overall health of aquacultured stock, especially carnivorous finfish – leading to heavy mortalities and in some cases lawsuits by aquaculture companies seeking compensation from feed companies for incurred losses. Most cases are kept discrete, such as not to damage the reputation of feed companies, although a case regarding Cleanseas Tuna (CST) in South Australia is acknowledged in annual reports and investor updates, being a publically listed company. CST is seeking compensation from feed companies due to high mortalities and poor growth rates in yellowtail kingfish, *Seriola lalandi*, as a result of inadequate levels of taurine in the diet (Cleanseas 2013b). Taurine is an essential amino acid that is not produced by fish and is essential in metabolic processes such as cholesterol production and osmoregulation. Another related symptom of the taurine deficiency included green livers, which was thought to be a result of vegetable based oil and protein in the diet (D'Antignana 2012).

The importance of selenium, taurine and Vitamin E supplementation has been recognised in studies on other domesticated animals such as swine, poultry and ruminants (Fekete et al. 2007). With the exponential increase in aquaculture worldwide, optimising fish diets has become a high priority for increasing growth rates, boosting resistance to diseases and parasites and environmental responsibility.

Estensoro et al. (2011) revealed that an increase of plant based oils, at an inclusion rate of 66%, in the diet of gilthead sea bream, *Sparus aurata*, led to a higher prevalence, faster establishment and increased severity of infections by the myxozoan *Enteromyxum leei*. Control fish were fed a diet with 100% fish oil and displayed superior health to those fed a high inclusion of vegetable oil in the diet. The signs of disease and emaciation were observed in the vegetable oil diet fish which exhibited lowered growth rates, poorer condition factors, lowered specific growth rates and lower haematocrit to those fish fed on fish oil based feeds.

Although not currently published, it is possible that immunostimulants and pro-biotics, including beta-glucans, could reduce the incidence and severity of myxozoan parasites. Aquaculturists are seeking alternatives to antibiotics as the resistance of pathogens has increased in recent years from overuse. This has driven the need for extensive research into the pro-biotics. Advantageous effects such enhanced immune response and increased growth rates in aquaculture species such as pink snapper, *Pagrus auratus*, yellowtail, *Seriola quinqueradiata*, and Atlantic salmon, *Salmo salar*, have been identified through the use of probiotics (Dalmo et al. 2008). It is hypothesised that pro-biotics may indirectly lower parasite incidence and severity through optimising the relationship between gut health and immunity.

Optimal diets, suited to the nutritional needs of aquaculture species are continuously being researched, refined and re-developed. It is highly likely that nutritionally balanced diet for YTK will assist to increase fish resistance to pathogens and parasites, including myxozoans.

### 2.5.3. Life Stage and Sexual Development of Finfish

Fish may be more susceptible to myxozoan parasites at certain life stages due to physiological and morphological changes. For example, juvenile salmonids under five months old are at higher risk of whirling disease depending on pathogen abundance and salmonid species. Susceptibility to whirling disease at this life stage is due to lack of bone formation allowing easy migration of *Myxobolus cerebralis* spores into the cranial and skeletal regions via the cartilage (Neudecker et al. 2012).

In a study on Atlantic salmon, *Salmo salar*, in Canada, sexually mature fish and reconditioned grilse were found to be more susceptible to infection by *Kudoa thyrsites* (St Hilaire et al. 1998). It was established that immature fish had a lower incidence of *Kudoa thyrsites*, whereas sexually mature fish had a higher incidence. A method to overcome the higher prevalence of infection and to increase quality of the produce is to cull second year fish or change production schedule and the harvest strategy to remove fish before reaching maturation (Dawson Coates et al. 2003).

When assessing the population of *Kudoa*-infected Pacific whiting, *Merluccius productus*, off the Canadian Pacific coast, Kabata et al. (1981) found no difference in prevalence in male or female fish. With regards to size of the fish and infection rate, Morado et al. (1986) discovered that there was a significant increase in prevalence of *K. thyrsites* and *K. paniformis* in Pacific whiting with size and weight of the fish.

### 2.5.4. Environmental Factors and Culture System

***Water Temperatures***

Environmental factors such as seasonal variation and water temperature may have an effect on the prevalence and life cycle of myxozoan parasites. Literature suggests that infection rates appear to increase during warmer periods of the year (Hallett et al. 1997, Palenzuela et al. 1999). Water temperatures may stimulate primary invertebrate hosts to release infective myxospores into the water column, leading to higher rates of infection. These mass releases may be synchronised with congregations of breeding, feeding or migrating wild fish, in order to increase the chances of the continuation of the cryptic lifecycle (Xiao et al. 1998). The infective stage of the myxozoans may be more motile in warmer conditions, allowing easier penetration, migration and maturation within the host (Potera 1997).

Moran et al. (1999a) found that there was a trend in seasonality of infection, with an increasing incidence of *Kudoa thyrsites* infections in Atlantic salmon during the warmer water temperatures of summer and autumn in British Columbia, Canada. It was noted that in the cooler water temperatures of winter and spring, infection rates were low or absent.

Kabata et al. (1986) sampled wild populations of Pacific whiting, *Merluccius productus*, along the west coast of the USA, from California north to Vancouver, Canada. It was found that distribution had an effect on myxozoan infestation, with higher prevalence of *Kudoa thyrsites* recorded in the northern distribution range (cooler water) compared to the southern limits (warmer water) of distribution. It was also assumed that the fish were infected in the breeding grounds of the warmer waters of California, prior to their migration north, based on analyses of age classes, sampling locations and observation of muscle tissue.

***Culture System***

Culture systems are an important consideration in the control of myxozoan parasites. Land-based facilities are superior in prevention of parasitic infections due to extensive water treatment, but have limited applications for growing out large volumes of fish. The universal presence of sea cage farms exhibits the cost-effectiveness and practicality of growing fish in open water bodies such as oceans, rivers and lakes (Folke et al. 1992). Regarding myxozoan infections, exposure to primary hosts is drastically increased when fish are grown out in open environments.

Comprehensive investigations of yellowtail kingfish parasite assemblages and potential health issues were carried out for the south Australian industry, which have aided parasite management immensely (Shepphard 2005, Hutson et al. 2007b). Myxozoan parasites were cited as low risk for YTK culture in South Australia due to cooler water temperatures but were deemed a significant risk if the growout of YTK was to be conducted in eastern Australia. These assumptions by the aforementioned authors were based on previous research by Lester (1982) who advocated that *Unicapsula seriolae* is prolific in warmer waters.

Prior to pilot scale growout of YTK in sea cages in Western Australia, one of the biggest risk factors was identified as the myxozoan, *U. seriolae*. Flesh from wild YTK was collected from the mid-west of Western Australia including the Abrolhos Islands, Geraldton, Kalbarri and Dongara to assess infection rates. Some of the fish were found to be free of flesh parasites, and there were incidences of fish with *U. seriolae* and *Kudoa spp.* infections at varying degrees of severity; from mild to heavily infected (Pers. comm. IOFA. November 2011).

Yellowtail kingfish from a hatchery and sea cage operation in Jurien Bay, Western Australia were found to be infected with two species of myxozoan parasites. Upon histological investigation after a mass mortality event, fish exhibited *Kudoa neurophila* in brain tissue and *U. seriolae* in the muscle tissue

at prevalence of 90% and 8% respectively. This was based on 50 fish sampled from the sea cage with the heaviest mortalities. Despite the parasitic load, no definitive diagnosis was found; it was likely that numerous cumulative factors contributed to the demise of the operation (Stephens et al. 2010). It is hypothesised that the infections developed in the hatchery prior to stocking at sea, and only became noticeable during the devastating mortality event. The seawater intake for the hatchery was located in a protected bay with minimal water movement and fine benthic sediment – the ideal habitat for primary invertebrate hosts such as polychaete worms (Pers. comm. Alan Savage. January 2012).

Table 2-5**:** Factors influencing the prevalence and severity of myxozoan infections in finfish and management strategies to minimise the effects of myxozoans.

| **Factors Influencing Prevalence and Severity of Myxozoan Infections** | **Management Strategies and Solutions** | **References** |
|---|---|---|
| Fish health and stress | - Maintain fish in optimal health through good husbandry practices<br>- Minimise stress in farming activities such as bathing, harvesting and stock movements.<br>- Regularly sample fish for pathological analysis and collect morphometric data for condition factor analysis. | (Fevolden et al. 1993)<br>(Moran et al. 1999a)<br><br>(Richter et al. 2000) |
| Feeding | - Use suitable feeds for the culture species i.e. avoid feeding carnivorous fish high inclusion rates of land animal proteins and plant based oils/protein in feeds. Be aware of trace minerals and protein requirements for optimal health<br>- Avoid underfeeding fish.<br>- Implement a stock feeding plan based on fish growth model and feed at optimal levels for the culture species. | (Estensoro et al. 2011)<br><br>(Oliva-Teles 2012 )<br>(Moran et al. 1999a) |
| Life Stage and Sexual Development | - Monitor stock for signs of sexual development<br>- Identify stages at which fish may be more susceptible to myxozoan parasites I.e. juveniles in a hatchery may be more inclined to contract whirling disease.<br>- Change production schedule and harvest strategies | (St Hilaire et al. 1998)<br>(Bartholomew et al. 2005)<br>(Dawson Coates et al. 2003) |

| Factors Influencing Prevalence and Severity of Myxozoan Infections | Management Strategies and Solutions | References |
|---|---|---|
| Environmental Factors | - Monitor water temperature to look for trends in infection rate and prevalence of myxozoan parasites.<br>- Investigate alternative sites, as spatial variation may influence distribution, as some benthic habitats may not support the invertebrate host.<br>- Fishing or harvesting when infection is least prevalent, locating and fishing uninfected stocks | (Halliday 1976)<br><br>(Langdon et al. 1992) |
| Culture Environment | - Likelihood of infection is significantly higher in ocean environments as opposed to land-based aquaculture facilities.<br>- Ocean sites may vary in suitability according to benthic sediment. It is likely that shallow sites with fine sand and silt may harbour more invertebrate hosts. | (Xiao et al. 1998) |
| Post-harvest handling | Refer to chapter 2.3 Flesh Quality in Finfish | |

## 2.6. Utilisation of Infected Fish Species

### 2.6.1. Bait, Stock Feed and Disposal

Although there are markets for myxozoan infected fish as bait or pet food, these applications are limited because of the constraints of disease management, intermittent or unpredictable supply and storage. It was common practice in the commercial fishing industry for bycatch and low value species to be disposed of at sea, whilst keeping high value species for processing and selling. However, due to the growing global awareness of overfishing and the preciousness of fisheries resources, the limited supply of fish protein is increasing in value and is now a much sought after commodity (Tacon et al. 2008).

### 2.6.2. Human Consumption

Some commercially important species, such as Pacific whiting (*Merluccius productus*) are downgraded to seafood analogue (surimi) products due to a very high prevalence of myxozoan parasites and high proteolytic activity (Seymour et al. 1994). The fisheries for this species is considered underutilised, so researchers have been investigating ways to capitalise on their lack of exploitation as a protein source. These fish are largely unsuitable for fillets, either frozen or fresh due to the high rate of muscle myoliquefaction due to myxozoan parasites (An et al. 1994). One such use is for the flesh of affected fish to be broken down using enzymes, salvaging the protein component for use in manufacturing of surimi for human consumption. Pacific whiting is a perfect candidate for surimi, as its traits include white muscle colour, mild flavour and low fat content (Mazorra-Manzano et al. 2008). A significant amount of research has gone into investigating the enzymes responsible for liquefaction in these species, finding enzyme inhibitors to retard the liquefaction and methods to set the gel which is required for surimi manufacturing (Samaranayaka et al. 2008).

### 2.6.3. Hydrolysates

Due to the expensive and wasteful practice of disposing fish waste and unusable infected fish into landfill, other methods for utilisation of this potentially high valuable protein waste have been developed. Dumping offal into the ocean from processing activities has also been outlawed due to public health concerns, biosecurity and attraction of predators/scavengers. Technology using enzymes for protein recovery has gained popularity and acceptance in recent years (Kristinsson et al. 2000), turning waste products into functional food additives and neutraceuticals (Samaranayaka et al. 2008).

In large scale aquaculture operations there is background level of mortality that is normal part of mass culture operations, typically 5 to 10 % over the harvest cycle. In addition to this there is also the real probability of catastrophic failure with mass mortalities due to disease, water quality problems or factors such as unsalable product from severe myxozoan infections. Underutilised commercial wild catch species such as Pacific whiting and other low value species, that have a propensity to become parasitised, can also be used to make a biosecure, high protein product for use as food additives.

Hydrolysates are desirable in aquaculture feeds due to the high digestibility and assimilation efficiency of the amino acids derived from hydrolysed proteins. The free amino acids are also thought to directly stimulate the chemosensory receptors of fish and crustaceans and so elicit an enhanced feeding response because of this stimulation (Refstie et al. 2004). The oils are separated from the protein for uses such as use in animal feeds (Bimbo et al. 1992), and recently much emphasis has been placed on the importance of fish oils in the maintenance of health and alleviating symptoms of many lifestyle diseases (McManus et al. 2009).

Functional fish protein hydrolysates from Pacific whiting have been produced for human consumption. Applications of these hydrolysates include increasing food storage stability, agents for increasing solubility, emulsifying, foaming

and dispersing functions in food products. These products are used in foods such as sausages, mayonnaise, salad dressings, beverages and creams (Pacheco-Aguilar et al. 2008).

Advantages of hydrolysates include long shelf lives, as they can be stored more readily than whole fish. The liquefied protein and fish oils are able to withstand high temperature treatments to manage any disease risk. Furthermore, the hydrolysates could be stored with refrigeration as a pasteurised product with the use of preservatives and antioxidants. They can also be dried for ease of use as a dietary component of aquaculture feeds.

Hydrolysates have been proven to reduce lipid oxidation in smoked salmon. A brine solution containing fish protein hydrolysate, from Atlantic salmon, injected into smoked salmon fish fillets was shown to reduce lipid oxidation during 6 weeks of cold storage (4°C) and 8 months of frozen storage (-18°C). Hydrolysates from the same species of fish can be used to regain the weight lost through cooking and processing of product such as smoked salmon. The hydrolysates are not required to be listed in the ingredients, as they are from the same source as the original product (Samaranayaka et al. 2008).

The process of enzymatic hydrolysis of these waste fish into a biosecure, valuable product is a relatively simple, low cost mechanism which converts an economic liability into a product with value.

# CHAPTER 3. MATERIALS AND METHODS

## 3.1. Collection and Storage of Samples

Yellowtail kingfish (YTK) samples were collected through various methods including two harvest trials, monthly collection during the sea cage grow out period and collection of wild fish. After collection, the fish were dissected and flesh was set aside for further testing. The methods of collection and storage conditions are described below:

### 3.1.1. Harvest Trial One

In November 2011 the first harvest trial was undertaken on a pilot scale sea cage farm in Geraldton Harbour, Western Australia, approximately 420km north of Perth (28.7792° S, 114.6144° E). Refer to figure 3.1 and figure 3.2.


Figure 3.1: Map of Western Australia showing the location of Geraldton in relation to the capital city of Perth. Source: Google Maps.


Figure 3.2: Map of Geraldton and surrounds where the majority of YTK samples were collected. Source: Google Maps.

Thirty (30) fish (weight 2.408g± 325 g, length 49.7cm ± 3.1 cm) from each three (3) different harvest methods were collected (n=90).

Harvesting involved manually dragging a crowd net (25m length of nylon netting used to harvest fish) across a 65m circumference cage where an unknown percentage of the total cage biomass was crowded prior to harvest. Once the crowd net was secured, fish were subjected to the following treatments:

- *Normal harvest (standard industry procedure)*: Fish were hand netted from the crowd and placed onto a harvest table on the boat, pushed individually through a percussive stunner, bled, tagged and transferred directly into seawater slurry.

- *Spinal Cord Destruction (SCD) harvest:* Fish were hand netted from the crowd and placed onto a harvest table on the boat, pushed individually

through a percussive stunner, a corer was used to remove a flesh plug between the fishes eyes and a stainless steel rod was inserted into the hole and down the spinal canal until all nerve activity in the fish ceased. Fish were then bled, tagged and transferred directly into seawater slurry.

- *Rested harvest:* Prior to crowding up the fish a tarpaulin was secured to the side of the sea cage. Fish were seine netted to the position of the tarpaulin and the crowd net was tightened and secured. The tarpaulin was pulled up and secured to engulf the contents of the crowd net. The volume of the water in the tarpaulin was calculated and a 25ppm dose of AQUI-S (Lower Hutt, New Zealand) was administered to anaesthetise the fish. Upon the fish reaching the desired sedation state where the fish had completely lost equilibrium, they were hand netted from the crowd and placed onto a harvest table on the boat, pushed individually through a percussive stunner, bled, tagged and transferred directly into seawater slurry.

Immediately after bleeding a small incision was made in the flesh of each fish located below the dorsal fin and above the lateral line on the left side to obtain a pH reading using a pH meter (waterproof pH Spear meter, Eutech instruments, Singapore). A new incision was made on the opposite side of the fish to measure pH at 24 hours and 48 hours post-harvest. Three insulated containers were used to separate the harvested fish from the 3 different harvest methods and each contained an identical amount of ice and seawater in the slurry.

### 3.1.2. Harvest Trial Two

The second harvest trial was undertaken at the same farm in Geraldton Harbour, WA in June 2012, with some minor changes to the initial experimental trial in November 2011. A total of sixty (60) fish (weight 1217g ± 301 g, length 43.9cm ± 3.2 cm) were collected with the same three (3) harvest methods (20 fish per method) listed in 3.1.1. The parameters of the two harvest trials, including crowd conditions are compared in Table 3.1 and Table 3.2 and explained in further detail in sections 3.2 – 3.11.

Table 3-1**:** Parameters measured in Harvest Trial 1 and Harvest Trial 2.

| **Parameter** | **Harvest Trial 1** | **Harvest Trial 2** |
|---|---|---|
| **Month of harvest trial** | November 2011 | June 2012 |
| **Number of fish sample** | 90 | 60 |
| **Harvest methods** | Normal, SCD and Rested | Normal, SCD and Rested |
| **Water temperature** *(refer to Section 3.11)* | 23°C (increasing into summer) | 21°C (decreasing into winter) |
| **Average weight of fish** | 2408.4g | 1217g |
| **Average condition factor of fish** *(refer to Section 3.2)* | 1.97 | 1.41 |
| **Age of the fish (days)** | 472 | 342 |
| **Flesh pH taken** *(refer to Section 3.1.1)* | Y (0, 24, 48h post-harvest) | Y (0, 24, 48h post-harvest) |
| **Rigor index performed?** *(refer to Section 3.5)* | N | Y (0,2,23,50h post-harvest) |
| **Post-Harvest Processing** | Filleted and vacuum packed after 24 hours in ice slurry. | Remained whole for 3 days then filleted and vacuum packed. |
| **Product temperature post-harvest obtained?** *(refer to Section 3.6)* | N | Y |

Table 3-2**:** Sampling of YTK for post-harvest quality and assessment of *Unicapsula seriolae* infestation in November 2011 (Harvest Trial 1) and June 2012 (Harvest Trial 2). Harvest Trial 1 required a total of 3 crowd events, ranging from 13 – 30 min crowd duration, to achieve the desired harvest total of 90 fish. Harvest Trial 2 required a total of 3 crowd events, ranging from 23 – 28 min crowd duration, to achieve the desired harvest total of 60 fish.

| | **Crowd #** | **Harvest Method** | **Time to set crowd (min)** | **Duration of crowd (min)** | **Number of fish sampled** |
|---|---|---|---|---|---|
| Harvest Trial One | 1 | Normal/SCD | 13 | 30 | 60 |
| | 2 | Rested | 12 | 13 | 20 |
| | 3 | Rested | 13 | 13 | 10 |
| | | | | **Total** | **90** |
| Harvest Trial Two | 1 | Normal | 15 | 23 | 20 |
| | 2 | SCD | 12 | 28 | 20 |
| | 3 | Rest | 9 | 23 | 20 |
| | | | | **Total** | **60** |

### 3.1.3. Monthly Collection

Cultured YTK samples from two hatchery reared cohorts (ACAAR 2010/2011) were collected fortnightly during the grow-out period in Geraldton. Refer to Figure 3.1 and Figure 3.2 above for map of Geraldton. Methods of collection included feeding the fish into a dense school and dip netting or using bait-less, barb-less hooks. The fish were lethally anaesthetised with AQUI-S at a dose rate of 200ppm, placed into ice slurry, filleted, vacuum packed, labelled and frozen. Table 3-3 and Table 3-4 summarise the monthly collections of YTK.

Table 3-3**:** Summary of monthly collection of cohort one YTK samples from Geraldton in 2011 – 2012.

| **Month** | **N** | **Mean Length (cm)** | **Mean Weight (g)** |
|---|---|---|---|
| Jan-11 | 10 | 33 | 605.2 |
| Feb-11 | 10 | 38 | 862.5 |
| Mar-11 | 10 | 38 | 805 |
| Sep-11 | 5 | 48 | 2088 |
| Aug-12 | 2 | 73 | 7400 |
| **Total** | **37** | | |

Table 3-4: Summary of monthly collection of cohort two YTK samples from Geraldton in 2012.

| Month | N | Mean Length (cm) | Mean Weight (g) |
|---|---|---|---|
| Feb-12 | 5 | 32 | 453 |
| Mar-12 | 10 | 38 | 787 |
| Apr-12 | 15 | 39 | 800 |
| May-12 | 10 | 42 | 966 |
| Jun-12 | 60 | 43 | 1013 |
| **Total** | **100** | | |

### 3.1.4. Wild Yellowtail Kingfish Collection

Wild YTK samples were collected from sources including commercial wetliner fishermen, seafood retailers and recreational fishermen. Where possible, the length and weight of the fish and the location of the catch were recorded. A summary of samples is below in Table 3-5.

Table 3-5: Summary of wild YTK samples from mid-west Western Australia

| Month | N | Mean Length (cm) | Mean Weight (kg) | Locality* |
|---|---|---|---|---|
| Jun-10 | 1 | - | - | Kalbarri ,WA |
| Aug-10 | 7 | 70 | - | Abrolhos Islands, WA |
| Apr-12 | 3 | 87 | 6.9 | Kalbarri, WA |
| May-12 | 2 | 57.5 | 3.2 | Abrolhos Islands, WA |
| Dec-12 | 1 | 98 | 13 | Dongara, WA |
| **Total** | **14** | | | |

***Refer to Figure 3.1 and Figure 3.2 for a map of the collection locations.

### 3.1.5. Control Samples

Farm raised South Australian YTK were purchased from a seafood retailer in Perth and cultured YTK were collected from Australian Centre for Applied Aquaculture Research (ACAAR) in Fremantle, Western Australia. Fish from these locations were expected to contain no *Unicapsula seriolae* infection due to cooler water temperatures (Hutson et al. 2007a), clean water sources (Langdon 1991) and previous YTK histological samples from ACAAR with negative results (Pers. Comm. F. Stephens. August 2012). A summary of the control samples is found below in Table 3-6

Table 3-6: YTK samples used as controls for detection of *U. seriolae*.

| Month | N | Mean Length (cm) | Mean Weight (g) | Source |
|---|---|---|---|---|
| Feb-12 | 2 | 31 | 462.5 | Fremantle WA |
| Feb-12 | 2 | 60 | 2715 | South Australia (cultured) |
| **Total** | **4** | | | |

### 3.1.6. Jurien Bay Yellowtail Kingfish Samples

Frozen YTK samples from an unsuccessful sea cage venture in Jurien Bay, WA were obtained from the Department of Agriculture and Food WA (DAFWA) Fish Health Section. The samples were collected in March 2009 and comprised of 45 fish in total. The average weight of the fish at time of collection was approximately 1000g. In 2010, a subsample of this cohort was sent by DAFWA to Dr. Robert Adlard of the Queensland Museum for formal identification of the myxospores infecting the fish.

### 3.1.7. Storage of Samples

Since collection, all samples were maintained at a temperature of -20 °C in reliable freezers. Samples for analyses were thawed on the day of examination. All care was taken whilst defrosting and preparing samples for analysis to maintain a temperature of less than 5 °C. This was monitored by taking core readings of the flesh with a hand held thermometer. Samples have been clearly labelled and are currently located at Curtin University CESSH in chest freezers.

## 3.2. Fish Measurement

Each fish collected was weighed and the fork length was measured and recorded. Fork length is the measurement from the tip of the snout to the fork of the tail fin. Additional measurements were recorded including the width of the fish at its widest point, and the height of the fish at its highest point. The length and weight were used to calculate the condition factor of each fish as adopted from Barnham et al. (1998) for salmonids. Due to the similar body shape of YTK to salmonids the following formula was used.

Condition factor (K) = $[100 \times W)/L^3]$, where W represents weight of the fish in grams and L represents the length of the fish in centimetres. The condition

index ranged from 0.9 for poor fish to values exceeding 2.5 for fish in excellent condition.

### 3.3. Rigor Index

Rigor index was performed on fish from the Harvest Trial 2 only. The method used was adapted from Iwamoto et al. (1987). Fish were placed on a flat table with the anterior portion of the fish, up to the pectoral fins, making contact with the surface of the table. The posterior of the fish overhung the edge of the table. The distance from the fork of the hanging tail to the horizontal line of the table was measured at harvest (time = 0h), and subsequent measurements were made at 2, 23 and 50 hours post-harvest. Between sampling periods, the fish were stored flat immersed in iced water.

Rigor index was calculated by using the following formula: Rigor index (%) = [D0-Dt)/D0] x 100, where D0 represents the initial pre-rigor state and Dt represents measurements at the time intervals listed above.

