Role of shock protein 70 (hsp70), ubiquitin and gill-associated virus in loss of production on prawn farms

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The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *National Statement on Ethics Conduct in Research Practice* (1997), the *James Cook University Policy on Experimentation Ethics, Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research Practice* (2001). The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review Committee (approval number A925)

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After years of saying ‘I will never do a PhD’, here I am.
There are so many people I would like to thank I will have to be epigrammatic or I will end up with a 1000 page thesis, and I only want to write one page as I have already done the Table of Contents, so here I go.
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ABSTRACT

Shock protein 70 (hsp70), ubiquitin (Ub) and gill-associated virus (GAV) were chosen as bio-indicators in an attempt to determine if they could be used to predict production of *Penaeus monodon* on a farm. To investigate the response of these bio-indictors with respect to changes in environmental factors, an ELISA for Ub was developed and previously developed ELISAs for hsp70 and GAV were optimised.

The utility of the ELISAs with respect to farm conditions, changes in the expression of hsp70 and Ub relative to health status, transportation and laboratory-induced hypoosmotic stress in cultured *P. monodon* was investigated. Protein expression as determined by ELISA, showed samples from the high yield pond had significantly lower optical density for hsp70 and Ub than the low yield pond ($p<0.001$, $p<0.001$ respectively). Transport ($p<0.001$, $p<0.05$) and osmotically stressed ($p<0.001$, $p<0.001$) groups showed a significantly higher response for hsp70 and Ub when compared to the control group. These results indicated that further investigations using farm data were justified.

A trial was undertaken in collaboration with a commercial prawn farm who supplied all the environmental and production data for the trial period. Two investigations were undertaken using this data. The first was to investigate changes in the hsp70, Ub and GAV responses in relation to environmental factors. There were significant correlations between all factors, the greatest number were associated with hsp70 (22 significant correlation coefficients) followed by GAV (18 significant correlation coefficients) and then Ub (17 significant correlation coefficients). In general the correlations between bio-indicators were positive and the environmental factors showed mostly negative correlations with the bio-indicators.

To determine the biological significance of these interactions, correlation analysis was conducted for each bio-indicator and environmental factor for all ponds daily from six days prior to sampling up to and including the day of sampling. The major environmental factors identified were pH (am) and salinity (am).

Morning pH was negatively correlated to hsp70 at day of sampling and four days prior to sampling with a dramatic correlation coefficient increase at five and six days prior to sampling. A similar pattern was noted with Ub. Salinity (am) was negatively correlated to hsp70, Ub and GAV at all days.

Principal component analysis was used in an attempt to better understand the underlying factors that explained the correlations and to reduce the data necessary for farmers to monitor. Five components were produced. Component one consists of four factors; days in pond, salinity (am), hsp70 and GAV. Components two to five consists of two factors in each component being temperature (am and pm) in component two, secchi (am and pm) in component three, pH (am and pm) in component and DO (am) and Ub in component five. The total cumulative variance explained by the five components was 74.3%.

The next study investigated changes in the hsp70, Ub and GAV responses in relation to production factors. There were significant correlations between all factors, the greatest number were associated with hsp70 and GAV (28 significant correlation coefficients) and then Ub (10 significant correlation coefficients). In general, correlations between hsp70 and GAV and production factors were positive. Survival was the only exception with negative correlations for hsp70 and GAV.

Production factors and the bio-indicators were subjected to principal component analysis. Two components were produced. Component one consisted of five factors, being days in pond, average body weight, yield, hsp70 and food conversion ratio. Component two consisted of two factors, survival and Ub. The total cumulative variance explained for the two components was 61.7%.

Discriminant analysis was performed to determine 1) if the bio-indicators and environmental factors could be used to distinguish between specified production outcomes and 2) which factors contribute most to these outcomes. Production factors were separated into the mutually exclusive categories of < or > 70% survival and < or > 7 tonnes/hectare (yield).

Using am data only, the number of factors required to correctly classify greater than 70% of the ponds for both survival and yield was reduced to seven and five respectively.

Of these, the factors most important in distinguishing between the categories were days in pond, hsp70, salinity and to a lesser extent, dissolved oxygen. Using classification and regression tree analysis, decision trees were developed for the production factors. A maximum average body weight of 23.9 g was predicted if the minimum morning salinity can be kept below 38 ppt and the morning pH can be kept below 7.8. A minimum average body weight of 13.4 g was predicted if the minimum morning salinity was >= 38 ppt and the prawns had been in the pond for < 129.5 days. A maximum survival of 85% was predicted if the morning dissolved oxygen level can be kept at >= 4.4 ppm and the optical density of GAV is >= 0.191. A minimum survival of 50.8% was predicted if morning dissolved oxygen levels are < 4.4 ppm and the minimum evening salinity is <32.8 ppt. Food conversion ratio appears to be largely dependent on days in pond. The best food conversion ratio (1.43) was predicted if the prawns were in the pond < 113.5 days and the worst (1.8) was if the prawns had been in the pond between 113.5 and 136.5 days and the morning pH was >= 7.95. A maximum yield of 8.01 tonnes/hectare was predicted if the prawns had been in the pond for >= 166.5 days and the minimum was 3.11 tonnes/hectare at < 119 days in pond. Secchi and GAV also played a role in yield outcomes. A maximum biomass of 6650 kg/pond was predicted if morning salinity was kept below 40 ppt and the prawns were in the pond for >= 168 days and a minimum biomass of 2740 kg/pond was predicted is morning salinity was above 41.5 ppt.

It is concluded that hsp70 may be a useful indicator relating to transport stress, survival and yield of *P. monodon* in a commercial setting. The results presented here show the successful development of statistical models based on environmental factors for the prediction of production outcomes that are both practical and interpretable at farm level. Continued investigation and development of predictive methods for production outcomes and profitability associated with prawn farms is recommended.

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ABTS	2,2’-azino-di-(3-ethylbenzthiazoline-6-sulphonic acid)
ABW	Average body weight
APC	Antigen presenting cell
ATP	Adenosine 5'-triphosphate
B	Blank
BCA	Bicinchoninic acid
C	Control
CART	Classification and regression tree
CC	Correlation coefficient
CMV	Cytomegalovirus
CWE	Control with water exchange
DAB	3,3’,-diaminobenzidine tetrahydrochloride
DIP	Days in pond
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DOS	Day of sampling
DUB	Deubiquitinating enzyme
EBNA-1	Epstein-Barr nuclear antigen 1
ELISA	Enzyme linked immunosorbent assay
FBS	Foetal bovine serum
FCR	Food conversion ratio
GAV	Gill-associated virus
HRPO	Horseradish peroxidase
HSE	Heat shock element
HSF	Heat shock protein transcription factor
hsp	Heat shock protein
HYP	High yield pond
JCU	James Cook University
LYP	Low yield pond
MAb	Monoclonal antibody
MARFU	Marine and Aquaculture Research Facility Unit

MHC	Major histocompatibility complex
MoV	Mourilyan virus
MSLP	Mean sea level pressure
MW	Molecular weight
NC	Negative control
OD	Optical density
OS	Osmotic stress
PAb	Polyclonal antibody
PBS	Phosphate buffered saline
PC	Positive control
PCR	Polymerase chain reaction
PG	Positive gill
ppm	Parts per million
ppt	Parts per thousand
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
RT	Room temperature
RT-nPCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SP	Shock protein
SUMO1	Small ubiquitin-related modifier 1
t/ha	Tonnes per hectare
TP	Total protein
TS	Transport stress
Ub	Ubiquitin
UCRP	Ubiquitin cross-reactive protein
UV	Ultraviolet
WSSV	White spot syndrome virus
YHV	Yellow head virus

CHAPTER 1

GENERAL INTRODUCTION

At present the global aquaculture of marine prawns is worth in excess of US$12 billion (FAO, 2007) with Queensland contributing approx AU$46.3 million to the industry (Lobegeiger and Wingfield, 2007). The growth of this industry is largely regulated and dramatically affected by infectious disease with the main causative agents being bacteria and viruses (Bachere, 1998). For example, the yellow head virus (YHV) epizootic that swept through Thailand in 1992 caused serious losses to prawn farmers resulting in lost production in the range of US$40 million (Flegel, 1997, Walker and Young, 2001). A pan-Asian epizootic caused by white spot syndrome virus (WSSV) followed in 1994, which caused even more serious losses for prawn farmers in the range of US$3 billion (Lundin, 1997). Thus prawn viral epizootics are of major concern to the global aquaculture industry causing sometimes-catastrophic consequences and usually significant commercial loss (Lightner and Redman, 1998). So the control of disease must become a global priority if prawn farming is to be ecologically and economically sustainable.

The invertebrate immune system has been shown to involve both humoral (utilising substances such as lysins, agglutinins and antimicrobial factors) and non-specific cellular defence mechanisms that interact to protect the organism from pathogen infection and repair tissue damage (Couch, 1977; Ratcliffe *et al.*, 1985; Thornqvist and Soderhall, 1997; Jayasree, 2001; Lee and Soderhall, 2002; Cooper, 2003; Loker *et al.*, 2004). In the case of bacterial infections, penaeid prawns have an immune response similar to other crustaceans. This system is largely based on haemocyte activity that includes an effective clotting system, phagocytosis, nodule formation and the incorporation of the prophenoloxidase pathway (Ratcliffe *et al.*, 1985; Bachere, 1998; Sung *et al.*, 1998; Lee *et al.*, 2001). These mechanisms work very well against bacterial and fungal pathogens.

At present, most literature describing crustacean immunity is based on studies performed on crabs and crayfish and little is known about how penaeid prawns deal with viral pathogens. Flegel and Pasharawipas (1998) believe that penaeid prawns are able to deal with viral pathogens by active "accommodation". It has been found that when a penaeid prawn encounters a virus for the first time, the virions replicate within cells until they burst free killing the cell and then move on to the next cell, eventually killing the prawn. Histology of these animals shows extensive necrosis similar to that seen in bacterial infections (Nadala *et al.*, 1992). However after a period, a proportion of the penaeid population is able to actively 'accommodate' these virions by phagocytising them in haemocytes and storing them in the lymphoid organ (Owens pers. comm.) without causing any adverse response. However, during times of stress these virions induce significant pathological changes leading to high mortalities (Fegan *et al.*, 1991).

Studies by Pasharawipas *et al.,* (1997) on penaeids showed that when previously encapsulated virions became pathogenic, the animals died from uncontrolled apoptosis rather than necrosis as seen in bacterial infections. This observation has led to some speculation about the mechanisms responsible for this sudden change in pathogenicity. It has been shown that during the wet season, periods of heavy rainfall reduce the culture pond salinity to a point where the animals become osmotically stressed. It is during this time that mortalities in cultured prawns can increase significantly, thus having a profound effect on the aquaculture industry and contributing to significant economic losses (Chanratchakool and Limsuwan, 1998). It is clear that stress plays some role in the pathogenicity of otherwise contained viruses.

A virus of concern to Australian penaeid culture is gill-associated virus (GAV). Gill-associated virus, a virulent pathogen of *Penaeus monodon*, has been described as having rod-shaped, enveloped, positive RNA virions closely resembling the morphology and cytopathology of yellow head virus from Thailand (Spann *et al.*, 1997).

Gill-associated virus has caused significant production losses to Australian aquaculture since 1996 and has been described as being endemic to both cultured and wild *P. monodon* along the east coast of Australia (Spann *et al.*, 1995; Cowley *et al.*, 2000; Cowley *et al.*, 2002; Spann *et al.*, 2003). Although GAV is highly prevalent and can be extremely pathogenic, it does not always cause disease and has been found as a chronic infection in otherwise healthy animals (Spann *et al.*, 1995; Spann *et al.*, 2003). Studies have indicated that environmental stress factors may initiate the acute stage which is then associated with high mortalities and production losses (Flegel and Pasharawipas, 1998; Vidal *et al.*, 2001). Recently, polyclonal and monoclonal antibodies specific for GAV were produced and an ELISA was developed for the detection of GAV (Munro and Owens, 2007) making high throughput testing affordable.

In addition to disease issues, rapid or dramatic changes in environmental factors can also have a major impact on production factors such as growth. One universal response to a range of environmental stressors is the synthesis of a group of highly conserved proteins commonly termed heat shock proteins (hsp) or, more appropriately, shock proteins (SP).
Briefly, SP have been shown to play a number of roles in the routine functioning of unstressed cells including the stabilisation of proteins at intermediate stages of folding, assembly, translocation across membranes including secretion, regulation and the targeting of proteins for degradation and removal (Hartl, 1996; Feder, 1999; Buchanan, 2000). These proteins are typically assigned to families on the basis of molecular weight (kDa), sequence homology and function and are highly conserved across taxa. The four main families are hsp90, the predominant hsp70 (Bukau and Horwich, 1998; Lewis *et al.*, 1999; Bukau *et al.*, 2006; Nicoll *et al.*, 2006), hsp60 and small hsp (Buchanan, 2000).

Research into shock proteins in aquatic invertebrates is increasing. A number of animals, in particular crustaceans including crayfish (Rochelle *et al.*, 1991; Sheller *et al.*, 1998; Cimino *et al.*, 2002), shrimp (Ravaux *et al.*, 2003), prawns (Cimino *et al.*, 2002; Selvakumar and Geraldine, 2005; Selvakumar *et al.*, 2005; de la Vega *et al.*, 2006) and lobster (Chang *et al.*, 1999) have been shown to respond to a variety of stressors. Upregulation of SP may be an indication of reversible protein change. However, increased or prolonged stress can result in the accumulation of irreversibly damaged proteins, at which time the ubiquitin-proteasome response is activated (Pickart and Eddins, 2004; Nandi *et al.*, 2006).

Ubiquitin (Ub) is a small protein composed of 76 amino acids present in all eukaryotic cells (Hershko and Ciechanover, 1998). As with stress proteins, the 53 ubiquitin sequences reported are highly conserved across a wide variety of species (Haas and Siepmann, 1997) and are involved in diverse cellular functions including regulating the cell cycle, cell growth and proliferation, endocytosis, DNA repair, embryogenesis, protein degradation, apoptosis and heat shock. Ubiquitin's structure, function and mechanisms have been comprehensively reviewed (Amerik and Hochstrasser, 2004; Pickart and Eddins, 2004; Nandi *et al.*, 2006). Briefly, the ubiquitin-proteasome system is largely responsible for the regulation of protein turnover in cells by closely regulating the degradation of specific proteins. The upregulation of ubiquitin and conjugated ubiquitin moieties can be considered indicators of an increase in protein turnover (Parsell and Lindquist, 1993; Hofmann and Somero, 1995; Amerik and Hochstrasser, 2004; Nandi *et al.*, 2006).

The vast majority of studies investigating the Ub system in aquatic invertebrates have focused on changes in the stress response of polyubiquitin in moult-induced muscle atrophy in land crabs (Mykles, 1996; Koenders *et al.*, 2002), in lobster (Shean and Mykles, 1995; Koenders *et al.*, 2002; Spees *et al.*, 2003) and responses of intertidal mussels (Hofmann and Somero, 1995), fresh water snails (Al-Khedhairy *et al.*, 2001) and lobster

(Spees *et al.*, 2002a; Spees *et al.*, 2002b; Spees *et al.*, 2003); to environmental stress. A molecular biomarker system using dot blot ELISA has been developed to assess the physiological status of grass shrimp (*Palaomenetes pugio*) (Downs *et al.*, 2001b) and gastropods (*Ilyanassa obsolete*) (Downs *et al.*, 2001a) when subjected to a range of stressors. The Ub antibody used in these latter two studies is said to detect both free ubiquitin and ubiquinated proteins, the collective levels of which were reported to be significantly higher after stress. We propose that targeting changes in the Ub monomer associated with stress may enable an overall view of cellular perturbations regardless of the fate of the protein or cause of the stress.

With the understanding that stressors (environmental and disease) have a profound effect on production outcome in penaeid culture, it is fundamental that quantifiable biological indicators (bio-indicators) of stress be developed to enable farmers to make informed management decisions thus improving production and profitability.

A range of physiological components including shock proteins, ubiquitin, blood glucose, ions, pH and osmolarity, lactate, osmolytes such as betaine, number/population of haemocytes, clotting time and others have been investigated in an attempt to find a set of bio-indicators that can be used to predict outcomes (Lewis *et al.*, 1999; Hall and de la Vega, 2003; Hyne and Maher, 2003; Dahlhoff, 2004). However the majority of the methods used are time consuming, costly, require highly skilled technicians and often kill the animal so the usefulness of each bio-indictor appears to be limited by these factors.

With the development of ELISAs for SP (Cimino *et al.*, 2002) and GAV (Munro and Owens, 2007) in *P. monodon* it is now possible to screen multiple samples quickly, at relatively low cost using non-lethal methods.

It is proposed that by using SP, Ub and GAV as indicators of stress and measuring changes in these bio-indicators in response to environmental changes at the farm level, it may be possible to predict production outcomes thus enabling the farmer to make informed management decisions.

Therefore the aims of this study are to:

1. Determine presence of ubiquitin in *Penaeus monodon* using SDS-PAGE and Western blotting techniques
2. Develop an ELISA to detect changes in ubiquitin
3. Optimise previously developed shock protein and gill associated virus ELISAs
4. Subject prawns to a range of stressors and determine changes in shock protein, GAV and ubiquitin
5. Use the assays to monitor farms and attempt to develop predictive plans for management intervention.

CHAPTER 2

REVIEW OF SHOCK PROTEINS AND UBIQUITIN AND THE STRESS RESPONSE IN AQUATIC ANIMALS

2.1 Introduction

It is proposed that the aetiology of many penaeid diseases is based on the fact that many animals normally carry a parasitic load including bacteria and viruses. Many pathogens such as bacteria and fungi can be controlled to some extent by improved husbandry techniques and the mechanisms by which penaeid prawns deal with these pathogens are well documented (Ratcliffe *et al.*, 1985). Relatively little is known, however, with respect to how the penaeid immune system deals with viral pathogens.

There is evidence to suggest that after an initial period of mortality due to viral infection of a naïve population, penaeid prawns are able to actively "accommodate" virions within their system without adverse effects (Pasharawipas *et al.*, 1997). Yet, when subjected to an environmental stress, such as sudden changes in temperature or osmolarity, these virions become pathogenic resulting in high mortalities (Flegel and Pasharawipas, 1998). At present the triggers for these phenomena are unknown.

Cultured penaeid prawns are often faced with highly stressful conditions. In most instances the prawn is cultured under relatively high densities in man-made structures such as tanks or ponds, fed an unnatural diet and faced with fluctuations in environmental parameters. Many of these stressors can be manipulated by the farmer and lessened by improved husbandry techniques and appropriate knowledge of the animals' biology (Owens and Evans, 1989). Two environmental factors in particular are almost impossible for the farmer to influence in prawn ponds and these are temperature and osmolarity.

These particular stressors can have a profound effect on penaeid aquaculture resulting in significant economic losses due to resultant viral epizootics (Chanratchakool and Limsuwan, 1998). Although the stress itself cannot be manipulated, the stress response of the animal may respond to manipulation. For example, many animals compensate for changes in internal osmolarity by accumulating organic osmolytes including *myo*-inositol, glycine betaine, and taurine (Burg *et al.*, 1997; Beck *et al.*, 1998). What mechanisms exist in penaeid prawns to reduce damage caused by environmental stress that may have an effect on immune functions?

A well documented response to environmental stress is the ability of all organisms to synthesise a group of proteins correctly termed shock proteins or commonly termed heat shock proteins. These proteins have a multitude of roles in both stressed and unstressed cells (Feder and Hofmann, 1999). One of the many tasks these proteins perform is to refold proteins that have been partially denatured due to stress.
However, when that stress becomes too great and the protein cannot be 'rescued' the ubiquitin (Ub) pathway is triggered. Ubiquitin tags proteins for degradation and removal thus contributing to the maintaining of homeostasis within the biological system.

It has been proposed that the delayed synthesis of SP by penaeid prawns during times of environmental stress may trigger a cascade of events resulting in the release of previously entrapped virions which then become pathogenic causing apoptosis and death (Owens and Liessmann, 2004).

2.2 Disease in Penaeid Prawn Aquaculture

Aquaculture can be broadly defined as the 'large scale husbandry or rearing of aquatic organisms for commercial purposes' (Landau, 1992). This type of farming has a long history, possibly dating back to the Egyptians in 2500 B.C.

Since this time, aquaculture has spread globally and has developed from extensive systems, or open systems (the environment is the farm and there is little or no intervention or control over environmental conditions), to intensive systems where all aspects of husbandry and environmental factors are carefully monitored and manipulated to achieve optimum growth and survival of the target organism (Landau, 1992).

The type of system used in modern times is largely dependant on the biology of the target organism and economic considerations. For example, oysters are farmed using extensive systems, crustaceans, such as penaeid prawns, are farmed using semi-intensive ponds and many fish species can be cultured in very high densities using intensive systems (Landau, 1992). Organisms grown using semi-intensive and intensive systems are subject to 'un-natural' environmental conditions (e.g. high stocking densities, reduced water quality, and often large fluctuations in osmotic pressure, dissolved oxygen and temperature). Consequently, they often show signs of stress which can lead to reduced growth and survival and increases in disease. It is under these conditions that the immune response is of critical importance.

2.2.1 The immune response of penaeid prawns.

Proteins are one of the fundamental molecules in all living organisms. These remarkable molecules accomplish an almost endless myriad of biological 'jobs' within an organism. Individual reactions of thousands of different proteins, whose intrinsic structures allow them to combine with absolute precision with other molecules, enable biological systems to function (Elliott and Elliott, 1997). Chemical reactions within the cell depend on protein enzymes that catalyse reactions at a speed and specificity that cannot be achieved by other means. Contractile tissues such as muscles depend on protein-protein interactions for movement, protein-DNA combinations are essential for gene control and the activities of hormones and nerves are dependant on interactions with protein receptors.

Solute transport across membranes is dependant on protein-solute interactions and the immune response relies on proteins that are able to recognise foreign bodies and protect the organism from damage. This list continues, encompassing most of the processes that are associated with a living organism (Abeles *et al.*, 1992).

Every organism must have mechanisms that can recognise and deal with foreign (non-self) or damaged cells. These mechanisms, collectively, are the immune response and they increase in complexity as the complexity of the organism increases. The most complex systems are those of mammals and higher vertebrates (Knox *et al.*, 1997).

The invertebrate immune system has been shown to involve both humoral (utilising substances such as lysins, agglutinins and antimicrobial factors) and non-specific cellular defence mechanisms that interact to protect the organism from pathogen infection and repair tissue damage (Couch, 1977; Ratcliffe *et al.*, 1985; Jayasree, 2001). These mechanisms are largely reliant on a group of cells called haemocytes that circulate within the haemolymph. These cells are the animal's primary line of defence, as to date, specific immune functions have not been documented in invertebrates. These cells are able to lyse, phagocytose and encapsulate foreign material (Millar and Ratcliffe, 1994). In the case of bacterial and fungal pathogens, penaeid prawns have an immune response similar to other crustaceans that is both efficient and rapid. When wounded, an effective clotting system responds with a rapid aggregation of haemocytes at the wound site. This seals the wound from the external environment and reduces the possibility of pathogen infection (Ratcliffe *et al.*, 1985). Those pathogens that are able to penetrate into the circulation are dealt with by other haemocytes. In penaeid prawns, when a foreign body has been recognized, the prophenoloxidase pathway (Cerenius and Soderhall, 2004) is triggered and its components are released from the haemocytes (Smith and Soderhall, 1986). Prophenoloxidase is converted to phenoloxidase through an enzyme cascade which can be triggered by several microbial polysaccharides, including β-1,3-glucan from

fungal cell walls, resulting in melanisation reactions (Smith *et al.*, 1984; Johansson and Soderhall, 1985; Aspan and Soderhall, 1991; Sung *et al.*, 1998). As well as producing highly reactive oxygen radicals such as hydrogen peroxide, which effectively oxidizes the pathogen, haemocytes are also responsible for phagocytosis and nodule formation or encapsulation. For larger foreign bodies, for example, a metazoan parasite or a large number of bacteria, phagocytosis is not efficient enough to deal with the infection, so a nodule is developed encapsulating the pathogen within several layers of cells (Cheng and Rifkin, 1970; Sung *et al.*, 2000). These mechanisms work very well under stable conditions, however when faced with rapid changes in environmental factors the system becomes compromised. For example, Wang and Cheng (2006a; 2006b) showed that rapid changes in temperature or salinity reduced the resistance of *P. monodon* to *Photobacterium damselae* subsp. *damselae* infection.

Increasingly severe exposure to pathogen attack will result in cell death. In general, cell death follows two well documented pathways, necrosis (non-programmed death) or apoptosis (programmed death). Necrosis is characterised by cellular swelling, random DNA fragmentation and lysis of the damaged cell resulting in the release of the cellular contents into the surrounding environment and inflammation. In comparison, apoptosis is a highly regulated process characterised by cytoplasmic shrinkage, membrane blebbing, protease and endonuclease activation, nuclear fragmentation and formation of apoptotic bodies. Apoptotic cells do not elicit an inflammatory response and are removed by phagocytosis (Janeway and Travers, 1997). At present, the prophenoloxidase pathway appears to be the most intensely investigated mechanism of penaeid immunity. However, very little is known about how penaeid prawns deal with viruses and most literature describing crustacean immunity has been based, until relatively recently, on studies performed on crabs and crayfish (Soderhall and Cerenius, 1992; Cardenas *et al.*, 2000).

Pasharawipas *et al.,* (1997) believe that penaeid prawns are able to deal with viral pathogens by active “accommodation”. It has been found that when a penaeid prawn encounters a virus for the first time, haemocytes phagocytose the virions. These virions then replicate within the cell until they burst free killing the cell and then move on to the next cell, eventually killing the prawn. Histology of these animals shows extensive necrosis similar to that seen in bacterial infections (Nadala *et al.*, 1992). Whilst studying the epizootics caused by YHV and WSSV however, Pasharawipas *et al.,* (1997) found that approximately 1.5 to two years after the initial outbreak, large numbers of active virions could be found within the tissue of surviving prawns, which did not cause pathology. This finding suggested a mechanism by which the prawn could actively ‘accommodate’ live virions without pathogenicity. This finding was further supported by Venegas *et al.*, (2000) whose investigations revealed that *P. japonicus* produced a quasi-immune response against WSSV.

The acquired protection started approximately three weeks post-infection and lasted for about one month, which was correlated with the presence of a humoral neutralising factor in the plasma component of the ‘immune’ animals (Wu *et al.*, 2002). However, the nature of the quasi-immune response is still unknown (i.e. could an innate immune response be unregulated or a type of adaptive immune response be induced?). Investigations by Owens (1993) clearly show virions within the haemocytes of hybrid penaeid prawns (*Penaeus esculentus* x *P. monodon*). From the figures presented, it is proposed that some of these virions had been recently phagocytosed and were being enclosed within vesicles. This proposal is further supported by investigations where it was shown that lymphoid organ spheroid cells of *P. monodon* ‘have the characteristics of exocytosed, granular haemocytes that have phagocytosed foreign material, particularly viruses’ (Anggraeni and Owens, 2000). It was hypothesised that these spheroid cells may be expelled during moulting.

During times of environmental stress, however, these previously sequestered virions induce significant pathological changes leading to high mortalities (Fegan *et al.*, 1991). Flegel and Pasharawipas (1998) have shown that innocuous YHV infections in some penaeid prawns can become pathogenic when the animals are subjected to stressors such as low dissolved oxygen concentrations or sudden pH changes but not by salinity shock. Conversely, Chanratchakool and Limsuwan (1998) showed that WSSV infections became lethal shortly after periods of heavy rain suggesting that a drop in salinity may be a trigger. Unfortunately, neither of these two studies mentioned the species of prawn investigated, so it is impossible to determine if there is a relationship between the type of stress and the species.
The study by Flegel and Pasharawipas (1998) showed that when these virions became pathogenic, the prawns died from viral triggered apoptosis rather than necrosis as seen in bacterial infections and during the primary viral infection. This finding was confirmed by Sahtout *et al.*, (2001) and Wu and Muroga (2004) during studies relating to WSSV.

These observations caused some speculation as to the mechanisms responsible for this sudden change in pathogenicity and as yet no definite triggers have been identified. It is clear from the available literature that stress plays some role in the pathogenicity of otherwise innocuous viruses. One universal response to environmental stress is the synthesis of a unique and universal group of proteins known as shock proteins (Feder and Hofmann, 1999).

2.3 Shock Proteins

Proteins are functional in their 3 dimensional structure (i.e. native structure). This structure is largely held in place by relatively weak hydrogen bonds. These bonds can be disrupted by a multitude of environmental stressors (heat, osmolarity, toxins, hypoxia, etc) that, when present at unfavourable levels, result in the denaturation of the tertiary structure.

This leads to loss of function of the proteins and consequently termination of all biological functions (Somero, 1995).

The denaturation of proteins exposes regions normally protected in the native protein by the tertiary structure. These exposed regions can bind to similar regions in other denatured proteins leading to protein aggregation. At worst this results in cytotoxic conditions. At best, it compromises the functional protein pool within the cell (Feder, 1999). The solution to this problem is the evolution of a repair and recycling system by which protein integrity can be maintained, effectively minimizing the problems which occur when proteins are in their non-native conformations.