### 3.4. Product Temperature Logging

Thermocron temperature loggers (OnSolution Pty Ltd, Australia) were inserted into the peritoneal cavity of one randomly selected fish from each harvest treatment after bleeding (n = 3). One external logger was attached to one fish in each harvest trial to monitor the temperature of the ice slurry and internal environment of the polystyrene cartons in which the fish were packed (n = 1). Four loggers in total were used.

## 3.5. Quality/Shelf-life Assessments

### 3.5.1. Drip Loss

Three fillets of each treatment from Harvest Trial 1 (n = 9) were weighed immediately after filleting (Wi = initial weight) and weighed again on days one, five, eight and eleven (Wf = final weight). Fillets were stored at 4°C in a sealed plastic bag. Total drip loss was calculated as a percentage of the previous sample weight: Drip loss (%) = [(Wi-Wf)/Wi] x 100.

### 3.5.2. Fillet Quality

Three fillets of each treatment from Harvest Trial 1 (n = 9) were evaluated by one trained research assistant using descriptive terminology based on a fillet quality index scheme developed for Icelandic cod, *Gadus morhua* (Bonilla et al. 2007). Samples were assessed on days one, five, eight and eleven for colour/sheen, texture, colour of the blood, gaping, odour and transparency. The quality index scheme is found below in Table 3-7.

Table 3-7: Quality Index scheme developed for fresh yellowtail kingfish fillets with skin (adapted from Bonilla et al. 2007).

| Quality | Parameter | Description | Score |
|---|---|---|---|
| Skin | Brightness | Iridescent Pigmentation | 0 |
| | | Rather Dull | 1 |
| | | Dull | 2 |
| | Mucus | Uniform, thin, transparent | 0 |
| | | Little thicker, opaque | 1 |
| | | Clotted, thick, yellow | 2 |
| Flesh | Texture | Firm | 0 |

| | | |
|---|---|---|
| | Rather soft | 1 |
| | Very soft | 2 |
| Blood | Bright red, not present | 0 |
| | Dull red | 1 |
| | Shadowy, brown | 2 |
| Odour | Fresh, neutral | 0 |
| | Seaweedy, marine, grass | 1 |
| | Sour milk | 2 |
| | Acetic, ammonia | 3 |
| Colour | White, greyish | 0 |
| | Some yellowish, a little pinkish | 1 |
| | Yellow, all over pink | 2 |
| Bright | Transparent, bluish | 0 |
| | Opaque | 1 |
| | Milky | 2 |
| Gaping | No gaping, one longitudinal gaping at the neck part of fillet | 0 |
| | Slight gaping less than 25% of the fillet | 1 |
| | Slight gaping, 25 - 75% of the fillet | 2 |
| | Deep gaping or slight gaping over 75% of the fillet | 3 |
| Quality Index (0 -18) | | |

Fish from Harvest Trial 2 were kept whole for 3 days prior to being filleted. If fish were to be kept whole for an extended period of time, the quality index for yellowtail kingfish was used to quantify quality (Boulter et al. 2009). This index uses skin, eyes and gills as the main quality attributes, with a focus on parameters such as colour, appearance and odour.

### 3.5.3. Microbiology

A piece of flesh from three fillets of each treatment from Harvest Trial 1 (n = 9) were cut to 10 g, diluted 1/10 using 90ml of maximum recovery diluent (MRD) (Sigma Chemical Co.) and homogenised using a stomacher (Seward 400, UK) for one minute. Serial dilutions were then made up to $10^4$ and both the $10^2$ and $10^4$ dilutions were distributed onto Plate count agar, Iron agar and Long and Hammer agar using a spiral plater (WASP, Don Whitely Scientific, England). Samples were incubated at 30 °C for two days, 25 °C for two days and 15 °C for five days before counting.

Plate count agar was used in a standard aerobic plate count to measure the total number of organisms that form visible colonies in food, water and waste water samples. Iron agar was used to identify hydrogen sulphide producing bacteria in fish samples. Long and hammer media allows for the detection of psychrotolerant bacteria commonly found on seafood, due to its low incubation temperature and high salt content. Agar plates were purchased pre-made from the PathWest pathology lab located at Royal Perth Hospital.

## 3.6. Myxozoan Spore Counting

### 3.6.1. Preliminary Spore Counting Methods

***Squash Technique***

The squash method was initially trialled to enumerate myxospores in YTK flesh and was based on the 'Myxosporean detection in fish' manual by Burger (2010a).

A 2cm² section (1cm depth) of muscle from the dorsal posterior quarter of the fish was excised, skinned and placed onto a glass plate. The muscle fibres were teased apart using two scalpel blades and finely diced. A small volume of saline was added to moisten the muscle and dicing continued to free potential spores into the saline. A second glass plate was placed on top and gentle pressure was applied while tilting the glass plates so that the saline solution trickled into a microfuge tube. As the trickling slowed, more pressure was applied to the plates to squeeze out most of the saline into the tube. The liquid was settled in the tube for about 2 minutes, allowing the spores to sink to the bottom of the tube. A small volume (~20 µl) of supernatant was extracted from the bottom of the microfuge tube and place on a microscope slide. A glass coverslip was applied and at 400x magnification the slide was scanned for myxospores. If myxospores were detected the saline sample was frozen for further analysis (morphological and DNA).

After a number of unsuccessful attempts using the squash mount to enumerate spores due to inconsistent results and difficulty identifying spores, alternative methods such as the muscle digest and histological spore count method were investigated.

***Digest Method***

Preliminary Methods for spore counting, using the digest method, were adopted from Zhou et al. (2009) and were as follows:

Two to five grams of skeletal muscle was extracted from the shoulder of fish and diced with two scalpel blades to the consistency of a fine paste. Duplicate samples of 100mg were weighed and placed into 15ml falcon tubes. After the addition of 10 mL 0.04 % w/v trypsin (Sigma Chemical Co.) in phosphate buffered saline, pH 7.4, the samples were placed on a vortex for 20 seconds. The samples were then incubated in a shaking water bath at 37°C for 30 minutes. The tubes were then placed on a vortex for 10 seconds and placed into the shaking water bath for a further 30 minutes at 37°C. The tubes were centrifuged at 1900 RCF for 20 minutes at 4°C. The supernatant was removed with a syringe and 200 µL of phosphate buffered saline was added to the pellet before shaking and vortexing to resuspend the spores. A pipette was used to transfer 10µL of the solution into a small eppendorf tube (0.5 ml) with 4 µL of 1% Neutral Red stain and was placed in eppendorf tube mixer for 2 seconds. A haemocytometer was loaded with resuspended solution using transfer pipettes and left to sit for 30 minutes to allow the spores to settle. The spores were counted in ten random squares on the haemocytometer using a phase contrast microscope at x 10 magnification, then at x 20 magnification. Spores appear as brown pencil marks as a result of the neutral red stain. If there were more than 50 spores per 1 $mm^2$ a dilution was done. If there were less than 15 spores per 1 $mm^2$ the process was repeated with less diluent.

The formula below was used to obtain a value (spores/gram) for each fish:

$$\text{Spore Count} = \frac{\text{Average count per small square} \times 90 \times \text{Final Volume (µL)}}{\text{Initial Weight (g)}}$$

Another digest method was trialled, using pepsin in place of trypsin, which is a similar proteolytic enzyme. The pepsin digest method used 6g pepsin, 10mL concentrated HCl, 0.6ml water mixed with 100mg of the flesh sample. The

samples were then incubated at 35°C for a total of one hour. The methods were the same as above regarding centrifuging the sample, removing the pellet, resuspending the spores and using a haemocytometer to count the spores. This method was discontinued due to the inability to replicate results and problems associated with muscle digestion.

### 3.6.2. Final Histological Spore Count Method

Pieces of skeletal muscles, approximately 1cm$^3$, were cut from the left dorsal anterior quarter, above the lateral line, of each fish collected. The flesh was placed into sample jars containing 10% neutral buffered formalin and then embedded in paraffin wax using standard histological techniques. 5µm sections of flesh were mounted on glass microscopy slides, stained with Giemsa (Sigma Chemical Co.) to highlight polar capsules within myxospores, and then had coverslips applied. Each slide was observed under an Olympus BX40 compound light microscope. The numbers of plasmodia were counted on 5 random low power fields (x100) across the slide and the number of spores across 10 random plasmodia were counted at higher magnification (x400). The average number of plasmodia and the average number of spores across the plasmodia were multiplied together to obtain a value. The average numbers of granulomas were counted for each sample, to assess whether there had been an immune response by the fish to combat the parasite. This method was chosen as the final method due to the simplicity, cost effectiveness and repeatability.

## 3.7. Protease Assays

### 3.7.1. Final Protease Assay Method

Protease assays were adapted from the methods of An et al. (1994) and Zhou et al. (2009) to detect proteolytic activity in uncooked YTK flesh.

A piece of flesh (6.3g) from each YTK fillet was minced manually with razor blades. Samples of the mince were placed into 10ml polypropylene centrifuge tubes (Thermo Fisher Scientific, Australia) and 20mM phosphate buffer at pH 7 was added at a ratio of 3:1 (2.1ml). The contents of the tubes were mixed with a stainless steel rod and then placed into an IEC Centra GP8R centrifuge at 1900 RCF for 20 minutes at 4°C.

The supernatant was recovered and adjusted to pH5.5. The reaction mixture containing 0.4g azocasein (Sigma Chemical Co), 1.25 mL McIlvaine's Buffer (0.2M sodium phosphate, 0.1M sodium citrate pH 5.5) and 0.75 mL of water was incubated at 55°C for 5 min in centrifuge tubes before adding 0.5ml of the supernatant or 0.5ml of the control (20mM phosphate buffer at pH7). The samples were then incubated at 55°C for 60 minutes, whereby the enzymatic reaction was halted by the addition of 0.4ml of cold trichloroacetic acid (Sigma Chemical Co). The samples were then held at 4°C for 15 minutes, to allow precipitation of unhydrolysed proteins, and then centrifuged at 1900 RCF at 4°C, to separate the supernatant which contained hydrolysed oligopeptides.

Absorbance of the supernatant was read at 450nm (Cary-50 UV-Vis spectrophotometer, Varian Inc, USA) in standard 3.5 ml plastic cuvettes after the addition of 0.18ml of 10N NaOH. Triplicates of each fish sample were assayed and averaged to produce a single reading expressed as $A_{450}$.

### 3.7.2. Validation and Verification of Protease Assay Methods

The enzyme methods were validated and verified through experiments including dilutional linearity, co-efficient of variation and variability of the enzyme assay. Supernatant leftover from the enzyme assays had been frozen and stored at -20°C and was used in the validation and verification of the assays

**Dilutional Linearity**

The dilutional linearity of the assay was plotted by serial dilution of supernatant from fish with high infection rates of *U. seriolae*. The supernatant was diluted with phosphate buffer at rates of ½ - 1/128 of the original. These dilutions were then used in the protease assay as described in section 3.8.1. Duplicates of the samples were processed and the results were compared, averaged and plotted on a line graph.

The fish used for these assays had known high infection rates due to severe myoliquefaction in the cooking and texture trials. Highly infected fish were chosen, as fish with moderate or low infections can lead to unreliable spectrophotometer readings when diluted to the lowest concentrations. The serial dilution can also reveal the sensitivity of the spectrophotometer, revealing the limits for the lowest readable absorbance. The significance of a high $R^2$ value and linearity is to verify the specificity and accuracy of the enzyme assay (Knaide et al. 2013).

**Co-efficient of Variation**

To measure the co-efficient of variation within the assay, flesh samples with known high, medium and absence of myxozoan infection rates were chosen. Each sample was prepared for enzyme analysis, as described in section 3.8.1,

and the resulting supernatant was used to repeat the assay multiple times (n=8 to n=12). The mean, standard deviation and coefficient of variation were calculated for each sample.

To calculate the coefficient of variation (CV), the standard deviation (SD) of the readings was divided by the mean, and was expressed as a percentage. The CV allows standardisation of the SD, which can then be compared to other CVs performed at different magnitudes of analyte concentration (Reed et al. 2002). Supernatant from fish with a low infection, moderate infection and high infection were used, and the number of times the assay repeated was 8, 12 and 10 times respectively. The number of times the assay was able to be repeated was limited by the amount of supernatant that was left over from previous assays.

**Intra-specific assay variability**

To measure the variation within each sample, fillets with known absence of infection and high infection were chosen for the protease assay. Seven sites on the fillet were chosen for enzyme analysis and the co-efficients of variation were compared (Figure 3.3). The enzyme absorbance between the anterior/posterior and the dorsal/ventral sections of the fillet were also compared.

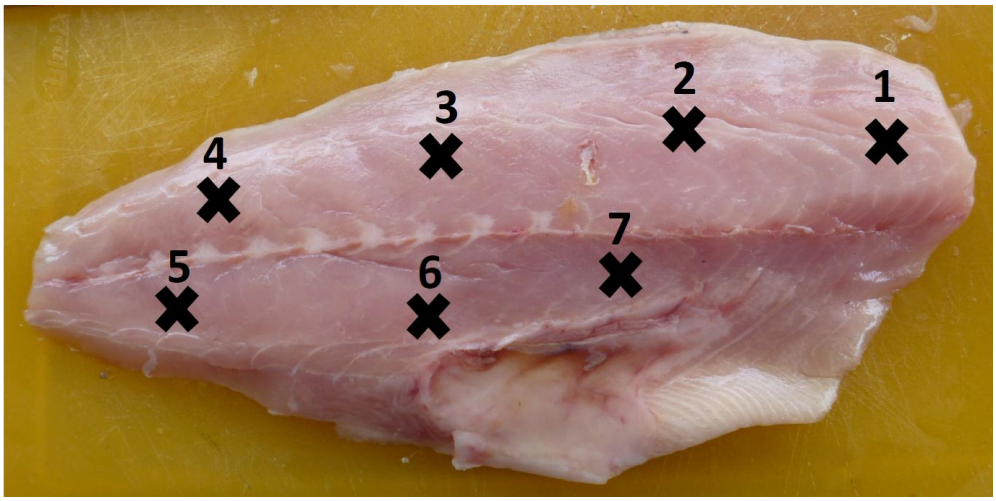



Figure 3.3: The position of the seven flesh samples taken from YTK fillets to measure the variability of enzyme activity across the fillets.

A total of four fillets from four different fish were used for the analyses. The samples comprised of two fish with low/no infections and two fish with high levels of infection. The infections rates were known to be high or low through previous cooking and texture experiments. The fish stated as having low/no infection had very low levels of myxospores and showed no signs of myoliquefaction during cooking, very firm texture and histology revealed that plasmodia containing *U. seriolae* were very sparse and seldom present in the samples.

**Enzyme Characterisation**

Characterisation of the enzyme (optimal pH and temperature) was not investigated. Based on existing literature, it was assumed that the enzyme found in *U. seriolae* is similar to that present in *Kudoa spp.* as identified in previous studies (An et al. 1994, Seymour et al. 1994, Zhou et al. 2009). The assay performed in this study was adequate for the purpose intended.

### 3.7.3. Investigation into Alternative Enzyme Activity Enumeration Methods

A brief investigation was undertaken into alternative methods for enumerating enzymatic activity. Methods investigated included a protein assay, and two methods using a refractometer to measure the amount of protein present in each sample. The purpose of undertaking these alternative methods were to seek a quick and easy field test that could be used on a fish farm, without the need for complex procedures and equipment. Refractometer method 1 and 2 were developed by the author of this thesis based on the theory that heated flesh containing parasites will undergo protein degradation. It is known that muscle containing high levels of parasites contain high levels of proteolytic enzyme. At the optimal temperature, due to high enzymatic activity, protein will be released into solution. Therefore higher parasite loads equate to higher protein readings. The refractometer is traditionally used to measure the specific gravity of plasma protein concentration (Calloway et al. 2002).

A total of eleven fish were used in this investigation into alternative enzyme/protein assay methods. Ten fish from the same cohort, harvested in June 2012, were used. These fish had varying degrees of *Unicapsula seriolae* infection from low to severe. A wild fish was used as the control and contained no myxozoan infection. A brief description of the three alternative methods is provided below.

**Protein assay**

This method was used to detect the amount of protein in a 250mg flesh sample after incubation at 55°C for 30 minutes in a water bath. One ml of phosphate buffer was mixed with the minced flesh prior to incubation. It was hypothesised that ‘free’ protein resulting from enzymatic breakdown of the muscle upon heating will be present in the sample. The samples with higher

infections of *Unicapsula seriolae* will release more protein into the supernatant and result in higher readings. The protein assay was undertaken at the Department of Agriculture and Fisheries WA (DAFWA) biochemistry department using standard protocols. The supernatant was measured in a spectrophotometer and the total protein levels are expressed in grams per litre. One of the issues with this assay is that the dynamic range of measurement is 30 – 120g/L. The highest recorded protein level in the YTK flesh was 7.9g/L, so sensitivity and accuracy was an issue with using this particular assay.

**Refractometer Method #1**

250mg of minced flesh was mixed with 1ml of phosphate buffer, and incubated at 55°C for 10 minutes in a water bath. After incubation, the samples were vortexed and centrifuged. A drop of supernatant was placed onto a refractometer and the specific gravity was recorded as g/100ml of serum protein. Minimal differences in protein levels were found between samples. Sensitivity may have decreased due to the amount of phosphate buffer and the short time of incubation. The samples may not have been adequately heated in the water bath to activate the enzymes within the myxospores and minimal breakdown of protein occurred.

**Refractometer Method #2**

The issues that arose in refractometer method #1 were rectified by decreasing the amount of buffer to 0.5ml, and increasing the incubation time to 30 minutes. The amount of flesh used was 250mg. This ensured that samples were heated thoroughly and allowed myoliquefaction in samples containing myxospores. The samples were centrifuged and a drop of supernatant was placed onto a refractometer and the specific gravity was recorded as g/100ml of serum protein.

## 3.8. Cooking and Texture Analysis

### 3.8.1. Preliminary Cooking and Texture Method 1

The first method trialled for cooking and texture analysis were adapted from Zhou et al. (2009) are as follows:

Skeletal muscle was extracted from the shoulder of the fish and minced with scalpels to the consistency of a fine paste. 3.6g of flesh was packed into a plastic tube (diameter 15 mm/ length 18 mm), wrapped with food-grade polyethylene film and tied off at each end. The tubes containing fish mince were pre-incubated in a water bath at 52 °C for 15 min. Upon completion of pre-incubation, the tubes were cooked for a further five minutes in boiling water. The tubes were then placed into ice slurry for five minutes and then left to further cool at ambient temperature for 15 -20 minutes. The plastic wrap was removed and a syringe plunger was used to extract the cooked flesh from the plastic tube. The cylinder of flesh was cut in half and objective assessment of the texture was undertaken using a TA-XT2i Texture Analyser (Stable Micro Systems, Haslemere, UK) which was interfaced to a computer. The meat tested was compressed by 7mm with an aluminium cylinder probe (diameter 5mm) with a test speed of 1 mm / sec and a 5kg load cell. Two tests were performed for each piece and the results were averaged to obtain a value, expressed as maximum compression force in grams.

### 3.8.2. Preliminary Cooking and Texture Method 2

Based on the results achieved with preliminary method 1 (section 3.10.1) the second method was trialled, in which pieces of flesh (rather than minced flesh) were used. The most common cooking methods in Australia for YTK are frying, baking and slow cooking and by keeping the flesh as a whole piece, it replicates the way it is cooked in commercial and residential kitchens.

Small squares of flesh (5cm² x 1cm thickness) were cut from each YTK fillet. The flesh was placed into a snap lock bag, air excluded, with a string and tag attached for identification.

Cooking parameters and times were trialled and ten samples were simultaneously placed into a water bath to:

1. Pre-incubate at 52 °C for 15 minutes and cooked at 65 °C for 5 minutes
2. Cook at 52 °C for 15 minutes
3. Cook at 52 °C for 10 minutes
4. Cook at 52 °C for 5 minutes

Objective assessment of the cooked flesh texture was undertaken using a TA-XT2i Texture Analyser (Stable Micro Systems, Haslemere, UK) which was interfaced to a computer. The meat tested was compressed by 50 % with a Warner Bratzler blade with a test speed of 1 mm / sec and a 5kg load cell. Two tests were performed for each piece and the results were averaged to obtain a value, expressed as maximum compression force in grams.

### 3.8.3. Preliminary Cooking and Texture Method 3

A standardised cooking and assessment method was developed from the two previous methods described above.

A 40mm diameter circular plastic attachment was trialled initially but due to some inconsistencies in the samples, such as piece size, angle of the cut surface of the flesh and breakage of the cooked flesh, it was found that a smaller probe was necessary and a 10mm diameter cylindrical attachment was used. The load on the texture machine was initially 25kg, which was then changed to a 5kg load after a few samples were tested, as the highest compression force encountered was approximately 2700g. Other parameters included a 30% travel into the sample, which was then changed to 50%. The speed of the probe into the samples was 1mm/second. The aim is to simulate

a human bite into a piece of cooked flesh and measure the hardness and resistance of the flesh in grams of maximum compression force.

### 3.8.4. Final Cooking and Texture Method

The methods were changed to cook smaller batches of fish (n=10) at a time and to immediately conduct a subjective assessment of the texture and then a measurement with the texture analyser. This prevented further degradation of the samples and simulated a realistic situation where the fish is consumed shortly after cooking. The time elapsed from finishing the cook to objective texture analysis ranged from 8 minutes to 25 minutes.

All pin bones and rib bones were removed from the flesh, as well as blood lines as the cooking trials was concentrated on the edible portions of the fish. Cooking temperatures were raised from 52°C to 55°C, as literature suggested that this was the optimal temperature for the highest proteolytic activity in myxozoans such as *Kudoa thyrsites* (Samaranayaka et al. 2008).

Small squares of flesh (5cm x 5cm x 1cm) were cut from each YTK fillet, after a period of frozen storage, and the weight was recorded. The flesh was placed into a snap lock bag (air excluded) with a string and tag attached for identification. Ten samples were simultaneously placed into a water bath set to 55°C for 10 minutes. Internal temperature of one piece per cook was taken immediately after cooking with a hand held thermometer.

After cooking, the flesh was removed from the bag and a knife was used to apply pressure on the corner of the flesh to test for flaking. A subjective texture scale based on (Patashnik et al. 1982) was developed to categorise the texture of each fish. See Table 3-8 for subjective texture assessment.

Objective assessment of the cooked flesh texture was undertaken using a TA-XT2i Texture Analyser (Stable Micro Systems, Haslemere, UK) which was interfaced to a computer. The meat tested was compressed by 50 % with an

aluminium cylinder probe (diameter 10mm) with a test speed of 1 mm / sec and a 5kg load cell. Two tests were performed for each piece and the results were averaged to obtain a value, expressed in grams of maximum compression force.

Table 3-8: Subjective texture scale used for cooked YTK flesh, based on Patashnik (1982).

| Score | Texture of Cooked Flesh | Appearance | Edibility | Example |
|---|---|---|---|---|
| 1 | Firm – Normal | Myotomes well defined. Will flake easily. | Good to eat.<br>Excellent texture.<br>"Normal" |  |
| 2 | Normal – Soft | Myotomes defined. Can flake although some softness. | Good to eat.<br>Slightly soft |  |
| 3 | Soft | Myotomes disappear when pressure is applied. Flaking apparent in some sections, mushy in others. | Poor texture.<br>Unacceptable to eat. |  |

| Score | Texture of Cooked Flesh | Appearance | Edibility | Example |
|---|---|---|---|---|
| 4 | Overly Soft | Myotomes disappear upon light pressure, although shape is still held. No flaking. | Unacceptable to eat. |  |
| 5 | Mushy/Pasty | Myotomes indistinguishable. No form. Pasty consistency. | Unacceptable to eat. |  |

### 3.9. Water Temperature Profile of Geraldton, Western Australia

Throughout the YTK grow out period in Geraldton, water temperature data was collected from a variety of sources including the Geraldton Port Authority (GPA) Seaview webpage, HOBO loggers attached directly to the sea cage and from IOFA staff with a hand held Oxyguard dissolved oxygen meter, which also measured temperature. Temperature data was collected to identify if there is any correlation between water temperature and severity of myxozoan infection.

## 3.10. Data analysis

Statistical analyses were performed in IBM SPSS Statistics 19 for Windows (SPSS Inc.), JMP 11.0 (SAS Inc.) and R (Development Core Team 2013). Prior to any analyses, data were analysed for normality and homogeneity of variances and transformed if necessary or, if transformation did not normalise the data, then non-parametric statistics were used. A significance level of 0.05 was used for all analyses.

### 3.12.1 Parasite prevalence

The prevalence of *Unicapsula seriolae* in fish populations was expressed as the proportion of samples with positive spore counts; with 95% confidence intervals calculated assuming a binomial distribution (Rózsa et al. 2000). Differences in prevalence between different populations of fish were tested using the Fisher exact test.

### 3.12.2 Univariate analyses

Means for continuous variables were compared among groups by one-way analysis of variance (ANOVA), followed by Tukey's HSD post-hoc test when significant difference between treatments were identified. Relationships among continuous variables were compared by Pearson product-moment correlation, Spearman's rank correlation or linear regression, as appropriate.

### 3.12.3 Multivariate analyses

Correlations among predictor variables that were considered to influence flesh texture were examined by principal components analysis (PCA) of the correlation matrix. After initial extraction, factors with eigenvalues greater

than 1 were retained and rotated to simple structure using the varimax criterion. Factor scores were related to flesh texture scores using a general linear model (GLM) approach. The full model using all three factors was fitted and the statistical significance of each factor tested by the difference in log-likelihoods of the full model and the model without the effect. Because GLM results will be influenced by collinearity among factor scores, a hierarchical partitioning approach was used to determine variable importance. $R^2$ values were hierarchically partitioned to determine the proportion of variance explained independently and jointly by each factor (Chevan et al. 1991, Mac Nally 2002), and the independent effect of each factor was calculated as the percentage of total independent contributions, using the R package hier.part (Walsh et al. 2013). The significance of independent effects was assessed with Z scores from a comparison of observed effects with those obtained after 500 randomisations of the data, using hier.part.