This is accomplished by a group of proteins correctly termed shock proteins or commonly termed heat shock proteins (Feder and Hofmann, 1999). This group of proteins have been termed heat shock proteins due to their discovery during early investigations where their expression was found to be induced by thermal shock (Lindquist, 1986). However, during the past decade further investigations have shown that shock proteins are induced or upregulated in response to a wide range of physiological perturbations or stressors (including changes in temperature, osmotic, oxygen, pH and chemical levels) and so in this literature review these proteins will be referred to as shock proteins (Feder and Hofmann, 1999; Lewis *et al.*, 1999; Prohaszka and Fust, 2004). These findings have shifted the focus of research to understanding the task of SP as molecular chaperones (Gething and Sambrook, 1992; Hartl, 1996).

The genes encoding SP are highly conserved. The level of amino acid homology between prokaryotic and eukaryotic hsp70 proteins (molecular weight of 70kDa), for example, is close to 50% (Parsell and Lindquist, 1993). These proteins are typically assigned to families on the basis of molecular weight (kDa), sequence homology and function (Appendix 1). The four main families are hsp90, hsp70, hsp60 and small hsp. Other families include hsp110, hsp100, hsp40 and hsp10 (Buchanan, 2000).

In eukaryotes, some of these families have multiple members differing in cellular location and function with many being constitutively expressed in unstressed cells and upregulated during times of stress (Feder, 1999; Feder and Hofmann, 1999).

In an unstressed cell, molecular chaperones play diverse roles in the successful stabilisation of proteins at intermediate stages of folding, assembly, translocation across membranes, secretion, regulation and the targeting of proteins for degradation and removal (Feder, 1999; Buchanan, 2000). Incorrect functioning of these activities may underlie a number of important human diseases (Thomas *et al.*, 1995). When faced with a stress event, SP gene expression is regulated by the interaction between heat shock protein transcription factors (HSF) and heat shock elements (HSE) (Figure 2.1) (Xiao and Lis, 1988; Wu, 1995)

There exists a large quantity of literature describing the location, function and structure of these factors in vertebrate systems, however relatively little is known about these factors in invertebrate systems (Robert, 2003). It has been shown that HSF1 plays a role in the stress response of marine snails and mussels. Buckley *et al.*, (2001) have quantified levels of HSF1 in thermally challenged intertidal mussels (*Mytilus*). Their results revealed that endogenous levels of HSF1 did not vary between winter and summer acclimated mussels after thermal stress although levels of hsp70 were significantly different. It is implied that significant changes in HSF1 levels is not needed to adjust the heat shock response during acclimation. A similar response was shown by Tomanek and Somero (2002) investigating the shock response in marine snails (*Tegula*).

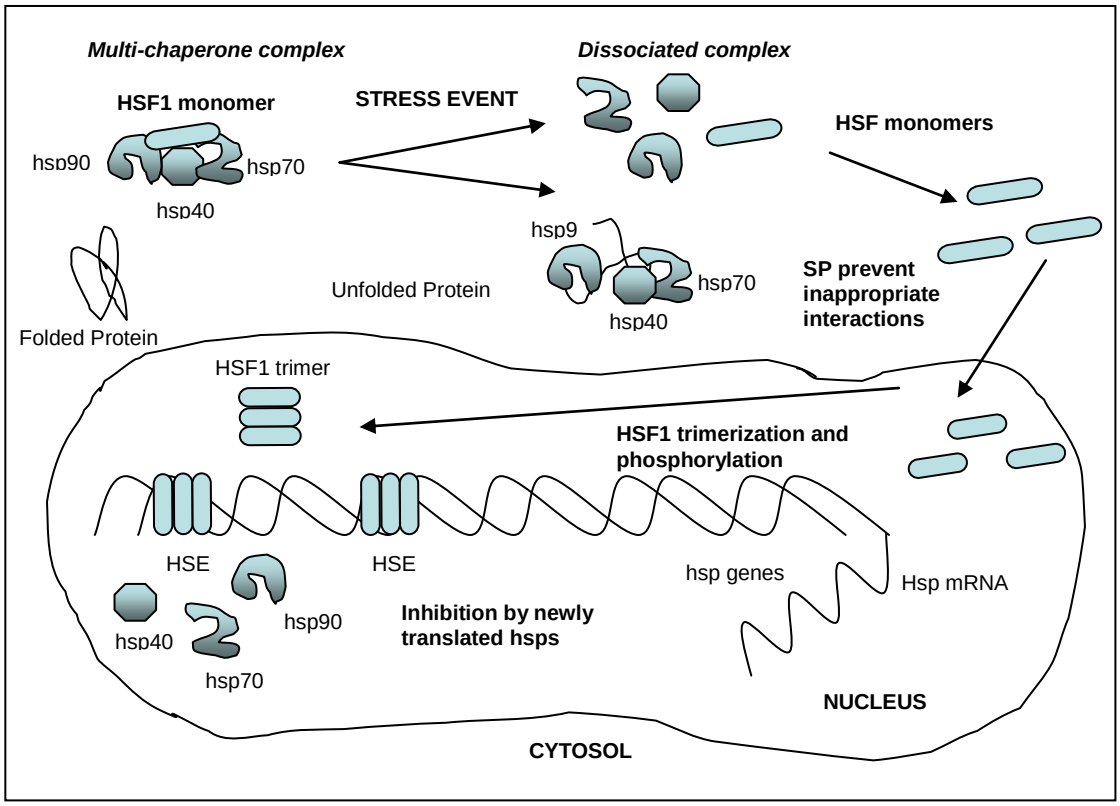


Figure 2.1: Model of the regulation of HSF1 in intertidal mussels and marine snails (modified from Buckley *et al.*, 2001; Tomanek and Somero, 2002) resulting in the synthesis of shock proteins. Under non-stressful conditions the inactive HSF1 monomer is found in the cytosol and is associated with a complex that consists of at least hsp70, hsp90 (in mussels and snails) and hsp40 (in snails). When thermally stressed the shock proteins disassociate from the complex and bind to proteins that have lost their conformational structure. The free HSF1 monomers move into the nucleus and bind to the HSE where they become hyper-phosphorylated to enable transcription. With the increase of shock protein production their binding to the HSF1 triggers its dissociation from the HSE thus leading to an inhibition in shock protein transcription.

For organisms that inhabit environments that are often biologically stressful, the roles of SP are of paramount importance. In the aquatic environment, for example, organisms such as those living in shallow waters (e.g. tidal pools, ponds, swamps, reefs) or in the intertidal zone must often cope with extreme fluctuations in environmental factors such as temperature, oxygen availability and osmotic concentrations (Webber and Thurman, 1991).

The array of literature present on shock proteins and molecular chaperones is immense. Reviews of this literature have allowed a number of important conclusions to be reached.

2.3.1 Current knowledge on shock proteins

The majority of research on molecular chaperones and SP leading up to present time has primarily focused on molecular, functional and evolutionary aspects elucidated by *in vitro* or laboratory based studies. Major interest has also been shown with studies investigating SP synthesis in diverse tissues, organs and individual organisms relating much of this to the organisms undergoing ecologically and evolutionarily relevant stresses. There is also increasing literature suggesting that certain SP play a role in both innate and adaptive immunity (Srivastava, 2002; 2004). Due to the strong interest in this field, the literature on molecular chaperones and SP is prodigious comprising in excess of 15,000 references (Feder and Hofmann, 1999).

For this reason, the present review will begin by identifying the salient conclusions evident from this literature (Feder and Hofmann, 1999; Robert, 2003):

*Molecular chaperones and shock proteins are a crucial aspect of all living organisms. Every organism studied to date has genes that encode and express these proteins.

*Molecular chaperones play a number of roles in the routine functioning of unstressed cells and are able to respond to every form of environmental stress that has been investigated.

*Some shock proteins have been shown to stimulate inflammatory cytokines, chemokines and co-stimulatory molecules through signalling pathways and play a role in both adaptive and innate immunity.

*The genes encoding shock proteins are highly conserved. Many are typically assigned to families on the basis of molecular weight (kDa), sequence homology and function: hsp110, hsp100, hsp90, hsp70, hsp60, hsp40, hsp10 and small hsp families. In eukaryotes some families have multiple members differing in cellular location and function.

*The importance of shock proteins cannot be underestimated however; they are collectively only one of the molecular mechanisms by which inducible stress tolerance is achieved.

It is no longer novel to investigate and find an as-yet-unexamined species expresses a SP in response to environmental stress unless the findings are to be used as the basis of further studies such as investigation of disease. These studies are made difficult as techniques such as genetic or experimental manipulation are often required or at least some kind of quantitative assay must be performed. The techniques being employed must be adapted from mammalian systems. In the study of aquatic organisms, the vast majority of investigations have used semi-qualitative assays such as Western blotting, a small percentage used genetic methods such as reverse transcription polymerase chain reaction (RT-PCR), Southern and Northern blotting and only one study used ELISA technology (Appendix 2). It is strikingly obvious that, although there is a huge collection of literature investigating most aspects of SP and the stress response, there is relatively little research with respect to the stress response seen when subjected to multiple stressors (Appendix 2). This has implications if studies involving cultured animals are to be undertaken, and so it is evident that further research in this area is needed.

In addition to this, very little literature could be found investigating SP and the stress response in penaeid prawns. As these animals are of significant economic importance to global aquaculture, it is suggested that further investigations in this area may provide valuable information.

2.4 Aquatic Temperature Stress

In the aquatic environment many organisms are able to use movement/behavioural methods to avoid stress inducing SP. For example, organisms that can burrow in the substrate are able to escape extremes in temperature; other animals are able to move in the water column or geographically to remain in favourable temperatures (Webber and Thurman, 1991). Sessile organisms that live in shallow waters (e.g. tidal pools, ponds, swamps, reefs) often cannot use these methods to reduce environmental fluctuations. As a consequence, thermal stress is the most common environmental stress encountered by these animals. The coral *Dendronephthya klunzingeri,* for example, showed upregulation of both hsp70 and hsp90 with a temperature change of only +/- 4^0C (Wiens *et al.*, 2000). Intertidal invertebrates are subject to much larger variations in temperature during tidal exposure with increases in body temperatures of up to 20-25^{0}C (Hofmann and Somero, 1995; Tomanek and Somero, 1999; Tomanek and Somero, 2000). The stress response reacts by increasing the production of many SP families (Tomanek and Somero, 1999). Tirard *et al.*, (1995) showed that when subjected to an increase of 20-28^{0}C from the acclimated temperature of 20^{0}C, haemocytes of *Crassostrea virginica* (oyster) responded with a dramatic increase in hsp70 (predominant protein for several days post-stress), hsp37, hsp34 and hsp32.

A similar response can be seen in crustaceans such as *Procambarus clarkii (*crayfish) (Rochelle *et al.*, 1991; Sheller *et al.*, 1998) and *Cherax quadricarinatus* (crayfish) (Cimino *et al.*, 2002) *Penaeus monodon* (prawn) (Cimino *et al.*, 2002; de la Vega *et al.*, 2006), *Rimicaris exoculata* (shrimp) (Ravaux *et al.*, 2003), *Artemia* (encysted brine shrimp) (Frankenberg *et al.*, 2000; Clegg *et al.*, 2000b), and *Homarus americanus* (lobster) (Chang *et al.*, 1999) with a significant increase in the synthesis of hsp70 and hsp90 when subjected to thermal stress.

An interesting study by Snyder and Rossi (2004) investigated differences in hsp70 levels expressed by *Anthopleura elegantissima* (Cnidaria) under natural conditions. It was found that hsp70 expression by polyps was high in shallower polyp clones than deeper clones. Clones exposed to a sunny, high intertidal zone expressed greater than three fold the hsp70 levels compared to those clones exposed to a foggy, high intertidal zone. This study is one of the very few performed *in situ*, so provides valuable data for conservative biology.

Even relatively mobile organisms, such as fish, face periods of thermal stress. For example, many species of desert fishes can undergo major temperature changes within a 24-hour period and have shown increased levels of hsp90, hsp70 and hsp30 (White *et al.*, 1994; Norris *et al.*, 1997a). Carpenter and Hofmann (2000) revealed a constitutively expressed 70kDa chaperone in the Antarctic fish *Tremaomus bernacchii* suggesting that despite the absence of environmental stress for at least 2.5 million years some of the 70kDa subunits have been maintained. In contrast, a study by Hofmann *et al.*, (2000) on thermal stress in *T. bernacchii* showed no synthesis of any size class of SP when subjected to an increase in temperature. The only other reference to an absence of SP synthesis in any other organism is *Hydra oligactis* (Bosch *et al.*, 1998). The reason/s for the absence of SP synthesis in these animals is yet to be determined. However, it has been suggested that perhaps “the absence of positive selection during evolution at stable sub-zero temperatures, dysfunctional genes for inducible SP, unstable messenger RNAs or the absence of a functional heat-shock factor” (Hofmann *et al.*, 2000), has lead to their absence. Another factor that may have had some influence on the latter study of *T. bernacchii* was that samples were shipped back to the USA, a trip taking three months, during which time sample integrity may have been compromised.

It has been suggested that SP may play a significant role in thermotolerance. When subjected to high or low temperatures such as those experienced during seasonal or daily changes, a number of studies have shown

significant differences in the synthesis and type of SP expressed (Schultz *et al.*, 1993; White *et al.*, 1994). Hofmann and Somero (1995) have shown that the levels of hsp70 are significantly higher in summer acclimated mussels (*Mytilus trossulus*) than in their winter acclimated counterparts. Similarly, seasonal changes in the levels of hsp70 evaluated in *Mytilus edulis* (mussel) showed increases during the warmer months and extremely low levels during the cold months. Seasonal changes also influence the induction temperature of SP synthesis. Buckley *et al.*, (2001) determined that the induction of hsp70 in thermally stressed *Mytilus trossulus* (mussel) was strongly influenced by the animal's thermal history. For example, mussels collected in the winter showed an induction threshold of temperature for hsp70 and hsp90 of 23^{o}C and for the mussels collected in the summer it was 28^{o}C. A similar pattern was shown by Buckley and Hofmann (2002) who demonstrated that acclimation temperature of the animal positively correlates to the induction temperature of hsp70 in the goby *Gillichthys mirabilis.* These findings also showed positive correlations with tolerance/survival to heat stress (Chapple *et al.*, 1998). For example, Clegg *et al.*, (1998) showed that prior heat shock can induce tolerance to otherwise lethal temperatures in *Crassostrea gigas* (oyster).

An increase in levels of SP during an increase in seasonal temperature is primarily to prevent protein aggregation, denaturation and to aid protein folding. This mechanism may also be important during times of increased growth, as is often the case during warmer temperatures, when enhanced levels of protein synthesis may require larger amounts of SP to chaperone the greater amount of ribosome-bound polypeptides (Hofmann and Somero, 1995). However, as environmental temperatures increase beyond the tolerance level, it is assumed that the metabolic cost of synthesising and maintaining protein integrity may become too great, compromising growth and survival. When the stress level reaches lethal levels, a signal that leads to programmed cell death, apoptosis, is activated and the animal is no longer able to survive. So the balance of SP synthesis may play a role in the habitat distribution of organisms (Tomanek and Somero, 1999).

The review of current literature clearly indicates that most aquatic organisms react to thermal stress in a similar manner with the synthesis of a particular group of SP during times of increased temperature and a decrease or lack of synthesis during times of decreased temperature. These findings suggest that during times of increased temperature the animal, especially poikilotherms, are in greater need of protein protection than during cold conditions. This would seem logical with most biological functions slowing with a decrease in temperature. Future research would seem repetitive if performed solely to demonstrate this phenomena with additional species unless for the purpose of using the information as a basis for other research.

2.4.1 Non-thermal stresses

Almost all non-thermal stress tested can induce SP synthesis. However, few studies have looked at the effect of these stresses in the natural environment or in the animal as a whole (Feder and Hofmann, 1999). This is biologically important as the expression and levels of SP synthesised vary depending on the type and number of environmental stresses encountered. For example, Tirard *et al.*, (1995; 1996) have investigated the effects of thermal and osmotic shock on SP synthesis in oyster haemocytes and found that both heat shock and hypoosmotic shock resulted in an increase in SP synthesis. Cold and hyperosmotic shock, however, did not alter protein synthesis.

The response to hypoosmotic shock was different to the response seen with hyperthermic shock, each stress synthesising a distinct set of SP. It has been proposed that this molecular adaptability may allow oyster haemocytes to maintain immune surveillance both during and immediately following episodes of environmental stress. A similar pattern was seen by Cimino *et al.*, (2002) who demonstrated a differing response in the synthesis of SP in *P. monodon* when hypoosmotically and hyperthermally shocked, each stress synthesising a distinct set of SP.

An interesting study by Schill *et al.*, (2003) revealed that female *Gammarus fossarum* (amphipod) were more sensitive to cadmium exposure than males with ‘drastically’ greater mortalities and at low cadmium concentration, ‘tendentially’ higher hsp70 mean levels compared to the control. All factors were kept constant in this experiment (i.e. age, size, moult stage, life stage etc.) except for those factors being investigated and no postulation was given for these outcomes. No other literature could be found showing differences in SP levels between sexes, however further research in this area is logical.

There has also been some very interesting work with respect to thermotolerance and ecological setting carried out on the hypoxia-tolerant *Artemia*. This small crustacean encounters extremes in environmental conditions during its life history and is very easily cultured under laboratory conditions. These factors have led to extensive research on the effects of long-term anoxia, desiccation, thermal, osmotic and chemical stress on the levels and expression of SP in these tiny animals.

Clegg (1997), Clegg *et al.,* (1999), Clegg *et al.,* (2000a), Liang *et al.*, (1997a), Liang *et al.*, (1997b) and Liang and MacRae (1999) have extensively investigated the synthesis and expression of large quantities of p26, a small heat shock/alpha-crystallin protein found in encysted *Artemia* embryos after periods of long-term anoxia. This particular protein has not been found in the adult *Artemia* suggesting that the presence of high levels of this protein may interfere with biological processes functioning in adults but not in cysts. It has been suggested that perhaps DNA synthesis and cell division may be most affected (Clegg *et al.*, 2000b).

Investigations have also shown an interaction between combined stressors and the synthesis of some SP. Dunlap and Matsumura (1997) showed that the synthesis of hsp70 by adult *Artemia* increased with thermal stress and further increased with the addition of lindane.

This study is one of the very few that was further investigated by combining changes in osmolarity with the addition of a number of chemical pesticides often present in the natural environment. The results clearly showed an increase in SP synthesis due to hypoosmotic conditions and a further increase in hsp70 synthesis due to combined chemical stressors.

A wide range of chemical stressors have been studied, many of which have concentrated on using the levels of SP produced as potential environmental biomarkers (Werner and Nagel, 1997; Kohler *et al.*, 1999; Schroder *et al.*, 1999; Kilemade and Mothersill, 2001; Snyder *et al.*, 2001; Lund *et al.*, 2002; Triebskorn *et al.*, 2002; Lund *et al.*, 2003; Dahlhoff, 2004). Most of the literature, using both *in vitro* and *in vivo* studies, showed increases in SP synthesis in organisms when they were exposed to stress under laboratory conditions. These results have been then applied as useful indicators of pollutants or toxins in the environment.

More studies are needed outside the controlled laboratory as organisms are often exposed to a multitude of stresses simultaneously. Some of these interactions result in SP expression when apparently non-harmful levels or conditions apply. For example, the levels of hsp90 were significantly increased in *Dendronephthya klunzingeri* (octocoral) that had been exposed to cadmium with the addition of thermal stress (Wiens *et al.*, 2000). Similarly, increased levels of hsp70 in freshwater sponges exposed to river water pollutants were further elevated when thermal stress was also imposed (Mueller *et al.*, 1995). Conversely, antagonistic or sparing interactions may also be present. For example, fathead minnows exhibit reduced levels of hsp70 during hypothermia when in the presence of toxaphene (Dunlap and Matsumura, 1997). This result perhaps indicates that during times of reduced or increased temperatures, the levels of some chemicals cannot be monitored accurately using SP levels.

The majority of the literature available on non-thermal stress relates to chemical stressors. Other environmental stressors, such as changes in salinity, for example, are of great importance to aquaculture and require further investigation.

It appears from the literature examined that aquatic animals react similarly to increased temperature and decreased osmolarity by synthesising SP. It is also clear that combined stresses can alter the synthesis and expression of SP. Given that the number and type of stressors imposed on the animal has a combined effect on the levels and expression of SP produced, caution must be taken with respect to *in vitro* and laboratory studies relating to SP as biomarkers.

2.5 Shock Proteins in Aquaculture

Very few studies have investigated the effect of SP on the stress response in commercially cultured organisms. These studies would seem difficult to perform due to the interactions of combined stressors imposed on a cultured animal at any given time. Some interesting work has, however, been carried out with respect to osmotic shock in anadromous fish such as salmon (*Salmo salar*) and the subsequent synthesis of SP. When smolt are transferred from freshwater to saltwater, osmoregulatory abilities were compromised resulting in a disruption of biochemical processes, stunting and sometimes death (Woo *et al.*, 1978). DuBeau *et al.*, (1998) showed that thermal shock prior to transfer to saltwater lead to the synthesis of hsp70 and consequently improved osmoregulatory mechanisms, resulting in higher survival of smolt.

Zarate and Bradley (2003) have shown that cultured one year old *Salmo salar* (Atlantic salmon) transferred from water at 15^{o}C to 26^{o}C for 15 minutes expressed increased levels of hsp70 mRNA (three fold increase from control levels), hsp90 mRNA (two fold increase from control levels) and levels of hsp30 increased two fold after 30 minutes thermal challenge.

Levels of hsp70, hsp90 and hsp30 were not significantly changed when these animals were exposed to other hatchery stresses such as feed deprivation, tricaine methanesulfonate (anaesthesia), capture stress, crowding stress, formalin, hypoxia and cold stress. A possible explanation for these results may be that SP are upregulated in response to protein degradation and the chemical concentration (i.e. formalin and tricaine methanesulfonate) were not high enough to be detrimental to the integrity of the fish. When using these chemicals the aim is to not cause any harm to the animal and so at working concentrations, SP expression was not induced.

Cold stress has not been shown to induce upregulation of heat shock proteins in crustaceans. However, a class of 'cold shock' proteins have been identified in a range of organisms including bacteria (Horn *et al.,* 2007), plants (Karlson and Imai, 2003), insects and fish (Ulusu and Tezcan, 2001). Some shock proteins shown to be synthesised in response to thermal stress may however, be involved in recovery from cold stress. For example, following cold shock *Drosophila melanogaster* larvae upregulate the same set of heat shock proteins that are noted in response to thermal shock (Burton *et al.,* 1988).
And Rinehart *et al.,* (2007) propose that the upregulation of several heat shock proteins are involved in cold survival during diapauses in insects.

Further research in this area is sorely needed; the results of which could be of significant economic importance to the aquaculture farmer.
Investigations should also include studies relating to the stress response during transport with a view to increase survival and product integrity particularly during export when transport times may be relatively long.

2.5.1 Shock proteins and disease

There exists a large quantity of literature which investigates the role of SP in mammalian disease. Studies have shown correlations between SP synthesis

and disease outcomes including cancer (Castelli *et al.*, 2001; Schueller *et al.*, 2001), rheumatoid arthritis (Blass *et al.*, 2001), Down syndrome (Yoo *et al.*, 2001), myocardial ischaemia (Li *et al.*, 2000) and inflammation and infectious disease agents such as bacteria and viruses (Barray *et al.*, 1990). Both advantageous and detrimental effects have been observed (see reviews Zinsmaier and Bronk, 2001; Zusman and Ben-Hur, 2001).

The molecular mechanisms are still unknown however it is believed that SP released from necrotic cells actively attract phagocytic antigen presenting cells (APC) into the region. Peptides are then presented to the incoming APC by the SP, chemicals are secreted, predisposing them to re-present the SP-delivered antigens in a way that encourages a reaction by immune cells. It is further believed that this system (compared with the B cell-driven humoral or antibody response) is better suited to attacking tumours and virally infected cells (Prohaszka and Fust, 2004). This hypothesis has been confirmed by a number of independent university laboratories (Goldman, 2002). At present, the relationship between SP expression and the immune response in teleosts and marine invertebrates is unclear (Deane *et al.*, 1999).

Some similarities may be present between teleosts and mammals as they have alike immune responses (i.e. both posses an adaptive immune response with immunoglobulins).
However, aquatic invertebrates such as crustaceans and molluscs (the vast majority of invertebrates cultured) are very different (Thornqvist and Soderhall, 1997).

It has been proposed that SP are part of an ancestral pathway that is the forerunner to, and independent of, the adaptive immunity of vertebrates. It is further proposed that the surface expression of SP may have provided a primordial system for presentation of cell surface antigens and may be widespread in present organisms (Robert *et al.*, 2001; Robert, 2003).

The evidence accumulating indicates that SP are recognised by different targeted cells and cells are activated by SP, and both processes are receptor mediated. This is an exciting area of research and warrants investigations in invertebrates.

There is increasing research into the use of SP as adjuvant-free vaccines in vertebrates. It has been shown that immunisation with a range of pathogen SP stimulates a robust immune response and provides protection against the disease caused by these pathogens (Suzue and Young, 1996). It has been proposed that strong memory responses are elicited and maintained by self SP or SP present in normal microbial flora. It is further proposed that this SP-specific immunological memory may structure as a first line of adaptive defence against pathogens (Murray and Young, 1992). This is an interesting area of research which warrants investigations in invertebrates as it may be of economic importance to the aquaculture industry.

As already mentioned, there is evidence suggesting that cultured penaeid prawns are dying from apoptosis associated with viral infection rather than necrosis. Mechanisms allowing these previously innocuous virions to become pathogenic appear to be triggered by a variety of environmental stressors, some of which the farmer can control or manipulate lessening the impact (Cowley *et al.*, 2000; Vidal *et al.*, 2001) (Table 2.1). These stressors include thermal and osmotic shocks which have been shown to result in the synthesis of SP. However, these are almost impossible for the farmer to control and so they are of significant importance.

Extremely high temperature stress could cause injuries such as DNA damage leading to mutation (King and Wild, 1983). Samali *et al.*, (1999) proposed that inhibition of SP synthesis may act as a cellular mechanism to ensure that repair of severely damaged cells does not occur and their results show that cell death (necrotic or apoptotic) and SP induction are mutually exclusive.

Samali *et al.*, (1999) hypothesis that apoptosis is caused by the lack of SP induction, rather than the consequence of SP induction (i.e. the failure of the protective effect of SP to protect and repair proteins leads to programmed cell death).

Table 2.1: Methods for the control of environmental stressors known to produce shock proteins on aquaculture farms.

ENVIRONMENTAL FACTORS PRODUCING SHOCK PROTIENS	CAUSES ON AQUACULTURE FARMS	CONTROL OF THESE FACTORS BY FARMERS
Low dissolved oxygen Clegg *et al*., (1997,1999, 2000[a])	Paddlewheel problems, algal bloom crashes, harvests	Regular maintenance of equipment, improved husbandry and handling techniques
pH	Extreme algal bloom fluctuations	Buffering
Osmotic shock Tirard *et al*., (1995,1996) Cimino *et al.*, (2002)	Salinity fluctuations due to periods of heavy rain or high evaporation	Almost impossible to control
Temperature shock (Many authors)	Cold snaps, heat waves	Almost impossible to control

Investigations have also shown that when cells are infected with virus the expression of SP is delayed following a stress event (Barray *et al.*, 1990). Results from Owens and Liessmann (2004) showed that SP synthesis in hypoosmotically challenged *P. monodon* resulted in two distinct groups: responders (i.e. a significant increase in SP levels after challenge) and non-responders (i.e. no significant increase in SP levels after challenge). There are two possibilities for this outcome: 1) As shown by Barray *et al.*, (1990) the upregulation of SP can be delayed in virally infected cells. Owens and Liessmann (2004) believe that it was probable that the experimental animals were virally challenged, 2) Samali *et al.*, (1999) proposed that SP upregulation may be halted in extreme stress events and cells become apoptotic in order to reduce the risk of cellular mutation.

The degree of stress (possibly a combination of the thermal or osmotic and viral stresses) was more than the animals could physiologically cope with and as a result SP expression was reduced. Those animals may possibly have died later as a result of viral associated apoptosis.

It has been proposed (Owens and Liessmann, 2004) that the delayed synthesis of SP in viral challenged animals may trigger a pathway (possibly linked to the interleukin 1 (α and β pathway) leading to a cascade of events ultimately resulting in DNA fragmentation and apoptosis. The majority of the components of this proposed pathway have been identified in penaeid prawns by researchers investigating other areas (Table 2.2). These theories are pertinent to an understanding of penaeid immunity and require further investigations.

Owens and Liessmann (2004) have suggested that if the stress from environmental challenge can be reduced during the delayed synthesis of SP, the resulting biological cascade will not be activated and the incidence of viral disease will decrease. Investigations have shown that the synthesis of SP can be reduced during times of osmotic stress by the accumulation of a number of naturally occurring organic osmolytes.

Table 2.2: Identified components for environmentally triggered apoptosis pathway in penaeid prawns 'accommodating' active viral pathogens. Modified from Owens (1999).