# CHAPTER 4. RESULTS

## 4.1. Confirmation of *Unicapsula seriolae* in WA YTK

Histological slides of YTK flesh showed myxospores were morphologically similar to *U. seriolae*, as described by Lester (1982). In 2010, flesh samples from Western Kingfish Limited, Jurien Bay, were sent by DAWFA to Dr. Robert Adlard of the Queensland Museum for formal myxospore identification and comparison with other *Unicapsula spp.* collected from Australia (refer to section 3.1.6 Materials and Methods). Subsequently, a scientific article was published in 2013 which confirmed that the myxozoan species infecting YTK was *U. seriolae* (Miller et al. 2013), and is presumed to be the same species infecting YTK from Geraldton, WA.

## 4.2. Validation and Optimisation of Methods

This section outlines the results of the preliminary methods. The first attempts at analyses were performed on fish from Harvest Trial One and it was discovered that due to the low severity of infection, the methods for detecting and enumerating *U. seriolae* appeared inadequate. Only when analysing fish from infected wild samples or Harvest Trial Two, it was discovered that the very high severity of the parasite allowed the methods to reveal their effectiveness.

### 4.2.1. Preliminary Spore Counting Trials

***Squash Technique***

The squash method was found to be inaccurate and unable to be repeated with consistent results. Inaccuracies may have arisen from the initial mincing, counting and variations in the amount of flesh used. All spores within the flesh may not have been released upon mincing and varied greatly within samples.

***Digest Method***

The digest method for counting of *U. seriolae* spores was time consuming and inaccurate. The counts were unable to be accurately repeated, the methods and equipment settings varied between researchers and effectiveness of the enzyme used to digest the flesh was questionable.

The microscope settings were unable to be standardised for each counting session. This was due to the microscope sustaining heavy use by the Department of Agriculture Parasitology Unit when not in use for this study. The settings on the phase contrast microscope could not be replicated for optimal viewing, even with the assistance of experienced users.

The optimal temperature for highest activity in trypsin is 37°C (Promega-Corporation 2013). It was also essential to incubate the samples containing trypsin at a lower temperature than the optimal activity temperature of the active enzyme, Cathepsin-L, found within the *U. seriolae* spores. Cathepsin-L is most active between 52° – 55°C (Samaranayaka et al. 2008). If the temperature of the incubation bath reached these temperatures, the spores would most likely rupture, leading to inaccurate spore counts. The temperatures of the water baths in the initial trials were not recorded.

When the histological spore counting method was trialled, it was compared with the spore counting using the digest method. There was no significant correlation between the spore counts using the digest method and the final histological counting method ($R^2 = 0.0068$; $P = 1.598$; $n = 23$). The differences were due to the inaccuracy of the digest methods, as replicated counts often varied greatly.

***Determination and Validation of Final Spore Counting Method***

The histological mounting and staining of the flesh with Giemsa was a more time efficient, accurate and cost effective method of counting spores. Using Giemsa stain, the polar capsules of *U. seriolae* are stained blue and the muscle tissue is stained pink, highlighting a clear contrast between the host tissue and the parasite. Stained sections of normal muscle and infected muscle are shown in Figure 4.1 and Figure 4.2.

The histological method of counting plasmodia and *U. seriolae* spores in Giemsa stained muscle tissue was easier and provided more reliable and repeatable results than the methods described previously. All subsequent spore count data refers to only to those obtained via histology.

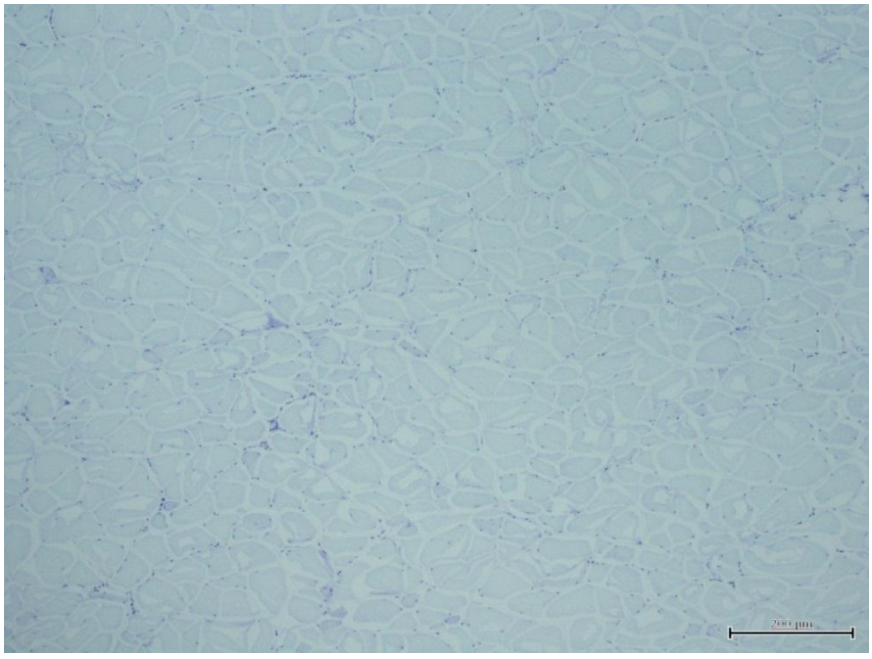


Figure 4.1: An example of a cross section of normal fish muscle (x100 magnification) with no myxozoan infection, stained with Giemsa. The scale bar is 200µm.

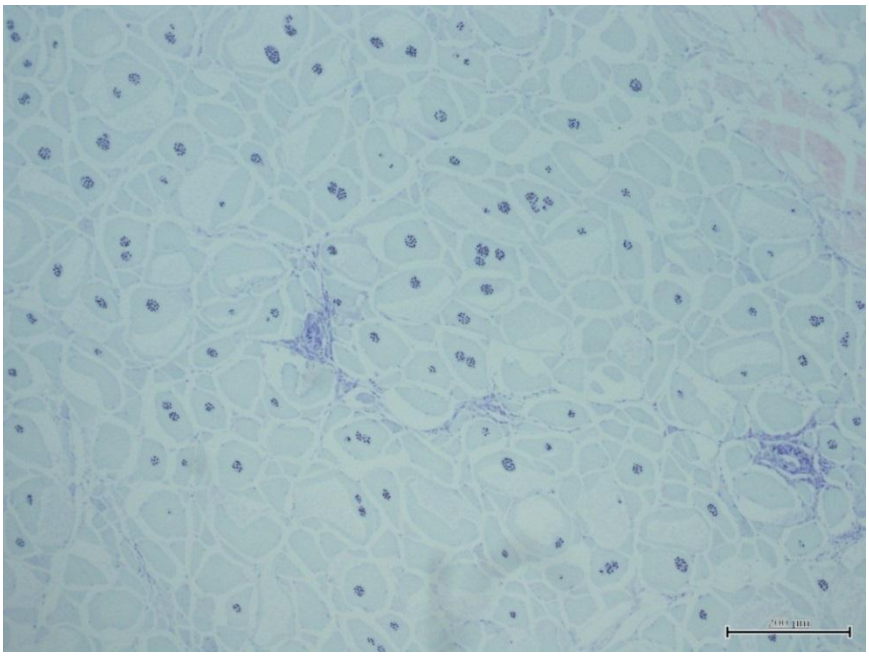


Figure 4.2: An example of a cross section of YTK muscle (x100 magnification) with a severe *U. seriolae* infection, stained with Giemsa. The scale bar is 200µm.

The immune responses of the fish were not taken into account when calculating the final number of spores (Figure 4.3 and Figure 4.4). Multiple plasmodia per muscle cell were observed in severely infected fish (Figure 4.5).

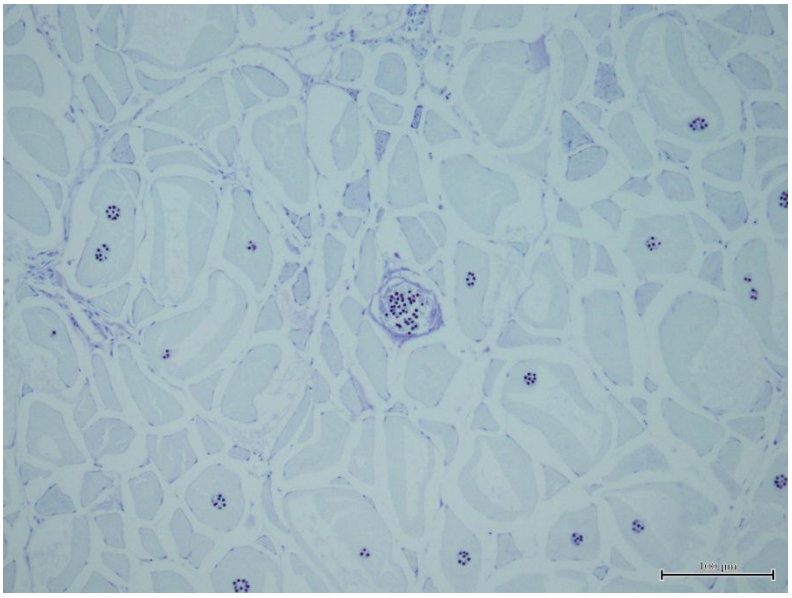


Figure 4.3: Granuloma formed around infected muscle cell. (Giemsa stain. x200. Scale Bar 100µm)

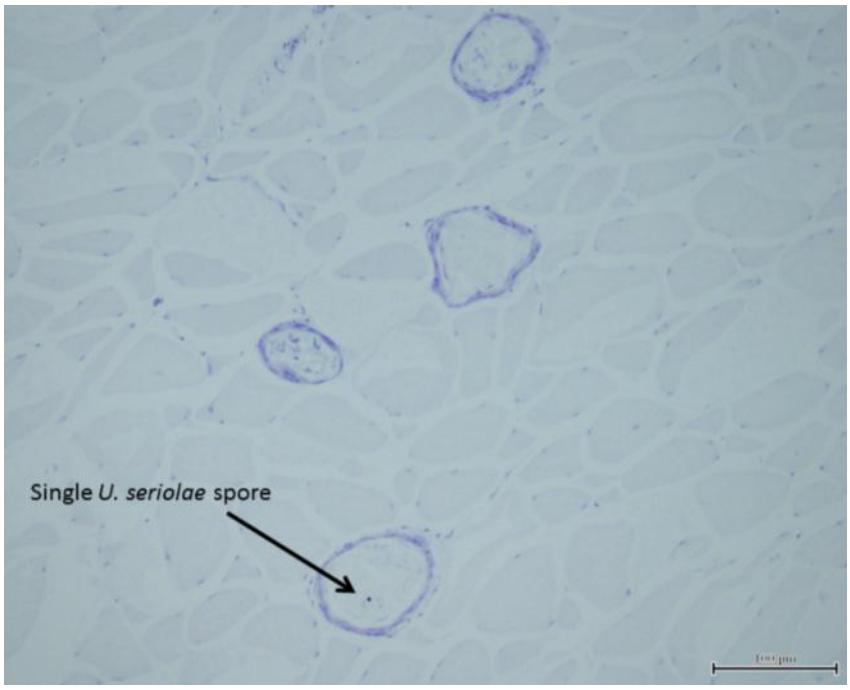



Figure 4.4: Multiple granulomas leaving no trace of *U. seriolae*, apart from a lone myxospore. (Giemsa stain. x200. Scale Bar 100µm).

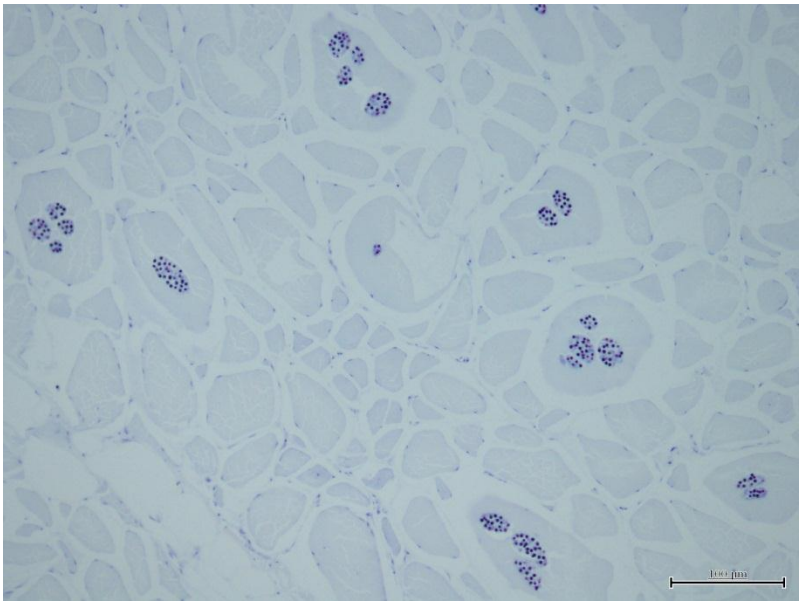


Figure 4.5: Multiple *U. seriolae* plasmodia per muscle fibre (Giemsa stain. x200. Scale Bar 100µm)

### 4.2.2. Preliminary Cooking and Texture Trials

***Preliminary Method 1***

This method involved mincing raw flesh, packing into plastic tubes and then wrapping in polyethylene film. The tubes were then pre-incubated at 52 °C for 15 min and cooked for five minutes in boiling water.

The 3.6g of flesh that was packed into the plastic tube did not fit properly prior to cooking, and during cooking the sample expanded and overflowed out of the sides of the tubing. The flesh after cooking was crumbly, contained gaps and was unable to retain its shape. When placed under the texture analyser, the section penetrated by the probe was not always flat, due to the sample seeping out the side before cooking. If the texture analyser first makes contact with an uneven surface, the reading is usually higher than if it were to hit a flat surface.

Each cooked cylinder of flesh was then cut in half (two 9mm sections) and the exposed face was subject to compression of 7mm by the texture analyser. This only allowed for a 2mm gap of flesh between the probe and the platform of the texture analyser. If the samples were not cut exactly into 9mm sections, then the probe would touch the platform and give erroneously high readings.

Consequently, the length of the tubing was increased to 20 mm in further trials, with the same result. No significant differences could be found between samples (oneway ANOVA, $F = 47.6$, $P = 0.976$, $n = 12$) of varying infection rates.

This cooking method was adopted from researchers investigating enzyme activity in fish that was destined to become surimi (Samaranayaka et al. 2007), a seafood analogue composed of low value fish products. These methods for processing the fish into a mince was not relevant to the context of the YTK in this project, which is predominantly used for sashimi and cooking as a fresh, unprocessed product.

***Preliminary Method 2***

Method number 3, cooking for 10 minutes at 52°C, resulted in the best suited flesh texture for analysis – neither undercooked or overcooked. Cooking methods one and two resulted in overcooked flesh and cooking method four was undercooked.

***Preliminary Method 3***

Based on preliminary method two, cooking flesh for 10 minutes at 52°C was found to be most effective. The experiment intended to replicate cooking in a commercial or residential setting, and it is very unlikely that fresh cooked fish will be consumed several hours post cooking, so texture analyses occurred as soon as possible after cooking. Cooked flesh samples with moderate *U. seriolae* infections degraded over time, leading to a softer overall texture. Leaving the flesh too long prior to texture analysis prompted the final method which changed to immediate analysis of the flesh after cooking.

***Determination of Final Cooking and Texture Method***

This method was simple, repeatable and consistent results were obtained. The cooking temperature was raised to 55°C for 10 minutes to represent the worst case cooking scenario which clearly revealed the effects of the parasite. All subsequent texture data refers to only to those obtained via the final method, as outlined in methods section 3.10.4.

### 4.2.1. Validation and Verification of Enzyme Assay

***Serial Dilution***

Serial dilution was attempted twice. The first attempt was only diluted to 1/32 of the original supernatant and the relationship with absorbance showed an $R^2$ value of 0.9481 (y = -0.2022x + 1.39). The $R^2$ value increased to 0.9927 (y = -0.1963x + 1.33) if an outlier at ¼ dilution were removed from the data set, showing that the assay is highly accurate.

In the second attempt at investigating the dilutional linearity of the enzyme assay, the supernatant was diluted up to 1/128 strength. The $R^2$ value was 0.9634 (y = -0.1186x + 0.98), but was raised to 0.9817 (y = -0.1199x + 0.97) with the main outlier at 1/16 dilution removed.

***Coefficient of Variation***

In terms of enzymatic activity, the lower the absorbance values, the higher the coefficient of variation. In the cases of fish with low or moderate infections, the enzyme absorbance readings were lower. Very low readings were more sensitive to subtle differences in absorbance, which caused the higher variability. The co-efficient of variation for low and moderately infected fish was 25% and 24% respectively. On the other hand, the fish with a high infection and high enzyme activity showed a lower variability at 7%. Figure 4.6 shows that despite the larger apparent individual variations in the absorbance, the overall variation was lower in the high infection fish than the subtle differences found in the low and moderately infected fish.

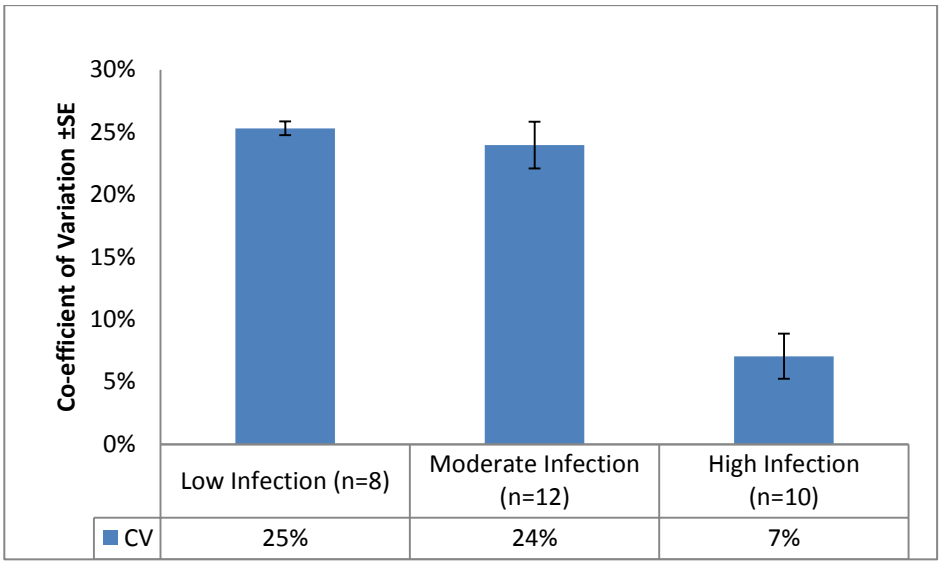



Figure 4.6: The results of the investigation into the coefficient of variation in absorbance readings fish with low, moderate and high infections. Variability decreased with increasing infection rate.

***Intraspecific Variability of Enzyme Assay***

The absorbance readings from all fish revealed that there was minimal difference between enzyme absorbance readings between the anterior (positions 1, 2 and 7) and the posterior (positions 3 – 6) of the fillet (n = 4), with respective values of 0.19 and 0.18. There was also minimal difference in the dorsal (positions 1 -4) and ventral positions (positions 5 – 7) of the fillets (n = 4), having values of 0.18 and 0.20 respectively. The absorbance values are shown in Figure 4.7. These results demonstrate that infections at moderate levels may be uniformly distributed throughout the flesh; although more samples are needed to strengthen the evidence for the basis of this statement.

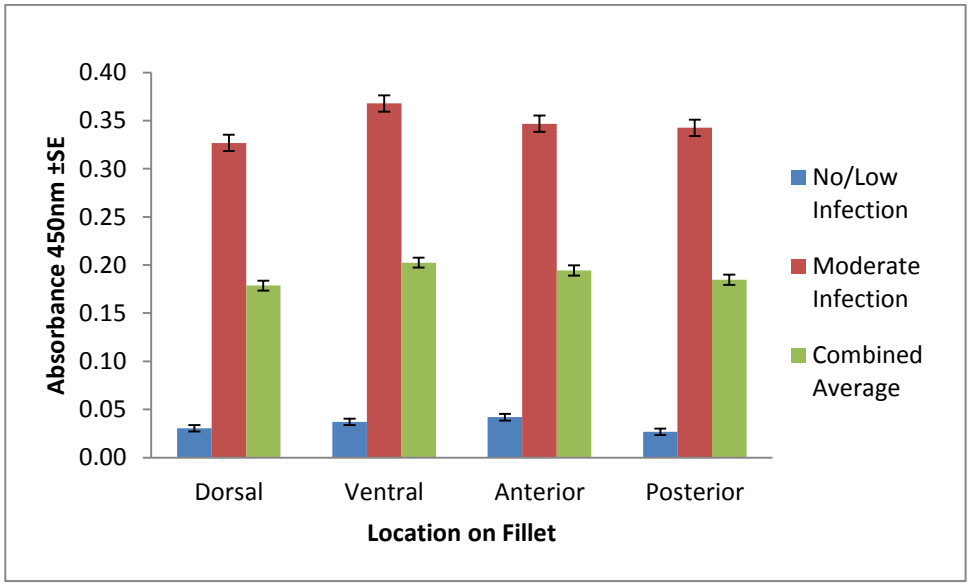



Figure 4.7: The differences in enzyme activity between the different locations on the fillet were negligible.

The infection appeared to be evenly distributed throughout the fish fillet, as demonstrated by the intraspecific enzyme assay. This confirms that the sampling position for histology, located on the dorsal anterior quarter of the fish, is a reliable indicator of overall infection levels of *U. seriolae* throughout the fish.

The mean absorbances in the two fish with no/low infections were 0.029 and 0.037, which is around the lower detectable limits of the assay method used. This is explained further in the dilutional linearity section. The moderately infected fish had absorbance values over ten times the uninfected fish. The combined coefficient of variation (CV) for the moderately infected fish was 31%, whereas the CV for no/low infected fish was 64%. The combined mean and variability in absorbance values for low and moderately infected fish are shown in Table 4-1.

Table 4-1: The mean, standard deviation and coefficient of variation in enzyme absorbance in low and moderately infected fish.

| | **Infection Intensity** | |
|---|---|---|
| **Parameters** | **Low (n=2)** | **Moderate (n=2)** |
| Mean Enzyme Abs (±SE) | 0.033 ± 0.02 | 0.344 ± 0.03 |
| St. Dev. | 0.02 | 0.11 |
| CV (%) | 64 | 31 |

***Alternative Enzyme Enumeration Results***

Upon investigating alternative methods for enumerating *U. seriolae* infections in YTK, the results of each method were compared by Pearson product-moment correlations against the enzyme assay Table 4-2. The highest correlation with the standard enzyme assay was in refractometer method two ($R^2 = 0.65$, $P = 0.003$, $n = 11$), followed by the significant but weakly correlated protein assay ($R^2 = 0.40$, $P = 0.036$, $n = 11$) and refractometer method one was insignificant ($R^2 = 0.16$, $P = 0.228$, $n = 11$).

Table 4-2: Regression analyses results of alternative enumeration methods, compared against the proteolytic enzyme assay.

| Method | $R^2$ Value | Regression Equation | N |
|---|---|---|---|
| Protein Assay | 0.40* | y = 0.1187x + 0.014 | 11 |
| Refractometer Method 1 | 0.16 | y = 0.8856x - 0.943 | 11 |
| Refractometer Method 2 | 0.65* | y = 0.4624x - 0.940 | 11 |

*Data displaying * are statistically significant ($P < 0.05$).*

## 4.3. Prevalence of *Unicapsula seriolae* in YTK Samples

If spores were present, even in very low numbers, fish were classified as infected. Control YTK collected from South Australia and ACAAR, Fremantle had no detectable myxospores in the flesh, but prevalence of infection was high in wild fish collected around Geraldton and in both cultured cohorts of YTK (Table 4-3).

Table 4-3: Infection levels and Intensity of *U. seriolae* assuming uninfected fish have spore count = 0 and infected fish have spore count = 1.

| Cohort | N | Prevalence | 95% CI |
|---|---|---|---|
| Wild YTK | 10 | 0.80 | 0.45 - 0.96 |
| Cohort 1 (2010) | 32 | 0.97 | 0.83 - 1.00 |
| Cohort 2 (2011) | 84 | 0.96 | 0.90 - 0.99 |
| All cultured (Cohort 1 & 2 combined) | 116 | 0.97 | 0.92 - 1.00 |

There was no significant difference in sample prevalence between wild fish and all cultured fish (Fisher exact test, P = 0.07) or between cohort one and cohort two cultured fish (Fisher exact test, P = 1.00), meaning that almost all wild and cultured fish were infected with *U. seriolae.*

YTK from the Jurien Bay operation, Western Kingfish Limited (WKL), were previously examined histologically by DAFWA in 2009; however, the results are not included in these analyses. The reason is due to differences in the previous DAFWA histological counting technique to the method used in this project, and time constraints preventing recounting the histological slides. The

data did provide information on the prevalence of *U. seriolae* in these YTK, which was 75% from a sample size of 44 fish.

### 4.4. Spore Counts

Total spore counts, when pooled, were much lower in cohort one than cohort two fish, with a mean spore count of 9.1 (n=32, SD=14) and 73 (n=85, SD=104) respectively (Figure 4.8). There was a significant difference in mean spore count levels among wild, cultured cohort one and cultured cohort two fish ($P < 0.001$),

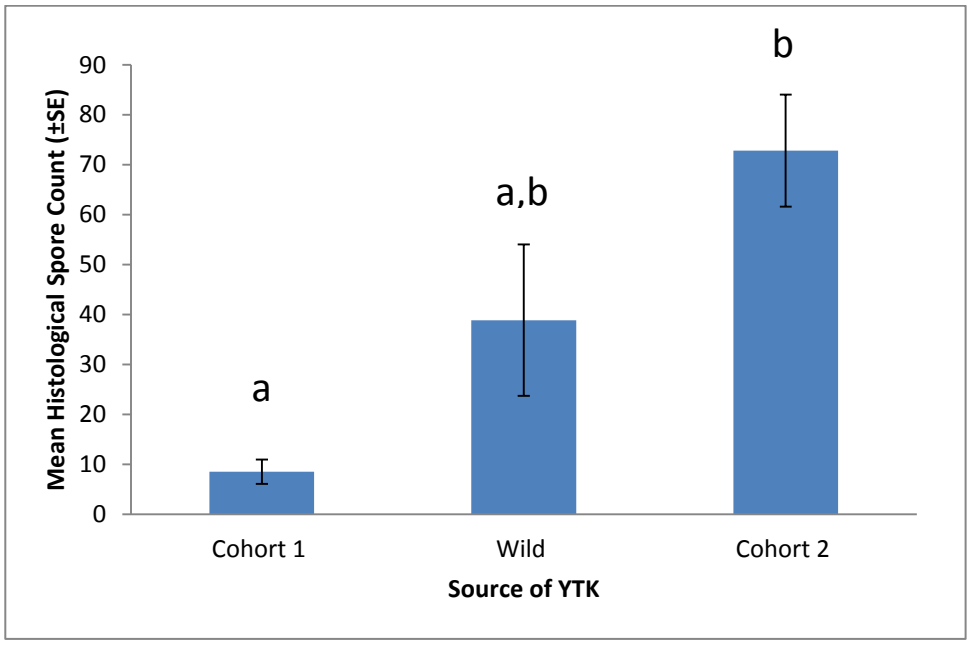



Figure 4.8: Comparison of mean histological spore counts from cultured and wild YTK. Data are means ± SE and columns sharing the same letter are not significantly different by Tukey's HSD test.

The highest spore count in cohort one fish was 53, whereas the highest count in cohort two fish was 488, which was collected during the growout period in the monthly sampling. The absence of an immune response was 60% in Harvest Trial Two fish (n=60) and 80% in Harvest Trial One fish (n=15).

## 4.5. Cooking and Texture Analysis Results

### 4.5.1. Pooled Texture Results

The results of the cooking and texture analyses are based on the final method, as outlined in methods section 3.10.4.

***Correlation between Subjective and Objective Texture Results***

When all samples (wild, cultured and harvest trials and control fish) were pooled, the subjective and objective texture analyses had a significant correlation (Spearman's non-parametric correlation; $F = -0.90$, $P < 0.001$, $n = 216$). This reveals that the texture score (objective assessment) can be used as a reliable measure of flesh texture, when the samples are cooked using the final method.

Objective texture scores differed significantly among subjective texture groups (one-way ANOVA, $F = 89.2$, $P < 0.001$, $n = 216$). The groups that could not be significantly differentiated were a score of 4 (overly soft) or 5 (mushy/pasty) (Figure 4.9).

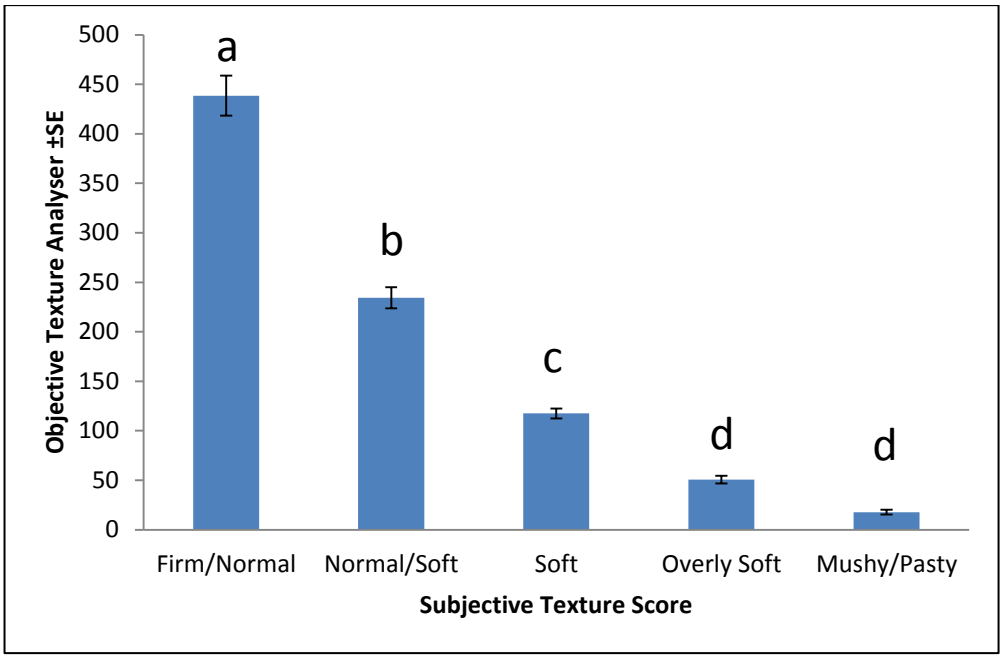



Figure 4.9: The relationship between the subjective texture scores and TA-XT2 texture analyser results (n=216). Data are means ± SE and columns sharing the same letter are not significantly different.

***Texture and Edibility***

Based on the subjective texture analyses, subjective texture scores of 1 and 2 was acceptable for consumption, a score of 3 was border line and scores of 4 and 5 were unacceptable to eat.

The breakdown of subjective scores into suitability for eating shows that 89% of fish in cohort one, fall within categories one (firm/normal texture) and two (normal/soft texture) and are deemed acceptable to eat. 9% had borderline texture and 2% were inedible. No incidences of complete myoliquefaction were encountered in cohort one fish (Figure 4.10).

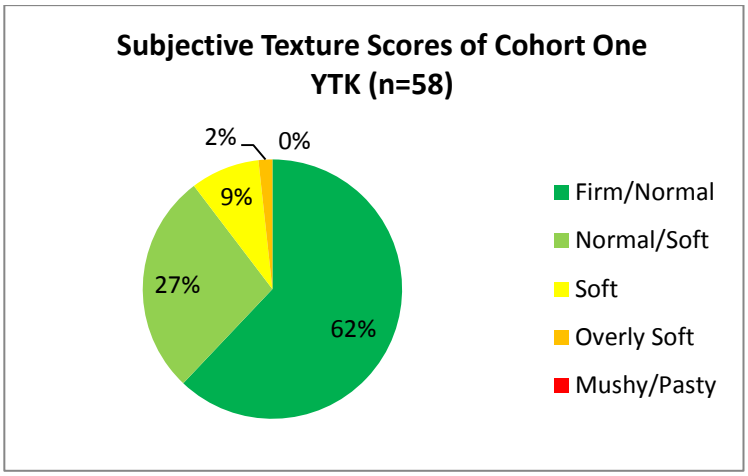



Figure 4.10: Breakdown of subjective texture scores from cohort one YTK.

Out of 151 fish from cohort two, 34% of fish were found within categories one and two, and classified as acceptable to eat. The majority of fish were in category three (44%), borderline soft and 22% (category 4 and 5) were considered unacceptable to be eaten. Of these unacceptable fish, 7% had a subjective texture score of 5, showing signs of complete myoliquefaction (Figure 4.11).

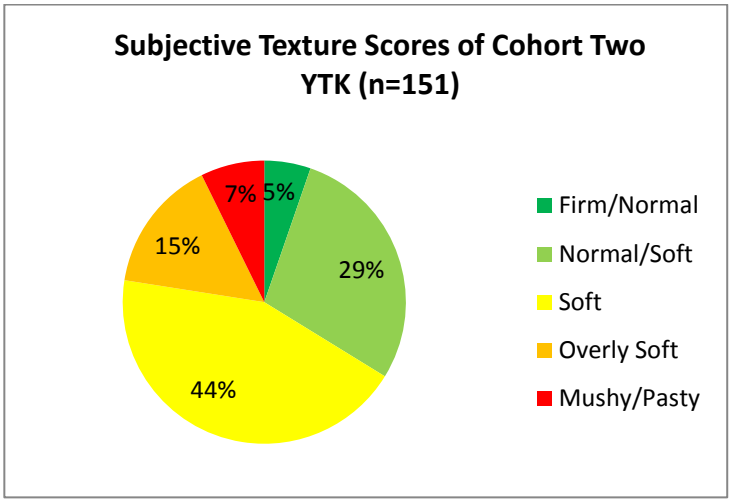



Figure 4.11: Breakdown of subjective texture scores from cohort two YTK.

62% of wild collected YTK fell within categories one and two and displayed firm flesh. 23% had borderline texture in category three and 15% were

unacceptable in category four. No complete myoliquefaction was observed in any wild fish (Figure 4.12).

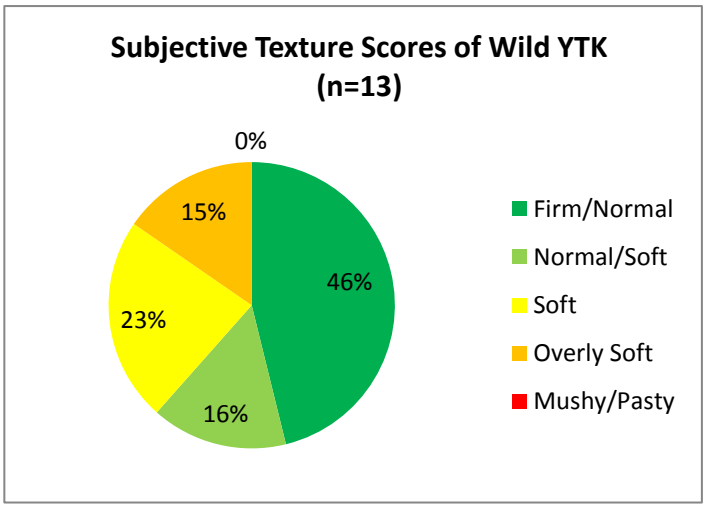



Figure 4.12: Breakdown of subjective texture scores from wild YTK

### 4.5.2. Raw Myoliquefaction

Out of all the raw flesh samples prepared for cooking (n=216), one sample displayed myoliquefaction upon defrosting. Of the two amberjack (*Seriola dumerili*) samples from the Abrolhos Islands, one had partially liquefied flesh when thawed at room temperature (Figure 4.13). The cooked texture of this fish had a subjective score of 4 and objective score of 50g of maximum compression force, but did not experience full myoliquefaction. The other amberjack contained no infection and scored 2 in the subjective assessment and 470g of force in the objective assessment. It was assumed that this fish had infection by a *Kudoa spp*., due to its tendency to liquefy flesh at low temperatures, but histology revealed that only *U. seriolae* was present in the flesh.

Figure 4.13: Amberjack flesh displaying myoliquefaction in the raw state after defrosting (right).

## 4.6. Enzyme Analysis Results

### 4.6.1. Enzyme Assay Results

All samples, including fish with no infection on the basis of histological spore counts, had enzymatic activity that was detected in the enzyme assay. Infected fish had significantly higher enzyme absorbance levels than uninfected fish (0.31 ± SE 0.03 compared with 0.07 ± SE 0.01; one-way ANOVA, F =7.840, P = 0.006, N = 121). In infected fish, there was a significant positive relationship between enzyme absorbance levels and spore count (Figure 4.14; linear regression analysis; $R^2$ = 0.76, F = 325.48, P <0.0001, n=112).

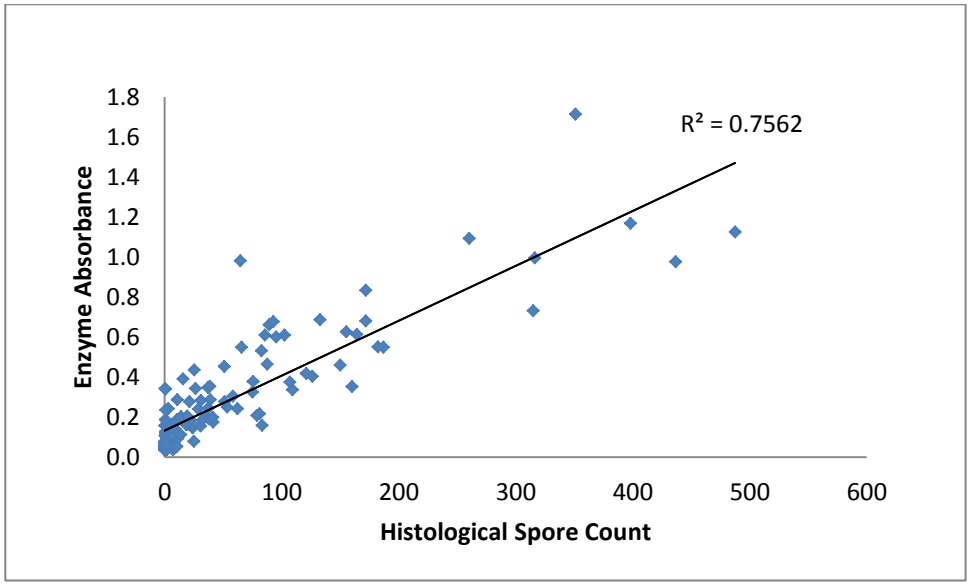



Figure 4.14: Bivariate fit of enzyme analysis by spore count.

## 4.7. Relationship between Fish Morphology, Environmental Factors, Spore Counts, Enzyme Levels and Texture

Hypothesised relationships between fish morphology, environmental factors, *Unicapsula* spore counts and flesh texture in YTK are shown in Figure 4.15 were confirmed in this study. These relationships were investigated in those fish that were not subjected to experimental harvest treatments (i.e. wild fish and cultured fish sampled prior to harvest), using texture analyser and subjective texture scores as response variables, and fish weight, fish length, fish condition score, fish age, days at sea, water temperature, histological spore count and enzyme level as predictor variables.

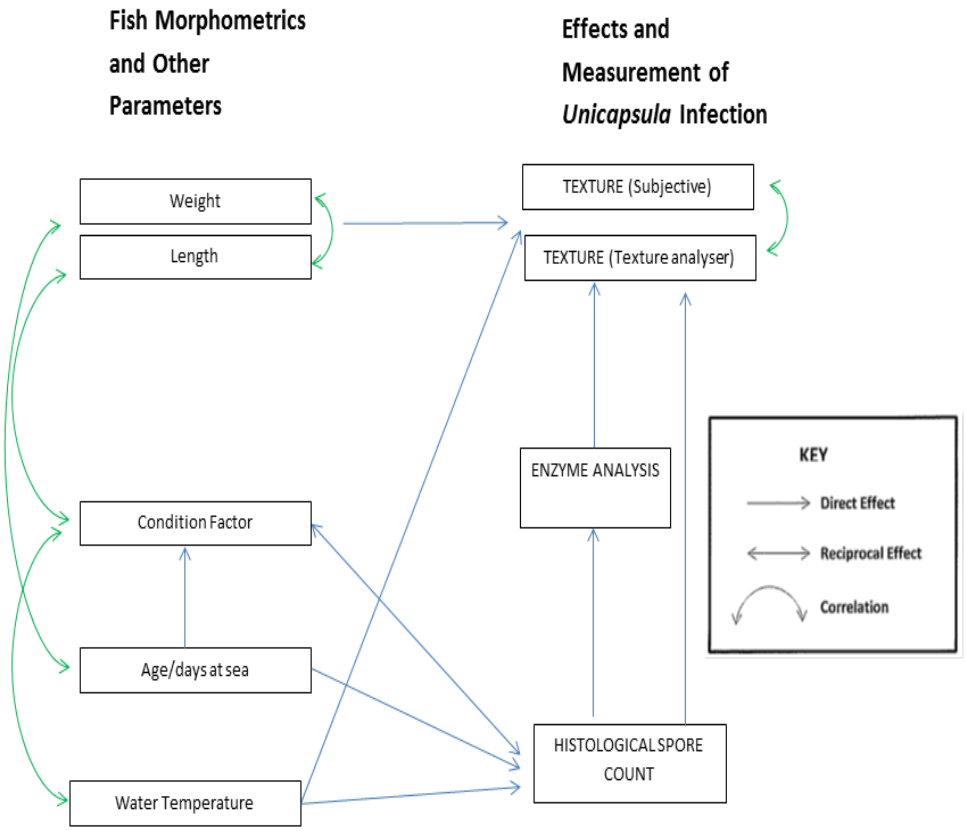



Figure 4.15: Hypothesised relationships between fish morphometrics, environmental factors and effects of *Unicapsula seriolae* on flesh texture in YTK.

### 4.7.1. Multivariate analysis of predictor variables

There were substantial correlations among many of the predictor variables. Principal component analysis (PCA) identified three factors with eigenvalues greater than 1, explaining almost 90% of the total variance in the data set; the factor loadings after rotation are shown in Table 4-4.

Table 4-4: Loadings for predictor variables, and % variance explained by the first three factors in PCA, following varimax rotation. Significant factor loadings shown in bold.

| Variable | Factor1 | Factor 2 | Factor 3 |
|---|---|---|---|
| Fish age | **0.98** | -0.03 | 0.18 |
| Days at sea | **0.98** | -0.03 | 0.19 |
| Fish length | **0.94** | -0.09 | 0.05 |
| Fish weight | **0.91** | -0.11 | 0.21 |
| Fish condition score | 0.06 | -0.19 | **0.84** |
| Mean water temperature | **-0.46** | 0.10 | **-0.71** |
| Spore count | -0.07 | **0.85** | -0.10 |
| Enzyme activity | -0.07 | **0.96** | -0.17 |
| Explained variance (%) | 48.2 | 21.5 | 15.1 |

Factor 1, accounting for 48.2% of total variance, was identified as an age/size factor, with significant positive loadings from fish length, weight, age and days at sea, and a smaller negative loading from water temperature, suggesting increased growth of fish at lower water temperatures. Factor 2, explaining a further 21.5% of total variance, had very high positive loadings from spore count and enzyme activity, indicating a strong relationship between intensity of infection with *U. seriolae* and proteolytic enzyme levels. Factor 3, explaining 15.1% of variance, reflected a strong negative relationship between fish condition score and mean water temperature during the growing period.

### 4.7.2. Relationship between predictor variables and texture analyser score

All three factors were significantly related to flesh texture. Simple bivariate regression analyses indicated that firmer texture was associated with older, larger fish (Factor1; Figure 4.16; $r^2$ = 0.32, F = 23.25, P<0.0001, n = 52), fish with lower spore counts and enzyme activity (Factor 2; Figure 4.17; $r^2$ = 0.23, F =15.33, P< 0.0001, n = 52) and fish with lower condition scores that were grown out at warmer temperatures (Factor 3; Figure 4.18; $r^2$ = 0.27, F =18.57, P <0.0001, n = 52).

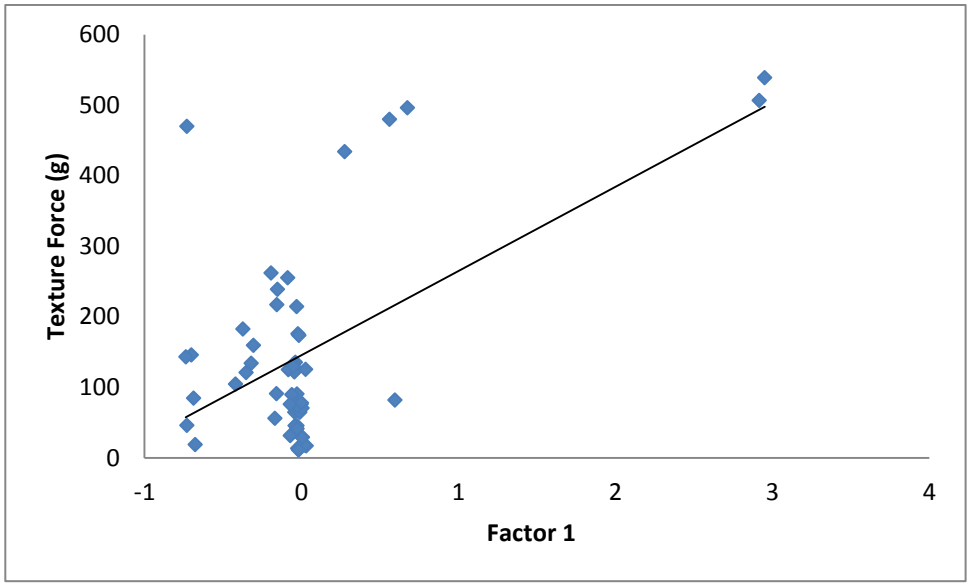



Figure 4.16: Linear fit of age/size and texture in YTK. Texture Analysis = 145.42073 + 119.38348*Factor1. $R^2$=0.32.

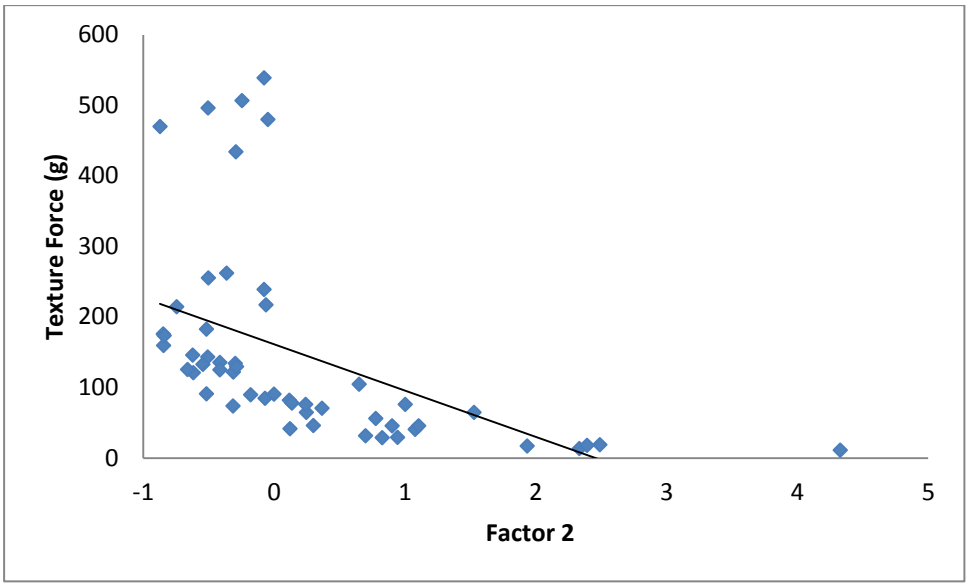



Figure 4.17: Linear fit of spore count/enzyme activity and texture in YTK. Texture Analysis = 161.21409 - 65.656996*Factor2. $R^2$=0.23.

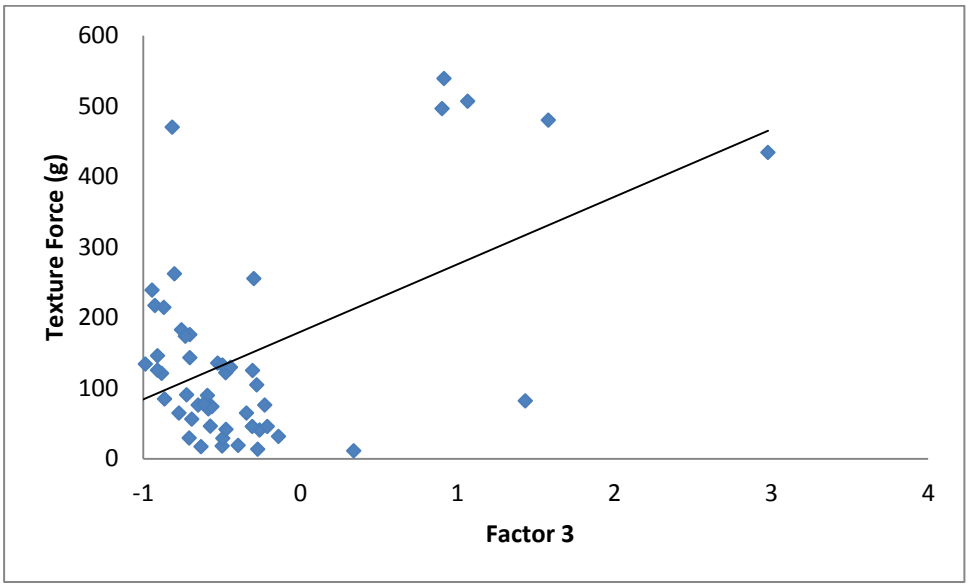



Figure 4.18: Linear fit of condition score/water temperature and texture in YTK. Texture Analysis = 179.78959 + 95.766113*Factor3. $R^2$=0.26.

Multiple regression analysis using a general linear model approach found a significant effect of each factor on texture. Because factors were inter-correlated, a hierarchical partitioning approach was used to determine the

relative importance of each factor in influencing flesh texture. Although all were significant, the independent effect of Factor 2 (Spore count/enzyme activity) (39.2%) was greater than Factor 1 (Age and size) (31.0%) and Factor 3 (Condition factor/water temperature) (29.8%).

### 4.7.3. Relationship between enzyme level, spore count and subjective texture score

Multivariate analyses demonstrated strong relationships between spore count and enzyme level, and between these variables and objective texture analyser score of fish flesh. There were significant differences among subjective texture categories in both enzyme levels (one-way ANOVA, $F = 165.6$, $p < 0.0001$, $n = 119$) and histological spore counts (one-way ANOVA, $F = 70.1$, $p < 0.0001$, $n = 108$). Post hoc analyses, however, showed that the enzyme assay was able to differentiate flesh among the soft, overly soft and mushy flesh categories, but not between the firm/normal flesh and the normal/soft flesh (Figure 4.19).