APOPTOSIS PATHWAY COMPONENT	EVIDENCE OF PRESENCE IN PRAWNS	REFERENCE
Shock Proteins	SP mRNA SP ELISA product	Moore and Lehnert (1998) Cimino *et al.*, (2002)
Interleukin 1α	Interleukin 1α RT-PCR product	Alcivar-Warren *et al.*, (1997)
Interleukin 1α β; 2;6, TNFa	All present in molluscs which are lower phylogenetically than prawns	Multiple authors
P35/38 inhibition of apoptosis in insect polyhedrosis viruses	Monodon baculovirus and baculovirus penaei producing polyhedrons	Multiple authors
Apoptosis	Apoptosis in viral infections	Flegel and Pasharawipas, (1998) Anggraeni and Owens (2000) Wu and Muroga (2004)

2.6 Osmotic Stress and Organic Osmolytes

Marine crustacean's exihibit a wide range of osmoregulatory capacities. Some tolerate relatively minor changes in salinty whilst others, such as *Artemia* sp can tolerate extreme variations (Charmantier and Charmantier-Daures, 2001). The mechanisms used by marine crustacea to maintain osmotic homeostatis include enzymatically catalysed ionic transport and control over the permeability of specific structures such as gills (Forest and von Baupel Klein, 2006).

Osmotic fluctuations in prawn ponds are almost impossible for the farmer to control to any great extent. In order to maintain homeostasis during times of fluctuating osmolarity, both prokaryotic and eukaryotic cells, almost universally, respond by accumulating compatible organic osmolytes such as inositol, betaine and taurine (Yancey *et al.*, 1982).
Most of the studies relating to these organic osmolytes have used vertebrate renal medullary cells *in vitro* and from these investigations a large amount of literature describing the structure and function of these substances has been generated (Burg, 1995; Burg *et al.*, 1997; Beck *et al.*, 1998).

Of interest were the findings of Petronini *et al.*, (1993) and Sheikh-Hamad *et al.,* (1994) that described the cellular accumulation of betaine and inositol. By adding high concentrations (2.5 - 25 mM) of one or both to the medium of the cell cultures, the increase in hsp70 mRNA levels was greatly reduced in response to hyperosmotic conditions. It has been suggested that the synthesis of SP in response to hyperosmotic conditions may precede the accumulation of organic osmolytes by several hours and that this mechanism may offer some protection to the cell while the organic osmolytes are accumulating (Cohen *et al.*, 1991). This is of special interest as it suggests a relationship between the synthesis of SP and the accumulation of organic osmolytes such as betaine.

Studies suggest that betaine in particular, when added to feed, may have positive effects on farmed animals during periods of osmotic stress by its ability to increase intracellular osmotic strength, replace inorganic ions in this function and protect enzymes from osmotic shock (Yancey *et al.*, 1982; Le Rudulier *et al.*, 1984; Kidd *et al.*, 1997). For example, in the aquaculture industry the addition of betaine to the feed of smolts of Atlantic salmon (Virtanen *et al.*, 1989), rainbow trout (Clarke *et al.*, 1994) and coho salmon (Castro *et al.*, 1998) resulted in reduced osmotic stress and mortality and enhanced the specific growth rate and food conversion ratio after saltwater transfer.

Commercial studies have suggested that the addition of betaine to the diets of penaeid prawns at a level of 1% (*P. monodon* and *P. vannamei)* improved the growth, food conversion ratio and survival of animals when compared to control groups (Finnsugar Bioproducts, unknown). A study by Saoud *et al.,* (2005) showed no significant difference in survival or growth of *P. vannamei* reared at extreme salinities (0.5 and 50 ppt) when fed with a feed supplemented 0.4% betaine. This result may have been due to the percentage of betaine supplementation. Personal experience has shown that betaine is extremely water soluable and added as a feed supplement can leach from the feed rapidly. It is crucial that the method used to supplement the feed overcomes this challenge. Ponpai *et al.,* (2007) showed that supplemented feed with betaine levels of 3% and 4% had a significant effect on both growth and immune response of *P. vannamei* when challenged with *Vibrio harveyi.* Likewise, studies performed at James Cook University (Owens and Liessmann, 2004) have shown that the addition of 1% betaine to the feed of *P. monodon* had a positive effect on the survival of prawns under viral challenge and had a beneficial or sparing effect when these animals were osmotically shocked.

These findings suggest positive outcomes that may have a significant effect on the penaeid prawn industry on a global basis.

However, the latter study was a one off, laboratory trial which, although giving valuable information, failed to answer a number of important questions including: 1) what concentration of betaine in the feed is both economically viable and effective 2) how long does betaine need to be fed to the prawns for it to be in sufficient levels 3) what other stressors (e.g. oxygen, temperature, pH etc) will betaine protect against. These questions can be answered cost effectively by laboratory trials. However, to determine the value of these findings from a commercial view, it seems logical that further research should be performed on commercial prawn farms

2.7 Ubiquitin

So what happens when stress results in the accumulation of irreversibly damaged proteins? At this point the ubiquitin (Ub) response is activated. The huge increase in information describing the Ub system in recent years has made it unrealistic to describe all the significant findings in this review. There are a number of comprehensive reviews that describe different aspects of the pathway (Hershko, 1988; Wilkinson, 1995; Haas and Siepmann, 1997; Ciechanover, 1998; Hershko and Ciechanover, 1998; Kornitzer and Ciechanover, 2000).

This review will summarise the enzymology, and known mechanisms involved in the ubiquitin- proteasome pathway and describe some important advances in the pathophysiology of the system.

Ubiquitin is a small shock protein (~8.5kDa) composed of 76 amino acids and is present in all eukaryotic cells (Garrett and Grisham, 1999). The structure of Ub is compact and tightly hydrogen-bonded to form a globular protein (Vijay-Kumar *et al.*, 1985). It is found throughout cells and can exist as either a free form or conjugated to other proteins.

As with shock proteins, the 53 reported Ub sequences are highly conserved across a wide variety of species (Haas and Siepmann, 1997). Ubiquitin is involved in diverse cellular functions. For example, Ub conjugation is involved in regulating the cell cycle, cell growth and proliferation, endocytosis, DNA repair, embryogenesis, protein degradation, apoptosis, heat shock and has been implicated in the immune response. This system is responsible for the regulation of protein turnover in cells by closely regulating the degradation of specific proteins. Such a role is crucial as cells can quickly eliminate a protein that serves a regulatory function in that cell (e.g. cyclins or a transcription factor needed for a particular gene expression). In addition, the degradation of particular regulatory proteins ensures that the biological cascade is shut-down (Hershko and Ciechanover, 1998).

The ubiquitin genes identified in eukaryotes typically exists in two forms: 1) the ubiquitin fusion gene, which encodes a single ubiquitin moiety fused to a ribosomal protein gene (Finley *et al.*, 1989) which are expressed constitutively in unstressed cells and 2) polyubiquitin chains, which consist of a linear repeat of ubiquitin molecules (Wiborg *et al.*, 1985; Baker and Board, 1987) and are induced in stressed cells (Fornace *et al.*, 1989; Finley and Chau, 1991).

It is important to understand that Ub itself does not degrade proteins. Its purpose is to act only as a tag that marks specific proteins for degradation. In short, proteins targeted for degradation are first tagged by conjugation with Ub molecules then these ubiquinated proteins are recognised and moved to the proteasome (26S) for degradation.

2.7.1 The ubiquitin-proteasome pathway

Protein ubiquitinylation is dependant on the sequential action of three enzymic families (Hershko and Ciechanover, 1998; Garrett and Grisham, 1999).

In short, ubiquitin is activated in its C-terminal glycine area (an ATP dependant step) by a specific ubiquitin activating enzyme, E1 (114 kDa) (Haas and Siepmann, 1997) (Figure 2.2).

Following activation, ubiquitin is then transferred from E1 to a cystine area of one of several ubiquitin conjugating enzymes, E2 (14-35 kDa), which then transfers the activated ubiquitin to a substrate-specific ubiquitin ligase, E3. The final step is catalysed by E3, the covalent attachment of ubiquitin to the substrate. A series of successive reactions using additional ubiquitin moieties results in the synthesis of a polyubiquitin chain. This polyubiquitinated substrate binds to a receptor in the 19S complex of the 26S proteasome resulting in the recycling of the ubiquitin molecules and degradation of the substrate to short peptides.

This pathway is further complicated by the existence of a number of deubiquitinating enzymes (DUBs). These enzymes cleave ribosomal ubiquitin fusion proteins and polyubiquitin chains from their respective genes as well as the polyubiquitin chains generated by the E1-E2-E3 cascade. In the latter case, DUBs can either rescue ubiquinated proteins before degradation by the proteasome by cleaving the polyubiquitin chain or cleave free chains that have been released from the tagged substrate following degradation by the proteasome (Mykles, 1997; Ciechanover, 1998; Mykles, 1999; Kornitzer and Ciechanover, 2000). So the activation of DUBs results in the inhibition of ubiquitin-mediated proteolysis.

A schematic ubiquitin-proteasome pathway is shown in Figure 2.2. The literature suggests that the organization of this system is hierarchal (Haas and Siepmann, 1997; Ciechanover, 1998; Hershko and Ciechanover, 1998): usually a single E1 enzyme is responsible for the activation of ubiquitin.

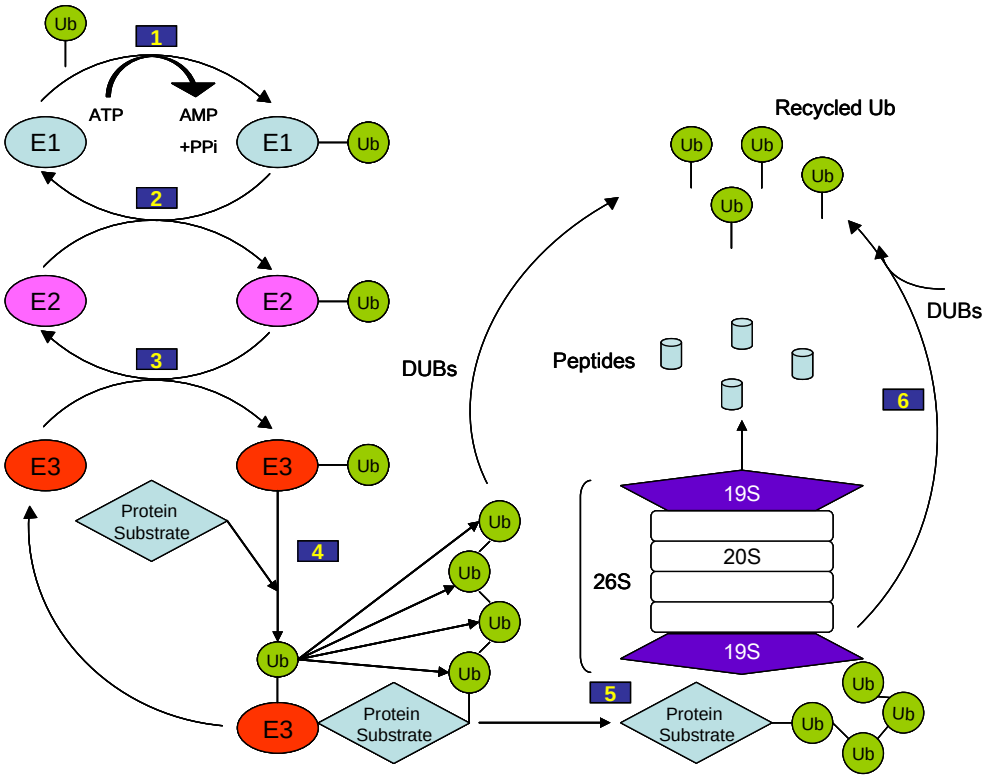


Figure 2.2: The ubiquitin-proteasome pathway. (1) Initially Ub is activated by E1 (using ATP). (2) The activated Ub is transferred to a member of the E2 family. (3) Ub is then transferred to a substrate-specific member of the E3 family. (4) A substrate-E3 complex is formed and through successive reactions, a polyubiquitin chain is synthesised. (5) The polyubiquitinated substrate binds to the Ub receptor subunit in the 19S complex of the 26S proteasome. The substrate is then degraded by the 20S complex resulting in short peptides. (6) Ub is made available to the system again by the action of isopeptidases.

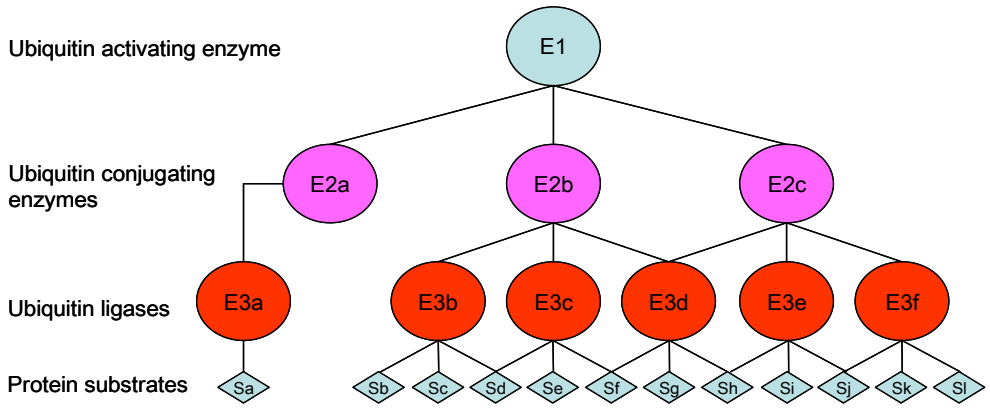


Figure 2.3: The proposed hierarchical organisation of the Ub conjugating system. A single E1 enzyme activates a Ub molecule and transfers it to one of several E2 enzymes. In some cases the E2 enzyme is E3 specific however, in most instances an E2 enzyme transfers Ub to one of several E3 enzymes. E3 enzymes can be either substrate specific or can recognise several substrates.

To date only a relatively few E3 enzymes have been identified and knowledge of these enzymes remains limited. Research in determining new E3 enzymes have been difficult partly due to the lack of sequence homologies between different types of E3s and their mode of action. Generally E3s can be defined as “an enzyme that binds, directly or indirectly, specific protein substrates and promotes the transfer of ubiquitin, directly or indirectly, from a thiolester intermediate to amide linkages with proteins or polyubiquitin chains” (Hershko and Ciechanover, 1998).

Four families of E3s have been described to date:
1) The main N-end rule E3, E3α and Ubr1p (its yeast homolog) (review by Hershko and Ciechanover, 1992) is a family containing two sites that bind either basic (Type I) or large hydrophobic (Type II) N-terminal residues of their substrates. They also recognise other non-N-end rule substrates such as unfolded proteins and some N-α-acetylated proteins and bind via a ‘body’ site that is not yet well characterised (Ciechanover, 1998; Hershko and Ciechanover, 1998). E3α is able to bind to a specific E2, facilitating the transfer of activated Ub from a specific E2 directly to the substrate protein. E3β (a related enzyme) is specific to proteins with small and uncharged N-terminal amino acid residues. The recognition mechanism of the N-end rule is highly conserved across eukaryotic evolution, however its’ physiological roles and substrates are unclear.

2) The second family of E3 enzymes are known as the HECT (homologous to E6-AP C-terminus) domain family. A large family of proteins that contain a HECT domain have been identified in a number of eukaryotic organisms (Huibregtse *et al.*, 1995). The C-terminal region that contains the ubiquitin binding cystine residue is highly conserved however, the N-terminal regions of the different HECT proteins are highly variable. This suggests that the N-terminal regions may be involved in the binding of specific substrates.
The first member of this family discovered, E6-associated protein (E6-AP) is required, together with human papillomavirus E6 oncoprotein, for the ubiquitination and degradation of p53 (a tumor suppressor protein).

Unlike E3α which facilitates the transfer of Ub from a specific E2 directly to the substrate protein, E6-AP does not bind directly to p53 instead it forms a ternary complex with E6 which recognises and binds both p53 and E6-AP (Ciechanover, 1998; Hershko and Ciechanover, 1998). Mutations of the E6-AP gene have been shown to be involved in some cases of the human hereditary disease Angelman syndrome (Kishino *et al.*, 1997; Matsuura *et al.*, 1997).

Members of the HECT domain family are involved in the targeting of a number of other specific substrates including RSP5 which conjugates the large subunit of RNA polymerase II in yeast (Huibregtse *et al.*, 1997), FUR4 uracil permease in yeast and NEDD4 in rat kidney epithelial sodium channels (mutations of this molecule in humans causes Liddle's syndrome) (Staub *et al.*, 1996).

3) The third family of E3 enzymes is the cyclosome (Sudakin *et al.*, 1995) or anaphase promoting complex (King *et al.*, 1995). This complex has E3 activity specific for cell-cycle regulator proteins such as mitotic cyclins, certain anaphase inhibitors and spindle-associated proteins that consist of a nine amino acid pattern (termed the 'destruction box'), all of which are degraded during mitosis (Hershko *et al.*, 1994).

4) The fourth family of E3 enzymes known as phosphoprotein-ubiquitin ligase complexes are involved in the degradation of other cell-cycle regulators such as the CDK1 (cyclin-dependant kinase) inhibitor, SIC1 (substrate inhibitor of cyclin-dependent kinase), or certain G1 cyclins (Ciechanover, 1998; Hershko and Ciechanover, 1998). This family involves phosphorylation of the substrate converting it to a form subject to the action of the ligase complex.

Due to the differences in sequence homology and mechanisms by which E3 enzymes carry out their two basic functions, substrate recognition and ubiquitin transfer, there is a substantial area of knowledge relating to the ubiquitin field that remains an enigma.

However, recent studies using proteomic tools including multidimensional chromatography, tandem mass spectrometry and data base searching (Denison *et al.*, 2005) indicate that this area of research is advancing rapidly.

A number of ubiquitin-like molecules have been discovered that show varying degrees of sequence homology to ubiquitin. For example, Parkin (a ubiquitin-like molecule that is larger than ubiquitin) has ubiquitin-like domains and is implicated in the pathogenesis of certain forms of Parkinson's disease in humans.

Other groups contain a higher sequence homology to ubiquitin and are involved in post-translational, single or multiple modifications of target substrates (Hochstrasser, 1998). For example small ubiquitin-related modifier 1 (SUMO1) can act as a ubiquitin antagonist by generating a degradation resistant protein and consequently protecting cells against anti-FAS, tumour necrosis factor α, induced apoptosis (Kamitani *et al.*, 1997). A second ubiquitin-like molecule, NEDD8, has been identified in mice and plants. Ubiquitin cross-reactive protein (UCRP) is an interferon-inducible protein that shows significant sequence homology to a tandem repeat of ubiquitin. UCRP appears to be involved in targeting proteins to the cytoskeleton (Loeb and Haas, 1994).

2.7.2 Proteasomes

Clearly proteins elicit some form of signal that is recognised by the Ub system however, it is still unclear what factors determine if a protein gets tagged by Ub for subsequent degradation by the 26S proteasome complex. Proteasomes are large oligomeric structures that possess proteolytic sites which degrade proteins resulting in oligopeptides approximately seven to nine residues long (Wilkinson, 1995; Coux *et al.*, 1996). Studies have shown that the proteasome is crucial for cell survival and proliferation.

In eukaryotic cells two forms of proteasome exist; the 20S proteasome and the 26S proteasome. Comprehensive reviews describing these complexes have been reported elsewhere (Hershko and Ciechanover, 1998; Forster and Hill, 2003). Briefly, the eukaryote 26S proteasome is composed of a 20S proteasome and the substructures termed 19S caps or PA700.
These structures work in concert by recognising and selecting ubiquitinylated proteins which they then unfold and transport to the proteolytic cavity of the 20S proteasome (Garrett and Grisham, 1999) where they are degraded to small peptides (Coux *et al.*, 1996; Forster and Hill, 2003). A number of studies have been undertaken to determine the structure, functions and biochemical properties of invertebrate proteasomes (Mykles, 1997; Mykles, 1999).

At present the mechanisms involved in substrate recognition and the entry of protein substrates into, and exit of peptides out of, the proteasome are not well understood. This is an area of research that is attracting much attention and continues to generate important discoveries.

2.7.3 The ubiquitin-proteasome pathway and disease

Considering the complexity of the ubiquitin pathway, it is no surprise that perturbations in the system may be involved in the pathogenesis of a number of diseases (both hereditary and acquired). There has been considerable research in the area of human disease however very little in invertebrate systems and none could be found relating to aquatic animals. The pathological states that are present in those systems studied can be divided into two broad groups: 1) conditions resulting from a loss in function, mutation or substrate that leads to stabilisation of certain proteins and 2) conditions resulting from a gain in function (i.e. accelerated degradation).

Examples of diseases include malignancies, cystic fibrosis, Angelman's syndrome, Liddle syndrome, Alzheimer's disease, Parkinson's disease, Lewy body diseases, amyotrophic lateral sclerosis, Creutzfeld-Jakob disease, Huntington disease, spinocerebellar ataxias and skeletal muscle atrophy (Ciechanover, 1998). An interesting study was undertaken using rats launched into space (Ikemoto *et al.*, 2001). The general aim of the study was to investigate muscle atrophy, a complication encountered by astronauts, induced by space travel. The results from this study showed that spaceflight, in particular weightlessness, increase the levels of polyubiquitin mRNA in rats and subsequently induced significant degradation of muscle contractile proteins resulting in muscle wasting. Unfortunately, the pathway that triggers the ubiquitin-proteasome system during spaceflight/weightlessness is still unclear so further research into muscle atrophy associated with space travel is required.

The immune and inflammatory responses involve interesting interactions between the ubiquitin pathway and viruses. In two instances the virus is able to exploit the system and remain undetected. The first example is the Epstein-Barr nuclear antigen 1 (EBNA-1) protein, which is found in healthy carriers for life.

Unlike other members of the EBNA family, EBNA-1 contains a region that inhibits the degradation of this protein by the ubiquitin system and so persists in the carrier possibly contributing to some of the virus-related pathologies (Levitskaya *et al.*, 1997). The second example involves the human cytomegalovirus (CMV) that encodes two proteins known as US2 and US11. These proteins are involved in the targeting of major histocompatibility complex (MHC) molecules for degradation. The viral mediated degradation of the MHC molecules halts the presentation of viral antigens and thus enables the virus to infect the immune system (Ciechanover, 1998).

It is clear from the magnitude of research and the knowledge gained that the ubiquitin pathway plays an important role in the pathogenesis of disease in humans. It would seem feasible then that a similar pattern might be discovered in other organisms. Of particular interest are those organisms of economic importance such as in aquaculture. Further research in this area would certainly increase our understanding of the mechanisms behind the pathogenesis of disease (in particular viral diseases) in aquatic organisms.

2.7.4 Ubiquitin research in aquatic invertebrates

Relatively few studies have investigated the ubiquitin system in aquatic invertebrates and none could be found relating to disease in these animals. Studies have shown that a range of aquatic species have genes that encode ubiquitin/polyubiquitin. For example, Pfeifer *et al.*, (1993) cloned the polyubiquitin cDNA from the marine sponge *Geodia cydonium* (one of the simplest multicellular eukaryotic groups of organisms). A comparison of the predicted amino acid sequence from the sponge with other organisms showed 93% (*Euplotes* a protozoa) and 99% (human) homology. This is an indication of the highly conserved nature of ubiquitin between the simplest of marine organisms and higher vertebrates.

The ubiquitin/proteasome pathway has been shown to carry out protein degradation in moult-induced muscle atrophy in land crabs (Mykles, 1996; Koenders *et al.*, 2002) and lobster (Koenders *et al.*, 2002; Spees *et al.*, 2003); and responses of intertidal mussels (Hofmann and Somero, 1995), fresh water snails (Al-Khedhairy *et al.*, 2001) and lobster (Spees *et al.*, 2002a; Spees *et al.*, 2002b) to environmental stress. In the crustacean studies, the majority of the research focused on changes in polyubiquitin levels with respect to muscle atrophy associated with moulting or muscle wasting due to lack of use. Crustaceans must moult many times during their life to enable growth.

In many species of large clawed crustaceans their claws are shaped such that they cannot withdrawn the muscle from the exoskeleton during moulting so they must undergo muscle atrophy to facilitate this process. Shean and Mykles (1995) used a partial-length clone (1.7kb) of polyubiquitin isolated from a lobster claw muscle cDNA library to investigate changes in polyubiquitin and E2 levels during a moult cycle in land crabs. The study showed an approximately five fold increase in polyubiquitin mRNA (2.7kb) and an approximately eight fold increase in ubiquitin-protein conjugates (>200kDa) during atrophy (moulting). Interestingly the level of E2 (16kDa) remained unchanged during the moult cycle. Koenders *et al.*, (2002) showed that levels of both polyubiquitin mRNA and ubiquitin-protein conjugates increased during claw muscle atrophy in both *Gecarcinus lateralis* (land crab) and *Homarus americanus* (American lobster). RT-PCR of polyubiquitin in the lobster claw muscle showed the amount of amplification product varied depending on moult stage. The highest amount occurred at early proecdysis with less at late proecdysis and the lowest levels during intermoult. Spees *et al.*, (2003) took this area of research further by investigating polyubiquitin and shock proteins (constitutive shock protein 70 [hsc70], hsp70, and hsp90) mRNA levels in claw and abdominal muscles from premoult and intermoult lobsters. The levels of polyubiquitin, hsc70 and hsp90 mRNA were significantly higher in premoult relative to intermoult claw muscle, while levels of hsp70 mRNA were unchanged (n=6).

A comparison between tissues showed higher levels of polyubiquitin mRNA in abdominal muscle during intermolt, conversely higher levels of polyubiquitin mRNA in claw muscle was noted during late proecdysis (n=1). These studies suggest care when investigating shock proteins (including ubiquitin) in either environmentally stressed or unstressed crustaceans as moult cycle appears to affect mRNA levels. However, increasing the number of animals used should reduce variability between moult stages in farm based *in vivo* studies. Of particular interest is a study by Mykles (1996) who has suggested the use of a crustacean model (land crabs) for spaceflight experiments.

Muscle atrophy is a serious problem associated with spaceflight and much research has been undertaken to solve this problem. Although mammalian models such as rats (Ikemoto *et al.*, 2001) have been used in this type of study, it is difficult to find a suitable model for spaceflight due to issues such as housing, feeding and waste removal to mention a few. The crustacean model overcomes many of these problems as they require minimal care during spaceflight; can go extended periods without food (one to two weeks); and can be maintained in a simple moist environment.
Of major importance are the structural and biochemical features and ubiquitin/proteasome pathway that crustaceans possess which are the same as those found in vertebrate skeletal muscle.

In addition to muscle atrophy, the ubiquitin response has been investigated in aquatic invertebrates under environmentally stressful conditions.
Al Khedhairy *et al.*, (2001) reviewed the effects of cadmium levels (linked to some human diseases) on gene expression in a range of invertebrates including the freshwater snail, *Biomphalaria arabica*.
It has been shown that the gene expression of hsp70 and polyubiquitin when stressed with cadmium is non-linear and is affected by cadmium concentration and duration of exposure.

If the stress response is considered, it would seem logical that upon first contact with the toxicant, proteins in the animal may become denatured and so the expression of hsp70 is induced to repair the damage. When the toxicant persists causing longer exposure and/or increased concentrations, the ubiquitin pathway is initiated and polyubiquitin genes are induced as was observed in *B. arabica*. Further work by Al-Khedhairy *et al.*, (2001) has shown that at lethal and sublethal cadmium concentrations, hsp70 gene expression was increased first (first phase). As exposure time and cadmium concentration increased polyubiquitin gene expression was induced (second phase). This pattern of gene expression may be a useful bio-indicator for environmental stress in a range of organisms.

This is an area of research that requires further research as it has the potential to be developed into a tool to measure the health of many species either in their natural habitat or in farmed situations. Indeed Downs *et al.*, (2001b) developed a molecular biomarker system using dot blot ELISA techniques to assess the physiological status of the grass shrimp (*Palaomenetes pugio*) when subjected to a range of environmental stressors. Factors symptomatic of a stressed or non-stressed state in *P. pugio* were investigated and they were hsp60, hsp70 (indicators of reversible protein damage/chaperoning), αB-crystallin homologue (an indicator for the level of cytoskeletal damage due to stress), lipid peroxide (an indicator of biological membrane perturbations and oxidative stress), total glutathione level, ubiquitin (an indicator of protein turnover), mitochondrial manganese superoxide dismutase (an indicator of mitochondrial oxidative stress), metallothionein (an indicator of metal toxicity) and cytochrome P-450 2E homologue (an indicator of xenobiotic stress). This is the only study found that investigated the ubiquitin monomer (conversely polyubiquitin) in a crustacean. However, the molecular size of the cross-reactive ubiquitin molecule was not mentioned in the paper.

Levels of ubiquitin, hsp60 and hsp70 were shown to significantly increase in response to high temperature (increased from 22°C to 38°C), cadmium chloride exposure (minimum 1μM, 5μM and 50μM consecutively), bunker fuel exposure (minimum 3 g/L ubiquitin and hsp60 only), diesel fuel exposure (minimum 2 g/L ubiquitin and hsp70 only) and atrazine exposure (minimum1 mg/L hsp60 and hsp70 only). This study was able to distinguish between stressed and non-stressed responses and was also able to discern between the different stressors. A number of limitations/considerations to this system were mentioned by Downs *et al.*, (2001b) including developmental stage/age of the grass shrimp, baseline data concerning the animal's response to combined stressors as opposed to individual stressors and seasonal variations in cellular parameters. These are all significant considerations and further research in this area is required to elucidate any mitigating factors.