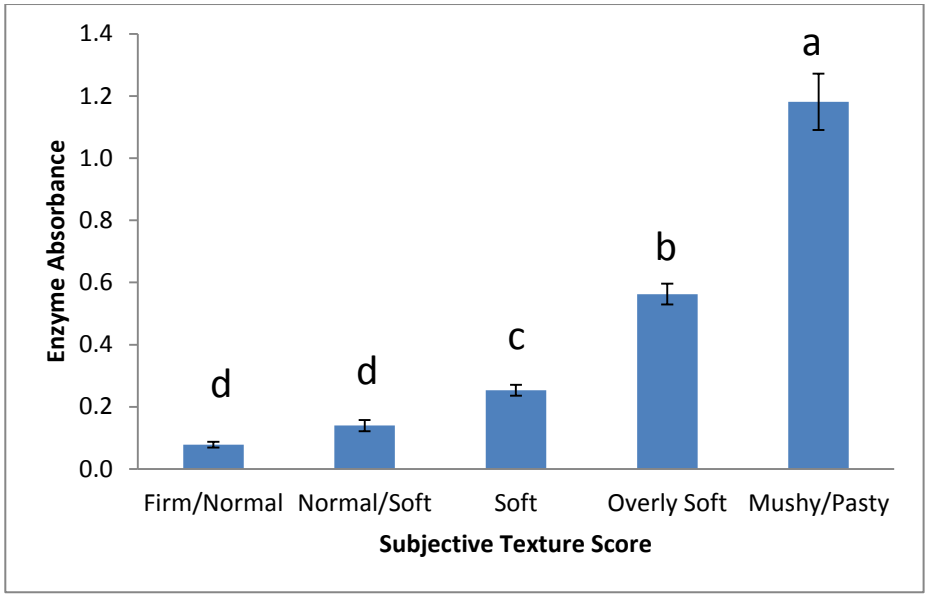



Figure 4.19: The relationship between the subjective texture scores and enzyme absorbance results (n=119). Data are means ± SE and columns sharing the same letter are not significantly different by Tukey's HSD test.

The histological spore count was able to differentiate samples among the overly soft and mushy flesh categories, but not between the categories of firm/normal flesh and normal/soft flesh (Figure 4.20). This probably reflects the fact that spore counting is more effective at detecting moderate to high myxozoan infections within fish flesh.

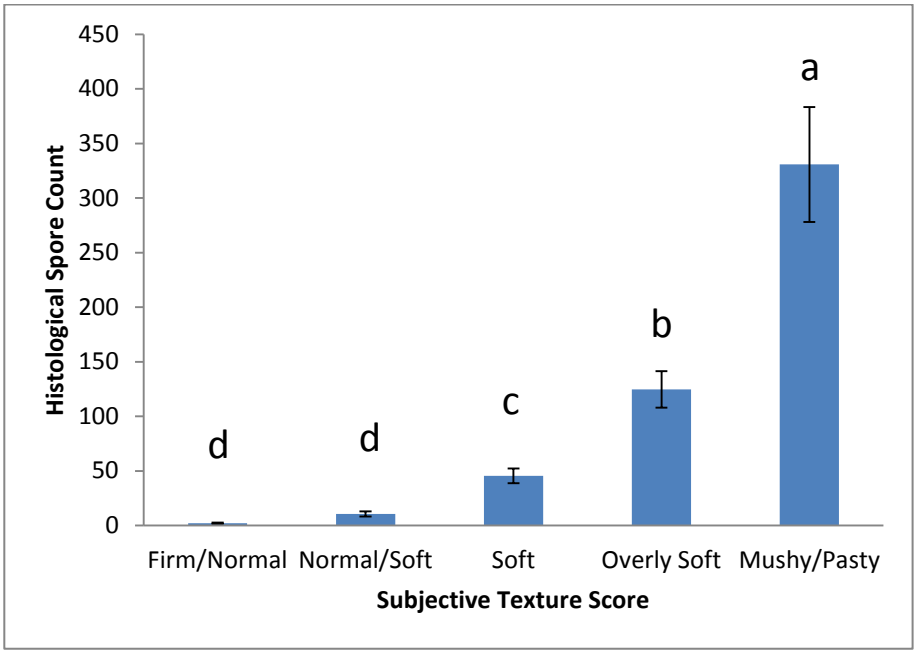



Figure 4.20: The relationship between the subjective texture scores and histological spore counts (n=108). Data are means ± SE and columns sharing the same letter are not significantly different by Tukey's HSD test.

A multivariate analysis of the non-experimental data from all collected fish was undertaken, which had a positive correlation of enzyme levels to spore count. The inter-factor correlation values were 0.85 and 0.96 respectively. These factors accounted for 22% of the variance found in the flesh texture. This relationship, demonstrated that fish with lower spore counts and enzyme levels have firmer texture (F Ratio = 15.3, P = 0.0003, n = 52).

The condition factor of the rested harvest during Harvest Trial Two was significantly higher than the normal harvest treatment and was not significantly different to fish in the SCD method (F Ratio = 3.4, P = 0.0389, n = 60) (Figure 4.21). This unexpected result can be attributed to experimental design and is addressed in the discussion.

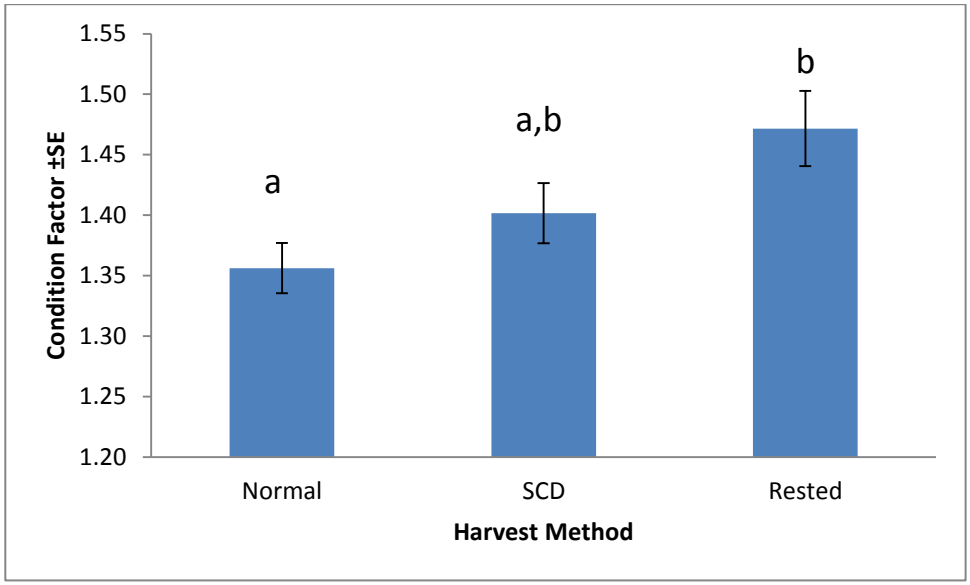



Figure 4.21: Condition factor and harvest treatment in Harvest Trial Two. Data are means ± SE and columns sharing the same letter are not significantly different.

## 4.8. Infection Thresholds for Acceptable Flesh Quality

Fish deemed unacceptable for consumption had the following traits:

- Subjective texture scores of 3 and above;
- Texture analyser scores of less than 100 grams of maximum compression force;
- Histological spore count values greater than 50; and
- Enzyme assay absorbances greater than 0.25.

Fish that experienced complete myoliquefaction upon cooking had subjective texture scores of 5, a texture analysis score of less than 25 g of force, histological spore counts of greater than 300 and enzyme assay absorbances greater than 0.90.

## 4.9. Impact of Harvest Methods on Quality of Yellowtail Kingfish

This section will outline the results of the quality parameters of harvest trial one and harvest trial two. Parameters including flesh texture, flesh pH, rigor index, product temperature, drip loss, fillet quality and microbiology collected from the fish post-harvest will be compared among experimental treatments; the hypothesised relationships among these variables are shown in Figure 4.22.

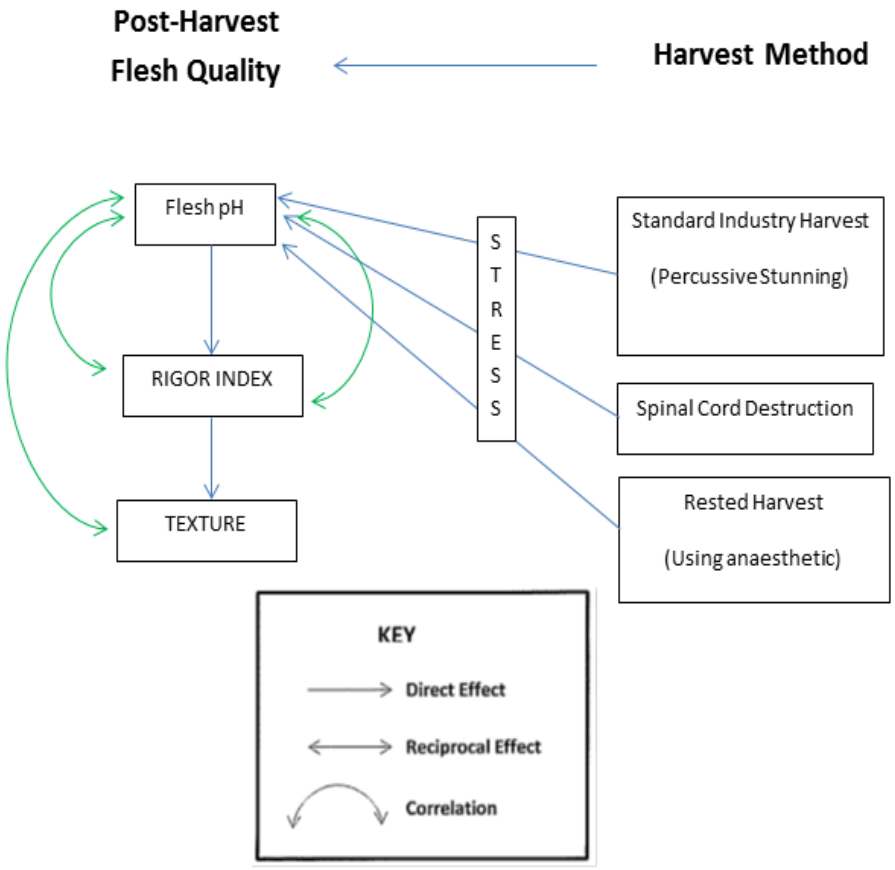



Figure 4.22: Hypothesised relationships for harvest methods and parameters tested.

### 4.9.1. Flesh Texture

***Harvest Trial One***

The objective texture results from Harvest Trial One showed that there were no significant differences in objective texture values between harvest methods (one-way ANOVA; F = 0.587, P = 0.571, n = 21) and there was a tight range in subjective texture scores (mean = 1.3, firm-normal flesh texture) across all fish, indicative of a low level of infection.

***Harvest Trial Two***

In Harvest Trial Two there was a much greater range of objective textures amongst fish (11g/force to 462g/force) and harvest type had a significant effect on texture (one-way ANOVA; F = 6.563, P = 0.003). The rested harvest fish had significantly firmer flesh (188 ±24 g) than the normal group (96 ±14g), although neither differed significantly from those of the SCD harvest group (133 ±16g) (Figure 4.23. The results of the Tukey HSD showed that the normal and rested harvest textures were significantly different (P = 0.002) and that there were no differences (P = 0.623) in normal and SCD harvest and SCD and rested harvest.

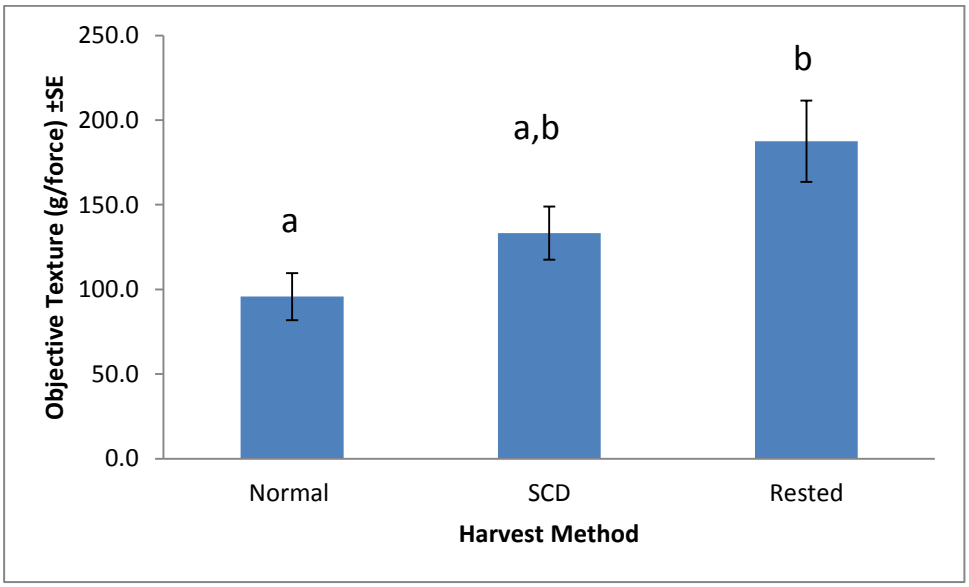



Figure 4.23: Objective texture score and harvest method in harvest trial two. Data are means ± SE and columns sharing the same letter are not significantly

different by Tukey's HSD test., showing significantly firmer flesh texture in the rested harvest treatment.

### 4.9.2. Flesh pH

***Harvest Trial One Flesh pH***

Results from Harvest Trial One showed a significant effect of harvest method of flesh pH measured immediately after harvest (one-way ANOVA, F = 7.64, P = 0.0009, n = 90). Post hoc analyses indicated no significant difference in pH between the normal and SCD harvest methods, and a significantly higher pH in these two treatments compared with the rested harvest (Figure 4.24).

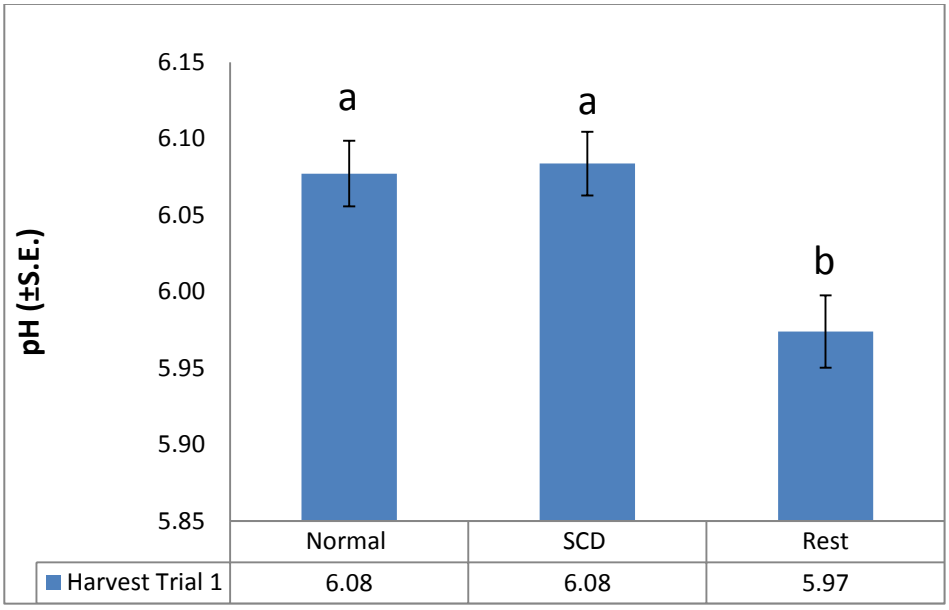



Figure 4.24: Flesh pH at Harvest Trial One showing rested harvest pH as significantly lower than the other treatments. Data are means ±SE and columns sharing the same letter are not significantly different by Tukey's HSD test.

The pH was measured again at 48 hours post-harvest (T=48) (

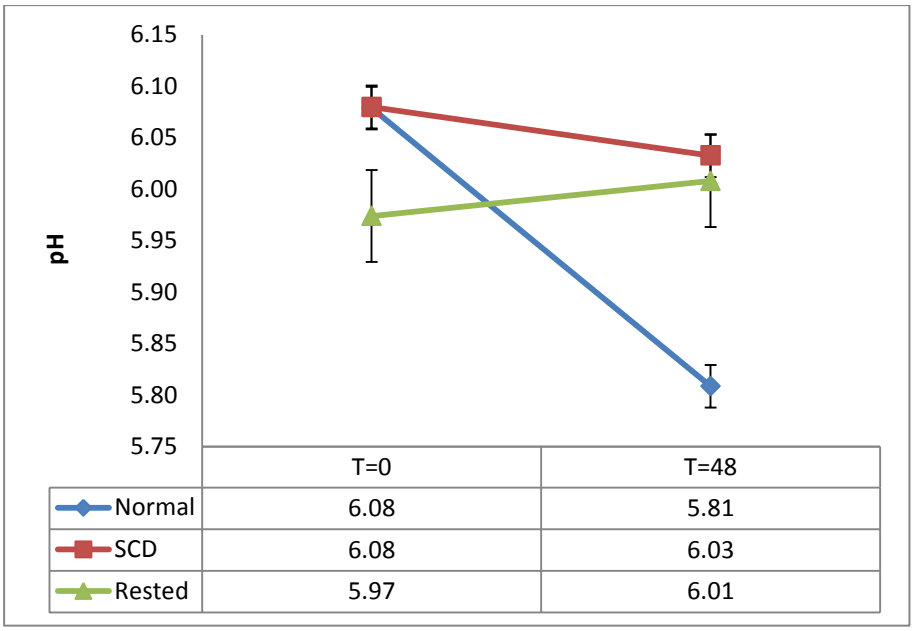



Figure 4.25). Obtaining the pH of flesh at 24 hours post-harvest was attempted, but complete results were unable to be obtained due to mechanical damage to the pH meter. Fish from the normal harvest treatment experienced the greatest change in pH, dropping from 6.08 to 5.81. The SCD harvest treatment experienced a slight drop in pH from 6.08 to 6.03 and the rested harvest fish slightly increased in pH over 48 hours, from 5.9 to 6.01.

6.15
6.10
6.05
6.00
5.95
5.90
5.85
5.80
5.75
pH
T=0
T=48
Normal 6.08 5.81
SCD 6.08 6.03
Rested 5.97 6.01

Figure 4.25: pH of YTK flesh at harvest and 48 hours post-harvest for the three different harvest methods used in Harvest Trial One. Data are means ±SE.

Three fish from each harvest method (n=9) were used for quality index measurements over a longer period of time, up to eight days post-harvest. Fillets were used for the measurements, as the fish were filleted one day post-harvest. The pH was taken from fillets at day one (1), five (5) and eight (8) and the mean pH did not change significantly over time. The largest difference in pH between day one and day eight was only 0.36, and occurred in the rested harvest treatment. Changes in the normal and SCD harvest treatments were negligible, with overall increases of 0.07 and 0.24 respectively.

***Harvest Trial Two Flesh pH***

Harvest Trial Two shows the same trend as Harvest Trial One. There was a significant effect of harvest method on flesh pH immediately after harvest (one-way ANOVA, $F = 7.35$, $P = 0.001$, $n = 61$). There was no difference in pH between fish from the normal and SCD harvests, but pH in fish from the rested harvest treatment was significantly lower (Figure 4.26).

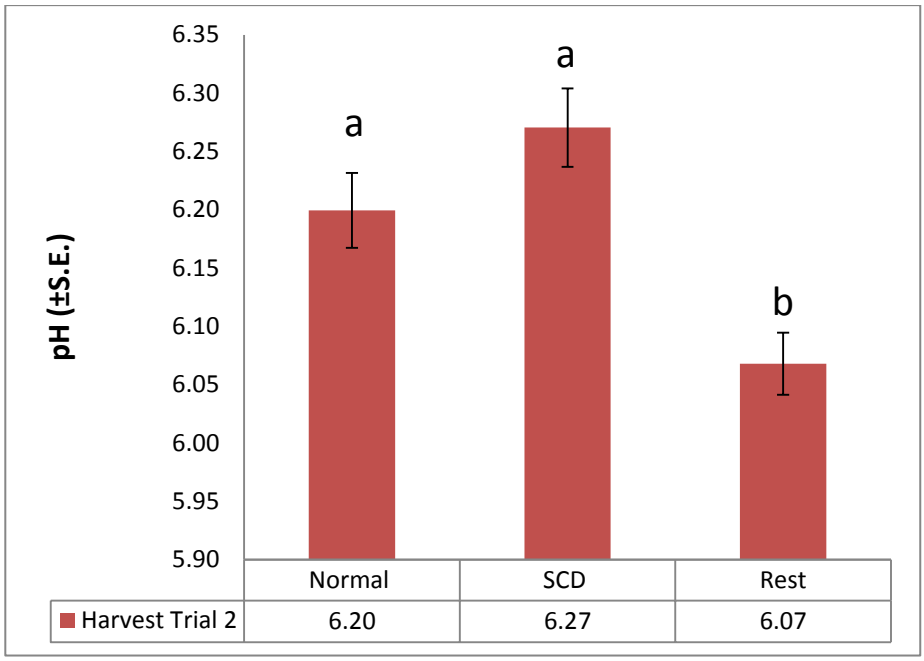
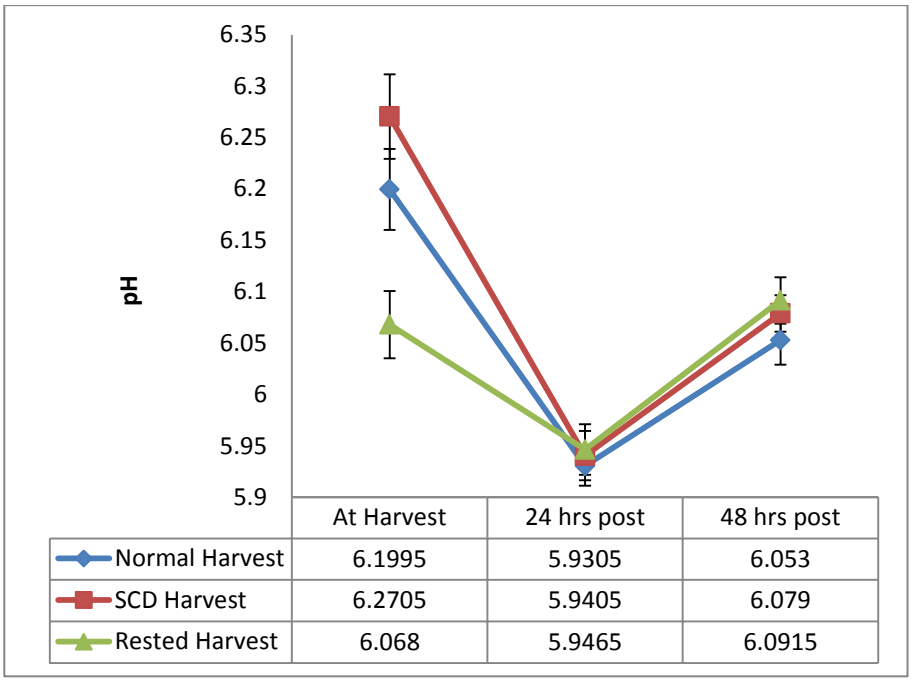


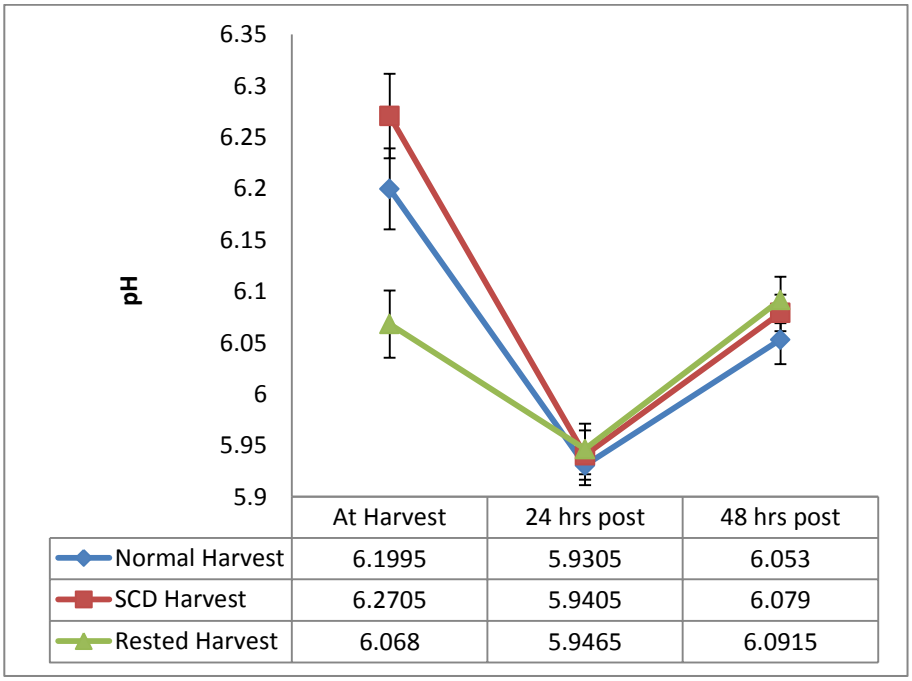

Figure 4.26: Flesh pH of YTK at Harvest Trial Two. Data are means ±SE and columns sharing the same letter are not significantly different.

The pH of skeletal muscle of whole fish was recorded at harvest and after 24 and 48 hours. Although the initial pH (T=0hr) for the three harvest methods were very different, the pH for each method was very similar after 24 and 48 hours (




| | At Harvest | 24 hrs post | 48 hrs post |
|---|---|---|---|
| Normal Harvest | 6.1995 | 5.9305 | 6.053 |
| SCD Harvest | 6.2705 | 5.9405 | 6.079 |
| Rested Harvest | 6.068 | 5.9465 | 6.0915 |

Figure 4.27).




| | At Harvest | 24 hrs post | 48 hrs post |
|---|---|---|---|
| Normal Harvest | 6.1995 | 5.9305 | 6.053 |
| SCD Harvest | 6.2705 | 5.9405 | 6.079 |
| Rested Harvest | 6.068 | 5.9465 | 6.0915 |

Figure 4.27: pH of YTK flesh at harvest and 48 hours post-harvest for the three different harvest methods used in Harvest Trial Two. Data are means ±SE.

### *Comparison of pH over Two Harvest Trials*

Both harvest trials have similar results, although overall Harvest Trial One has lower pH levels across all harvest treatments. In both trials, the rested harvest demonstrated a lower flesh pH than both the normal and SCD harvest methods. A summary of the mean pH values is provided in Table 4-5. In both trials the initial pH of the rested harvest fish was lower than in the other two groups. In Harvest Trial Two, the initial pH was higher than in the first harvest trial but the final pH after 48 hours was not significantly different except for the normal method in Harvest Trial One.

Table 4-5: Summary of the pH values over time from Harvest Trial One and Harvest Trial Two.

| | | Flesh pH | | |
|---|---|---|---|---|
| | **Harvest Method** | **Time = 0** | **Time = 24** | **Time = 48** |
| Harvest Trial One (n = 90) | Normal | 6.08+0.11 | - | 5.81*+0.11 |
| | SCD | 6.08+0.11 | - | 6.03+0.24 |
| | Rested | 5.97*+0.13 | - | 6.01+0.11 |
| Harvest Trial Two (n = 60) | Normal | 6.20+0.17 | 5.93+0.08 | 6.05+0.10 |
| | SCD | 6.27+0.18 | 5.94+0.10 | 6.08+0.08 |
| | Rested | 6.07*+0.14 | 5.94+ 0.10 | 6.10+0.10 |

Values are presented as means ± standard error (SD). *indicates a significant difference ($P<0.05$) between treatments.

### 4.9.3. Rigor Index

A rigor index analysis was only performed on fish from Harvest Trial Two (n=61). There was a significant difference in rigor index among treatments (one-way ANOVA; F = 3.58, P = 0.03, n = 60). There was no significant difference between the normal and SCD treatments; whereas the rested treatment approached rigor more rapidly 3 hours post-harvest (Figure 4.28). This result was to be expected, as this group had the lowest pH at the point of harvest.

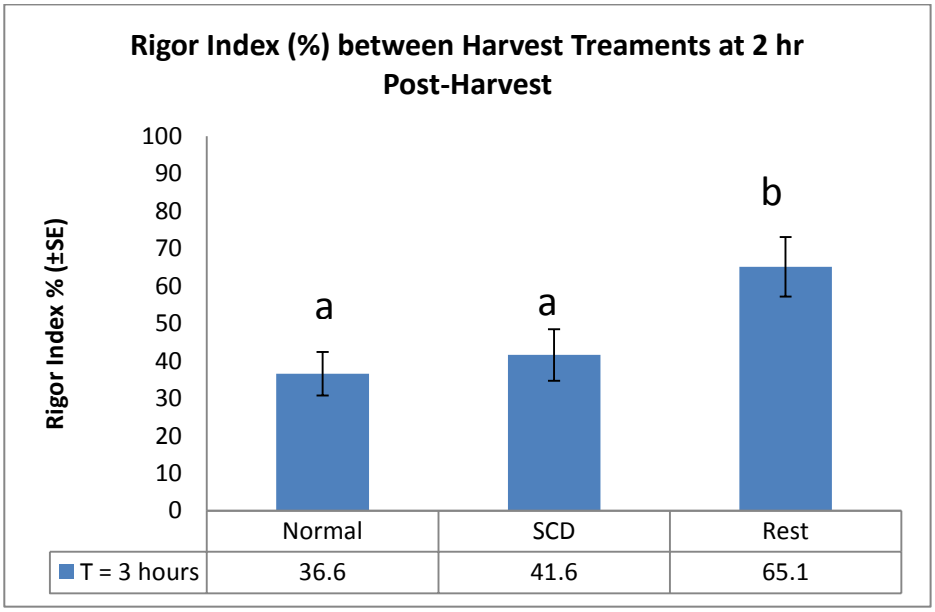



Figure 4.28: Graph showing the rigor index from fish in Harvest Trial Two, showing that fish from the rested harvest treatment progressed into a state of rigor mortis more rapidly than those in the normal and rested harvest treatments 3 hours post-harvest. Data are means ±SE and columns sharing the same letter are not significantly different by Tukey's HSD test.

Two hours post-harvest the fish had started entering a state of rigor mortis. At 23 hours post-harvest, the higher rigor index shows that the fish are either entering full rigor mortis, or the rigor mortis is resolving. At 50 hours post-

harvest, the fish were starting to overcome rigor mortis (

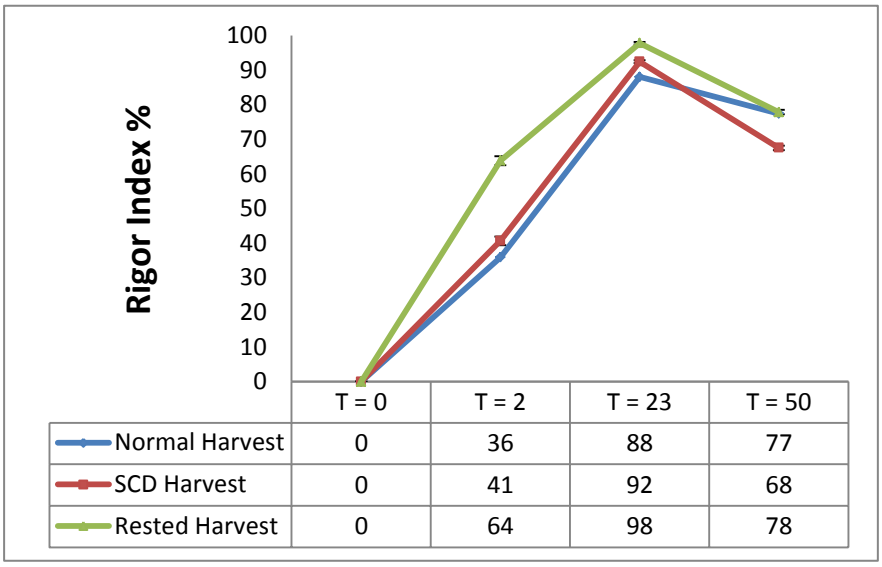



Figure 4.29).

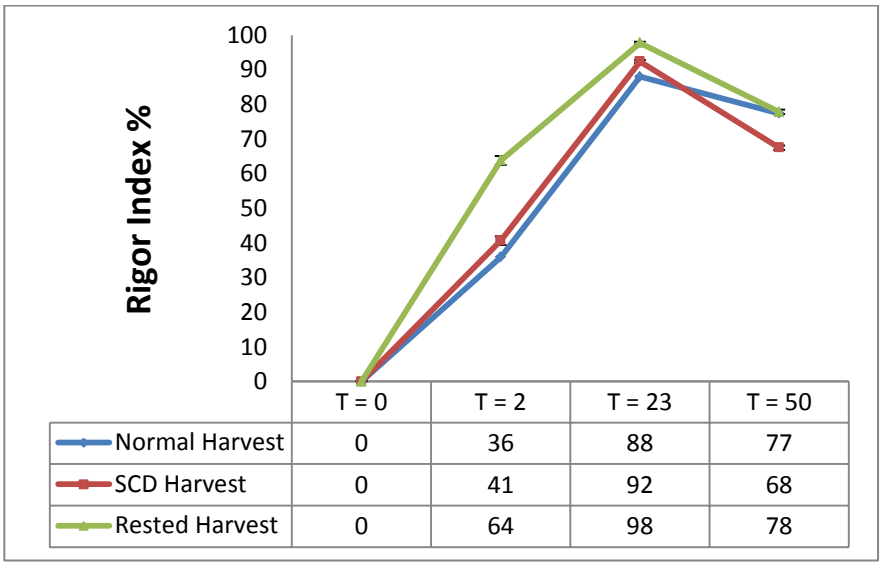



Figure 4.29: The progression of rigor mortis over time in YTK in different harvest treatments.

The onset of rigor mortis was influenced by flesh pH at harvest. Fish with lower initial pH entered into a state of rigor more rapidly than fish with a higher pH. Fish with pH of 5.9 and 6.0 are shown to enter into advanced stages of rigor mortis faster than fish with a higher flesh pH. The lowest initial

pH of 5.9 had reached rigor mortis stage of 87% after 3 hours, whereas pH 6.1 – 6.7 ranged from 17% - 39% (Figure 4.30).

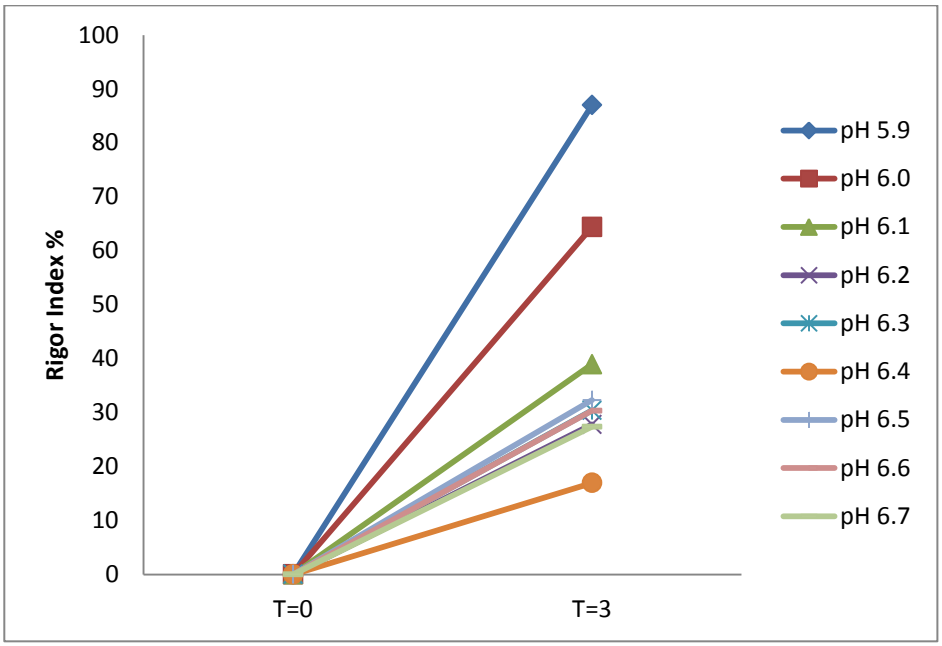



Figure 4.30: Change in rigor index in the first 3 hours post-harvest, related to flesh pH.

### 4.9.4. Product Temperature

The recording of temperature of the first harvest trial fish was attempted, but the loggers were unable to be retrieved before the fish fillets were placed into long term freezer storage. Harvest trial two temperature logging was successful and temperatures were recorded for four days post-harvest in whole YTK and within the storage environment. The graph of post-harvest product temperature is shown in Figure 4.31 and Figure 4.32.

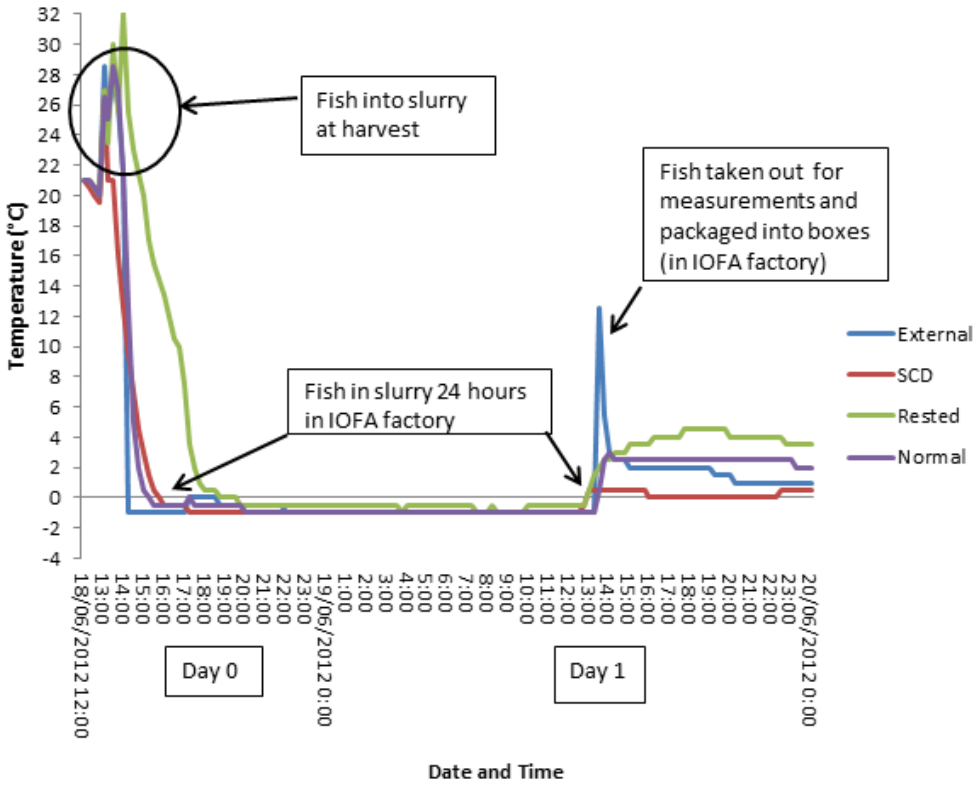



Figure 4.31: Product temperature 0 – 24 hours post-harvest.

The graph shows the ambient air temperature (28-30°C) prior to inserting the logger into the fish, followed by a significant decrease in temperature as the fish are placed into the ice slurry. The temperature on the day of harvest was 25.9°C, and the slight increase in temperature may be due to the loggers being exposed to the sun.

During the first 24 hours post-harvest, the ice slurry was maintained below 0°C. Upon the initial insertion of the logger into the gut cavity of the fish, the rested harvest fish was slower to chill down to the slurry temperature compared to the other two fish. The reason for this was that the weight of the rested harvest fish was higher (1.90kg), as opposed to the normal and SCD harvested fish (1.04kg and 1.02kg respectively), therefore taking longer for the temperature to drop.

When the fish were removed from the slurry at 24 hours for measurements and packing into boxes, the external tag showed a sharp spike in temperature to 12.5°C. The rise in temperature in fish after packing into polystyrene boxes ranged from 1°C in the SCD harvested fish to 4°C in the rested harvest fish. The temperature of the product was maintained less than 5°C, which is within food safety limits (according to the Food Safety Information Council of Australia).

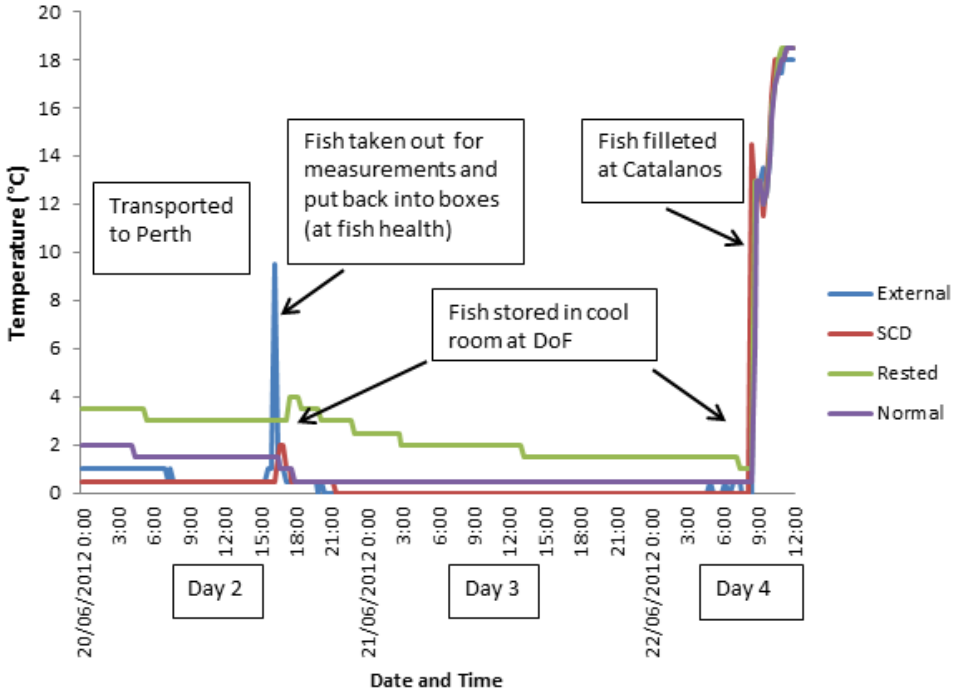



Figure 4.32: Product temperature 48 – 96 hours post-harvest.

After approximately 24 hours of the product being in polystyrene boxes and transported to Perth, the temperatures of the fish remained stable. The fish were unpacked at 48 hours post-harvest to obtain a pH reading and a rigor index measurement, which resulted in a slight spike in temperature of the fish, still remaining under 5°C. The fish were repacked and remained in polystyrene boxes in cold storage for another 36 hours before the fish were filleted, vacuum sealed and placed into freezer storage.

### 4.9.5. Drip Loss

Fillets from Harvest Trial One were tested for drip loss. The drip loss of vacuum packed fillets did not vary between harvests (Figure 4.33) until day eight where there was a 4.31% difference between the normal harvest and the rested harvest. By day eleven there was no difference between the SCD and rested harvests. The greatest drip loss from each harvest was observed on day eight and ranged from 3.25% (normal harvest) to 7.56% (rested harvest). The vacuum packs reduced the amount of weight lost over time.

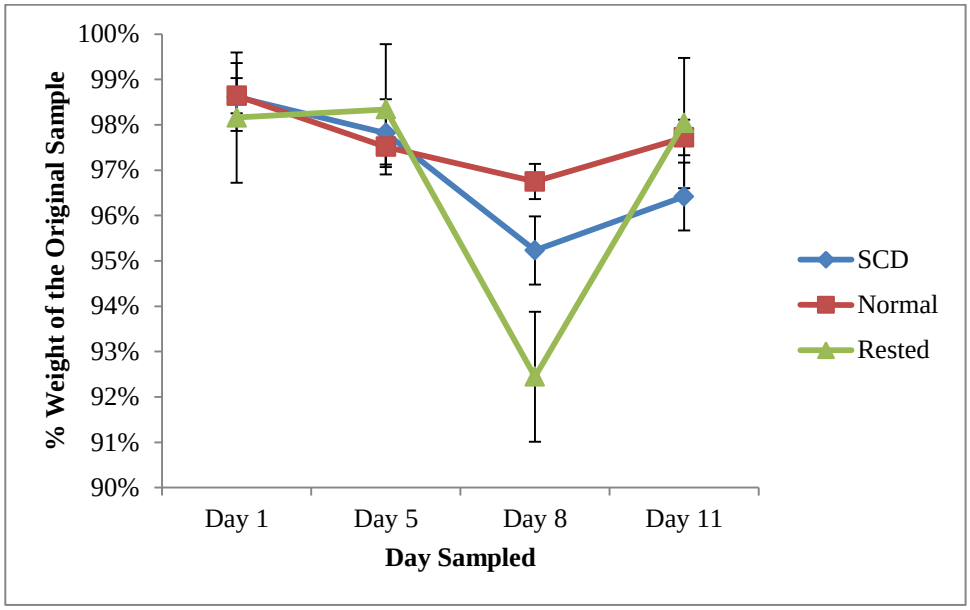



Figure 4.33: Fillet weight over time expressed as a percentage of the original fillet weight (n = 9).

### 4.9.6. Fillet Quality

The quality index scheme showed that the texture of the fillet became soft by day eight for SCD and rested harvests. Normal harvest fillets remained firm until day eleven. Gaping was observed on all harvest treatments from day five.

The colour of fillets darkened to brown by day five for the SCD and rested harvests and day eight for the normal harvest. Blood colour did not brown until day eleven regardless of harvest method or storage. Brown blood in the fillet renders an unacceptable appearance to consumers.

For the fillets stored in their vacuum packaging, a fishy smell was observed on day eight and a citrus odour on day eleven for rested and SCD harvest fillets respectively. Normal harvest fillets remained with a neutral odour by day eleven. Fillets that had been opened previously were observed to have a fishy odour by day eight (SCD and normal harvests) and neutral odour by day eleven (rested harvest). Therefore according to odour, vacuum packed rested harvest fillets were rendered unacceptable by day eight, SCD harvest by day eleven and normal harvest after day eleven. Open SCD and normal harvest fillets were rendered unacceptable by day eight and rested harvest fillets after day eleven.

Therefore, according to the colour of the fillet and blood, odour and texture, fillets will be pronounced as acceptable or unacceptable by consumers. From the above observations, vacuum packed normal harvest fillets gaping appeared from day five, but the colour did not become unacceptable until day eight, vacuum packed SCD and rested harvest fillets both turned brown and had apparent gaping by day five, reducing the likelihood of a sale after this point. Opening the packs and storing them wrapped had no impact on the colour, texture, gaping or odour of the fillets observed.

### 4.9.7. Microbiology

The Standard Plate Count (SPC) on YTK fillets did not increase by one log over the six days measured in any harvest method. By day eleven, all three harvests' counts were still below the specification set by major supermarkets on all seafood products which is equal to 1,000,000 CFU/g (Figure 4.34). Storing fillets in their vacuum packs reduced the growth on the plate count agar to the extent that all three harvest methods remained under 1,000,000 CFU/g by day eleven. There were no significant differences in plate growth between the three harvest treatments.

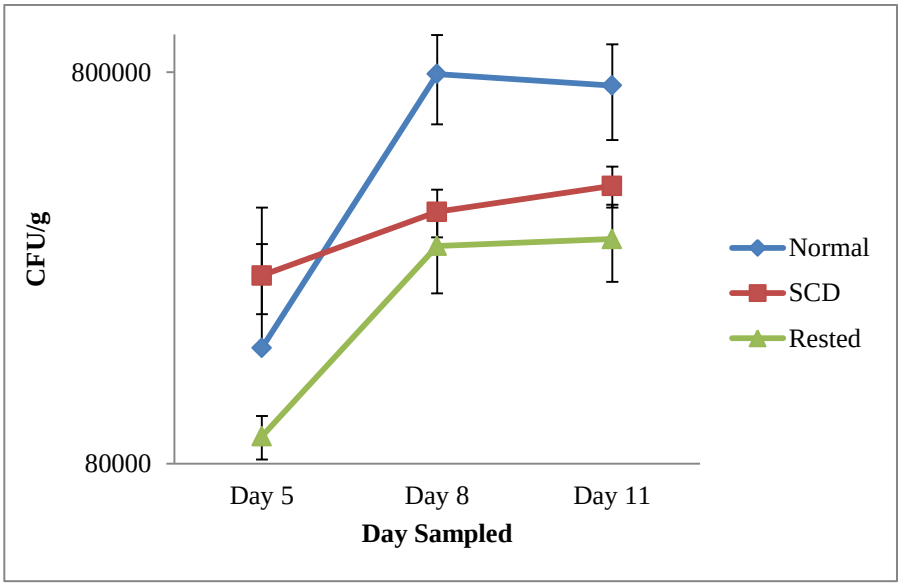



Figure 4.34: Bacterial count (SPC) from YTK fillets up to 11 days post-harvest in each harvest treatment.

Storing vacuum packed fillets sealed for as long as possible before opening successfully reduced the microbiology counts as well as keeping drip loss to a minimum. Fillets also needed to be used within five to eight days before becoming soft and unattractive. SCD harvest had significantly lower bacterial

growth on the fillets than normal and rested harvests at day 11 (

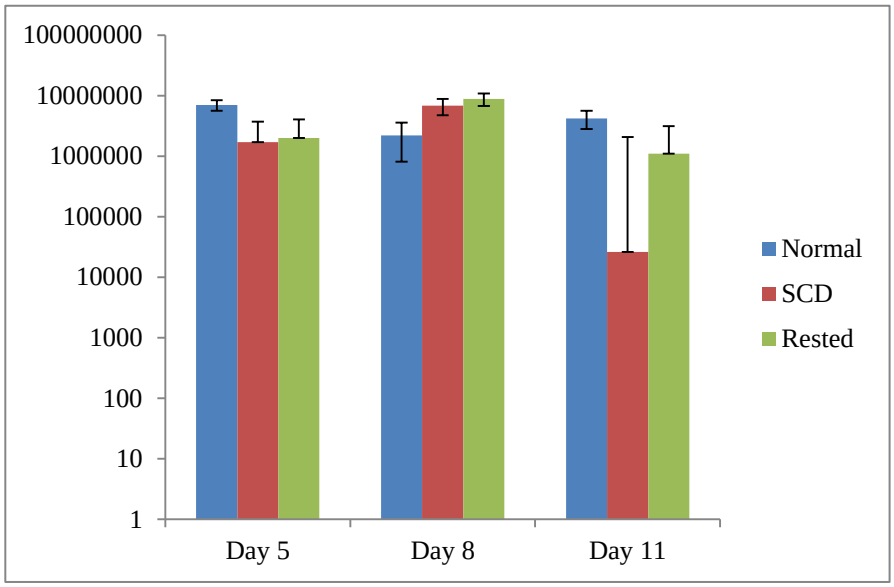



Figure 4.35).