Hofmann and Somero (1995) investigated the relationship between environmental temperature and protein damage in the intertidal mussel *Mytilus trossulus*. To do this, changes in the levels of ubiquitin conjugates and hsp70 from gill tissue were measured using Western blotting techniques. The results showed that the summer acclimated mussels had higher levels of ubiquitin conjugates and hsp70 than those of the winter acclimated animals (n=4). Similarly mussels collected from the intertidal zone had higher levels of these two proteins compared to those collected from the submerged zone.

Using homologous molecular probes, Spees *et al.*, (2002a) investigated changes in the levels of hsp70, hsp90 and polyubiquitin mRNA associated with thermal acclimation and stress in *H. americanus*. Mussels were split into two groups: ambient temperature acclimated and cold acclimated animals. Both groups were heat shocked with an increase in water temperature of 13ºC. Samples from the hepatopancreas and the abdominal muscle were taken at several times during the stress and at several recovery times (n=4). Significant increases in all three components and both tissue types were found in the ambient acclimated group with highest levels seen at two hours of heat stress followed by six to12 hours recovery.
Although both tissue types showed significant increases, the hepatopancreas showed greater induction of polyubiquitin mRNA than the abdominal muscle. Not mentioned in this study but indicated by the pattern of gene expression over the trial, is the induction of hsp70 genes in the abdominal muscle soon after heat shock (0.25 hours) followed later by the induction of polyubiquitin genes (two hours). This appears to agree with Al-Khedhairy *et al.*, (2001). In contrast to the ambient acclimated animals, the cold acclimated group treated with the same stress procedure showed no significant increases in any of the genes investigated.

As mentioned earlier, osmotic shock appears to be linked to mortalities in crustaceans, particularly under cultured conditions. In a similar study to Spees *et al.*, (2002a), Spees *et al.*, (2002b) investigated stress-responsive gene expression in osmotically stressed *H. americanus*.

Again, homologous molecular probes were used to investigate changes in the levels of hsp70, hsp90 and polyubiquitin mRNA in abdominal muscle and hepatopancreas during hypo- (reduced from 32ppt to 16ppt using distilled water) and hyper- osmotic stress (increased from 32ppt to 48ppt using sea salts). Samples were taken at several times during the treatments. There was increased induction of hsp70, hsp90 and polyubiquitin mRNA in the abdominal muscle at treatment times of 30, 60 and 120 minutes in both hypo- and hyper-osmotic groups. Interestingly, in all instances the hypoosmotic groups had higher levels of mRNA than the hyper-osmotic groups, and levels of mRNA were highest in the 30 and 60 minute groups. Polyubiquitin and hsp90 mRNA levels were measured in the hepatopancreas under the conditions mentioned above. Polyubiquitin mRNA levels were significantly induced in the 60 minute hypoosmotic group but not in the hyper-osmotic group, conversely hsp90 mRNA levels were significantly induced in the 120 minute hyper-osmotic group. It appears that in this study, the abdominal muscle was more sensitive than the hepatopancreas to osmotic stress.

As with previously mentioned shock protein studies, the vast majority of ubiquitin/proteasome studies used molecular tools in their investigations. This leaves a large area of research open to investigations using proteomic tools. This is an exciting area of research which is still only in its infancy with respect to the knowledge to be gained with further research.

2.7.5 The role of the ubiquitin-proteasome pathway and apoptosis

As mentioned previously, viral triggered apoptosis has been linked to high mortalities in environmentally stressed prawns. Studies on both the ubiquitin/proteasome pathway and the apoptotic pathway (largely regulated by caspases) have produced evidence to suggest exchanges between these pathways.

The first study to indicate the involvement of the ubiquitin/proteasome pathway in apoptosis was research into the programmed cell death in intersegmental muscles in the hawkmoth *Manduca sexta* (Schwartz *et al.*, 1990). Polyubiquitin mRNA dramatically increased in intersegmental muscles when these muscles were committed to degeneration. Injection of 20-hydroxyecdysone, which delays degeneration in this system, prevented the increase in polyubiquitin mRNA, indicating a role in cell death without the influence of other stressors.

Further evidence for the involvement of the ubiquitin/proteasome pathway in apoptosis comes from studies of thyroxine-induced apoptosis of the tips of tadpole tail (*Rana catesbeiana*) (Phillips and Platt, 1994), dystrophin-deficient mice (Sandri *et al.*, 1995), murine lymphocytes treated with teniposide, the colonial ascidian (*Botryllus schlosseri*) and human lymphocytes treated with γ-irradiation (Orlowski, 1999). In all of these studies, the levels of ubiquitin/polyubiquitin were increased in tissues undergoing apoptosis.

There are however, studies that do not support these findings. For example, during apoptosis levels of ubiquitin/polyubiquitin were not increased in:
R. catesbeiana tail tips treated with cycloheximidethe (Phillips and Platt, 1994), dying cells in the compound eye of *Drosophila*, rat sympathetic neurons undergoing apoptosis due to deprivation of nerve growth factor and dexamethasone induced apoptosis in T-cells (Orlowski, 1999).
The abdominal ganglia of *M. sexta* showed increased ubiquitin levels in some neurons not undergoing apoptosis and no increase in ubiquitin levels in some neurons undergoing apoptosis (Orlowski, 1999). Theses conflicting studies suggest that the involvement of the ubiquitin/proteasome pathway in apoptosis appears to be cell and environment specific.

Considering the importance of the ubiquitin/proteasome pathway in protein degradation and turnover it seemed probable that the proteasome play a role in degrading the contents of cells undergoing apoptosis.

Indeed, a number of studies have supported this hypothesis by showing changes in the 26S proteasome concurrently with increased levels of ubiquitin in cells undergoing apoptosis (Jones *et al.*, 1995; Orlowski, 1999; Wojcik, 2002). The discovery of proteasome inhibitors has enabled a more direct evaluation of the role proteasomes play in apoptosis. Many studies have used proteasome inhibitors to investigate this pathway and have shown that in some cells proteasome inhibition promotes apoptosis, while in other cells it protects cells from apoptosis (Orlowski, 1999; Wojcik, 2002).

It appears from the literature that proteasome inhibition of apoptosis is predominately found in primary cell cultures such as thymocytes (Grimm *et al.*, 1996; Beyette *et al.*, 1998; Hirsch *et al.*, 1998) and neurons (Sadoul *et al.*, 1996). These investigators have postulated that aspects of these differentiated, non-dividing cells are a determinant, and it was only in actively proliferating cell lines that apoptosis was induced (Orlowski, 1999). It has also been suggested that these conflicting results may be due to the concentrations of inhibitory agents used or to differential activation of kinases and certain shock proteins (Orlowski, 1999; Wojcik, 2002). At present it is still unclear why some cells are sensitive to proteasome inhibition and why some cells are not, reflecting the complexity of these systems. Due to the complexity of these systems, it is important that *in vivo* studies be undertaken.

2.8 Conclusion

It is evident from this review that there exists almost no literature investigating the mechanisms allowing the immune system of penaeid prawns to cope with viral pathogens. The mechanisms involved in viral ‘accommodation’ and the triggers that ultimately lead to viral-associated apoptosis are also largely unknown.

However, it is obvious that environmental stress (particularly temperature and osmotic stress) affects the pathogenicity of viruses in cultured penaeid prawns that show evidence of viral ‘accommodation’ (Chanratchakool and Limsuwan, 1998). The significance of this must then be the animal’s response to environmental stress, which results in the production of shock proteins.

The almost endless investigations regarding shock proteins demonstrate that the synthesis of these proteins is a universal mechanism for coping with environmental stress. Early investigations concentrated on the structure and function of these proteins, and later ecological and evolutionary aspects were studied. These studies have contributed greatly to the understanding of shock proteins when subjected to individual stressors. A significant finding from this review was the lack of quantitative assays for investigations relating to shock proteins. The methods used are relatively costly, time consuming and require laboratories and trained technicians. From an aquaculture point of view, these methods are not practical in the field. Cimino *et al.*, (2002) developed the first and to date, the only ELISA enabling the qualitative analysis of changes in hsp86 levels in thermally stressed *C. quadricarinatus* and *P. monodon* and hypoosmotically stressed *P. monodon*. This assay is relatively cost effective, can screen multiple samples fast, can be modified to detect changes in other shock protein levels and as such, is more appealing to the farmer.

Relatively few studies have investigated the effects of combined stressors outside laboratory conditions. It is, however, evident from these investigations that interactions do exist among multiple stressors leading to changes in both the type and levels of shock protein expressed (Mueller *et al.*, 1995; Wiens *et al.*, 2000). This is an important observation if studies relating to cultured animals are to be of use. The discovery that shock proteins play a significant role in both vertebrate and invertebrate innate immunity is exciting.

Although the mechanisms/pathways are not well understood, shock proteins have been shown to be major players in generating specific anti-tumour, anti-viral and anti-bacterial protective immunity in mammals, This may have important implications for the development of vaccines for economically important cultured animals (i.e. fish and crustaceans).

Of particular interest is the finding that osmotic stress often leads to shock protein synthesis (Tirard *et al.*, 1995; Tirard *et al.*, 1996; Cimino *et al.*, 2002). This stressor has also been suggested as having a role in viral disease episodes in cultured prawns (Chanratchakool and Limsuwan, 1998). These findings suggest that there may be a relationship between the syntheses of shock proteins due to osmotic shock and disease episodes in penaeid prawns.

The viral accommodation theory of Flegel and Pasharawipas (1998) has been substantiated by others investigating WSSV and the proposal of Owens (2003) requires further investigations. However if proven, then farmers may have some control over viral epizootics. For example, if a farmer is aware that his prawns are accommodating viruses and he has knowledge of the environmental factors that may trigger the synthesis of shock proteins and consequently the apoptotic cascade, he can ensure that the range of these factors are not exceeded.

There is no doubt that further studies in this area are of utmost importance if a greater understanding of penaeid immunity is to be achieved.

In summary:

- Shock proteins and ubiquitin have been discovered in a variety of aquatic invertebrates however, only one quantitative assay has been developed to measure SP levels.
- The roles shock proteins and ubiquitin play in crustacean immunity remains an enigma as does the mechanisms and pathways involved.
- It is still unclear how crustaceans deal with viral infection and investigations are made difficult as basic tools such as cell lines have not been developed.
- Betaine may aid in reducing osmotic stress and as such, possibly reduce viral epizootics in cultured penaeid prawns.

With viral disease having such a significant affect on the global aquaculture for penaeid prawns, and virtually no literature available on the mechanisms or pathways involved in this animal's immune response, it is imperative that investigations combine and expand to achieve a better understanding.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 Introduction

This chapter will outline the materials and methods commonly used throughout this project. Techniques used only once for particular experiments and were not commonly used will be addressed in the relevant chapters.

3.2 Laboratory Trials

3.2.1 Experimental animals

Juvenile *P. monodon* (average wet weight of 17g, average length of 110mm) were obtained from Pacific Reef Pty Ltd (located in Ayr, 100 kilometres south of Townsville). All prawns, randomly captured by cast netting, were removed from 1.5 m deep, one hectare earthen ponds where they had been held for approximately three months. Captured prawns were placed into an 800 L aquatic transporter previously filled with pond water and transported to JCU. Water was oxygenated using bottled oxygen supplied via one 0.5 m air stone.

3.2.2 Experimental system

The experimental recirculation system comprised eight modules of nine, 50 L rectangle, high quality food grade plastic tanks (opaque blue to reduce visual stress) (Figure 3.1). For experimental trials, seven tanks per module were used, six for holding prawns and one as a biological filter.

Seawater was purchased through the Marine and Aquaculture Research Facility Unit (MARFU) at James Cook University (JCU), and transported via tanker truck to JCU infectious aquatic diseases facility where it was stored in 10000 L outdoor covered plastic tanks.

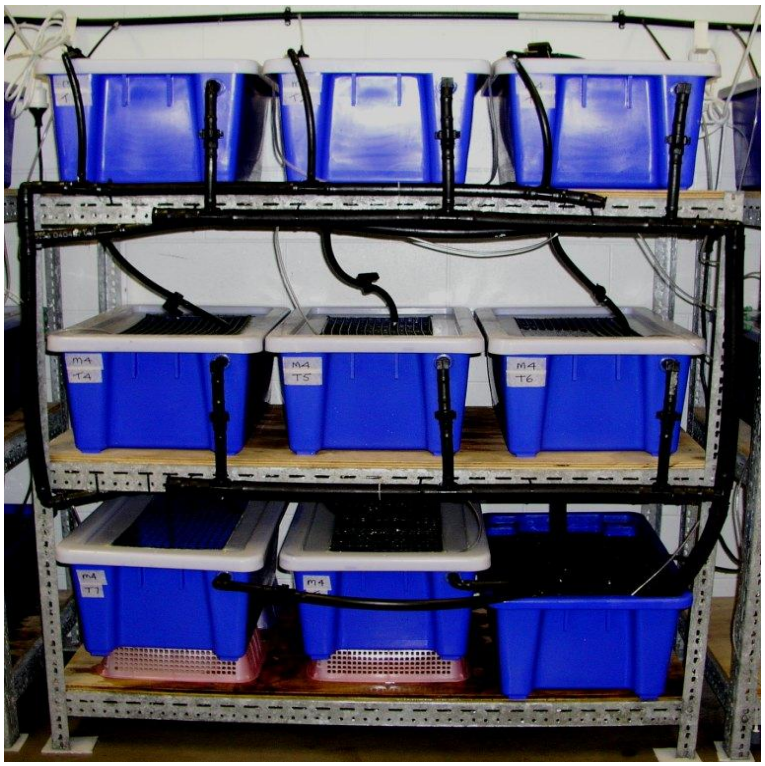


Figure 3.1: Holding and experimental system showing one complete module consisting of nine, 50L tanks, eight tanks for holding animals and one tank used as a biological filter (bottom right tank*).*

Air was supplied by compressor (Medo, Niho Kohki Co., Ltd., Japan) and was recirculated by PVC tubing. Air hoses and air stones were attached at intervals along the PVC tubing supplying air to all tanks via regulators and one grey air stone per tank. One biological filter per module was used to aid in the breakdown of nitrogenous wastes within the system. These filters consisted of a 50 L tank filled to a capacity of 75% with small PVC off cuts. New filters were 'seeded' by placing approximately ¼ cup of dry prawn pellets (C. P. 4003 starter 2, Thailand) in a stocking and suspending this in the biofilter. Ammonia and nitrite levels were tested daily using commercial kits and accompanying procedures (Aquarium Pharmaceuticals, Canada, Inc) until the ammonia levels had reached 1-2 ppm.

Each biofilter was then spiked with 10ml of Cycle™ (Nutrafin, USA) which is a concentrated solution of beneficial bacteria designed to colonize nitrifying biofilters enabling the system to convert ammonia to nitrite and then nitrite to nitrate. Prawns were introduced into the system once the ammonia and nitrite levels had returned to < 1 ppt.

Submersible pumps (EHEIM, H_{max}m, 3.1) delivered water from the biological filter through PVC tubing into each tank. Each tank was fitted with an individual water delivery pipe and an internal standpipe enabling waste water and debris to be removed from the bottom of each tank. The average water flow for the complete system was 4.4 L per hour. Water from the tanks drained back into the biological filter via PVC tubing to complete the system. All stress was minimised for a minimum acclimation period of 22 days prior to experimental procedures.

3.2.3 Husbandry

Upon arrival at JCU the prawns were housed in 50 L rectangle, high quality food grade plastic tanks. Prawns were matched for size within tanks and held at five prawns per tank.

The salinity for all holding tanks was kept at 35 ppt (+/- 2ppt) and a temperature of 26°C (+/- 1°C) for the duration of this study unless otherwise stated. To maintain the salinity level, tap water that had been aerated for a minimum of 48 hours was used as necessary. Water quality factors were monitored daily using commercial saltwater ammonia and nitrite detection kits as described above. Prawns were fed a commercial diet specific to penaeid prawns (C. P. 4003 starter 2, Thailand) two times per day. Tanks were cleaned of excess feed, moults, faecal strings and other debris before the morning feed or as required.

3.3 Sample Collection and Preparation

3.3.1 Sample collection

3.3.1.1 Laboratory trial sample collection

All experimental prawns were sacrificed by immersion in chilled water until non-responsive (Animal Ethics Approval number A925). The wet weight and length of the prawns were recorded and all pleopods were surgically removed. Scissors were used to remove the pleopods at the protopodite-endopodite joint. To avoid deterioration, all samples were immediately placed into 2ml screw top vials (Trace Plastics) with 0.75ml of 1mm zirconia/silica beads (Daintree Scientific, Tasmania) and 1ml chilled phosphate buffered saline (PBS) (Na_2HPO_4 10 mM, NaH_2PO_4 10 mM, NaCl 150mM, pH 7.2) then stored at -20°C unless otherwise stated.

To develop a positive control (PC) for the assays used in subsequent investigations, all of the pereiopods were removed from the osmotically stressed (OS) group (see below) and placed into 40ml chilled PBS and frozen at -20°C until analysed (< five days).

3.3.1.2 Field trial sample collection

To avoid damage and unnecessary stress during surgery, prawns were gently restrained and the procedure completed in the shortest possible time.

Scissors were used to remove four pleopods as above per prawn from alternate sides of the abdomen to avoid comprising their swimming action. All prawns were returned to their respective ponds immediately after sampling. Field samples were stored on ice during transportation to JCU.

3.3.2 Sample preparation

All samples were thawed overnight at 4°C then homogenized at 36 oscillations/second using a MiniBead Beater96 (Daintree Scientific, Tasmania).

Samples from the laboratory trials were homogenized for three mins, three times then centrifuged (Eppendorf 5804 R) at 4444 *g* (5000 rpm) for 12 mins. The pellet was discarded and the supernatant was centrifuged at 15294 *g* (12000 rpm) for 15 mins. The resulting pellet was discarded and the remaining supernatant was stored at - 80^{o}C until used for protein analysis. Samples from the farm trials were homogenized for four mins, two times. Samples were then centrifuged (Eppendorf 5804 R) at 2844 *g* (4000 rpm) for 10 mins. The pellet was discarded and the supernatant was centrifuged at 2844 *g* (4000 rpm) for 30 mins. The resulting pellet was discarded and the remaining supernatant was stored at - 80^{o}C until used for protein analysis. The pereiopod samples were semi-thawed and homogenised using a chilled mortar and pestle. The homogenate was centrifuged (Eppendorf 5804 R) at 4444 *g* (5000 rpm) for 15 mins. The pellet was discarded and the supernatant was centrifuged at 15294 *g* (12000 rpm) for 15 mins. The resulting pellet was discarded and the remaining supernatant (PC), was placed into 1ml aliquots and stored at - 80^{o}C until used for protein analysis.

3.4 Protein Estimations

3.4.1 Protein assay for bicinchoninic acid

The protein concentrations of samples were determined spectrophotometrically using a commercially available kit (BCA Protein Assay Reagent kit, Pierce, U.S.A). This system combines the Lowry method (1951) in which the reaction of protein with Cu^{2+} (cupric ions) in an alkaline medium yields Cu^{+} (cuprous ions) with bicinchoninic acid (BCA), a highly sensitive and selective detection reagent for Cu^{+} (Smith *et al.*, 1985).

A purple colour reaction product is formed when protein samples are assayed with BCA (Wiechelman *et al.*, 1988). The coloured product, formed by the interaction of two molecules of BCA with one Cu^{+}, is water-soluble and exhibits a strong absorbance at 562 nm allowing the spectrophotometric quantitation of protein concentrations in aqueous solutions.

A calibration curve was set up according to the Standard Protocol supplied with the kit using bovine serum albumin (BSA) in PBS to provide standards of 25, 125, 250, 500, 750, 1000, 1500 and 2000 μg/mL. Unknown samples were diluted 1/8 or 1/10 using PBS, and the working colour reagent was prepared by mixing 50 parts of Reagent A with one part of Reagent B.

The microtitre plate protocol was used. Triplicate samples of 25μL of the BSA standards, blanks and unknowns were pipetted into wells of a 96-well plate (IWAKI, Crown Scientific or Sarstedt depending on availability). Two hundred microlitres of working colour reagent were then added to each well and samples were mixed for 30 s. The microtitre plate was incubated at 37°C for 30 min and the absorbances measured using a plate reader (Labsystems Multiskan EX, Finland) and Labsystems Genesis V3.00 BCA protocol computer program. From this data a standard curve was drawn, and the protein concentrations of the unknown samples were determined.

3.5 Protein Electrophoresis

Protein profiles were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a method modified from Laemmli (1970). The method used for all gels and Western blots was the Bio-Rad Mini-PROTEAN 3 system and all samples were in their denatured form.
All gels were commercially available pre-cast tris-tricine gradient gels (10% - 20%) (LifeGels, Monarch Medical, Australia catalogue # NT21-1020) unless otherwise stated.

All samples were diluted 1:1 with sample buffer (Monarch Medical, Australia catalogue # BG-125) excluding ß-mercaptoethanol in 1.5 mL microfuge tubes (Sarsdent, Australia), mixed well, boiled for five min and 30 μL of sample added to each lane.

To determine the molecular weight (MW) of the individual bands, 5 μL of broad range molecular weight markers (Progen, Australia catalogue #SM0671) were added to one spare well per gel. The gels were electrophoresed at 140V at room temperature (RT) until the dye front had reached the bottom of the gel. Gels were then stained overnight with Gradipure (Monarch Medical, Australia catalogue # SG-021) at RT on a rocker then destained using 6% ethanol at RT on a rocker. Molecular weights of the sample protein bands were calculated using a calibration curve of molecular weight of standards vs the natural log of the distance migrated by the bands (Westermeier, 1993).

3.6 Western blots

3.6.1 General technique for protein transfer

Protein samples were electrophoresed using the SDS-PAGE technique (Section 3.5). However, instead of staining, the gels were loaded into gel holders (Bio-Rad Laboratories, Sydney) in direct contact with polyvinylidene fluoride (PVDF) membrane.

Proteins were transferred at 40V for one hour at 4ºC to polyvinylidene fluoride (PVDF) membrane Immobilon-P^{SQ} (Millipore, Australia catalogue # ISEQ-10100, pore size 0.2μm) in tris-tricine running buffer (Monarch Medical, Australia catalogue # BG-168). Western blots were conducted according to the manufacturer's protocol for rapid immunodetection of blotted proteins without blocking (Millipore publication RP 562, Australia).

All samples were transferred a minimum of three times using each monoclonal antibody (MAb) individually on each blot to ensure specificity of the MAbs.

The transillumination method described in the Immobilon-P transfer membrane user guide (Millipore, Australia) was used to determine protein transfer efficiency and for visualisation of the protein bands. This method is non-destructive, reversible and will not interfere with subsequent protein analysis.

3.6.2 Immunostaining of Western blots

The membranes (blots) were dried overnight at RT. Once thoroughly dried the blots were placed in 50 ml tubes with the gel side of the blot facing in and incubated with 2 ml of the appropriate primary antibody per tube diluted in TEN-TC buffer (pH 8.0 TropBio Pty. Ltd., Townsville) for one hour. It is important to ensure an even distribution of the antibody solution across the membrane so the incubations were carried out at RT on a rocker/roller. The membranes were washed twice for one min each time using fresh PBS. Goat anti-mouse conjugated to horseradish peroxidase (HRPO) (TropBio, Townsville) was added (2 ml per tube) at a concentration of 1/500 and blots were incubated for one hour at RT on a rocker/roller followed by washing as above.

The substrate 3,3',-diaminobenzidine tetrahydrochloride (DAB) (Progen, Australia catalogue # 34002) was added and the development reaction was halted at the desired level (usually achieved in five to 10 min) by the addition of distilled water.

CHAPTER 4

DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DETERMINATION OF UBIQUITIN (Ub) AND OPTIMISATION OF AN ELISA FOR THE DETECTION OF SHOCK PROTEIN 70 (HSP70) IN *PENAEUS MONODON.*

4.1 Introduction

Shock proteins are a group of highly conserved proteins that are a crucial aspect of all living organisms. Briefly, SP have been shown to play a number of roles in the routine functioning of unstressed cells including the stabilisation of proteins at intermediate stages of folding, assembly, translocation across membranes, secretion, regulation and the targeting of proteins for degradation and removal (Hartl, 1996; Feder, 1999; Buchanan, 2000) (Section 2.3). These proteins are typically assigned to families on the basis of molecular weight (kDa), sequence homology and function and are highly conserved across taxa. The four main families are hsp90, the predominant hsp70 (Bukau and Horwich, 1998; Lewis *et al.*, 1999; Bukau *et al.*, 2006; Nicoll *et al.*, 2006) hsp60 and small hsp (Buchanan, 2000). In eukaryotes, some of these families have multiple members differing in cellular location and function with many being constitutively expressed in unstressed cells and up-regulated during times of stress (Feder, 1999; Feder and Hofmann, 1999).

Upregulation of SP may be an indication of reversible protein damage. However, increased or prolonged stress can result in the accumulation of irreversibly damaged proteins, at which time the ubiquitin-proteasome response is activated (Pickart and Eddins, 2004; Nandi *et al.*, 2006) (Section 2.7.1).

Ubiquitin is a small shock protein (~8.5kDa) composed of 76 amino acids and is present in all eukaryotic cells (Garrett and Grisham, 1999). The structure of Ub is compact and tightly hydrogen-bonded forming a globular protein (Vijay-Kumar *et al.*, 1985). It is found throughout cells and can exist as either free form or conjugated to other proteins (Section 2.7).

As with shock proteins, the 53 reported ubiquitin sequences are highly conserved across a wide variety of species (Haas and Siepmann, 1997). Ubiquitin is involved in diverse cellular functions. For example, Ub conjugation is involved in regulating the cell cycle, cell growth and proliferation, endocytosis, DNA repair, embryogenesis, protein degradation, apoptosis, heat shock and has been implicated in the immune response. This system is responsible for the regulation of protein turnover in cells by closely regulating the degradation of specific proteins. Such a role is crucial as cells can quickly eliminate a protein that serves a regulatory function in that cell or remove proteins irreversibly damaged as a result of stress (Fornace *et al.*, 1989; Finley and Chau, 1991). The ubiquitin genes identified in eukaryotes typically exist in two forms: 1) the ubiquitin fusion gene, which encodes a single ubiquitin moiety fused to a ribosomal protein gene (Finley *et al.*, 1989) and 2) polyubiquitin chains, which consist of a linear repeat of ubiquitin molecules (Wiborg *et al.*, 1985; Baker and Board, 1987; Fornace *et al.*, 1989; Finley and Chau, 1991) (Section 2.7). The upregulation of ubiquitin and conjugated ubiquitin moieties can be considered indicators of an increase in protein turnover (Parsell and Lindquist, 1993; Hofmann and Somero, 1995; Amerik and Hochstrasser, 2004; Nandi *et al.*, 2006).

In the study of aquatic organisms, the vast majority of investigations relating to shock proteins have used semi-qualitative assays such as Western blotting, a small percentage used genetic methods such as RT-PCR, southern and northern blotting and only two studies used ELISA (Section 2.3.1).

To date only one study could be found that investigated ubiquitin in crustaceans. This study showed changes in ubiquitin levels in grass shrimp (*Palaeomonetes pugio*) in response to a range of stressors using dot blot ELISA (Downs *et al.*, 2001b). A number of studies have investigated polyubiquitin in crustaceans, with a particular focus on lobsters undergoing moult-induced muscle atrophy (Shean and Mykles, 1995; Koenders *et al.*, 2002; Spees *et al.*, 2002a; Spees *et al.*, 2002b; Spees *et al.*, 2003) (Section 2.7.4). The majority of these studies utilised genetic assays such as RT-PCR and northern blotting and only one study used Western blotting.

Each method has limitations and often the method chosen is largely dependent on the skill of the researcher and the availability of appropriate equipment or, more importantly, the sensitivity of the detection system and the ability to quantitate the substance of interest (Weeks and Woodhead, 1988). For example, RT-PCR is a qualitative method and is costly, requires a high degree of skill and extensive laboratory equipment.

Southern and northern blotting methods are semi-quantitative techniques (determines increases or decreases in abundance) that are also very sensitive but are relatively time consuming and expensive. Western blotting is sensitive, generally precise but is time consuming, often complex and in most cases enables only a semi-quantitative result (Madigan *et al.*, 1997). The often lengthy and complex procedures that are common to the techniques mentioned mean that it is not practical to process large numbers of samples at any given time. As a result, the investigation of ubiquitin in aquatic organisms has remained largely semi-quantitative. Determination of stress levels in cultured species would provide the culturist with additional information likely to enhance the efficiency of their production. Thus, there is a great need to have available a practical, sensitive, cost-effective assay that allows the testing of large numbers of samples quickly.

The ELISA meets all of these requirements, and with the availability of automated detection and quantification methods for use with microtitre plate assays, is able to detect and quantify target antigens or antibodies quickly (Weeks and Woodhead, 1988; Madigan *et al.*, 1997; Edwards *et al.*, 1999). Cimino *et al.*, (2002) developed the first and to date, the only ELISA enabling the qualitative analysis of changes in the level of one SP (86 kDa) in thermally stressed *C. quadricarinatus* and *P. monodon* and hypoosmotically stressed *P. monodon*. This assay is relatively cost effective, can screen multiple samples fast, can be modified to detect changes in other SP levels and as such, is more appealing to the farmer. However, no microtitre plate ELISA has yet been described in the literature for the detection of ubiquitin in crustaceans.

Although the shock protein ELISA previously developed by Cimino *et al.*, (2002) is able to detect changes in the levels of an 86 kDa SP in *P. monodon* and *C. quadricarinatus*, due to time constraints during its' development the assay was not fully optimised. This is a crucial component in the effective development of any assay. For example, Cimino *et al*., (2002) determined appropriate sample homogenate and diluent, maximum total protein concentration per microtitre well, protein form, blocking vs no blocking and type of microtitre plate. However, it is important to determine optimal MAb and MAb conjugate dilutions to reduce the chances of non-specific binding and wasting of expensive MAbs. Another crucial component in the development of any ELISA which has not be previously developed, is the addition of an effective positive and negative control to ensure the assay is not flawed (Edwards *et al.*, 1999).

The aims of this investigation were two fold:

1) Develop and optimise an ELISA technique suitable for the detection and quantification of ubiquitin in *P. monodon* using a commercial monoclonal antibody.
2) Further optimise the previously developed ELISA for the detection of a SP in *P. monodon*.

4.2 Materials and Methods

4.2.1 Experimental animals

Prawns were collected and transported as described in Section 3.2.1. All prawns were held in the experimental system (Section 3.2.2) as described in Section 3.2.3. For this investigation only samples from the osmotically stressed group (Section 5.2.6) were used.

4.2.2 Sample collection and preparation

Pleopod and PC samples were collected (Section 3.3.1.1) and prepared (Section 3.3.2).

4.2.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein separation by SDS-PAGE was performed as described in Section 3.5. As a comparison of protein profiles PC, un-stressed (C), and OS pleopod samples were investigated using SDS-PAGE. To compare protein profiles, pleopod and PC samples were diluted in PBS to achieve 20μg total protein per 30μl well. These gels were then stained with Gradipure as described in Section 3.5.

To compare immuno-reactive proteins using Western blotting techniques, pleopod and PC, samples were diluted in PBS to achieve 70μg total protein per 30μl well. SDS-PAGE was performed as described in Section 3.5 without staining. These gels were used in Western blot analysis.

4.2.4 Western blot

As a comparison, the pleopod and PC samples were investigated using Western blotting techniques. Sample proteins separated by SDS-PAGE (Section 4.2.2) were transferred to 0.2μm PVDF membrane by Western blotting (Section 3.6.1). After using the transillumination method to visualise protein bands, the blot was thoroughly dried in preparation for immunostaining (Section 3.6.2). Blots were incubated with the mouse anti-human ubiquitin MAb at a dilution of 1:500 (2μg/ml) or mouse anti-human hsp70 MAb at a dilution of 1/5000. A goat HRPO conjugate was used at a dilution of 1:500 for all blots. Detection of the antigen was visualized using the substrate DAB.

4.2.5 Antibodies

The antibodies used in this investigation were:

1. Commercially available mouse anti-human ubiquitin MAb (Table 4.1) (R&D Systems, catalogue #MAB701). This MAb detects ubiquitin and ubiquitin+1 (more prevalent than ubiquitin in humans with Azheimers Disease or Downs Syndrome).
2. Commercially available mouse anti-human hsp70 MAb (Table 4.1) (Affinity BioReagents, catalogue #MA3-006). This MAb detects several members of the hsp70 family including the stress induced hsp72 and constitutively expressed members.
3. The labelled secondary antibody used in all procedures was a goat HRPO conjugate (TropBio, Townsville).

All antibodies were stored at -20°C.

Table 4.1: Description of the monoclonal antibodies (MAb) used in the development and optimisation of an ELISA for the detection of ubiquitin and hsp70 in *P. monodon*.

MAb Isotype/ Clone	Clone	Catalogue Number	Reconstitution	Dilution	Western Blot
Mouse αhuman ubiquitin IgG/2B	83406	MAB 701	100μl of 0.2μm filtered PBS was added to lyophilized sample. Vortex to resuspend **(1mg/ml)**	**1/50 (20μg/ml)** Reconstituted sample was added to 4900μl conjugate diluent (TropBio, Townsville)	Dilute 1/50 x 1/10 to achieve 1/500 = **2 μg/ml**
Mouse αhuman hsp70 IgG1	3A3	MA3-006	50μl of 0.2μm filtered distilled water was added to lyophilized sample. Vortex to resuspend	**1/50** Reconstituted sample was added to 4950μl conjugate diluent (TropBio, Townsville)	**1/5000**

4.2.6 ELISA

4.2.6.1 Indirect ELISA

The indirect ELISA performed with excess antibody is quick and reliable, and can be used to determine relative levels of the antigen in an unknown sample. The principle is based on the binding of the antigen to the well surface. A MAb is added which binds to sites on the antigen. A labelled second antibody is then added that binds to the primary antibody and can be quantified by measuring the intensity of the coloured product produced in the presence of a colorimetric substrate (Harlow and Lane, 1988; Crowther, 1995) in this case, 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) (Figure 4.1). After a set incubation period, OD levels are measured using a dual absorbance of 414 nm and 492 nm on a plate reader (Labsystems Multiskan EX, Finland) using the Labsystems Genesis V3.00 protocol computer program.

Briefly, an absorbance of 414 nm is optimal for measuring the colour intensity of the ABTS reaction. The second wavelength of 492 nm is outside the absorbance range for ABTS. Once the microtitre plate is measured at 414 nm then the values from the 492 nm measurements are subtracted from the first to give a corrected value, which is used in subsequent calculations.

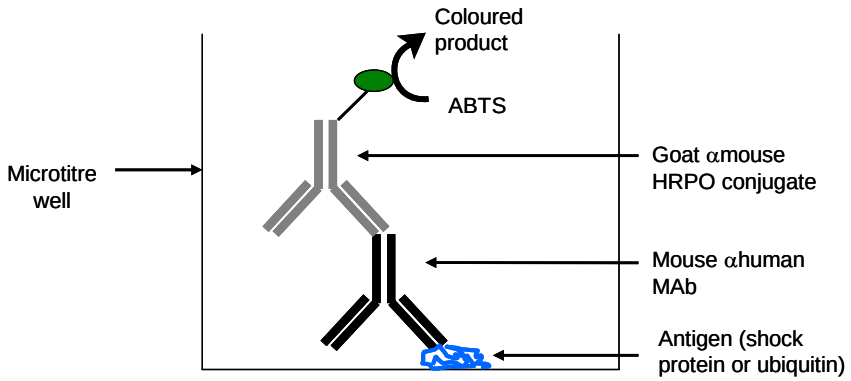


Figure 4.1: Schematic representation of an indirect ELISA. Sample is bound to the well surface. Monoclonal mouse anti-human antibody binds to antigen. Goat anti-mouse conjugate binds to the mouse MAb and with the addition of the chromogenic substrate, ABTS, a green colour will form with an intensity related to the amount of bound conjugate and as such a measure of antigen can be determined.

4.2.6.2 Assay validation

To precisely determine the levels of hsp70 and Ub in samples, an homologous antibody and a calibration curve from a pure sample is desirable (Hamilton and Adkinson, 1988; Harlow and Lane, 1988). To date, no homologous antibodies or pure SP or Ub standards have been isolated or purified for *P. monodon*. Therefore, the results are based on relative quantitative differences in OD measurements due to differences in hsp70 and Ub levels detected by the MAbs determined by ELISA (Hamilton and Adkinson, 1988). To ensure the validity and reproducibility of the assays a positive control was developed as a ‘range finder’. An assay with a positive control OD outside the optimum range would be considered to have failed and the assay repeated.

Standard concentrations curves were generated for both hsp70 and Ub MAbs using the developed positive control with doubling dilutions from 4μg to 500μg total protein.

4.2.6.3 Microtitre plates investigated for shock protein ELISA

Three types of microtitre plates were used in this investigation: 1) IWAKI Glass tissue culture plates; flat bottomed, 96 well, polystyrene, coated (Crown Scientific, Australia catalogue # 3860-096), 2) NUNC-Immuno plates, flat bottomed, 96 well, polystyrene, MaxiSorp coated (Medos; Australia catalogue # NUN4-42404), 3) NUNC-Immuno plates, flat bottomed, 96 well, polystyrene, MediSorp coated (Medos; Australia catalogue # NUN4-67320)

The NUNC plates used in this investigation were sample plates and so only one of each was available. The MediSorp plates were not available until after the first titration had been completed.

4.2.6.4 ELISA for detection of hsp70

The development of the SP ELISA by Cimino *et al.*, (2002) provided the following outcomes: 1) Using pleopod tissue provides a non-lethal sampling technique 2) The sample was used in its reduced form 3) A blocking step to be included 4) PBS used as sample homogenate 5) Carbonate/bicarbonate used as sample diluent 6) Maximum total protein concentration per microtitre well 7) Protocol for coating microtitre plates with antigen.

Checkerboard titration 1:

A checkerboard titration was undertaken to determine the following factors (Figure 4.2): 1) Total protein concentration, 2) hsp70 MAb concentration, 3) conjugate MAb concentration, 4) negative control (NC) concentration, 5) ABTS incubation time. OD measurements were recorded at one, four and 16 hour intervals, 6) microtitre plate type (IWAKI and MaxiSorp only)

All positive control samples were included in quadruplicate. Individual plates were tested for the extent of non-specific binding using conjugate control wells or more commonly termed, blank wells (B). Blank wells contained no sample. However, all other steps were conducted as with the test wells. The negative control (NC) tested was foetal bovine serum (FBS).

Figure 4.2: Experimental design for the development of the indirect ELISA for the detection of a SP in *P. monodon*. All procedures were replicated on the IWAKI and MaxiSorp plates. 'B' = blank control wells, FBS = foetal bovine serum, TP = total protein concentration.

All procedures followed the protocol below:

1. Dilute samples in PBS to either 0.6μg/μl or 0.3μg/μl.
2. Place diluted samples into a 90°C water bath for five mins then cool.
3. Mix all samples 1:1 with carbonate/bicarbonate buffer (pH 9.6 TropBio Pty. Ltd., Townsville) (i.e. 50μl sample plus 50μl buffer).
4. Add 100μl of sample/buffer mix to each well of a microtitre plate (see Figure 4.3 for plate configuration) to achieve a final total protein concentration of either 30μg or 15μg per well.
5. Cover plate with a single sheet of paper towel and place into a 37°C incubator overnight. Ensure all wells are completely dry before proceeding.
6. Add 125 μL of blocking buffer (pH 7.9 TropBio Pty. Ltd., Townsville) to each relevant well (Figure 4.3) and incubate for one hour at 27°C.
7. Flick off excess blocking buffer and dry plate for one hour at 37°C.
8. Add 100 μL of diluted MAb to each well (Figure 4.3) and incubate for one hour at 27°C.
9. Remove excess MAb solution and rinse wells twice with TEN-tween buffer (TropBio Pty. Ltd., Townsville).
10. Add 100 μL of goat anti-mouse HRPO conjugated antibody at a dilution of 1/1000 or 1/2000 to appropriate wells and incubate for one hour at 27°C.
11. Remove excess MAb solution and rinse all wells six times with TEN-tween buffer.
12. Add 100 μL of ABTS to each well and incubate in the dark at 27°C.
13. Record OD (Section 4.2.5.1).

Titration 2:

Using results from the first checkerboard titration, a second titration was undertaken using MaxiSorp and MediSorp plates to compare microtitre plates and to optimise the ABTS incubation time. OD was recorded at one, four, six, eight, and nine hour intervals. All PC samples, NC samples and blank control wells were included in quadruplicate. Each procedure was replicated on all plate types and all procedures followed the protocol described in Section 4.2.5.4.

4.2.6.5 Microtitre plates investigated for ubiquitin ELISA

Three types of microtitre plates were used in this investigation: 1) IWAKI Glass tissue culture plates, flat bottomed, 96 well, polystyrene, coated (Crown Scientific, Australia; catalogue # 3860-096), 2) NUNC-Immuno plates, flat bottomed, 96 well, polystyrene, PolySorp coated (Medos; Australia catalogue # 4-46140), 3) NUNC-Immuno plates, flat bottomed, 96 well, polystyrene, MediSorp coated (Medos; Australia catalogue # NUN4-67320).

4.2.6.6 ELISA for detection of ubiquitin

Checkerboard titration 1:

A checkerboard titration was undertaken to determine the following factors (Figure 4.3): 1) Total protein concentration, 2) protein form, 3) blocking verses no blocking, 4) Ubiquitin MAb concentration, 5) ABTS incubation time. OD were recorded at one, four and 16 hour intervals and 6) microtitre plate type (IWAKI and PolySorp only).

All procedures followed the protocol described in Section 4.2.5.4 (excluding step 2 for native samples) using appropriate total protein concentrations and MAb dilutions. All samples were included in quadruplicate. Due to the cost of the Ub MAb and the complexity of this checkerboard titration, it was not feasible to investigate more than one conjugate dilution at this time. Thus, the conjugate was trialed at 1/2000 to reduce the cost associated with the development of this ELISA.

Figure 4.3: Checkerboard titration 1 for the development of the indirect ELISA for the detection of ubiquitin in *P. monodon*. Goat αmouse conjugate was used at 1/2000. All procedures were replicated on both the IWAKI and PolySorp microtitre plates. TP = total protein concentration.

4.3 Results

4.3.1 SDS-PAGE

SDS-PAGE (Section 4.2.2) of control and OS pleopod and PC (pereiopods) samples revealed differences in protein profiles. The majority of bands in all samples were represented by relatively fine lines. However, two large haemocyanin sub-units (Figueroasoto *et al.*, 1997) spanning the 70 – 81 kDa molecular weight region were observed (Figure 4.4).

The control and PC samples showed very similar protein profiles with the exception of an additional band at 100 kDa in the control sample and additional bands at 130 kDa and 14 kDa in the PC sample. The OS sample showed down-regulation of bands at approximately 170 kDa and 100 kDa when compared to the control and PC samples.

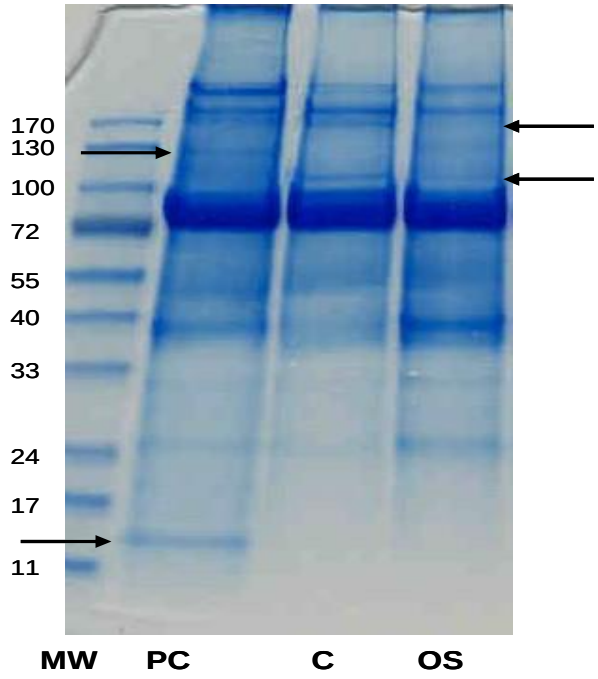


Figure 4.4: Protein profile of *P. monodon* positive control (PC), control (C) and hypoosmotically stressed (OS) pleopod samples as shown by a pre-cast tris-tricine Gradipure stained gradient gel (10-20%). Arrows indicate down-regulation of bands at 170 kDa and 100 kDa in the OS sample when compared to the C sample and additional bands at 130 kDa and 14 kDa in the PC sample. Molecular weight (MW) markers are in kDa.

4.3.2 Western blotting

Western blotting of OS pleopod and PC samples (70 μg/30 μL well) using the Ub MAb (2μg/ml) and hsp70 MAb (1/5000) resulted in bands at a MW of 72 kDa (immuno-reactive with the hsp70 MAb only) and 10 kDa (immuno-reactive with the Ub MAb only) in both samples as visualised by the addition of DAB (Figure 4.5). An additional band was detected in the PC sample only at an approximate MW of 170 kDa.

This additional band was not detected in blots incubated with the Ub MAb alone or in blots incubated with a sample total protein concentration of 20μg/well (data not shown).

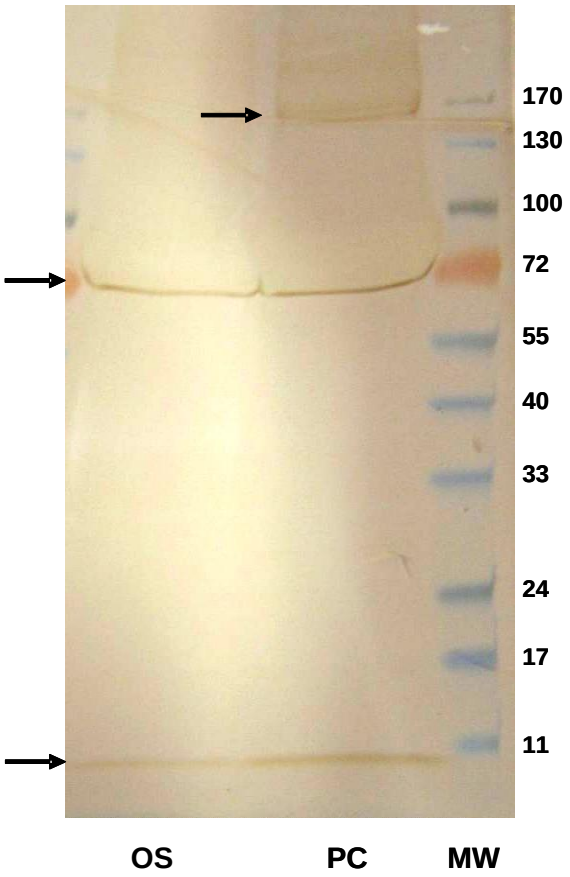


Figure 4.5: Western blot of hypoosmotically stressed *P. monodon* pleopod (OS) and positive control (PC) samples. Blot was incubated with the Ub MAb (2μg/ml), hsp70 MAb (1/5000) and conjugate (1/500). Arrows indicated immuno-reactive bands at 72 kDa (hsp70) and 10 kDa (Ub) in both samples. An additional band is visible at 170 kDa in the PC sample only. Molecular weight (MW) markers are in kDa. This combined blot was produced for photographic purposes only.

4.3.3 ELISA

4.3.3.1 Assay validation

Standard curves were generated for hsp70 and Ub using the PC sample and two pleopod samples randomly chosen (Figures 4.6 and 4.7 respectively).

The optimal total protein concentration of PC for both MAbs was between approximately 16μg and 63μg. A total protein concentration of 30μg was chosen, being in the optimum range.

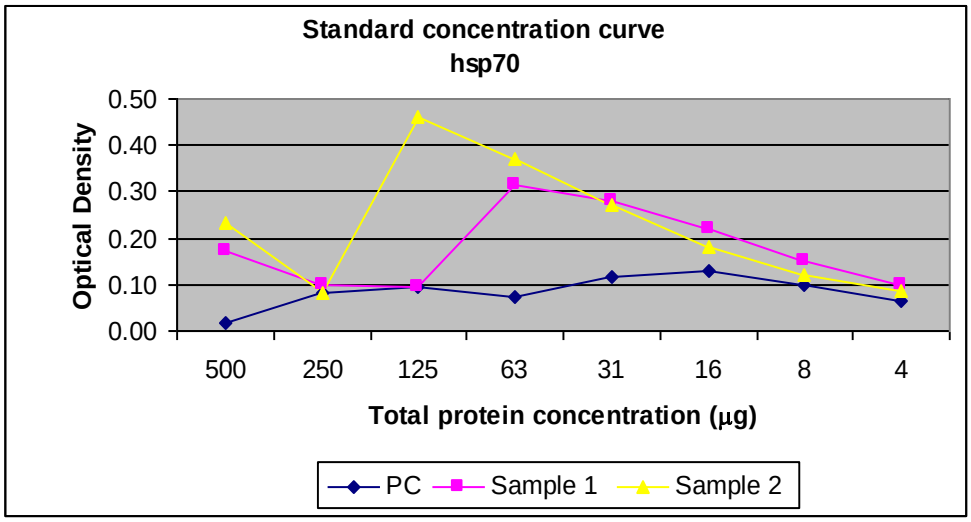


Figure 4.6: Standard concentration curve for hsp70 using *P. monodon* positive control (PC) sample (pereiopod origin) and two random samples termed sample 1 and sample 2 (pleopod origin). All data based on average OD.

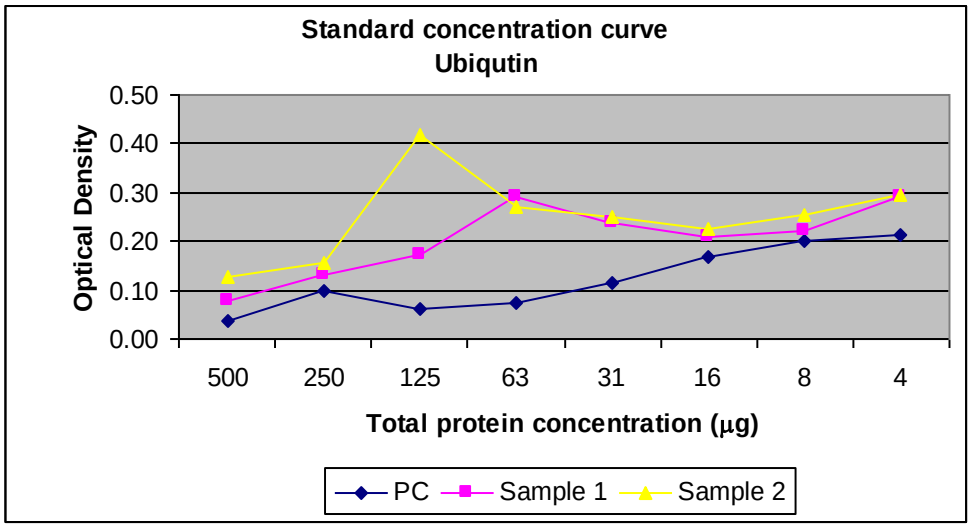


Figure 4.7: Standard concentration curve for ubiquitin using *P. monodon* positive control (PC) sample (pereiopod origin) and two random samples termed sample 1 and sample 2 (pleopod origin). All data based on average OD.

4.3.3.2 Hsp70 checkerboard titration 1

A summary of the outcome of the different checkerboard titrations using PC samples and conjugate concentrations of 1/1000 and 1/2000 is shown in Table 4.2 and Table 4.3 respectively (all results are average OD).

Table 4.2: Summary of the average OD recorded from the hsp70 checkerboard titration. The positive control sample was in its reduced form, with blocking buffer. MAb conjugate at 1/1000 dilution. Displayed times represent the hour after the addition of ABTS. The highest acceptable OD recorded is highlighted.

Conjugate 1/1000	30μg Total Protein/well			15μg Total Protein/well			
Hsp70 MAb concentration	**1/4000**	**1/5000**	**1/6000**	**1/4000**	**1/5000**	**1/6000**	**Plate type**
1 hr	0.251	0.22	0.215	0.194	0.171	0.15	**IWAKI**
	0.306	0.265	0.223	0.216	0.195	0.177	**MaxiSorp**
4 hr	0.451	0.393	0.402	0.335	0.302	0.263	**IWAKI**
	0.541	**0.476**	0.408	0.393	0.351	0.317	**MaxiSorp**
16 hr	0.385	0.342	0.334	0.305	0.27	0.237	**IWAKI**
	0.459	0.413	0.357	0.348	0.314	0.288	**MaxiSorp**

Table 4.3: Summary of the average OD recorded from the hsp70 checkerboard titration 1. The positive control sample was in its reduced form, with blocking buffer. MAb conjugate at 1/2000 dilution. Displayed times represent the hour after the addition of ABTS.

Conjugate 1/2000	30μg Total Protein/well			15μg Total Protein/well			
Hsp70 MAb concentration	**1/4000**	**1/5000**	**1/6000**	**1/4000**	**1/5000**	**1/6000**	**Plate type**
1 hr	0.134	0.105	0.093	0.111	0.111	0.096	**IWAKI**
	0.216	0.179	0.159	0.15	0.129	0.117	**MaxiSorp**
4 hr	0.233	0.179	0.158	0.194	0.191	0.165	**IWAKI**
	0.377	0.316	0.288	0.259	0.233	0.207	**MaxiSorp**
16 hr	0.209	0.165	0.147	0.176	0.174	0.153	**IWAKI**
	0.338	0.29	0.261	0.234	0.217	0.192	**MaxiSorp**

The average PC OD on the IWAKI plates were consistently lower than those noted on the MaxiSorp plates with all investigated hsp70 MAb, conjugate MAb, protein concentrations and ABTS incubation times. The highest average PC OD were noted on the MaxiSorp plate at a total protein concentration of 30μg/well, hsp70 MAb concentration of 1/4000, conjugate concentration of 1/1000 and an ABTS incubation time of four hours (Table 4.2).

Considering the cost of the hsp70 MAb, the difference in OD between the wells incubated with 1/4000 and 1/5000 was not deemed meaningful. Therefore a dilution of 1/5000 for the hsp70 MAb was used in subsequent investigations. A conjugate concentration of 1/1000 resulted in OD measurements consistently higher than relative wells containing a conjugate concentration of 1/2000 so this concentration will be used in subsequent investigations.

The OD for the NC and blank wells were comparable and did not change dramatically across either dilution or ABTS incubation time (data not shown). For ease of use a dilution of 1/16 of the NC was used in subsequent investigations.

It was found that ABTS incubation times of one and four hours resulted in a dramatic increase in PC OD on all plates. By 16 hours, OD in sample wells had decreased (data not shown).

The optimal conditions for this ELISA as determined by titration 1 are:
1) 30μg total protein per well, 2) hsp70 MAb concentration of 1/5000, 3) conjugate MAb concentration of 1/1000, 4) NC dilution of 1/16, 5) ABTS incubation time between one and four hours and 6) MaxiSorp plate.

4.3.3.3 Hsp70 titration 2

Using the optimal conditions numbered 1-4 for the SP ELISA determined in Section 4.3.3.2, a summary of the outcome of checkerboard titration 2 is shown in Figure 4.8 (all results are averaged OD). The OD for both plates tested increase dramatically between the one and four hour ABTS incubation times. Between the four and six hour incubation times a slight increase in OD was noted. No appreciable change in OD for either plate was noted at eight hour incubation. OD for both plates decreased marginally at the 10 hour incubation time. At all recorded incubation times the OD for the MaxiSorp plate were slightly higher than those recorded for the MediSorp plate. This was due to high OD from one blank control replicate on the MediSorp plate. The optimal time to record OD for the determination of hsp70 using the ELISA was between four and eight hour ABTS incubation time. Due to cost and availability, MediSorp plates were used for further investigations.

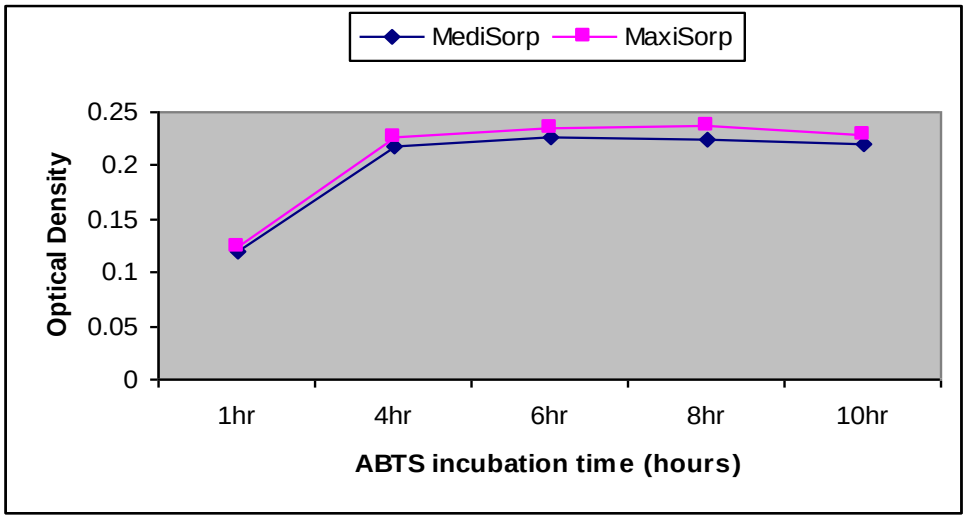


Figure 4.8: Checkerboard titration 2 for the optimisation of the ABTS incubation time using the ELISA for the detection of hsp70 in *P. monodon*. All data based on average optical density of 414, 492nm.

The optimal conditions for the determination of hsp70 in *P. monodon* using the ELISA are the same as Section 4.3.3.2 except that 1) ABTS incubation time between four and eight hours and 2) MediSorp plates were changed.

All future hsp70 ELISA investigations will use the above conditions.