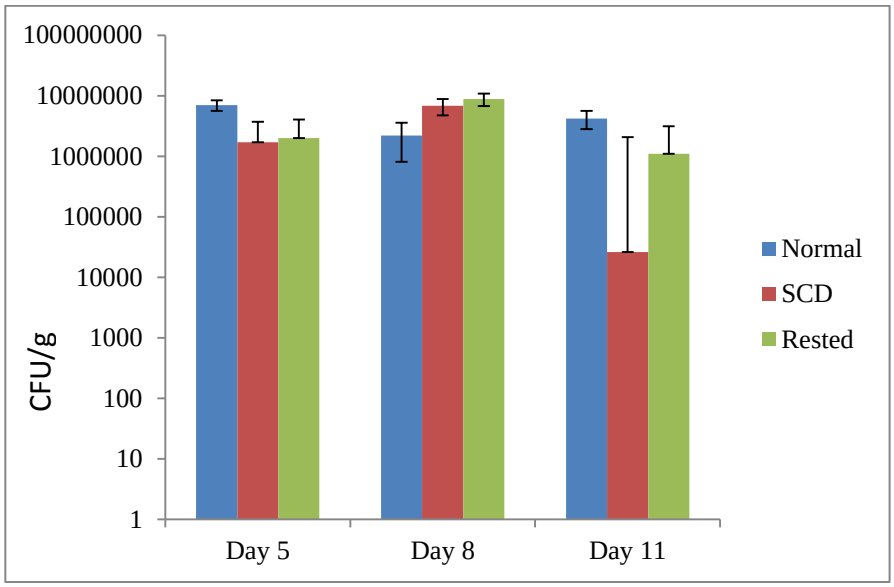



Figure 4.35: Spoilage organism (*Shewanella*) count taken on Iron Agar plates on day 11. YTK fillets from each harvest method were compared for bacterial counts.

# CHAPTER 5. GENERAL DISCUSSION

The major consequence of myxozoan parasites on finfish is poor post-mortem flesh quality, resulting in economic losses in both the wild and aquaculture sectors (Moran et al. 1999b, Gómez et al. 2014). The most documented aquaculture sector having a long history of problems associated with myxozoan parasites is the British Columbian Atlantic salmon, *Salmo salar*, industry. In this industry, *Kudoa thyrsites* has caused serious problems in terms of poor flesh quality since the early 1990s, and it appears to have worsened over time (Morisson 2010). It was reported that the effects of *Kudoa thyrsites* has cost the industry an estimated USD$30 - $40 million annually. (http://www.intrafish.com/. Date accessed 20/8/13). An article in Marine Harvest's Canadian Wharfside magazine states that "Diagnostic tests for detecting and quantifying infection level have been poor and tools to mitigate infection effects on flesh quality are based on best practices and gut feelings only and therefore less than reliable" (Morisson 2010).

Myxozoans have also been recognised as a threat to the Japanese aquaculture industry, not only through soft flesh but also a threat to human health from the newly discovered *Kudoa septumpunctata* (Matsukane et al. 2010, Grabner et al. 2012). *K. thyrsites* has a wide host range but had been previously undetected in any cultured fish in Japan until 2004, when Japanese flounder, *Paralichthys olivaceus*, and Chinese sea bass, *Lateolabrax sp.* were found to be infected (Yokoyama et al. 2004). With the exponential growth in aquaculture worldwide, and a broad host range, it is likely that myxozoans will pose a threat to not only established sectors, but upcoming aquaculture industries (Gómez et al. 2014).

YTK farming in Australia is a relatively new industry in Australia. In the South Australian YTK industry, established in the year 2000 (Kolkovski et al. 2004), there has been extensive research into monogenean parasites such as the skin

fluke, *Benedenia seriolae*, and the gill fluke, *Zeuxapta seriolae*, as these parasites are capable of causing significant stock losses (Ogawa 1996). Myxozoan parasites, on the other hand, have previously been identified as low risk and low prevalence in YTK farming in South Australia (Shepphard 2005), and subsequently have been a low research priority for industry. To date, there have been no published reports of *U. seriolae* in any fish from South Australia or New Zealand (Stephens et al. 2010).

Hutson et al. (2007b) surveyed 50 yellowtail kingfish, *Seriola lalandi*, from NSW and Victoria, Australia. No flesh myxozoans were found, although two myxozoan species, *Ceratomyxa buri* and *C. seriolae,* were discovered in the gall bladders of the Victorian fish. Despite the lack of myxozoan parasites infecting the muscle of YTK in cooler waters of Australia, it is noted that in cultured fish in South Australia myxozoans have been found infecting the heart, renal system and skeletal muscle. The undescribed myxozoan in fish muscle was associated with histological lesions and muscular inflammation (D'Antignana 2012).

However, in Western Australian YTK production, there was anecdotal evidence that myxozoan infection did have the potential to adversely impact the flesh quality of the product. Incidences of *U. seriolae* were recorded in the Western Kingfish Limited (WKL) operation in 2009 (Stephens et al. 2010). Due to the venture failing prior to fish being harvested, it was never ascertained whether flesh quality was unsatisfactory due to *U. seriolae* infection.

Hence, until the study reported here, no known studies have investigated the extent and severity of *U. seriolae* infection in cultured and wild YTK in WA and the subsequent consequences on flesh quality. The most relevant literature on myxozoans in YTK in Australia was published over thirty years ago when *U. seriolae* was described in wild YTK in eastern Australia by Lester (1982).

## 5.1. Prevalence of infection by *Unicapsula seriolae* in WA farmed and wild YTK.

*Unicapsula seriolae* was found in almost all samples analysed from WA waters, with the prevalence in wild and cultured fish at 80% and 97% respectively. The high incidence of *U. seriolae* in all YTK samples collected in WA may be further evidence that this parasite may be common throughout the Indo West Pacific region (Miller et al. 2013).

Control fish collected from South Australia and ACAAR, Fremantle harboured no flesh parasites. In South Australia, this is likely to be due to cooler waters, with the samples being sourced from areas beyond the southern limits of this species of myxozoan (Lester 1982). At ACAAR, the absence is possibly due to a combination of cooler water and natural filtration of the culture water through a deep limestone bore. Extracting water via this method will avoid the habitat of the invertebrate host which is likely to be a muddy, silty or sandy environment (Wolf et al. 1984). Lester (1982) observed a 62% prevalence (16 of 24 fish infected) of *U. seriolae* in YTK from Brisbane, Queensland. Cultured YTK from Jurien Bay, WA also displayed a high prevalence at 75%.

The survey by Hutson et al. (2007b) did not detect any *U. seriolae* in NSW or Victoria, most likely due to the high latitude of the sampling (Greenwell Point, 34°S and Killarney, 38°S), whereas Lester (1982) sampled at a latitude of approximately 27°S (Brisbane, QLD), which is similar to Geraldton's latitude of 28°S on the west coast of Australia. The water temperature profiles at these latitudes are significantly warmer than southern NSW and Victoria (Global sea temperature. http://www.seatemperature.org/. Date accessed 25/08/13). The YTK collected from the south coast NSW by Hutson et al. (2007b) examined squashes of fresh muscle tissue, and muscle was also examined histologically using standard H & E stain. If *U. seriolae* myxospores were present in low numbers in these fish, it may be possible that they were not detected. Low level *U. seriolae* infection is difficult to detect with the squash

method and standard histological staining does not adequately highlight polar capsules of myxospores.

Another species of *Unicapsula*, *U. pflugfelderi,* in the flesh of a potential aquaculture species striped seabream, *Lithognathus mormyrus,* and picarel, *Spicara smaris,* in the Mediterranean had a prevalence of 46% and 63% respectively. No myoliquefaction was observed, although infection was found to be of high intensity based on the presence of visible pseudocysts in the flesh of some fish (Alama-Bermejo et al. 2009).

Despite the very high prevalence of *U. seriolae* in wild and cultured fish (80% - 97% respectively), infection levels (as measured by histological analysis) varied greatly. Cultured fish collected from cohort one had low level of infections (mean 9.1) and displayed minimal detrimental effects on flesh texture. Fish collected from cohort two demonstrated a large variation in infection levels (range 0 - 488, mean 73) and, when the infection levels were high, soft texture became apparent.

In explaining the differences between infection rates, it is noteworthy that prior to being harvested, the second cohort of cultured fish from Geraldton had recently been immunocompromised from factors including dietary inadequacies and adverse water quality events which resulted in heavy mortalities (DoF. Fish Health Reports, July 2012). Inferior feeds, discovered to contain high levels of plant based protein and oil, also may have contributed to the poor status of this cohort of fish (Pers. Comm. IOFA. Feb 2013). The stress of these events may have led to higher susceptibility and infection rates by *U. seriolae* or possibly that infection from *U. seriolae* contributed to the fish failing to thrive. Chronic stress in fish was previously found to reduce lymphocyte activity and numbers through increased levels of corticosteroids, leaving fish vulnerable to infections by pathogens or parasites (Barton et al. 1991).

Lester (1982) noted that there was never more than one myxozoan plasmodium per muscle cell in wild YTK, whereas this study revealed that

severely infected cultured YTK had up to four plasmodia per muscle cell in cohort two fish. Severely infected fish with *U. pflugfelderi* had one plasmodium per muscle cell (Alama-Bermejo et al. 2009).

High prevalence (>60%) in a related parasite, *K. thyrsites*, was observed in cultured Atlantic salmon during smoltification, a process where a salmonid undergoes physiological changes to adapt to seawater from freshwater (Björnsson et al. 2011). Stressful events such as these have been linked to increased severity of myxozoans (Moran et al. 1999a). Links between poor diet (containing high inclusions of plant-based oils) and higher prevalence and increased severity on myxozoans have been made in gilthead seabream, *Sparus aurata*, infected by *Enteromyxum leei* (Estensoro et al. 2011), which holds similar parallels to harvest cohort two YTK that were sampled in this study.

The point in time at which the cultured fish in Geraldton became infected by *U. seriolae* is unknown. However, it is known that the fish were myxozoan free when leaving the hatchery in Fremantle. ACAAR have not had any accounts of *U. seriolae*, or any other myxozoan parasite, in any of their stock to date (Pers. Comm. Dr G. Partridge. Nov 2012). Prior to dispatching juvenile fish from the hatchery, a health certificate from DoF Fish Health section is required before the fish are released into the marine environment.

Two cohorts of YTK were stocked into sea cages in Geraldton in September 2010 and 2011 respectively. *U. seriolae* was first observed histologically in both cohorts of YTK within four months of the fish entering the oceanic environment. Myxospores were detected in muscle tissue in January (mid-summer) during the fortnightly health checks by the DoF Senior Fish Health Pathologist (DoF. Fish Health Reports. Jan 2010/11). It is highly likely that the parasite entered the fish shortly after being at sea; however, this was unable to be proven due to lack of regular weekly samples for PCR testing. Histological signs of infection in Atlantic salmon, *Salmo salar*, have been documented to occur as early as nine weeks post-exposure to *Kudoa thyrsites*.

In the same experiment, PCR picked up infection six weeks after exposure (Moran et al. 1999a).

## 5.2. Correlation between spore counts, protease activity and texture.

This study showed a relationship between flesh texture, myxozoan parasite numbers and enzyme activity in YTK. This relationship has also been demonstrated in a number of publications concerning different species of fish and myxozoan parasites (Dawson Coates et al. 2003, Samaranayaka et al. 2007, Zhou et al. 2009). An earlier documented attempt by Patashnik et al. (1982) was unsuccessful in correlating proteolytic enzyme activity with a subjective visual parasite intensity scale, measuring the presence of macroscopic cysts in raw flesh. A scale of one to five was developed, with categories three, four and five considered grossly parasitised (Patashnik et al. 1982).

Initial spore counting and protease activity methods used in this study were adapted from studies concerning species such as Pacific whiting, *Merluccius productus* (Zhou et al. 2009). The abundance, underutilisation and susceptibility to myxozoan infection of Pacific whiting led to research into characterising the enzyme responsible for post-harvest myoliquefaction (An et al. 1994). The majority of the catch is processed into fish mince before conversion into surimi.

YTK on the other hand, is utilised as sashimi or cooked fillets in Australia. The study by Dawson Coates et al. (2003) concerning Atlantic salmon, *Salmo salar*, was considered more relevant to the texture analysis in YTK, despite being conducted on raw flesh. Compression with a cylindrical probe was found to be the most practical measurement for flesh hardness in YTK rather than shear force, which is conducted using a warner-bratzler blade (Einen et al. 1998). The compression method is also less destructive and more than one measurement can be made on a piece of flesh (Sigurgisladottir et al. 1999). Since *U. seriolae* does not appear to result in raw flesh myoliquefaction, cooked flesh was used in this study to display the effects of the parasite.

St Hilaire et al. (1997a) demonstrated correlations between infection intensity and raw flesh texture in Atlantic salmon, *Salmo salar*. The muscle digest method was used and proved to be accurate and repeatable unlike in the current study.

Similar significant correlations between the subjective and objective texture scores were found in this study using cooked flesh. Subjective assessment, using a texture scale from 1 - 5, was adequate to predict of flesh texture when compared with the objective analysis. Despite the subjective scale not being able to differentiate between texture scores of 4 and 5, this is of little consequence to the practical application of this method, as both texture scores were unacceptable for consumption. In another study, the histological counting method was correlated with a subjective texture assessment and could accurately predict the effects of *Kudoa thyrsites* on raw flesh quality (Dawson Coates et al. 2003).

Some seafood retailers in Western Australia check for mushy flesh in yellowtail, *Seriola spp.*, prior to selling to consumers. A section of flesh is cut from the fish and cooked to reveal any signs of soft flesh. If myoliquefaction is present, the fish are discarded (Pers. Comm. Seafresh Innaloo. November 2012).

To overcome issues with measuring and correlating infection intensity, adding in a third method of enumeration into the current study allowed clear differentiation between infected and uninfected fish. Through enzyme analyses, it was revealed that enzyme levels in uninfected fish were significantly lower ($P = 0.006$) than those with very low levels of infection.

The aim of measuring the myxozoan derived proteolytic enzyme activity within the fishes flesh was to determine if there was a relationship between the number of spores and the rate of enzyme activity. The correlation between high enzyme activity and high spore count was proven in this study.

The major proteolytic enzyme in fish flesh and myxozoan parasites, cathepsin-L, can be released from myxospores upon physical damage, poor storage temperatures and cooking (Whitaker et al. 1991). These factors will directly influence the final texture of fish flesh. Funk et al. (2008) discovered that enzyme activity was six times higher in fish infected with *Kudoa sp.* fish than uninfected fish. Clear cut comparisons such as these were unable to be made in this study, as a small number of fish found to contain no infection had enzyme levels that overlapped with fish having very low levels of infection. This had minimal impact on the results, as the P-value of 0.006, demonstrated a clear distinction between infected and uninfected fish. The measurement of enzymatic activity in this study was very accurate, repeatable and effective in predicting infection levels.

High protease enzyme activity in fish flesh usually indicates a high parasite load, although high enzymatic activity can also be linked to post-mortem autolysis of the muscle without any parasite interaction (Martinez et al. 2011).

The intraspecific enzyme assays undertaken from different parts within the same fish in this study showed that the infection of *U. seriolae* in YTK was evenly distributed. This gave confidence that the histological sampling position of YTK flesh represented infection over the whole fish. Lester (1982) also found that infected muscle fibres were generally evenly distributed throughout YTK, although, unlike the present study, he did report a slight increase in frequency towards the posterior of the fish. In contrast, Dawson Coates et al. (2003) reported that *Kudoa thyrsites* myxospores were not uniformly distributed throughout the flesh and it was necessary to sample 6 – 8 sites per fish for histological spore counting. Reasons for variations in parasite distribution throughout the flesh were unable to be identified; however, they are likely to be specific to the species of myxozoan and host fish.

Correlated with the spore counts, it was determined that lower intensity of infection equated to firmer flesh texture. Overall, within the cultured fish it

was found that cohort two had much softer cooked flesh texture than fish from cohort one. Cohort one had firmer more desirable texture which was attributed to a lower intensity of infection with *Unicapsula seriolae*.

During the cooking trials in this study, it was observed that fish with moderate infections should be immediately consumed after cooking without allowing the fish to rest or cool significantly. Flesh textures in borderline acceptable fish (subjective texture score of 2/3) were found to degrade to inedible during the cooling period. This post-cooking change in flesh texture could be due to the continuation of enzymatic activity after rapid heating and successive cooling through the optimal temperature (55°C) for highest enzymatic activity (Samaranayaka et al. 2007). The internal flesh temperatures in this study ranged from 52 - 54°C immediately after cooking for ten minutes at 55°C. It is likely that the internal temperature of the cooked flesh was not high enough (70°C) and exposure to heat was not long enough (15 minutes) to deactivate the proteolytic enzymes (Nelson et al. 1985).

Temperature would be of higher importance if YTK experienced myoliquefaction at low storage temperatures, as does Atlantic salmon, *Salmo salar*, infected with *Kudoa thyrsites*. A study by Dawson Coates et al. (2003) demonstrated that fillets in the raw state, chilled for six days on ice, resulted in 80% of fillets being unsuitable for consumption. These fillets displayed various stages of degradation and some fillets had severe myoliquefaction. Langdon (1991) showed that cultured mahi mahi, *Coryphaena hippurus*, infected with *K. thyrsites* from Albany, WA displayed myoliquefaction in the raw state after 48 hours storage at 4°C.

The impact of storage temperature on YTK flesh quality and rupturing of *U. seriolae* myxospores is likely to be negligible, unless the product has undergone severe temperature abuse as observed in the wild amberjack, *Seriola dumerili* in this study. No other cases of raw myoliquefaction were seen in the YTK, as the fish were maintained at low temperatures until analyses were performed. The logging of fish from Harvest Trial Two

demonstrated that whole fish maintained optimal food safe temperatures between -1°C and 4°C (FSANZ 2006) over the four days of chilled storage. It is apparent that *U. seriolae* doesn't cause soft flesh in YTK until cooked, which was confirmed in the study by Lester (1982).

Another factor besides myxozoan parasites that could account for soft flesh texture in YTK is 'burnt meat syndrome'. Japanese yellowtail, *Seriola quinqueradiata*, are prone to a condition referred to as 'burnt meat' in raw flesh resulting in white, grainy, soft and watery meat which is unsuitable for human consumption (Mora et al. 2007). The cause has been attributed to high environmental temperatures, low muscle pH and high stress during harvest (Liang et al. 2012). Higher levels of lactic acid were found to be produced at higher temperatures and a muscle pH of 5.5 – 5.9 coincided with the incidence of burnt meat (Mora et al. 2007). Spinal cord destruction was the best method in minimising stress and reducing the incidence of this syndrome (Liang et al. 2012). The addition of oxygen to blood bins upon bleeding fish reduced the incidence of burnt meat. The theory behind this is yellowtail continue to respire until complete physiological death, which has an impact on flesh pH and subsequent flesh quality (Shioya et al. 2011).

The South Australian YTK industry had similar flesh quality issues in 2008, with reports of soft flesh, old appearance and gaping by customers. An investigation into the effects of maturation and harvest stress were undertaken, with minimal differences in quality detected between males and females and stressed and unstressed fish (Carragher et al. 2009). There is a possibility that these fish may have been affected by burnt meat syndrome, due to the absence of myxozoan parasites in the flesh.

Differentiating soft flesh caused by burnt meat syndrome and myxozoan parasites is simple if the infection rate of *U. seriolae* is able to be enumerated. Otherwise, in the absence of myxozoan parasites, factors such as pre-harvest stress, low flesh pH, high water temperatures at harvest and product

temperature post-harvest are contributing factors to burnt meat in *Seriola spp* (Mora et al. 2007).

Similar conditions to burnt meat have been observed in terrestrial animals including pork and poultry, which is referred to as pale, soft, exudative (PSE) (Bendall et al. 1988). Genetic manipulation of animals for traits such as faster growth and higher yields has resulted in animals that are more susceptible to stress (Cassens 2000). Mitigation methods to reduce effects of this syndrome include minimising overall stress prior to slaughter, bolt stunning and rapid chilling of product post-slaughter (Adzitey et al. 2011). These findings could be considered when refining methods for harvesting and handling YTK.

Despite extensive research into soft flesh issues for the last half century, the livestock industry is still struggling to find solutions to consistently produce quality meat (Cassens 2000). This demonstrates the complex interactions between the environmental, physiological and metabolic factors that need to be understood in order to create an acceptable meat or seafood product.

The multivariate factor analysis of the data collected in this study revealed that the biggest variations in texture were due to spore count/enzyme activity (39.2%), followed by age and size (31.0%) and water temperature/condition factor (29.8%). This confirms the hypothesis that spore counts and associated enzyme activity had the greatest impact on texture (An et al. 1994). The factors were ranked by relative importance, as determined by the percentage independent contribution of each factor to total explained variance, from hierarchical partitioning of $R^2$ values. To explain the seemingly low $R^2$ values for spore count/enzyme activity (0.23), age and size (0.32) and water temperature/condition factor (0.27) (results section 4.7.2), each factor is largely independent from the others, so with three factors, there is only about 33% of variance to be explained by each. If observing the $R^2$ values from this context, they are relatively high. The $R^2$ values give the proportion of total variance in the dependent variable (texture) explained by the independent variable (factor).

From the analyses, it is apparent that the factors determining texture in yellowtail kingfish are complex and that there are many factors influencing texture, not just infection with *U. seriolae*. There also is a multifaceted relationship between flesh pH, rigor mortis, drip loss and texture, which is discussed in further detail below. In addition to the aforementioned factors of age/size and water temperature/condition factor, soft texture has also been attributed to other factors such as fish species, fat content, amount and properties of proteins and handling stress prior to slaughter (Hultmann et al. 2004b).

By investigating multiple avenues of parasite infection effects, acceptable thresholds of infection for ensuring acceptable product quality were determined. Acceptable textures included subjective texture scores of one and two, texture analyser scores of greater than 100 grams of maximum compression force, histological spore counts under 50 and enzyme absorbance values under 0.25.

The correlation of these parameters will be an important reference tool for researchers or fish farmers to ascertain if product is suitable for placement onto the market. Fish can be sampled by the farm, prior to harvesting, using the simpler methods such as the subjective texture analysis or opt to send muscle samples to a laboratory for histological analysis. Ideally, a simple field method for immediate feedback on the level of infection would be ideal, and this idea is further elaborated in section 6.2.

## 5.3. Relationship between Fish Morphology, Environmental Factors, Spore Counts, Enzyme Levels and Texture

### 5.3.1. Water temperature/seasonal fluctuation

Whilst a correlation was found between texture and water temperature, the results indicate that there is no seasonal effect on infection rates by *U. seriolae* in YTK in mid-west WA. Although it was not proven in this study that higher water temperatures lead to an increased prevalence of *U. seriolae*, it was revealed that the condition factor and growth of YTK decreased as temperature increased beyond their optimum temperature. Physiological stress on the fish when placed outside optimal temperatures ranges may reduce overall condition of the fish; in turn increasing susceptibility to parasites (Landsberg et al. 1998).

The ideal temperature range for YTK culture is between 18°C - 24°C (Miller et al. 2011) and the overall average water temperature throughout the growout period in Geraldton, WA was 22.9°C in cohort 1 (2010/11) and 23.7°C in cohort 2 (2011/12). Geraldton water temperatures may seem ideal for YTK culture; however, unseasonably high summer water temperatures were experienced by both cohorts with average February temperatures of 28.0°C and 27.5°C respectively (Source: Geraldton Port Authority water temperature data). These high temperatures negatively affected feeding and growth over the summer months (Pers. comm. IOFA. June 2012).

It was noted in previous studies that YTK may be more susceptible to myxozoa in warmer waters compared to cooler waters of Australia (Lester 1982, Hutson et al. 2007a). The high prevalence of infected YTK in the current study combined with the samples from South Australia, being devoid of *U. seriolae* infection supports this theory, as well as the lack of flesh myxospores found in southern NSW and Victorian YTK (Hutson et al. 2007b). Unfortunately samples of wild YTK from the south coast of Western Australia were unable to be collected to determine the geographical range/extent of *Unicapsula* infected YTK.

Implications of rising water temperatures in Western Australia could include extended distribution ranges of common myxozoan parasites such as *Unicapsula seriolae* and *Kudoa thyrsites*. This may result in potential infection of previously unsusceptible fish species of economic importance. In the summer of 2010/2011, Western Australia experienced a significant warm water event, with average sea surface water temperatures increasing to 3°-5°C above average. This heat wave lasted around one week and led to numerous fish kills and significant range extensions of 'tropical' species (Pearce et al. 2013).

### 5.3.2. Condition Factor

Despite a correlation between texture and condition factor, the enzyme levels/spore counts in YTK were not correlated with condition factor, and therefore it is not a useful indicator for predicting the intensity of infection. Some fish with excellent condition factors were found to have high levels of infection and examples of fish in poor condition were devoid of infection. No literature relevant to condition factor and myxozoa infecting musculature was found. However, Estensoro et al. (2011) demonstrated that infection by *Enteromyxum leei*, infecting the intestinal tract of gilthead seabream, *Sparus aurata*, lowered growth and condition factor significantly.

### 5.3.3. Age and Immune Response

Through histological analysis, it was revealed that the majority of YTK sampled in this project displayed either no immune response, or minimal response to even the most severe infections of *U. seriolae* (DoF. Fish Health Reports, 2011 - 2012). There were no signs of an immune response, as granulomas in the flesh, in 80% of fish from Harvest Trial One and 60% of fish from Harvest Trial Two.

Anecdotally, it has been observed that yellowtail kingfish may 'grow out' of the *U. seriolae* infections and the parasites disappear over time. One potential

mechanism for this response is an immune response in which granulomas are formed as a result of connective tissue, histiocytes and fibroblasts encapsulating the infected muscle fibre. Aggregations of phagocytes are present in order to destroy the contents of the myxozoan plasmodia (Morado et al. 1986). Aggregations such as these were observed histologically in a very small number of cultured fish from Geraldton which appeared to be overcoming moderate to severe infections (DoF. Fish Health Reports. Oct – Dec 2011). Similarly, a lack of immune response was also documented in the striped seabream, *Lithognathus mormyrus,* and picarel, *Spicara smaris,* containing infection of the musculature by *Unicapsula pflugfelderi* (Alama-Bermejo et al. 2009).

It appears that the myxozoan plasmodia may reach a critical mass and excrete metabolic wastes into surrounding tissue before it is detected and acted upon by the immune system (Morado et al. 1986). The increasing size of the fish may 'dilute' the infection as the fish grows and the formation of granulomas around plasmodia eventually acts to destroy viable myxospores (Pers. Comm. Dr Fran Stephens. January 2013). The result of this effect as the fish grows lessens the severity of the infection and therefore decreases the chances of myoliquefaction over time. In the YTK collected in this study, it is highly possible that infection occurs only once in the early stages of the fishes life, as there is no evidence of re-infection.

There have been examples of cultured YTK that have overcome infections but have accumulated scar tissue in the muscle from the development of multiple granulomas (DoF. Fish Health Reports, Sept. 2011). Granulomas formed as a response to myxozoan parasites in Pacific whiting, *Merluccius productus,* increased the amount of connective tissue in the muscle (Morado et al. 1986). It is thought that scar tissue formation in YTK may result in tougher flesh post-harvest, although this could prove to be advantageous for firmer flesh which is desirable in cooked YTK.

The findings of firmer texture in older, larger YTK were found to be different to those reported in a study of Pacific whiting, *Merluccius productus*, in western USA and Canada. Older Pacific whiting were found to have higher infection rates (Kabata et al. 1986) which resulted in softer texture (Kudo et al. 1987). It was also found in cultured Atlantic salmon, *Salmo salar*, in British Columbia that older salmon or reconditioned fish (that have reabsorbed gonads) possessed very high levels of myxozoan infection and unacceptable flesh quality. Sexually mature fish were 13.6 times more likely to be infected than immature fish. This was overcome by harvesting fish prior to maturity and grading out reconditioned fish (St Hilaire et al. 1998). The difference may have been due to the increase in levels of cortisol in the fish during maturity, a stress hormone that has been proven to suppress the immune system (Pickering et al. 1989). Cohort one fish (2.5kg) from the current study were found to be approaching maturity, with some male fish being observed as ripe (DoF Fish Health Report. Oct 2011). There was no evidence to sugest that YTK approaching maturity had higher infections rates, as older and larger fish had firmer texture.

In Japan, where consumer preference is for raw flesh, factors influencing raw flesh texture in yellowtail, *Seriola quinqueradiata*, include muscle lipid content and collagen content which vary with the anatomical location and season (Thakur et al. 2002). A direct relationship between texture of flesh in fish and the size of muscle fibres has been established, with firmer flesh having smaller muscle fibres (Hatae et al. 1990). It is unknown whether the size of muscle fibres of YTK increase or decrease with age and size, but this may also have an impact on overall flesh texture as the fish ages.

## 5.4. Impacts of Harvest Strategy on Flesh Quality in YTK fillets

### 5.4.1. Flesh pH, Rigor Index and Drip Loss

The impact of different harvest strategies on quality aspects of YTK were explored in this study. The treatments were a comparison of common harvest methods including normal harvest, spinal cord destruction (SCD) and rested harvest using anaesthetic.

It was demonstrated that different harvest methods have an effect on the post-harvest pH, texture and rigor mortis in YTK. The overall lower pH in fish from Harvest Trial One could be explained by the higher amount of available glycogen in the muscle at the time of harvest. A factor in the pH of flesh post-mortem is the amount of resting glycogen reserves, which are likely to have been high due to regular feeding and good health of the fish (Korhonen et al. 1990). The condition factor and health status of cohort one fish was much higher than cohort two, as they experienced overall better health and growth, which is explained in section 5.1.

The rigor index was only measured in Harvest Trial Two, and the rested treatment displayed significantly faster onset of rigor than the other treatments. Ideally, the slow onset of rigor mortis will result in firmer flesh with less drip loss (Wilkinson et al. 2008). Rigor mortis progression is also affected by factors including physiological and temperature conditions after death, whereas starvation and fatigue have also been observed to hasten the onset of rigor mortis (Ashie et al. 1996).

Erikson et al. (2006) created a pH scale for assessing handling stress on Atlantic salmon, *Salmo salar*. The scale classified rested salmon as having a muscle pH at harvest of 7.4 ±0.1, partially stressed salmon 7.1 ±0.1 and exhausted salmon 6.8 ± 0.1. The pH usually declines to a final pH of about 6.3 after storage on ice in salmon (Einen et al. 2002). Initial flesh pH in YTK in this study was found to be significantly lower (6.0 – 6.3) than the *Salmo salar* values. Stressed YTK in the rested harvest crowd had pH values of

approximately 6.0. Initial pH values of cultured seabass, *Dicentrarchus labrax*, from the Mediterranean were *ca* 7.3, slaughtered by immersion into iced seawater (Taliadourou et al. 2003). Stien et al. (2005) demonstrated that Atlantic cod, *Gadus morhua*, in a stressed state had an average initial pH of 7.0, whereas anaesthetised fish had a significantly higher flesh pH of 7.3.
The differences in initial flesh pH may be the result of species to species variation, with fish being highly exhausted prior to sampling and/or anoxia during crowding, as yellowtail have been previously shown to have a high oxygen demand (Hishida et al. 1998).

It is suggested that the YTK may have been exhausted prior to sampling based on their reaction to the anaesthetic, which was the opposite of that reported for other species i.e. barramundi (Wilkinson et al. 2008), with excessive struggling prior to being adequately anaesthetised. Other fish, such as Atlantic salmon, *Salmo salar*, when anaesthetised slowly and quietly progress into a deep state of sedation (Robb et al. 2000). The increased activity in YTK will have depleted ATP levels in the muscle, reduced muscle pH and in turn hastened the progression into rigor mortis (Stien et al. 2005). Despite the anaesthetic causing apparent exhaustion and low flesh pH, it seems to have resulted in minimal impact on flesh texture.

There was no difference in drip loss between harvest treatments. If drip loss were measured in Harvest Trial Two, it would be assumed that rested fish would experience higher drip loss due to lower initial pH, as water holding capacity is greatly reduced. The inability to retain water in flesh is a function of the denaturation and degradation of proteins, as well as disruption of the muscle structure through excessive contraction (Rodríguez et al. 2008).

Fish in better condition have higher glycogen stores in the muscle, which is known to reduce pH in the muscle via the production of lactic acid (Korhonen et al. 1990). It was observed in both trials also that fish in the rested harvest treatment struggled excessively upon contact with anaesthetic, which caused

lactic acidosis and a lower overall pH. In Harvest Trial Two, the flesh texture of the rested harvest fish was firmer, followed by SCD and normal harvest.

Overall, normal harvest, using pneumatic stunner and bleeding, was found to be the most appropriate method for optimal YTK flesh quality. Although the desirable trait of firmer flesh was observed in the rested harvest, quality parameters such as shelf life and quality index may be compromised by the lower pH, higher drip loss and faster progression into rigor mortis (Erikson et al. 2008, Hultmann et al. 2012).

To gain an insight into the stress levels of the fish at harvest, pH measurements are recommended to be taken throughout the harvest to measure flesh pH. If pH is found to be low compared to baseline levels at harvest, methods can be adjusted to increase the pH.

### 5.4.2. Product Temperature, Fillet quality and Microbiology

Overall, it was found that cooked flesh texture was the best indicator of YTK product quality as impacted by myxozoan infeciton, as other quality parameters including temperature, fillet quality and microbiology were unable to be linked to the effects of *U. seriolae*.

Whole fish have a longer shelf life than filleted fish, as demonstrated by Taliadourou et al. (2003), which used sensory evaluation and microbiology to assess the shelf life of sea bass, *Dicentrarchus labrax*. Fillets on ice had a shelf life of 8-9 days, whereas whole fish on ice lasted 12-13 days. The YTK industry primarily sells fish to large seafood retailers in capital cities that distribute the fish to restaurants, which generally use YTK in the raw state for sashimi. Standard methods used by industry are adequate for maintaining chilled temperatures to the domestic markets, as demonstrated by the temperature logging experiment in this study.

Carragher et al. (2009) concluded that “yellowtail kingfish is a robust fish that, although stressed by harvest procedures, shows little, if any, deleterious impact on the quality and shelf life of the resulting chilled product.” Despite the differences in flesh quality indicators between harvest treatments in pH, early onset of rigor mortis and increased drip loss in this study, it appears that the above statement may align with the results found in this study.

## 5.5. Recommendations to Industry for minimising the effects of *Unicapsula seriolae*.

With the possible development of a commercial YTK industry in WA, the infection rates have shown that there is a high risk of impact on quality caused by myxozoan parasites. The outcomes of this research may be utilised by the aquaculture industry to assist in mitigating the impact of myxozoan infections on product quality. The following recommendations are for aquacultured finfish operations that are susceptible to myxozoan parasites, based upon existing literature and results from this study.

### 5.5.1. Stocking and Harvesting

- Stock in late summer/early autumn when water temperatures are warm. This will allow fish to experience good initial growth in the warm water, then stable growth throughout winter. Cooler water temperatures also slows down the reproduction of monogenean parasites such as skin and gill flukes (Lackenby et al. 2007)
- It is likely that the invertebrate host of *U. seriolae* is a polychaete worm that lives in the benthos of sandy/silty environments (Yokoyama 2003). If possible, stock fish in deeper sites which may result in less exposure to infective actinospores released by the worms.
- Conduct regular net changes and remove fouling from mooring ropes and seacages, as fouling may harbor the primary hosts.
- Add oxygen to the crowd nets when harvesting to alleviate lactic acidosis, maintaining a higher flesh pH.
- Reduce stress during crowding by netting less fish; remove and dispatch fish quickly from the crowd net.
- Add oxygen to the blood bin at harvest. This has been proven to improve flesh quality, as yellowtail respire until complete physiological death (Shioya et al. 2011).

### 5.5.2. Feeding

- Minimise the use of plant based oils and proteins in feed. High inclusions will lead to decreased immunity in fish and compromise liver function, leaving fish susceptible to infection (Ogawa 1996). Estensoro et al. (2011) found that carnivorous fish fed on a predominantly plant based diet had more severe and progressive myxozoan infections than those without.

### 5.5.3. Fish and Environmental Sampling

- Sample fish regularly after stocking i.e. every two weeks when under 500g. Retain flesh samples to monitor the onset and progression of the infection on the population. PCR will pick up myxozoan infection sooner than histological analysis.
- Collect and preserve polychaete worms found on fouling on mooring ropes, benthos around the seacages for PCR. If myxozoans are present in the worms – regular physical removal of fouling may be beneficial.
- Observe the species of wild fish in the vicinity of the sea cages. It has been proven that species such as damsel fish and sergeant majors are carriers of myxozoans. Collect if possible for PCR.
- Collect benthic samples to monitor faunal assemblages – may assist in identifying the host.

## 5.6. Limitations of the Research

***pH Measurements***

During Harvest Trial One, the measurement of flesh pH 24 hours post-harvest was incomplete due to mechanical damage to the pH meter after it was dropped. Subsequent pH measurements at 48 hours post-harvest were undertaken with a different pH meter, and slightly different methods. The speartip meter used in the initial measurements was designed for use in the meat industry and was placed directly into the flesh, whereas a standard pH meter was used on minced flesh due to the standard bulb shaped probe. This type of probe needs complete immersion in the medium to be effective. Difference in results are thought to be insignificant as both meters were cleaned between samples and calibrated regularly.

***Rigor Mortis***

Due to time constraints, the rigor measurement was only performed at 0, 2, 23 and 50 hours post-harvest. Ideally rigor mortis measurements would have been taken at 0, 2, 5, 10, 15 and 20 hours to observe the progression and resolution over time. However, the results displayed the expected trends at 3 hours post-harvest based on the initial flesh pH at harvest and were adequate for analyses. There was a clear trend showing that fish with lower initial pH had a faster onset into rigor mortis.

***Spore Counting***

The average diameter of *U. seriolae* spores is approximately 5.5µm (Lester 1982) which made counting very difficult in the digest methods, even for seasoned parasitologists (Pers. Comm. DoFWA. May 2012). Larger myxozoan species such as *Kudoa thyrsites,* ranging from 13 – 16 µm (Whipps et al. 2003) are much more obvious to detect and easier to count.

The histological spore counting method was more consistent and faster than the previously tried methods, such as the squash technique and the digest method, and allowed accurate enumeration of *U. seriolae* infection rates. The digest method was expected to produce accurate counts but proved to be difficult due to the small size of *U. seriolae*, undigested muscle segments (Figure 5.1), air bubbles, fat globules and other debris (Figure 5.2) obscuring accurate counts.

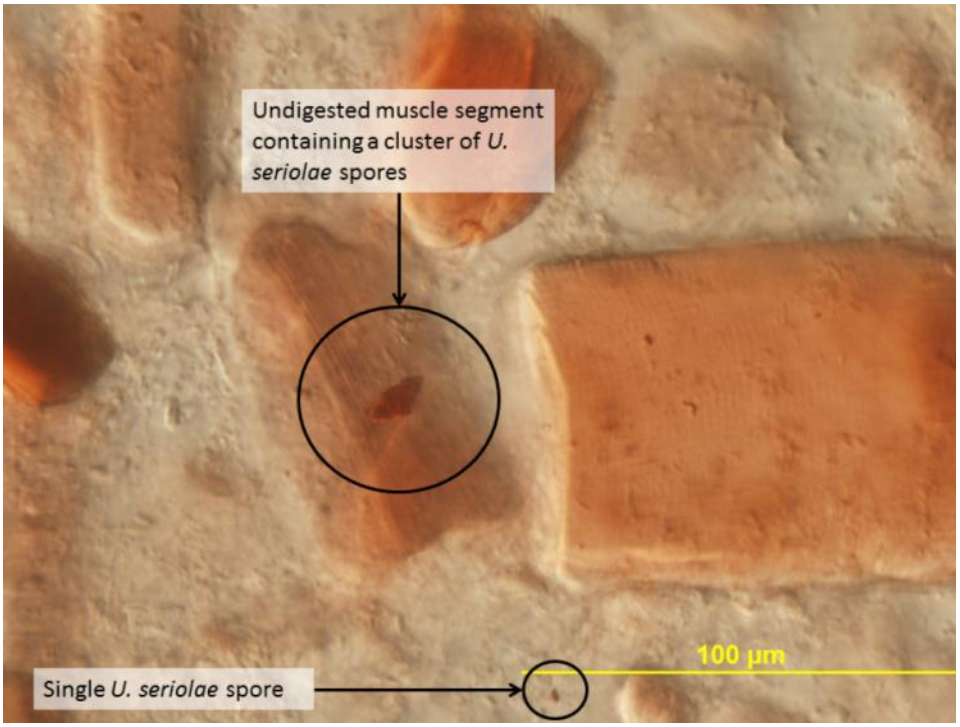



Figure 5.1: A cluster of *U. seriolae* spores embedded within a segment of muscle, as opposed to a single spore that has been released from the muscle tissue (x400). This clearly shows that the muscle was not completely digested by the trypsin in the preliminary digest method.

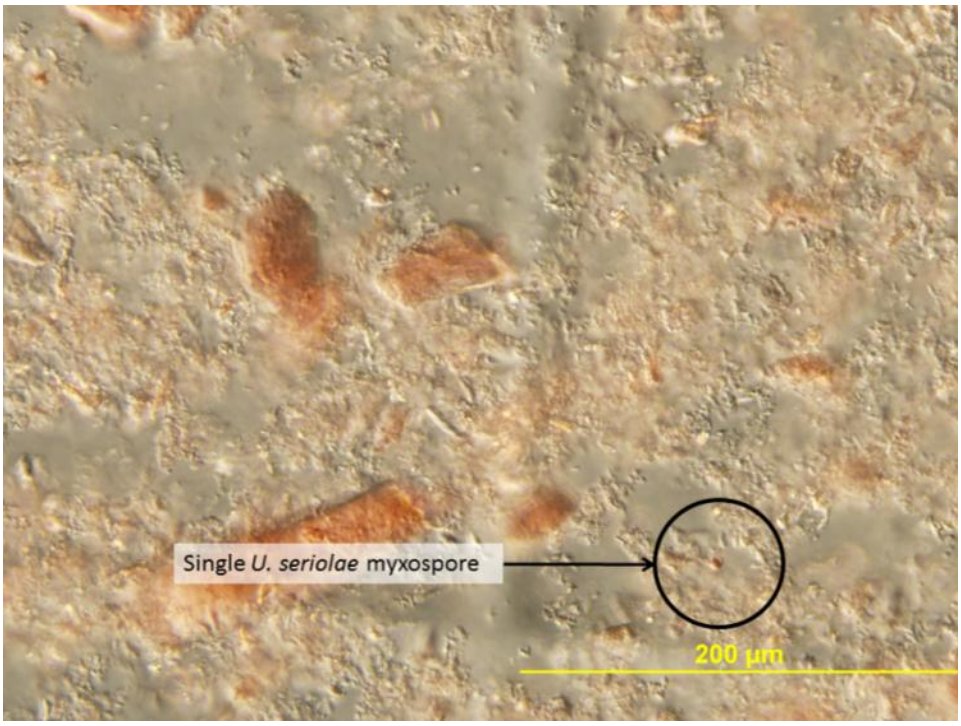



Figure 5.2: This picture (x200) demonstrates the undigested cellular debris in the samples which made spore counting difficult and time consuming in the preliminary digest method. Circled is an example of a single myxospore amongst the muscle segments.

The slight variation in size and specific gravity of the spores resulted in the spores being suspended on different planes within the solution. This resulted in the microscope needing to be continually adjusted to bring the spores into focus. The constant focal adjustment of the microscope whilst counting added to the difficulty of maintaining an accurate count. If the sample digested well and spores were on the same plane, counting was easier, faster and more accurate (Figure 5.3). The problems associated with the digest methods were overcome by using the histological spore counting method which proved to be accurate, repeatable and faster.

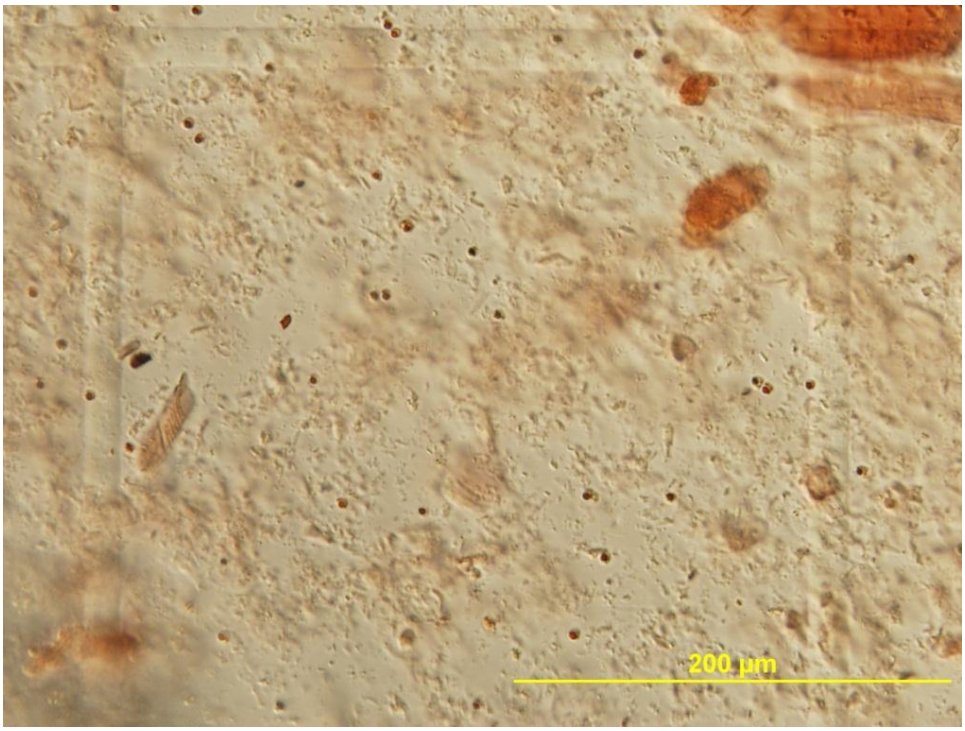



Figure 5.3: An example of a well digested sample in the preliminary digest method (x200 magnification).

***Texture Analysis***

During the texture analyses, it was observed that the myotomes (muscle fibres) in firmer flesh fish flaked and slid when pressure was applied from the texture analyser probe. Although the values obtained were still high, the value may not have been as high as possible due to the decrease in force as the flesh slid. This was observed in 3 of the samples but did not significantly affect the results. The samples that experienced the slippages were from larger wild fish (5kg+) in which the flesh was cut on a different plane to the majority of the cultured fish.

***Quality Parameters***

The small sample sizes (n = 3 per harvest treatment) used for fillet quality and microbiology in Harvest Trial One were found to be inadequate for statistical analyses. In addition to the small sample size, the low infection intensity of

this cohort of fish would not have been sufficient to reveal any effects of the parasite. Quality parameters were not performed on whole fish from Harvest Trial Two, as they were kept whole for 4 days prior to being filleted. After processing, the fillets were immediately frozen to prevent spoilage.

***Condition Factors***

During both harvest trials, it was found that there was a difference in condition factors between harvest methods. The condition factors increased with each crowd and treatment. One crowding of the sea cage per treatment was used in both harvest trials and order of sampling remained unchanged in both trials also – normal harvest, SCD and rested harvest. Only when analysing the data it became clear that the first and second crowds of the cage pulled out slower, weaker and smaller fish, whereas the last crowd for the rested harvest resulted in healthier and better conditioned fish. This was reflected in the significantly lower flesh pH in rested harvest fish in both trials. The design of the harvest trials did not factor this into the experiment.

***Confidentiality***

There are restrictions on intellectual property arising from this research due to a two year period of commercial confidentiality on this project. This has prevented the publication of scientific articles.

# CHAPTER 6. Conclusions and Recommendations

## 6.1. Conclusions

The hypotheses (Ho):

1. “that there is a quantifiable relationship between *U. seriolae* spore counts, enzyme levels and flesh quality in YTK”; and
2. “that environmental conditions and harvest strategies impact on *U. seriolae* infection in YTK”

Were accepted, based on the results obtained from the experiments conducted in this study.

The objectives for this research were as follows:

1. Confirm identification of *Unicapsula seriolae* species seen in cultured WA YTK.
2. Quantify the rate and prevalence of infection by *U. seriolae* in WA farmed and wild YTK.
3. Understand and quantify the relationship between *U. seriolae* infection and product flesh quality in YTK fillets**.**
4. Correlate *U. seriolae* spore count with other parameters including protease activity and texture of cooked YTK fillets.
5. Investigate other parameters such as water temperature, fish size and condition factor that may influence the prevalence of *U. seriolae.*
6. Identify the effects of harvest, handling and stocking strategies to limit the occurrence and economic impacts of myoliquefaction caused by *U. seriolae* in YTK.

The following summary indicates that the objectives of the research have been achieved:

1. The myxozoan species infecting cultured and wild YTK in WA was confirmed as *Unicapsula seriolae* (Objective 1).
2. No differences in the prevalence of *U. seriolae* in cultured and wild YTK in mid-west WA were found. The prevalence of the parasite was very high in all sample groups collected (>80%) (Objective 2).
3. Cooked flesh texture was the only product quality attribute that could be correlated with the effects of *U. seriolae* infection (Objective 3).
4. Parameters including proteolytic enzyme activity, cooked flesh texture and spore counts were highly correlated (Objective 4).
5. High infection rates of *U. seriolae* resulted in soft flesh texture through elevated enzymatic activity. No infection or low intensity of infection equated to firmer flesh (Objective 4).
6. Fish with higher condition factors had firmer texture and larger, older fish also had firmer texture (Objective 5).
7. During periods of lower water temperatures, fish experienced increased growth and higher condition factors (Objective 5).
8. Water temperature had minimal effect on *U. seriolae* infection rates (Objective 5).
9. Cooked flesh texture was the best overall indicator of YTK product quality (Objective 6).
10. Harvest method had a significant effect on flesh texture, flesh pH and the onset of rigor mortis (Objective 6).

## 6.2. Recommendations for Future Research

Based on the outcomes from the current research, the following recommendations are made for future research:

1. The lifecycle of *U. seriolae*, including its primary invertebrate host/s and the environment/s where it is most predominant remains to be explored. All assumptions on its lifecycle have been based on literature regarding *Kudoa spp*.
2. Investigating alternative myxozoan enumeration methods. A simple and rapid protein enumeration technique has potential to be developed as a useful field test (section 3.8.3). This test could be used to gain an insight into prevalence and severity of myxozoan infections on fish farms by measuring the optical density of supernatant containing 'free protein' after incubation. The enzyme study has demonstrated a good correlation with the enzyme assay, which may be further strengthened with refining the sensitivity and accuracy. With a more in-depth investigation, it could easily be applied to aquaculture operations as it requires minimal equipment, expertise and time. It could also be developed as non-lethal and non-destructive, as 250mg of muscle can easily be obtained from an anaesthetised fish.
3. Other alternatives for predicting the intensity of *U. seriolae* infection at the farm level could include a bench top method such as a lateral flow device. These tests use monoclonal antibodies developed specifically for the enzyme responsible for soft flesh, i.e. cathepsin L. If significant levels of the enzymes are detected, the antibodies will react with the enzymes to create a colour change on the device. The strength of the colour would vary depending on the severity of the infection. Specific kits can be developed overseas for a relatively low cost.

4. Due to the complex relationships of factors affecting post-harvest quality parameters in YTK, a series of controlled harvest trials is suggested. To further investigate flesh quality, without the influence of myxozoan parasites, it is suggested that harvest trials are undertaken in a controlled and parasite free environment, such as ACAAR, Fremantle, WA.
5. Cooking strategies to reduce the effects of the parasite on texture were not undertaken in these trials, as the cooking results were focused only on the varying degrees of effects of myxozoan parasites on flesh texture. It has been suggested that deep fat frying at high temperatures or microwaving the flesh are more effective methods of maintaining ideal flesh textures, even with high infections by myxozoan species (Lester 1982, Patashnik et al. 1982, Nelson et al. 1985).

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