4.3.3.4 Ubiquitin checkerboard titration 1

No reaction was detected using native protein or in the absence of blocking buffer at any of the ubiquitin MAb concentrations on any of the plates (data not shown). A summary of the outcome of the different checkerboard titration using reduced PC samples with blocking buffer is shown in Table 4.4 (all results are average OD).

Table 4.4: Summary of the average optical density recorded from the Ub checkerboard titration 1. The positive control sample was in its reduced form, with blocking buffer. MAb conjugate was at 1/2000 dilution. Displayed times represent the hour after the addition of ABTS. The highest OD recorded is highlighted.

Conjugate 1/2000	30μg Total Protein/well			15μg Total Protein/well			
Ub MAb concentration	**1/1000**	**1/2000**	**1/4000**	**1/1000**	**1/2000**	**1/4000**	**Plate type**
1 hr	0.095	0.062	0.045	0.096	0.067	0.049	**IWAKI**
	0.122	0.083	0.061	0.119	0.086	0.067	**PolySorp**
4 hr	0.167	0.106	0.073	0.167	0.115	0.077	**IWAKI**
	0.215	0.143	0.102	0.205	0.144	0.109	**PolySorp**
16 hr	0.149	0.101	0.072	0.149	0.107	0.073	**IWAKI**
	0.204	0.142	0.104	0.192	0.138	0.104	**PolySorp**

The average OD on the IWAKI plates were consistently lower than those noted on the PolySorp plates.

It was found that ABTS incubation times of one and four hours resulted in a dramatic increase in OD on all plates. By 16 hours, OD in sample wells had decreased. The highest average OD was noted on the PolySorp plate at a protein concentration of 30mg/well, Ub MAb concentration of 1/1000 at four hours ABTS incubation (Table 4.4).

The optimal conditions for this ELISA using a conjugate concentration of 1/2000 were determined to be: 1) 30μg total protein per well, 2) sample in reduced form, 3) blocking, 4) Ub MAb concentration of 1/1000, 5) ABTS incubation time between one and four hours and, 6) PolySorp plate.

4.3.3.5 Ubiquitin checkerboard titration 2

Using the optimal conditions numbered 1-3 for the Ub ELISA determined in Section 4.3.3.4 a summary of the outcome of checkerboard titration 2 is shown in Table 4.5 (all results are average OD).

The highest average PC OD were noted on the MediSorp plate at a Ub MAb concentration of 1/1000 and conjugate concentration of 1/1000 (Table 4.5). However, considering the cost of the Ub MAb, the difference in OD between the wells incubated with 1/1000 and 1/2000 was considered acceptable. Therefore a dilution of 1/2000 for the Ub MAb was used in subsequent investigations.

A conjugate concentration of 1/1000 resulted in OD consistently higher than relative wells containing a conjugate concentration of 1/2000 so this concentration will be used in subsequent investigations.

Table 4.5: Summary of the average optical density (OD) recorded from the Ub checkerboard titration 2. Displayed times represent the hour after the addition of ABTS. The highest OD using a Ub MAb dilution of 1/2000 and conjugate dilution 1/1000 between the ABTS incubation times of four and eight hours.

	Conjugate 1/1000		Conjugate 1/2000		
	Ub MAb 1/1000	**Ub MAb 1/2000**	**Ub MAb 1/1000**	**Ub MAb 1/2000**	**Plate Type**
1 h	0.311	0.193	0.169	0.114	**MediSorp**
	0.270	0.171	0.162	0.104	**MaxiSorp**
4 h	0.578	0.358	0.313	0.212	**MediSorp**
	0.520	0.343	0.319	0.205	**MaxiSorp**
6 h	0.599	0.372	0.328	0.218	**MediSorp**
	0.537	0.351	0.326	0.201	**MaxiSorp**
8 h	0.588	0.369	0.323	0.217	**MediSorp**
	0.530	0.343	0.327	0.204	**MaxiSorp**
10 h	0.565	0.355	0.310	0.212	**MediSorp**
	0.508	0.333	0.313	0.204	**MaxiSorp**

The OD for Ub at the different ABTS incubation times (Figure 4.9) shows a similar pattern to that reported for hsp70 in Section 4.3.3.3 (Figure 4.8). The OD increased dramatically between the one and four hour ABTS incubation times. Between the four and six hour incubation times a slight increase in OD was noted. These measurements then decreased at eight and 10 hour incubation times. At all recorded incubation times the OD for the MediSorp plate were slightly higher than those recorded for the MaxiSorp plate. The optimal time to record OD for the determination of Ub using the developed ELISA was between four and eight hours ABTS incubation time.

The MediSorp plate produced comparatively higher OD than the MaxiSorp plate for wells incubated with Ub MAb 1/2000 and conjugate at 1/1000 so will be used in all further investigations.

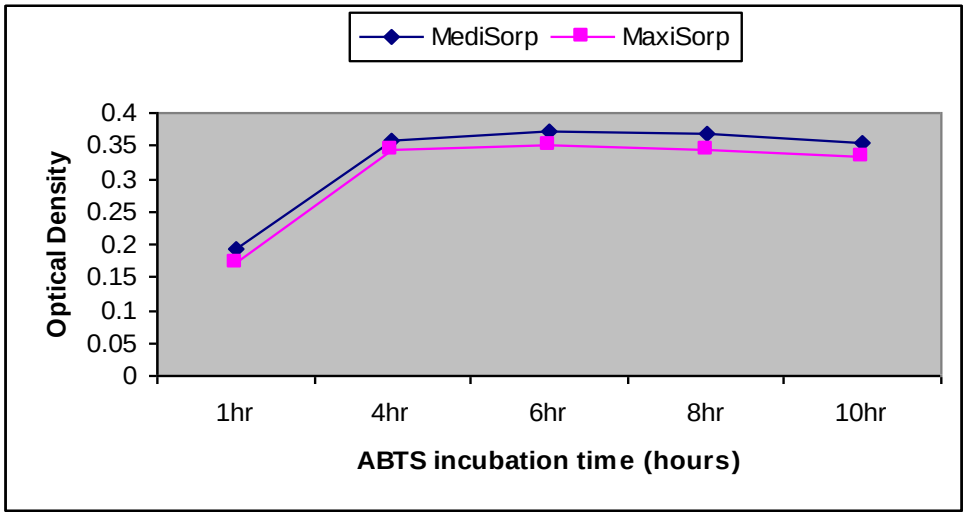


Figure 4.9: Checkerboard titration 2 for the optimisation of the ABTS incubation time using the ELISA for the detection of Ub in *P. monodon*. All data based on average optical density of 414, 492nm.

The optimal conditions for the determination of Ub in *P. monodon* using the ELISA were:

1) 30μg total protein per well, 2) sample in reduced form, 3) blocking, 4) Ub MAb concentration of 1/2000, 5) conjugate concentration 1/1000, 6) NC dilution of 1/16, 7) ABTS incubation time between four and eight hours, 8) MediSorp plate.

4.4 Discussion

In this study the previously developed ELISA for the detection of a SP in *P. monodon* was optimised and the first indirect ELISA was developed for the detection of ubiquitin in *P. monodon*. These assays will provide a high throughput, relatively cost effective means for detecting relative changes in hsp70 and ubiquitin levels in stressed and unstressed *P. monodon*.

The differences in the protein profiles described in Section 4.3.1 are an indication of the complexity of the biological system investigated. Differences in protein profiles between the control and osmotically stressed samples (pleopod) and positive control samples (pereiopod) may be due, in part, to the difference in tissues. The study conducted by Cimino *et al.*, (2002) is to date, the only previous study describing and comparing protein profiles in stressed and unstressed *P. monodon* (pleopod samples). The down regulation of the 100 kDa band in the hypoosmotically stressed sample is in conflict with that study whose investigations showed an up regulation of this band in hypoosmotically stressed *P. monodon*. Several differences in the reagents used in the SDS-PAGE between this current study and Cimino *et al.*, (2002), including the use of ß-mercaptoethanol in the sample buffer and different gel types, may account for this inconsistency. It is also possible that the differences in protein profiles between these two studies may be a result of an underlying response to an undetermined stressor (e.g. disease, previous transport stress). The major aim for using SDS-PAGE in this investigation was to determine immuno-reactive isoforms to the hsp70 and Ub MAbs using Western blotting. So further speculation regarding differences in protein profiles would require further investigations focused in this area.

Several studies differ in the number and MW of the immuno-reactive hsp70 isoforms in aquatic invertebrates (Section 2.7.4). For example changes in the hsp70 response in gastropods when exposed to stressors showed immuno-reactive isoforms at approximately 72 kDa and 74 kDa MW (Tomanek and Sanford, 2003) and several immuno-reactive bands between 70 and 73 kDa MW (Downs *et al.*, 2001a). In *Mytilus galloprovincialis* (mussel) and *Haliotis rufescens* (abalone) three hsp70 isoforms were detected at MW 67, 70 and 74 kDa (Snyder *et al.*, 2001). Tirard *et al.,* (1995) used the same hsp70 MAb as the present investigation to determine changes in hsp70 levels in haemocytes of heat-stressed oysters (*Crassostrea virginica*) and found that the MAb was immuno-reactive with two isoforms of hsp70 at 70 kDa and 71 kDa MW.

However, changes in the hsp70 response in the fresh water prawn *Macrobrachium malcolmsonii* when exposed to thermal (Selvakumar and Geraldine, 2005) and pesticide (Selvakumar *et al.*, 2005) stressors and in *P. monodon* when exposed to thermal and osmotic stress (de la Vega *et al.*, 2006) showed a single immuno-reactive isoform at the 70 kDa region, a finding supported by the current investigation.

Western blotting of the pleopod and PC samples with the hsp70 MAb showed a single band at 72 kDa MW. An additional band was detected at 170 kDa in the PC sample. This band was not detected in pleopod samples, blots incubated with the Ub MAb only or in blots where the total protein concentration in the SDS-PAGE gel was 20μg/well. This suggests that either the hsp70 MAb is immuno-reactive with two isoforms (72 kDa and 170 kDa) in hypoosmotically stressed leg (pereiopod) samples or, possibly, that the hsp70 MAb is cross-reactive with one isoform at 72 kDa and the band at 170 kDa is the result of insufficiently denatured protein when total protein concentrations in SDS-PAGE gels are high (>70μg/well). It is proposed that differences in tissue type used in this current investigation may have contributed to the differences indicated by Western blotting.

It is suggested that differences in gel type and MW markers may contribute to the apparent inconsistency between the current and previous investigations (Cimino *et al.*, 2002) and it is proposed that the immuno-reactive protein found in *P. monodon* belongs to the hsp70 group and may not be significantly different from hsp70 conformational epitopes found in other invertebrates. This protein should be more appropriately referred to as a member of the hsp70 family and will be termed hsp70 in all subsequent chapters.

A single band at 10 kDa MW was revealed in both the PC and pleopod samples. This band was immuno-reactive with the Ub MAb only. A number of studies have investigated polyubiquitin in crustaceans and the Western blotting results have shown cross-reactive bands in the high MW range (~200 kDa) (Section 2.7.4).

However only one study could be found investigating ubiquitin. Downs *et al.*, (2001b) developed a molecular biomarker system using a dot blot ELISA to assess the physiological status of grass shrimp (*Palaomenetes pugio*) when subjected to a range of stressors (Section 2.7.4). Significant increases in ubiquitin, hsp60 and hsp70 levels were observed after stress. The molecular size of the cross-reactive ubiquitin molecule was not mentioned so a comparison between studies could not be made.

The hsp70 MAb used in this present study was cross-reactive with a single 72 kDa isotype. The immunogen for the hsp70 MAb used for this present study was human recombinant hsp70 over expressed in *Escherichia coli* and the host/isotype was mouse IgG_1. The study conducted by Downs *et al.*, (2001b) used a hsp70 MAb whose immunogen was a chicken hsp70/hsp90 complex and the host/isotype mouse IgG_{2a} which was cross-reactive with two to three isotypes between 70-72 kDa. The use of both these SP MAbs in a sandwich ELISA may increase the sensitivity and specificity of the assay and should be investigated further.

Using the ELISA developed in this investigation; the first ever concerning the Ub response, as opposed to the polyubiquitin response, in crustacea and the first to use an ELISA to elucidate changes in the Ub response to stress in *P. monodon*, it is now possible to test a large number of samples quickly, easily and at relatively low cost. It also provides a means to screen farmed and wild populations for changes in hsp70 and Ub levels and may serve as a basis for possible health management strategies in times of stress.

CHAPTER 5

EFFECT OF TRANSPORTATION, DISEASE AND HYPOOSMOTIC STRESS ON HSP70 AND Ub EXPRESSION IN *PENAEUS MONODON*.

5.1 Introduction

Penaeid prawns are cultured extensively on a global scale and are economically important to the aquaculture industry (Section 2.1). In most instances these animals are cultured under relatively high densities in man-made structures such as tanks or ponds, fed an unnatural diet and face fluctuations in the environment. Changes in water quality such as ammonia, pH, salinity and temperature levels have an adverse effect on the health of crustaceans (Roch, 1999; Cheng *et al.*, 2003a; Hall and de la Vega, 2003; Du *et al.*, 2006). With an appropriate knowledge of the prawn's biology and by modifications to existing management practices, the culturist can manipulate most of these factors to lessen the effect of the stressor (Owens and Evans, 1989). However, salinity is almost impossible to control, particularly in tropical areas subject to monsoonal downpours. Changes in salinity can have a profound effect on penaeid aquaculture resulting in decreased growth rates (Bray *et al.*, 1994) and at worst, significant economic losses due to resultant viral epizootics (Flegel and Pasharawipas, 1998) (Section 2.4.1).

The transportation of live crustaceans (particularly lobsters, crayfish, crabs and prawns) forms a valuable part of the fisheries and aquaculture industries. During capture, handling and transport animals are often subject to the same environmental/water quality stressors as mentioned earlier plus physical and mechanical damage as a result of rough handling, packing, overcrowding and disease (Paterson and Spanoghe, 1997; Taylor *et al.*, 1997; Vijayakumaran and Radhakrishnan, 1997).

This can result in significant economic loss due to low survivability and poor quality or moribund animals (Taylor *et al.*, 1997; Vijayakumaran and Radhakrishnan, 1997; Fotedar *et al.*, 2006). For example, a recent study by de la Vega *et al.,* (2004) showed that handling stress can lead to a rapid increase in gill-associatted virus infection in *P. monodon.*

When faced with rapid changes in environmental factors, the animal typically responds by producing or up-regulating shock proteins (Section 2.3). Prolonged or increased stress can result in the activation of the ubiquitin pathway (Section 2.7).

Research on shock proteins in aquatic invertebrates is increasing. A number of animals, in particular crustaceans including crayfish (Rochelle *et al.*, 1991; Sheller *et al.*, 1998; Cimino *et al.*, 2002), shrimp (Ravaux *et al.*, 2003), prawns (Cimino *et al.*, 2002; Selvakumar and Geraldine, 2005; Selvakumar *et al.*, 2005; de la Vega *et al.*, 2006) and lobster (Chang *et al.*, 1999) have been shown to respond to a variety of stressors (Sections 2.4-2.6).

The vast majority of studies investigating the Ub system in aquatic invertebrates have focused on changes in the stress response of polyubiquitin in moult-induced muscle atrophy in land crabs (Mykles, 1996; Koenders *et al.*, 2002) in lobster (Shean and Mykles, 1995; Koenders *et al.*, 2002; Spees *et al.*, 2003) and responses of intertidal mussels (Hofmann and Somero, 1995), fresh water snails (Al-Khedhairy *et al.*, 2001) and lobster (Spees *et al.*, 2002a; Spees *et al.*, 2002b) to environmental stress. A molecular biomarker system using dot blot ELISA has been developed to assess the physiological status of grass shrimp (*Palaomenetes pugio*) (Downs *et al.*, 2001b) and gastropods (*Ilyanassa obsolete*) (Downs *et al.*, 2001a) when subjected to a range of stressors. The Ub antibody used in these latter two studies is said to detect both free ubiquitin and ubiquinated proteins, the collective levels of which were reported to be significantly higher after stress.

We propose that targeting changes in the Ub monomer associated with stress may enable an overall view of cellular perturbations regardless of the fate of the protein or cause of the stress.

An additional factor that impacts on the quality of the results gained from this area of research is the number of animals tested. In some investigations as few as six animals were sampled and in one investigation only one animal was sampled (Section 2.7.4). These small sample sizes may have a significant impact on the interpretation of results due to individual animal variations. If statistical analysis of the results is to be undertaken it is important to determine the minimum number of samples required for the results to be biologically and statistically relevant.

There have been no documented studies comparing changes in SP or Ub levels between animals in their natural environment and those held under controlled laboratory conditions, or in animals before and after transportation. It is proposed that the use of these stress related bio-indicators may provide the basis for an effective management tool enabling the culturist to make informed decisions from the minimum number of samples. The study described here is a foundation investigation. As the hsp70 assay has been previously validated (Cimino *et al.*, 2002) this part of the study establishes that the ubiquitin assay is meaningful under three common stressful regimes (transport, salinity and disease status) and further extends the data pertaining to hsp70.

The aims of this chapter were to use ELISA to:

1. Determine appropriate number of field samples needed to produce statistically relevant data.
2. Investigate changes in the hsp70 and Ub response in commercially important *P. monodon* subject to: 1 a) transport and 1 b) hypoosmotic stress under laboratory conditions
3. Compare hsp70 and Ub levels in *P. monodon* collected from high yield (healthy) and low yield (moribund) ponds *in situ*.

5.2 Materials and Methods

5.2.1 Experimental animals

Penaeus monodon were collected and transported as described in Section 3.2.1.

5.2.2 Experimental system

As described in Section 3.2.

5.2.3 Experimental design

Three stress regimes, disease, transport and hypoosmotic stress, were chosen to investigate differences or changes in the hsp70 and Ub response during or after a stress event. Figure 5.1 illustrates the sampling scheme.

5.2.4 Disease stress

Two ponds were selected by the farmer. One pond was considered to be a high yield pond (HYP) containing healthy prawns. The second pond was considered to be a low yield pond (LYP) containing moribund prawns around the pond edge. A total of 44 prawns from each pond were sampled *in situ.* These prawns were returned to their respective ponds immediately after sampling (Figure 5.1).

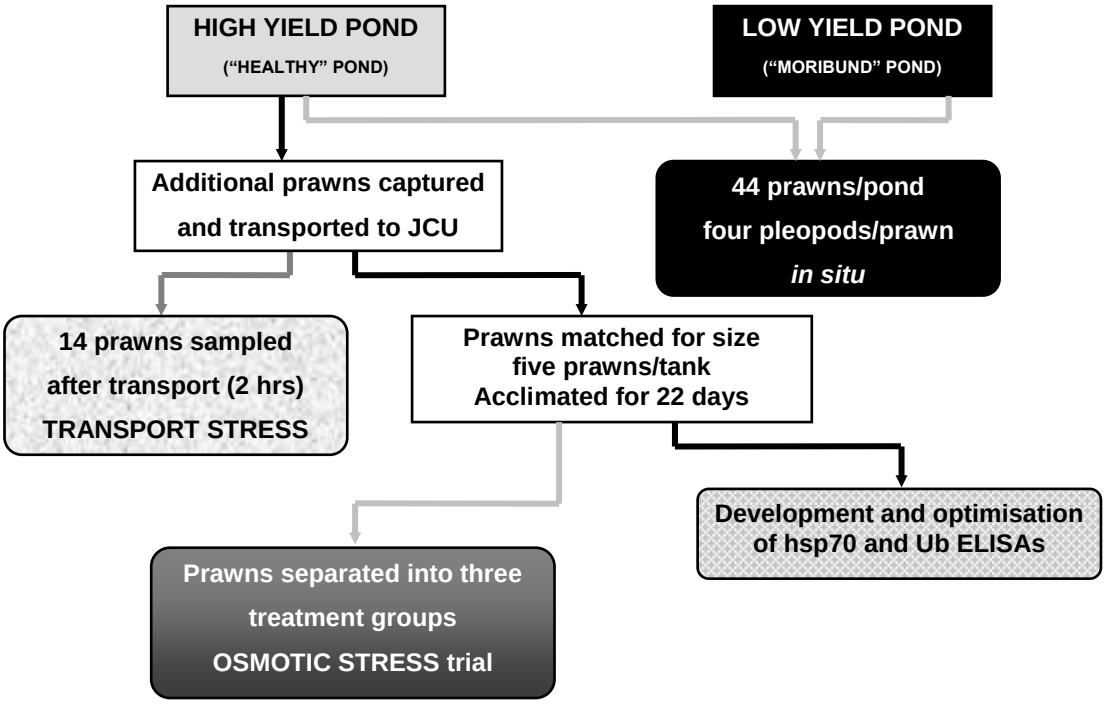


Figure 5.1: Schematic representation demonstrating the sampling scheme associated with the different stress treatments. Prawns from two ponds were sampled *in situ* (high yield and low yield ponds). Additional prawns from the high yield pond were transported to JCU where 14 were sampled immediately. The remaining prawns were placed into the experimental system for an acclimation period. During this time samples were taken and used for the development and optimisation of the hsp70 and Ub ELISAs. Remaining prawns were used later in the osmotic stress trial.

5.2.5 Transport stress

An additional 180 prawns from the same high yield pond (Section 5.2.3) were captured and placed into an 800 L aquatic transporter previously filled with pond water. Water was oxygenated using bottled oxygen supplied via one 0.5 m air stone and prawns were transported by road to the holding facility. Upon arrival at the holding facility (two hours transport time), 14 prawns were randomly chosen and pleopod samples were collected and stored (Section 2.5). These samples were termed transport stress (TS) (Figure 5.1). During capture, handling and transportation prawns were subject to a range of stressors common to this procedure including physical stress due to handling, air exposure, water turbulence and possible changes in water temperature, dissolved oxygen, pH and ammonia concentrations.

5.2.6 Hypoosmotic stress

All remaining prawns were matched for size and held at five prawns per tank in the experimental system. Stress was minimized for a settling in period of 22 days prior to the hypoosmotic stress trial (Figure 5.1).

Prawns were separated into three treatment groups at 24^{o}C (Figure 5.2): 1) Control. Salinity was kept constant at 35ppt. 2) Control with water exchange. To determine if water exchange had an effect on the levels of ubiquitin and/or hsp70, the same water exchange procedure as the hypoosmotic stressed group was followed. Salinity was kept constant at 35 ppt. 3) Hypoosmotic stress. The salinity of the stress treatment tanks was decreased from 35 ppt to 15 ppt over 40 minutes by the addition of tap water that had been aerated for 48 hours prior to eliminate chlorine.

The treatment tanks were maintained at this salinity for two hours. The salinity of the treatment tanks was then increased to the control salinity of 35 ppt over 30 min by the addition of seawater (35 ppt). The prawns were held at the control salinity for a recovery period of six hours, and then they were sampled.

5.2.7 Sample collection and preparation

As described in Sections 3.3.1 and 3.3.2.

5.2.8 ELISA

The ELISA developed in Chapter 4 was used in this investigation. The ABTS incubation time for all assays was seven hours. The estimation of total protein concentration within the samples was achieved using a BCA (Section 3.4.1). Duplicate positive (Section 4.3.3.1), negative (foetal bovine serum) and blank controls were included on each ELISA plate.

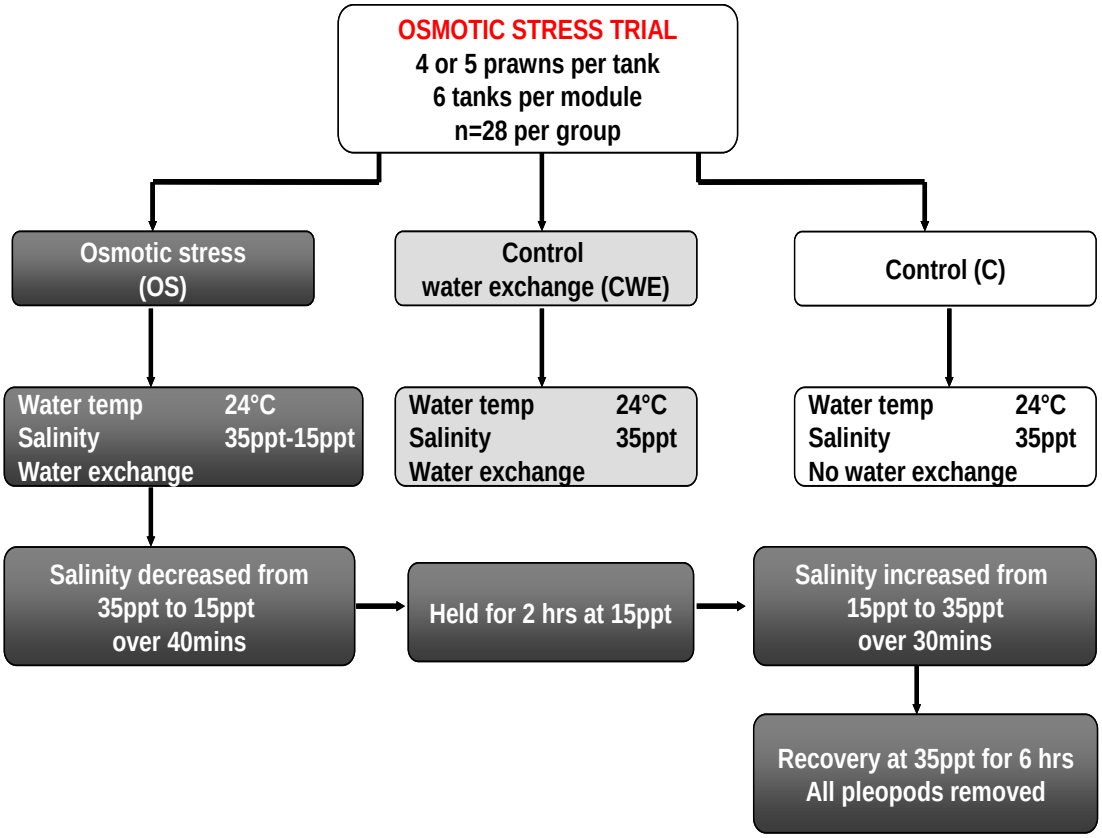


Figure 5.2: Schematic representation showing the experimental design associated with the osmotic stress trial. Prawns were separated into three treatment groups being osmotic stress, control and control with water exchange.

5.2.9 Determination of sample size

To determine the number of samples required to produce statistically relevant data, a simple line graph was used to plot:

- number of samples vs coefficient of variation (CV)
  (where CV = standard deviation/mean)

This analysis was performed using the average OD from the high yield pond samples obtained using the Ub and hsp70 ELISA.

5.2.10 Statistical analysis

All statistical analyses were performed using the computer program SPSS 12.0.1 for Windows. All reported measurements were based on average OD. Q-Q plots were used to determine normality of the data. If the data was not normally distributed, data was natural log transformed or non-parametric statistics were used. Univariate Analysis of Variance ($\alpha = 0.05$) was used to determine significant differences in hsp70 and Ub OD from the ELISA of low yield pond and transport stressed samples. Univariate Analysis of Variance and least significant difference (LSD) post-hoc analysis ($\alpha = 0.05$) was used to determine significant differences in Ub OD from the hypoosmotic stressed samples. With hsp70 OD in the hypoosmotic stressed samples, non-parametric Kruskal-Wallis and Mann-Whitney post-hoc analysis was used to determine significant differences.

5.3 Results

5.3.1 Animal husbandry

During the 22 day holding period, a total of 30 *P. monodon* were removed from the system. Of these, 20 were mortalities and 10 were unaccounted for (possibly cannibalism). This equates to a mortality rate of 25%. Mortalities occurred sporadically during this time, the highest number of bodies found occurring two and three days post-stocking (Figure 5.3).

A considerable number of prawns (86) moulted throughout the holding period. From days one to six post-stocking, moult numbers were low (one to two moults per day) (Figure 5.3). From day seven, the numbers increased dramatically to reach a peak of 17 at day 10. Moults then decreased quickly to two at day 15. From days 15 to 22, moults returned to low levels (zero or one moult per day). It is not uncommon for moults to be completely consumed by the prawns before they were recorded so the actual numbers of moults may have been greater than recorded.

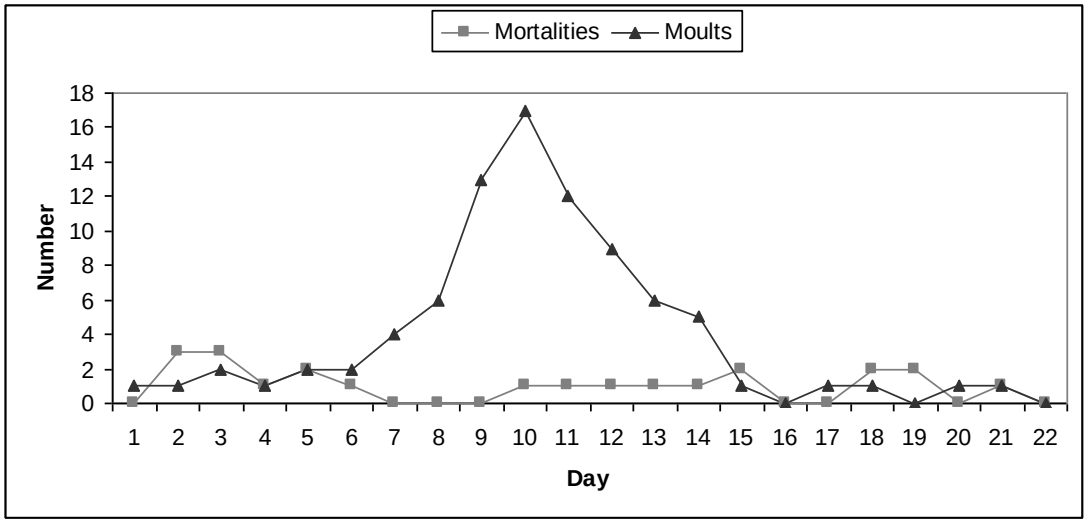


Figure 5.3: Plot of *P. monodon* mortalities and moults per day during the 22 days prior to the osmotic stress trial. Points represent the combined mortalities and moults found from all tanks in the system.

The water temperature increased from 26°C at day one post-stocking to a high of 27°C at day five. The water temperature decreased to 22°C at day 13 and then increased to 24°C at day 22.

5.3.2 Determination of sample size

The hsp70 and Ub ELISAs were plotted as number of samples vs coefficient of variation. The variation stabilised and became asymptotic after 25-30 samples (Figure 5.4). Therefore, 30 samples were chosen to represent an effective sample size.

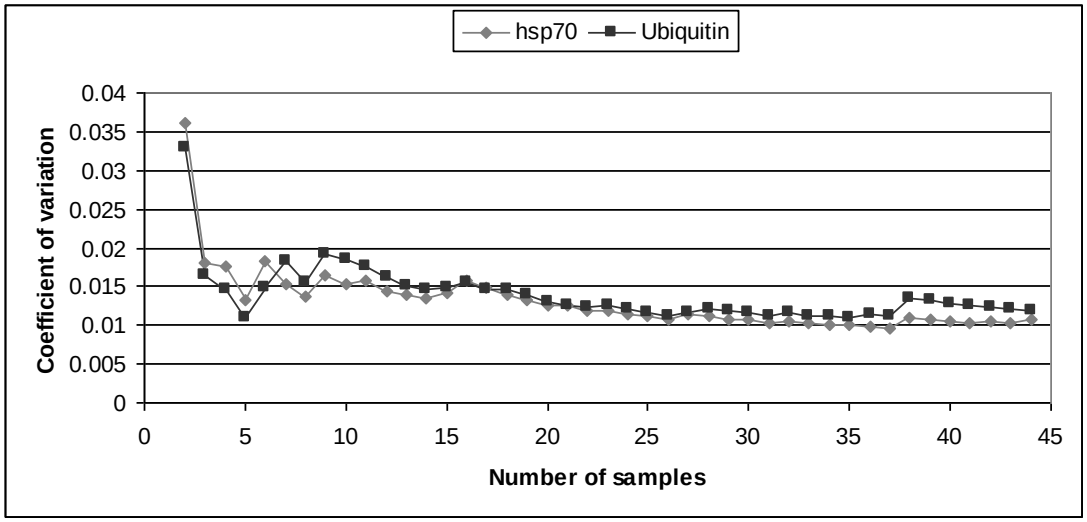


Figure 5.4: Plot of average hsp70 and Ub OD in pleopod samples as determined by ELISA in high yield pond *P. monodon* (n=44). Absorbance measured at 414 and 492nm.

5.3.3 Statistical analysis for hsp70 and Ub ELISAs

5.3.3.1 High and Low Yield ponds

For samples from the low yield and high yield ponds, there were significant differences between ponds for hsp70 ($F=21.972$, $df=1,175$, $p<0.001$) and Ub ($F=46.929$, $df=1,175$, $p<0.001$).

The samples from the LYP had a significantly higher average OD for hsp70 and Ub (mean=0.454, 0.501 respectively) than those from the HYP (mean=0.392, 0.436) (Figure 5.5).

5.3.3.2 Transport stress

There was a significant difference in OD between samples obtained from the transport stressed group and samples obtained from the high yield pond for hsp70 ($F=187.522$, $df=1,115$, $p<0.001$) and Ub ($F=4.715$, $df=1,115$, $p<0.05$). The transport stressed group had significantly higher OD for hsp70 and Ub (mean=0.760, 0.474 respectively) than the high yield pond (mean=0.392, 0.436) (Figure 5.6).

5.3.3.3 Osmotic stress

For hypoosmotic stress, there was a significant effect on hsp70 due to salinity ($\chi^2=39.373$, $df=3$, $p<0.001$). There was no significant difference ($p=0.594$) in OD between the control (mean = 0.505) and control-water exchanged groups (mean = 0.496). However, a significant difference ($p<0.05$) in OD was found between the control and hypoosmotically stressed groups (mean = 0.542) and the control-water exchanged and hypoosmotically stressed groups ($p<0.05$) (Figure 5.7).

With Ub, the pattern was slightly different. Overall there was significant effects due to treatment ($F=12.450$, $df=3,261$, $p<0.001$). There was a significantly ($p<0.05$) lower average OD in the control group (mean = 0.445nm) when compared to the control-water exchange group (mean = 0.480nm) and hypoosmotically stressed groups (mean = 0.518nm) ($p<0.001$). The control-water exchange group showed a significantly ($p<0.01$) lower OD than the hypoosmotically stressed group (Figure 5.7).

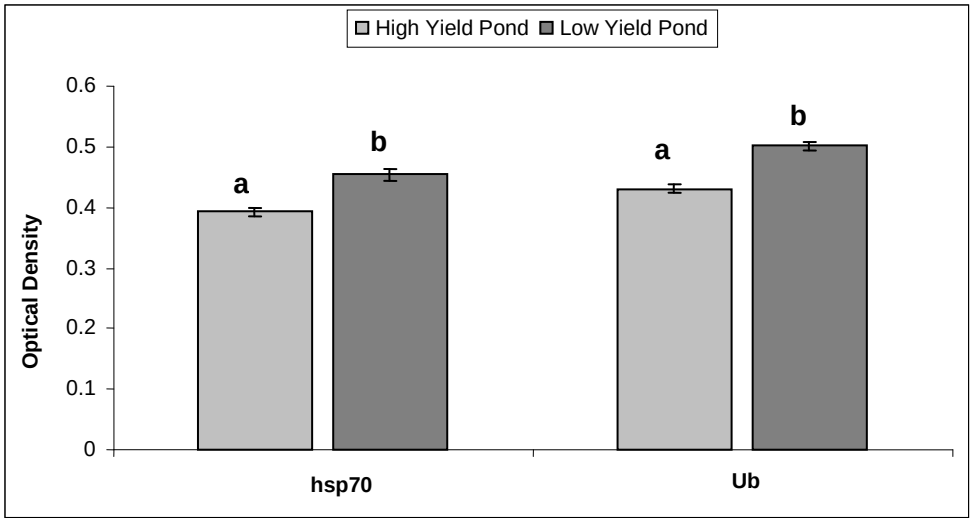


Figure 5.5: Comparison of average hsp70 and ubiquitin (Ub) OD in pleopods of *P. monodon* as determined by ELISA in a high yield pond, and a low yield pond. Same letters above each column indicate no significant difference between treatments (+/- SE). All treatments n=44. Optical density measured at 414 and 492nm.

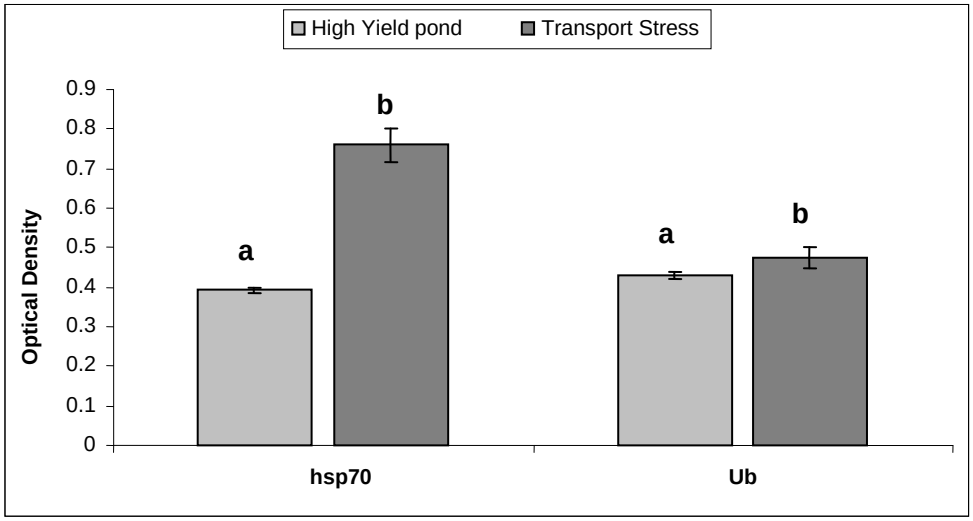


Figure 5.6: Comparison of average hsp70 and ubiquitin (Ub) OD in pleopod of *P. monodon* as determined by ELISAs in high yield pond, and transport stressed samples. Same letters above each column indicate no significant difference between treatments (+/- SE). High yield pond n=44, Transport stress n=14. Optical density measured at 414 and 492nm.

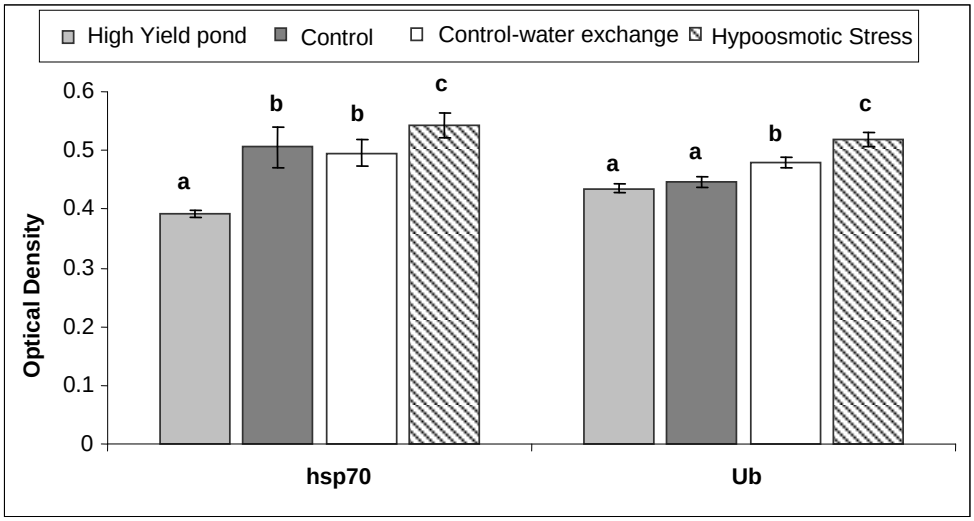


Figure 5.7: Comparison of average hsp70 and ubiquitin (Ub) OD in pleopod of *P. monodon* as determined by ELISAs in high yield pond, control, control water exchange and osmotic stressed samples. Same letters above each column indicate no significant difference between treatments (+/- SE). High yield pond n=44; control, control water exchange and hypoosmotic stressed n=28. Optical density measured at 414 and 492nm.

5.4 Discussion

Cultured penaeid prawns are often subject to rapid decreases in osmolarity during monsoonal rains and, at times, this has a significant impact on the health and survival of the animal. Despite the enormous amount of literature available on the SP response in a variety of organisms, very little is known about the SP response in aquatic crustacea. Most of the existing data on aquatic invertebrates have been gathered from work on bivalve molluscs, *Artemia* and freshwater crayfish (Appendix 2). The Ub response is even less well studied in aquatic organisms with the vast majority of studies investigating the role of polyubiquitin associated with moulting in crayfish (Section 2.7.4).

A large quantity of literature investigating the role of SP and Ub in mammalian disease exists. However, very little research has been undertaken relating to the role of SP in invertebrate disease and none could be found regarding the role of Ub in invertebrate disease.

Cheng *et al.,* (2007) described an upregulation of hsp70 mRNA in Pacific abalone (*Haliotis discus hannai*) following challenge with the pathogenic bacteria *Vibrio anguillarum*. A study by de la Vega *et al*., (2006) investigated the effect of thermal stress on *P. monodon* infected with gill-associated virus. Western blotting showed a significant increase in hsp70 levels in animals subjected to an increase in water temperature of 6°C. A hyperthermically stressed group also showed a decrease in the rate of GAV replication. The investigators suggest that a hyperthermic stress, and a consequent increase in hsp70, may reduce the rate of GAV replication in *P. monodon.* Although an interesting investigation, the sample size for each treatment group was less than 10, so care must be taken when interpreting the results.

The ELISAs were able to detect differences in the levels of hsp70 and Ub in samples collected from a culture pond that appeared to be healthy and productive (high yield pond) and one that was undergoing a disease episode as determined by the presence of moribund animals at the pond edge with low productivity (low yield pond). Our results show significantly higher hsp70 and Ub OD in the low yield pond samples when compared to the high yield pond samples. This suggests that prawns from the low yield pond were experiencing higher levels of cellular stress, indicated by higher OD for hsp70, and an increase in protein turnover, indicated by higher OD for Ub, than those animals in the high yield pond. There are a plethora of possibilities for the differences between ponds described here including differences in environmental and water quality factors (e.g. water temperature, salinity, pH, dissolved oxygen, nitrogenous compounds) and possibly health status (e.g. bacterial, viral or fungal disease) most of which have been shown to induce a SP response in crustacea (Hall and de la Vega, 2003). Further investigations using a greater number of ponds and detailed environmental and production data are required to determine the commercial utility of these bio-indicators.

With regard to further examining the hsp70 and Ub ELISAs, questions as to their usefulness in determining stress in transport and disease events were posed. In aquaculture and fisheries the transportation of live animals, both locally and internationally, is common. However, compromised health and low survival due to stress during and after transportation can have a significant economic impact (Spanoghe and Bourne, 1997; Taylor *et al.*, 1997; Vijayakumaran and Radhakrishnan, 1997; Coyle *et al.*, 2005). In the trial reported here, the OD obtained from both the hsp70 and Ub ELISAs were significantly higher in the transport stressed samples when compared to the samples collected *in situ* from the high yield pond. The method of capture, handling and transportation employed here has been routinely used to transport crustaceans and fish for several years. Although water quality factors were not measured during transport, the water used for transportation and the transported prawns were sourced from the same pond and as such minimal stress was due to rapid changes in water quality factors. The ambient air temperature was approximately 26ºC which may have lead to an increase in water temperature during transportation. In addition to this, capture, water turbulence and changes in dissolved oxygen during transportation were the most likely factors resulting in the increase in the hsp70 and Ub response. This demonstrates that these stress indictors are associated with capture, handling and the transport of *P. monodon* and as such, it is proposed that hsp70 and Ub may be useful bio-indicators in this area. Monitoring changes in their response could be an indication of the extent of stress experienced. Further research in this area may enable the farmer to predict an outcome regarding the animal's health and fitness (measured by a range of factors including changes in appearance, taste, ability to recover and morbidity) after specific handling and transport regimes. The sampling procedure described here is non-destructive and the ELISA is rapid and cost effective making this assay commercially viable. To validate the utility of these findings in greater depth, investigations are required in a situation more indicative of those encountered in a commercial setting.

During the subsequent holding period of 22 days in this investigation, a 25% mortality rate was recorded for the additional prawns that were held in the experimental system. The highest number of mortalities occurred two and three days post-stocking suggesting that the initial high mortality was due primarily to the physiological stress from handling and transportation. Subsequent mortalities were sporadic with no apparent correlation with other stressors such as moulting. All the mortalities found had been partially consumed by the other prawns in the same tank and it is assumed that the 10 unaccounted for prawns had been completely consumed. This cannibalistic behaviour is not uncommon in *P. monodon* and is one of the husbandry difficulties associated with holding this species under experimental conditions. It is possible that the prawns had an underlying disease and the stress from handling and transportation compromised their immune response resulting in sporadic mortalities. The experimental system used in this investigation was designed to minimize stress, for example, dark blue tanks to reduce visual stress, controlled water quality and environmental factors and minimal noise in the facility. However, it is suggested that partitioned tanks to stop prawn-prawn interactions or a maximum of three prawns per tank would reduce mortalities due to cannibalism and associated stress.

A comparison of hsp70 and Ub levels in the high yield pond and control groups showed no significant difference in Ub OD between these two groups (Figure 5.6). However, significantly higher hsp70 OD were recorded in the control group indicating that the stress response in the prawns kept in the experimental system was elevated. The most probable explanation for this is due to prawn-prawn interactions as previously discussed.

To date, this is the first investigation concerning the Ub response, as opposed to the polyubiquitin response, in crustacea and the first to use an ELISA to elucidate changes in the Ub response to stress in *P. monodon*.

Spees *et al*., (2002b) found significant increases in polyubiquitin mRNA levels in lobsters (*Homarus americanus*) as a result of both a decrease and increase in salinity. Our findings show a significant increase in the OD obtained from the Ub and hsp70 ELISAs to a decrease in salinity. We propose that changes in salinity have an impact on protein turnover and consequently may reduce fitness and growth. This may be important if future investigations can determine that changes in Ub or hsp70 levels correlate with productivity in prawn farms.

The investigation described here is the first stage of a larger investigation aimed at developing management tools to enable the farmer to make informed decisions regarding health and productivity at a commercial level The results have shown that for *P. monodon* the ELISAs are able to detect changes in the hsp70 and Ub response as a result of transport and hypoosmotic stress. Of more significance is the finding that the ELISAs detected differences in the hsp70 and Ub response between a ‘healthy’ and a ‘diseased’ pond. This result suggests that these bio-indicators may be useful measures of significant stress at the farm level and may provide a means of determining when a pond is in danger of failing.

CHAPTER 6

FARM INVESTIGATION OF CHANGES IN HSP70, Ub AND GILL-ASSOCIATED VIRUS (GAV) EXPRESSION IN *PENAEUS MONODON* WITH RESPECT TO ENVIRONMENTAL FACTORS.

6.1 Introduction

Many pathogens such as bacteria and fungi can be controlled to some extent by improved husbandry techniques and the mechanisms by which penaeids deal with these pathogens are well documented (Ratcliffe *et al.*, 1985; Roch, 1999; Lee and Soderhall, 2002; Loker *et al.*, 2004). Relatively little is known, however, with respect to how the penaeid immune system deals with viruses (Loker *et al.*, 2004) (Section 2.2.1).

It has been shown that a wide range of stressors can elicit a stress response in aquatic organisms with the upregulation or synthesis of shock proteins (Section 2.4) and ubiquitin (Section 2.7.4). It has also been shown that combined stresses can alter the synthesis and expression of SP (Section 2.4). It is clear that environmental stress, particularly temperature and osmotic stressors, can have a profound effect on penaeid aquaculture resulting in decreased growth rates (Bray *et al.*, 1994) and at worst, significant economic losses due to resultant viral epizootics (Flegel and Pasharawipas, 1998) (Section 2.5). At present most SP and Ub investigations in aquatic organisms have been conducted under laboratory conditions. Given that the number and type of stressors imposed on the animal have a combined effect on the levels and expression of SP, and cultured prawns are subject to a number of stressors at any given time, further investigations should be conducted under culture conditions.

Gill-associated virus, a virulent pathogen of *P. monodon*, has been described as having rod-shaped, enveloped, positive RNA virions closely resembling the morphology and cytopathology of yellow head virus from Thailand (Spann *et al.*, 1997). Gill-associated virus has caused significant production losses to Australian aquaculture since 1996 and has been described as being endemic to both cultured and wild *P. monodon* along the east coast of Australia (Spann *et al.*, 1995; Cowley *et al.*, 2000; Cowley *et al.*, 2002; Spann *et al.*, 2003). Although GAV is highly prevalent and can be extremely pathogenic, it does not always cause disease and has been found as a chronic infection in otherwise healthy animals (Spann *et al.*, 1995; Spann *et al.*, 2003). Studies have indicated that environmental stress factors may initiate the acute stage which is then associated with high mortalities and production losses (Flegel and Pasharawipas, 1998; Vidal *et al.*, 2001). Recently, polyclonal and monoclonal antibodies specific for GAV were produced and an ELISA was developed for the detection of GAV (Munro and Owens, 2007) making high throughput testing affordable.

Penaeid prawns are commonly farmed using semi-intensive methods, usually in 1.5 m deep, one hectare earthen ponds exposed to changes in environmental conditions (Appendix 3). At present there is no accurate way to predict when a crop is in danger of failing and the farmer must rely on changes in feeding patterns and visual health checks of the prawns. Large ponds make this a difficult task due to high amounts of suspended solids in the water column making the water very murky. Often high mortalities are not evident until a full blown epizootic is underway. In addition to this, preventative methods of disease control in prawn culture, such as “vaccinations”, have not been successfully developed. And so disease control in prawn aquaculture remains extremely difficult.

It is known that environmental stress can trigger disease outbreaks, so it is proposed that the use of a generic stress indicator will enable farmers to make informed management decisions. Therefore, in addition to hsp70 (an indicator of cellular perturbations) and Ub (an indicator of protein turnover), GAV has been included as a disease indicator in this trial.

This chapter aims to:

1) Apply the ELISA to investigate changes in the levels of the bio-indicators hsp70, Ub and GAV in farmed *P. monodon* during growout.
2) Apply the ELISA to investigate correlations between levels of the bio-indicators and environmental factors.

6.2 Materials and Methods

6.2.1 Experimental animals

Penaeus monodon were obtained from Pacific Reef Fisheries Pty Ltd beginning on 13th December 2005. All animals were randomly captured by cast netting and were placed into a 50L plastic container previously filled with pond water. Water was oxygenated using bottled oxygen supplied via one 0.5 m air stone.

6.2.2 Experimental design

A total of 11, 1.5 m deep, one hectare earthen ponds were chosen by the farmer and 30 prawns per pond were captured and sampled weekly until normal harvest or emergency harvest. The number of days the prawns had been in the pond at first sampling, total number of sample weeks and total days in pond are summarised in Table 6.1. Water temperature (°C), salinity (ppt), pH, dissolved oxygen (ppm) and secchi (bloom depth measured in cm) were measured twice daily (am and pm) by the farmer and any visual changes in prawn health in the sampled ponds were recorded.

Table 6.1: Summary of ponds sampled weekly. Pond numbers were assigned according to sampling regime. DIP = days in pond.

Pond	DIP Sample week 1	DIP Final sample week	Total Sample weeks
1	118	174	9
2	90	153	10
3	118	181	10
4	120	190	11
5	120	155	6
6	121	156	6
7	117	166	8
8	117	152	6
9	114	184	11
10	92	148	9
11	92	148	9

6.2.3 Sample collection

To avoid damage and unnecessary stress during surgery, prawns were gently restrained and the procedure completed in the shortest possible time (<50 minutes per pond).

Scissors were used to remove four pleopods at the protopodite-endopodite joint per prawn (representing one sample) from alternate sides of the abdomen to avoid comprising their swimming action. To avoid deterioration, each sample was immediately placed into an individual 2ml well of a deep-well microtitre plate (Daintree Scientific, Tasmania catalogue # P-DW-20-C) containing 1ml of 1mm zirconia/silica beads and 1ml chilled PBS. All samples were placed on ice and transported to JCU where they were stored frozen at -20°C. All prawns were returned to their respective ponds immediately after sampling.

6.2.4 Sample preparation

Samples were thawed overnight at 4°C then homogenized at 36 oscillations/second using a MiniBead Beater96 (Daintree Scientific) for four mins, two times. Samples were centrifuged (Eppendorf 5804 R) at 2844 *g* (4000 rpm) for 10 min. The pellet was discarded and the supernatant was centrifuged at 2844 *g* (4000 rpm) for 30 min. The resulting pellet was discarded and the remaining supernatant was stored at - 80^{o}C until used for protein analysis.

6.2.5 Antibodies

The antibodies described in Section 4.2.5 were used for the detection of hsp70 and Ub. A polyclonal antibody (PAb) (from chicken's serum) and three monoclonal antibodies (termed 3A-4, Ak5-11 and 3K3-4, derived from mouse ascitic fluid) against GAV were shown to give acceptable titres by Munro and Owens (2007) and were trialled in this study.

The labelled secondary MAb used in all procedures was goat anti-mouse HRPO conjugate (TropBio Pty Ltd, Townsville).

6.2.6 ELISA

The estimation of total protein concentration within the samples was achieved using a BCA (Section 3.4.1).The HRPO conjugate was used at a dilution of 1/1000 for all assays.

6.2.6.1 ELISA for detection of GAV

The ELISA used to detect GAV in this study was developed by Munro and Owens (2007). The tissue type (gill) and the sample preparation used in that previous investigation differed from this current study, therefore a checkerboard titration for each of the three MAbs using two random samples (S1 and S2) from the same pond and sample week and the PC sample (described in Section 3.3.1) was undertaken to determine: 1) if the sample preparation for the detection of hsp70 and Ub impeded the usefulness of the GAV ELISA, 2) which was the most appropriate GAV MAb, 3) what GAV MAb concentration should be used and 4) sample protein concentration (Figure 6.1).

The positive gills (PG) and negative controls were gill filaments from *P. monodon* and *P. merguiensis* respectively that tested positive and negative for GAV using reverse transcription-nested polymerase chain reaction (RT-nPCR). These controls were included on each plate at a dilution of 1/128. Flat bottom 96 well microtitre plates (Sarstedt, Australia catalogue # 82.9923.148) were used for all GAV ELISAs.

Individual plates were tested for the extent of non-specific binding using conjugate control wells or more commonly termed, blank wells. Blank wells contained no sample. However, all other steps were conducted as with the test wells.

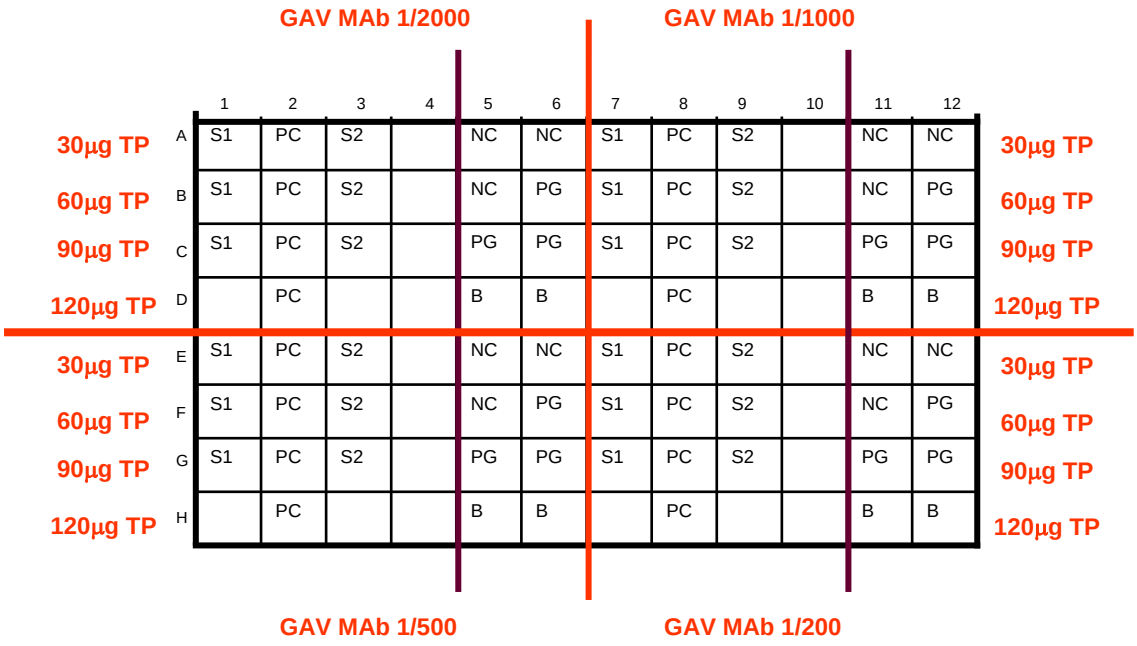


Figure 6.1: Checkerboard titration design for the optimisation of the ELISA for the detection of gill-associated virus (GAV) in *P. monodon*. All procedures were replicated for each of the three monoclonal antibodies tested. S1 = sample 1, S2 = sample 2, PC = positive control (pooled pereiopods), PG= positive control obtained from RT-nPCR positive *P. monodon* gills at 1/128, NC = negative control obtained from RT-nPCR negative *P. merguiensis* gills at 1/128, B = blank control, TP = total protein concentration per well.

All procedures followed the protocol below:

1. Bring diluents, buffers and ABTS to room temperature before use.
2. Dilute PAb 1/80 in carbonate buffer (TropBio Pty Ltd, Townsville) and add 50μl to each well of a 96 well, flat bottom microtitre plate
3. Cover plates to reduce evaporation and incubate overnight at room temperature.
4. Flick off excess PAb and wash one time with TEN-TW buffer (TropBio Pty Ltd, Townsville).
5. Add 100μl post-coating buffer (TropBio Pty Ltd, Townsville) to each well and incubate at 27°C for two hours.
6. Flick off post-coating buffer and dry plates at 37°C for one hour.
7. Dilute samples and controls in TEN-TC buffer (TropBio Pty Ltd, Townsville).
8. Add 50μl of diluted sample and controls per well and incubate for one hour at 27°C.
9. Flick off excess sample and wash three times with TEN-TW buffer.
10. Dilute GAV MAb in TEN-TC buffer. Add 50 μL to each well and incubate for one hour at 27°C.
11. Flick off MAb and wash three times with TEN-TW buffer.
12. Dilute goat anti-mouse HRPO conjugated antibody in TEN-TC buffer to a dilution of 1/1000. Add 50 μL to each well and incubate for one hour at 27°C.
13. Remove excess MAb solution and rinse all wells six times with TEN-TW.
14. Add 50 μL of ABTS to each well and incubate in the dark at room temperature for one hour.
15. Measure optical density (OD) at a dual absorbance of 414nm and 492nm.

6.2.6.2 ELISA for detection of hsp70 and Ub

The ELISA developed in Chapter 4 was used to determine changes in the hsp70 and Ub response in this investigation (protocol Appendix 4). The ABTS incubation time for these assays was six hours.

6.2.7 Statistical analysis

All statistical analysis was performed using the computer program SPSS 12.0.1 for Windows. Significant changes in average hsp70, Ub and GAV OD for each pond between weekly sampling times were determined using either Univariate Analysis of Variance (α=0.05) or the non-parametric Kruskal-Wallis tests depending if the conditions for normality were met. Correlation analysis was used to determine significant associations between the three bio-indicators (i.e. hsp70, Ub and GAV) and environmental factors.

Principal component analysis was used as a data reduction method. To determine if this analysis was appropriate, the Bartlett's test of sphericity and the Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy were calculated. Factors with correlations less than 0.5 were not included in the analysis. Due to a correlation coefficient of greater than 0.990 for salinity am and pm across most ponds the latter was excluded from this analysis (Appendix 8). Kaiser's criterion was used to determine the minimum number of components that adequately represents the relationships between the factors. The Varimax rotation was used in an endeavour to interpret the generated data.

6.3 Results

6.3.1 Checkerboard titration for GAV

Three monoclonal antibodies to GAV were titrated with *P. monodon* samples. All MAbs were immuno-reactive and results from the checkerboard titration showed a similar trend between all three MAbs (data not shown). In general optical densities increased with an increase in total protein concentration and MAb dilution. However, the MAb achieving the highest optical density measurements across the plate for the GAV checkerboard titration was 3A-4 (Figure 6.2). Although MAb 3A-4 produced higher OD at 1/500 and 1/200 dilutions, to reduce assay costs a dilution of 1/1000 was deemed suitable. Sample 1 had noticeably lower OD than sample 2 across the plate and the positive control (PC) had high OD, exceeding the PG sample.

	GAV MAb 1/2000						GAV MAb 1/1000					
	S1	PC	S2				S1	PC	S2			
30µg TP	0.173	0.756	0.510		0.013 NC	0.015 NC	0.175	0.720	0.639		0.018 NC	0.018 NC
60µg TP	0.313	0.772	0.542		0.012 NC	0.498 PG	0.324	0.875	0.638		0.015 NC	0.691 PG
90µg TP	0.367	0.905	0.588		0.542 PG	0.487 PG	0.401	0.905	0.654		0.549 PG	0.642 PG
120µg TP	NS	1.010	NS		0.010 B	0.012 B	NS	1.092	NS		0.013 B	0.014 B
30µg TP	0.177	0.710	0.545		0.016 NC	0.017 NC	0.225	0.752	0.630		0.033 NC	0.039 NC
60µg TP	0.279	0.946	0.593		0.017 NC	0.578 PG	0.323	1.063	0.661		0.034 NC	0.614 PG
90µg TP	0.405	1.040	0.572		0.477 PG	0.584 PG	0.519	1.037	0.748		0.617 PG	0.677 PG
120µg TP	NS	1.144	NS		0.012 B	0.020 B	NS	1.181	NS		0.025 B	0.025 B
	GAV MAb 1/500						GAV MAb 1/200					

Figure 6.2: Optical densities recorded from the GAV MAb (3A-4) checkerboard titration. GAV PAb used at 1/80, MAb conjugate at 1/1000. S1 = sample 1, S2 = sample 2, PC = positive control (pooled pereiopods), PG= positive control obtained from RT-nPCR positive *P. monodon* gills at 1/128, NC = negative control obtained from RT-nPCR negative *P. merguiensis* gills at 1/128, NS = no sample, B = blank control, TP = total protein concentration per well.

It was concluded from the titration that in all subsequent GAV ELISAs: 1) the MAb 3A-4 be used at a dilution of 1/1000, 2) total protein concentration for all samples will be 90μg per well and 3) due to a lack of sufficient PG sample, the PC sample will replace the PG sample on all plates as a positive control. The full protocol is described in Appendix 5.

6.3.2 Statistical analysis for hsp70, Ub and GAV ELISAs

Comparisons of means revealed significant differences between sample weeks for hsp70, Ub and GAV. Line graphs produced using average OD for all three bio-indicators revealed differing response patterns over the sampling period with individual ponds (Appendix 6). Due to the complexity of this trial, only common patterns across ponds will be discussed. Detailed analysis is below (Section 6.3.3 onwards). In general, hsp70 and GAV OD increased and Ub OD decreased notably at the first two to four sample weeks across ponds. At the final sample week, hsp70 and GAV OD were generally higher than sample weeks one to three and Ub OD remained stable or decreased slightly over this period.

Histograms using OD for the bio-indicators were also produced. No obvious patterns could be deduced from the hsp70 or Ub histograms. However the GAV histograms showed a similar pattern across all ponds (Figure 6.3). Generally, the frequency of samples with low OD decreased with a corresponding increase in the frequency of samples with higher OD over one to three weeks. This was followed with an abrupt increase in the frequency of samples with low OD for usually one week. This pattern was repeated until harvest.

6.3.3 Correlation analysis

To determine major trends across all ponds, correlation analysis was performed for each individual pond, bio-indicator and environmental factor over all days. Daily environmental data was averaged over each sample week. There were significant correlations between all factors (Table 6.2). The greatest number of significant correlations was associated with hsp70 (22) followed by GAV (18) and then Ub (17). Of interest was days in pond with 18 significant correlations with most pertaining to hsp70 and GAV. In general the correlations between bio-indicators were positive and the environmental factors showed mostly negative correlations with stress indicators.

To determine the biological significance of these interactions, correlation analysis was conducted for each bio-indicator and environmental factor for all ponds (grouped) daily from six days prior to sampling up to and including the day of sampling. Average positive and negative correlation coefficients (CC) were calculated for each factor (Appendix 7) and factors with > three significant values over the seven days for hsp70 (Figure 6.4), Ub (Figure 6.5) and GAV (Figure 6.6) are shown.

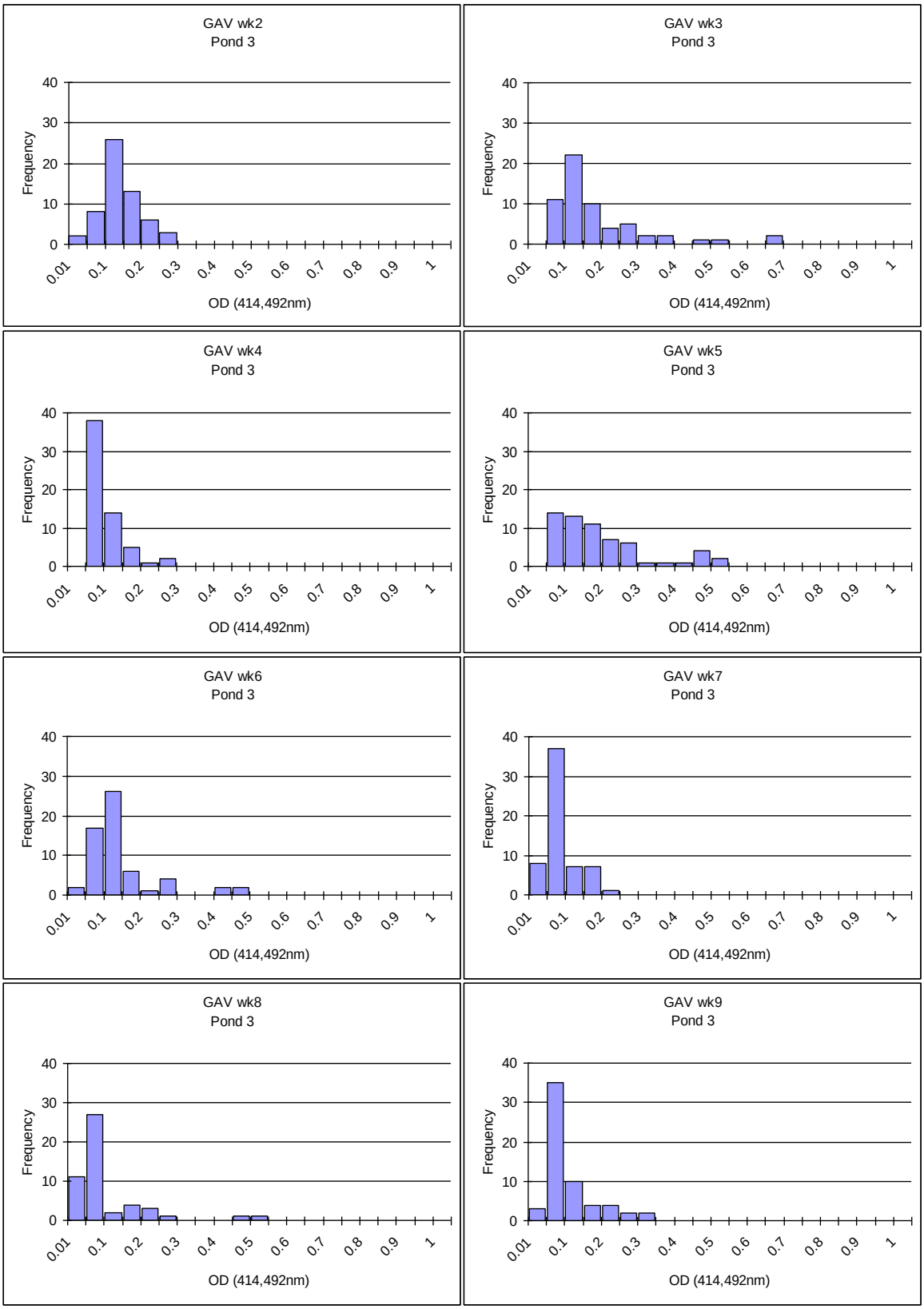


Figure 6.3: Histogram generated from the GAV ELISA results for Pond 3 illustrating the change in the frequency of OD across weeks. A similar trend was noted across all ponds.

Table 6.2: Significant correlation coefficients for hsp70, Ub and GAV with environmental factors for individual ponds (grouped weekly). Coloured numbers represent individual ponds. Bold numbers represent: 1) number of significant correlations, 2) positive and negative average correlation coefficients (calculated separately). DO = dissolved oxygen

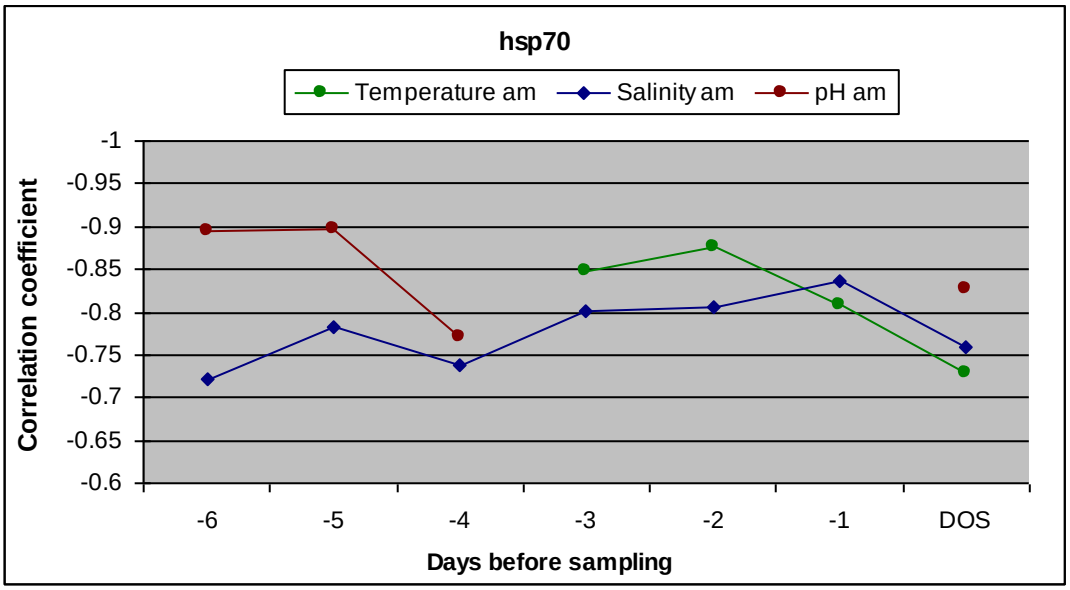
	hsp70	Ubiquitin	GAV
Ubiquitin **3 significant correlations**	**0.729, 0.844, 0.834,** **3, 0.802**		
GAV **4 significant correlations**	**0.835, 0.681, 0.787** **3, 0.768**	**0.822,** **1, 0.822**	
Days in Pond **18 significant correlations**	**0.741, 0.742, 0.847, 0.824, 0.773, 0.841, 0.803** **7, 0.796**	**0.723,-0.853, 0.698, 0.679,** **4, 0.700, -0.853**	**0.941, 0.913, 0.885, 0.943, 0.698, 0.894, 0.905** **7, 0.883**
Temp (°C) am **3 significant correlations**	**-0.729** **1, -0.729**	**0.858** **1, 0.858**	**-0.825** **1, -0.825**
Temp (°C) pm **1 significant correlations**		**0.879** **1, 0.879**	
Salinity (ppt) am **6 significant correlations**	**-0.859** **1, -0.859**	**-0.810, -0.776** **2, -0.793**	**-0.835,-0.847,-0.914** **3, -0.865**
Salinity (ppt) pm **5 significant correlations**	**-0.834** **1, -0.834**	**-0.779, -0.755** **2, -0.767**	**-0.857, -0.911** **2, -0.884**
pH am **3 significant correlations**	**-0.789, -0.674** **2, -0.732**	**0.761** **1, 0.761**	
pH pm **3 significant correlations**	**0.822** **1, 0.822**		**-0.704, -0.667** **2, -0.686**
DO (ppm) am **4 significant correlations**	**-0.743** **1, -0.743**	**-0.690** **1, -0.690**	**-0.667, -0.708** **2, -0.688**
DO (ppm) pm **3 significant correlations**		**-0.874, -0.786** **2, -0.830**	**0.908** **1, 0.908**
Secchi (cm) am **1 significant correlations**		**-0.654** **1, -0.654**	
Secchi (cm) pm **3 significant correlations**	**-0.835, -0.744** **2, -0.790**	**-0.722** **1, -0.722**	
TOTALCorrelation coefficients	**22**	**17**	**18**

Pond 1 (n=8)
Pond 2 (n=9)
Pond 3 (n=9)
Pond 4 (n=8)
Pond 5 (n=6)
Pond 6 (n=6)
Pond 7 (n=8)
Pond 8 (n=6)
Pond 9 (n=11)
Pond 10 (n=9)
Pond 11 (n=9)

6.3.3.1 Correlation analysis for hsp70

A negative correlation between hsp70 and water temperature (am) was significant at three (CC -0.848), two (CC -0.877) and one (CC -0.809) day prior to sampling and day of sampling (CC -0.729) (Figure 6.4). The correlation coefficient maximised at two days then decreased to day of sampling. pH (am) was significantly correlated at six (CC -0.896), five (CC -0.896) and four (CC -0.771) days prior to sampling and day of sampling (CC -0.828). Salinity am and pm (pm data not shown) were significantly negatively correlated to hsp70 with similar trends. Salinity (am) was negatively correlated to hsp70 at all days. The correlation coefficients increased from six days prior to sampling (CC -0.721) to a maximum at one day (CC -0.836) prior to sampling followed by a small decrease at day of sampling.

Figure 6.4: Plot of significant correlation coefficients showing correlations between hsp70 and the environmental factors water temperature (temperature am), salinity (am) and pH (am) daily from six days prior to sampling to the day of sampling (DOS).



6.3.3.2 Correlation analysis for ubiquitin

The trends for Ub were largely non-contiguous and therefore messy. A negative correlation between Ub and pH (am) was significant at six (CC -0.851), four (CC -0.819), three (CC -0.938) and one (CC -0.823) day prior to sampling (Figure 6.5). The highest correlation coefficient was noted at three days prior to sampling. pH (pm) was negatively correlated at six (CC -0.913), five (CC -0.889), three (CC -0.819) and two (CC -0.998) days prior to sampling with high correlation coefficients at five and six days prior to sampling and an extremely high correlation coefficient two days prior to sampling.

Salinity (am) was significantly negatively correlated to Ub at all days. A gradual increase in negative correlation coefficients from six (CC -0.754) days prior to sampling until a peak at four (CC -0.863) days prior to sampling thereafter a gradual decline occurred until day of sampling (CC -0.753).

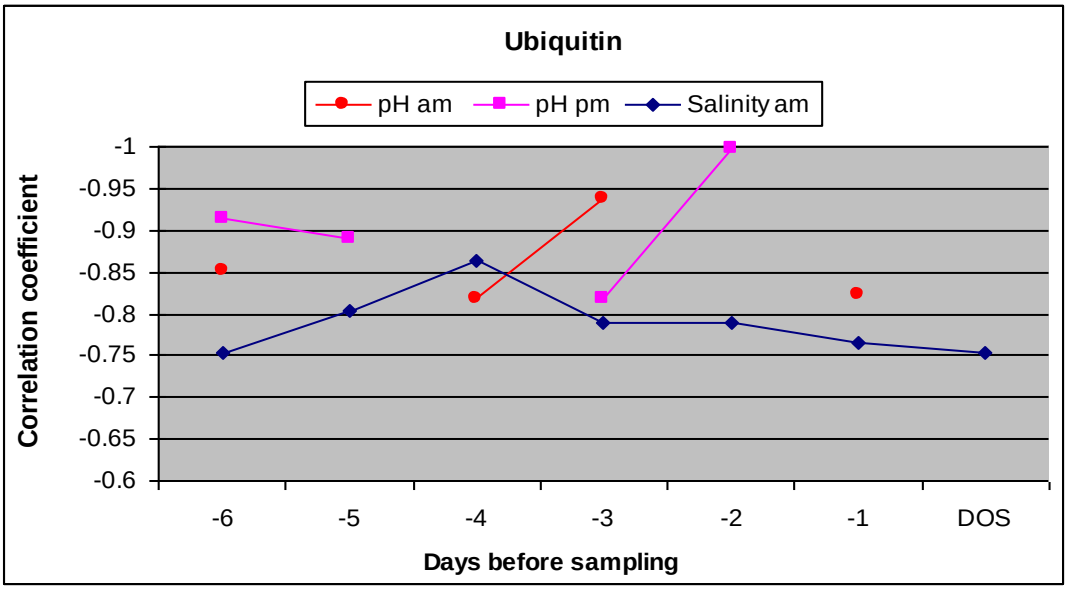


Figure 6.5: Plot of significant correlation coefficients showing correlations between ubiquitin and the environmental factors salinity (am) and pH (am and pm) daily from six days prior to sampling to the day of sampling (DOS).

6.3.3.3 Correlation analysis for gill-associated virus

A negative correlation between GAV and salinity (am) was significant at all days (Figure 6.6). The correlation coefficient was greatest at six days prior to sampling (CC -0.921), decreasing to a minimum at four (CC -0.832) and three (CC -0.831) days prior to sampling. An increase in correlation coefficients followed at two (CC -0.904) and one (CC -0.902) day prior to sampling, decreasing slightly at day of sampling (CC -0.883).

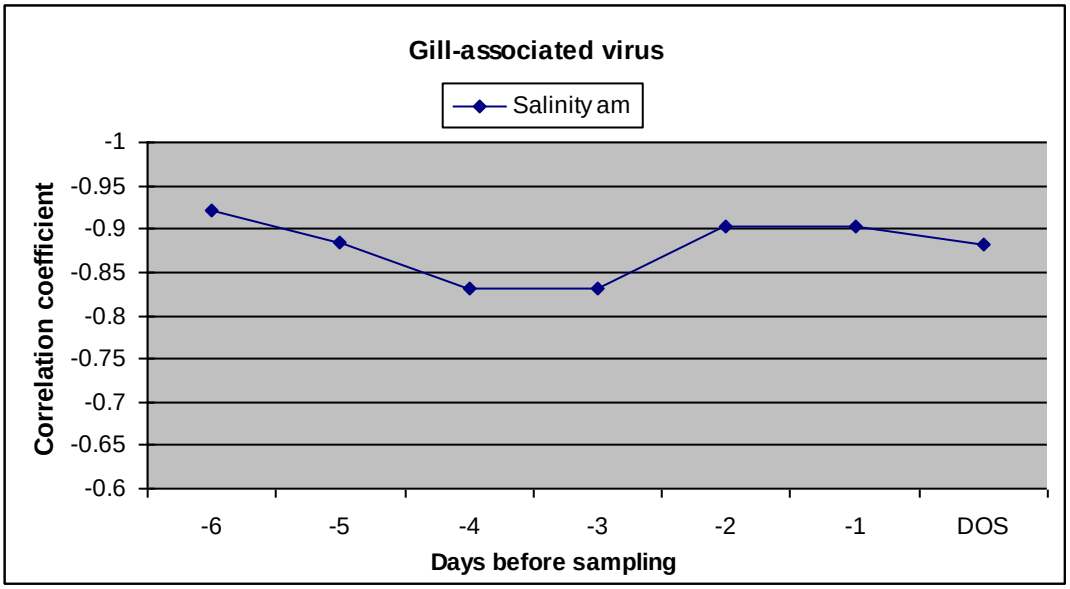


Figure 6.6: Plot of significant correlation coefficients showing correlations between gill-associated virus and salinity (am) daily from six days prior to sampling to the day of sampling (DOS).

6.3.4 Principal component analysis

As the correlation analysis was still very complex, other data reduction analyses were trialled. The Bartlett's test of sphericity was significant at $p<0.001$ and Kaiser-Meyer-Olkin measure of sampling adequacy was 0.542 (Appendix 8) indicating that principal component analysis was a suitable analysis.

To determine the minimum number of components that adequately represents the relationships between the factors, Kaiser's criterion was used. A total of five components with an Eigenvalue of greater than or equal to 1.0 were generated representing 74.3% cumulative variance explained (Appendix 8). To facilitate interpretation of the analysis, factor loadings of less than 0.5 will not be discussed.

Using the Varimax rotation method, a component matrix was generated. The output produced showed five components incorporating all factors except DO (am) (Table 6.3). Component one consisted of four factors; DIP, salinity (am), hsp70 and GAV with factor loadings of 0.885, -0.766, 0.665 and 0.505 respectively. Components two to five consisted of two factors in each component being temperature (am and pm) in component two with factor loadings of 0.933 and 0.927 respectively, secchi (am and pm) in component three with factor loadings of 0.952 and 0.915 respectively, pH (am and pm) in component four with factor loadings of 0.890 and 0.871 respectively and DO (am) and Ub in component five with factor loadings of -0.794 and 0.671 respectively.

The total variance explained is detailed in Table 6.4. Component one accounted for 22.6% of the total variance, followed by 19.4% for component two, 14.3% for component three, 10% for component four and 8% for component five with a total cumulative variance explained of 74.3%.

Table 6.3: Component matrix generated from average weekly data using principal component analysis for hsp70, Ub, GAV and environmental factors. Output produced five components incorporating all factors except dissolved oxygen am. Only correlations above 0.5 are included in the analysis. Salinity pm was excluded from the analysis.

Rotated Component Matrix[a]

	Component				
	1	2	3	4	5
DIP	.885				
Salinityam	-.766				
hsp70	.665				
GAV	.505				
DOam					
Tempam		.933			
Temppm		.927			
Secchipm			.952		
Secchiam			.915		
pHam				.890	
pHpm				.871	
DOpm					-.794
Ub					.671

Extraction Method: Principal Component Analysis.
Rotation Method: Varimax with Kaiser Normalization.

a. Rotation converged in 5 iterations.

Table 6.4: Total variance explained from average weekly data using principal component analysis for hsp70, Ub, GAV and environmental factors from five components. Salinity pm was excluded from the analysis.

Total Variance Explained

	Rotation Sums of Squared Loadings		
Component	Total	% of Variance	Cumulative %
1	2.514	19.338	19.338
2	2.037	15.666	35.004
3	2.019	15.528	50.532
4	1.788	13.751	64.283
5	1.305	10.035	74.319

Extraction Method: Principal Component Analysis.

6.4 Discussion

To date, this is the first investigation concerning the hsp70, Ub and GAV response in any crustacean within a commercial setting. The successful development of ELISAs for the analysis of changes in hsp70, ubiquitin and GAV in *P. monodon* has provided quantitative assays able to screen large numbers of samples quickly at relatively low cost.

Statistical analysis of the hsp70, Ub and GAV response within ponds showed significant differences between sample weeks. Line graphs of the bio-indicators (hsp70, Ub and GAV) for individual ponds showed unique stress response patterns with some common trends across ponds. However, there were no obvious patterns when compared to graphs of environmental factors. As previously discussed (Section 2.4) the hsp70 response in particular, is sensitive to many individual and/or combined stressors. Under culture conditions the prawns are subject to a range of stressors at any given time so what may appear to be minor event as gauged by graphs using averaged data may have a significant effect on the stress response or *visa versa*. For example, a decrease in salinity from 41ppt to 30ppt over four days was recorded at week five for the majority of the ponds (data not shown). However, no common significant effect was detected from the line graphs or the ANOVA across ponds. This may be a result of using the weekly rather than daily sampling regime. Further investigations are required to determine what rate or level of change is significant and if this response has a significant effect on production.

To reduce the complexity of the data, correlation analyses were performed on individual ponds for all three bio-indicators and all environmental factors over grouped sample weeks. Of particular interest was days in pond with seven of the 11 ponds analysed having highly significant correlations with hsp70 and GAV. To date there have been no published investigations describing the hsp70 or GAV response under culture conditions.

However, a study conducted by Munro *et al.,* (2008) provides strong evidence to suggest that prolonged culture in this type of system results in increased levels of stress and viral load. This finding may be of significance when investigating production outcomes on farms. In general the correlations between bio-indicators and the environmental factors showed mostly negative correlations. However, no substantive conclusions could be reached from this analysis.

To determine the biological significance of these interactions, correlation analysis was conducted for each bio-indicators and environmental factor from six days prior to sampling up to and including the day of sampling. Of particular interest was pH and salinity. Morning pH (am) was negatively correlated to hsp70 at day of sampling and four days prior to sampling with larger correlation coefficients at five and six days prior to sampling. A similar pattern was noted with Ub. This pattern is most likely related to the farms liming regime of the ponds. Briefly, in semi-intensive prawn culture the presence of high levels of plant and phytoplankton activity can result in high pH during the day (due primarily to photosynthesis) and low during overcast days or at night if the pond water is not buffered (Landau, 1992). These diurnal pH fluctuations can have a profound effect on productivity (Allan and Maguire, 1992). To correct this, lime is added, usually weekly, to stabilise the pH at an acceptable level. Liming was performed on the day of sampling in this investigation, resulting in an increase in pH and a subsequent decrease in hsp70 and Ub over the following few days (i.e. six to four days prior to sampling). The data shown here indicated that levels of hsp70 and Ub decreased one, two and three days after liming (i.e. six, five and four days prior to sampling) after which there was no significant correlation. This information has been reported back to the collaborating farm with the recommendation that liming of ponds be performed twice weekly to ensure pH levels remain stable.

Salinity (am) was negatively correlated to hsp70, Ub and GAV at all days. The highest correlation coefficients for hsp70 were at two and one day prior to sampling, for Ub at four days prior to sampling. This pattern is consistent with the fact that the SP response is a relatively quick response. However if the stress persists, the Ub response is activated as protein denatures.

Gill-associated virus presented with a different pattern with a decrease in correlation coefficients four and three days prior to sampling. The decrease in the correlation coefficient for GAV seen at four and three days prior to sampling is pertinent as studies, including this present study as shown by the histograms, have indicated that moderately infected prawn ponds show a pattern of initial increase in average GAV load followed by a period of low GAV load after mortalities (i.e. mortalities of the heavily infected prawns lowers the correlation coefficient). This pattern repeats until mass mortalities or harvest. It is proposed that the addition of an osmolyte such as betaine in the feed may have a significant impact on reducing the stress response associated with changes in salinity.

The bio-indicators and environmental factors were subject to principal component analysis in an attempt to better understand the underlying factors that explain the correlations and to reduce the data necessary for farmers to monitor. Five components were extracted; however only components one and five contained factors measuring different parameters. Days in pond, salinity, hsp70 and GAV all displayed strong factor loadings in component one. These four factors were grouped together due to the commonalities that they share and in addition to the results from the correlation analysis it evident that they are intrinsically related. Ub and dissolved oxygen (pm) displayed strong factor loadings in component five. It is possible that this component may be measuring survival/health, however further investigations are required to determine this. Under this scenario, environmental factors and bio-indicators were placed into components based on commonalities however interpretation of the results is difficult and no convincing conclusions could be reached from the analysis.

Furthermore the amount of factors to be measured by the farmer is not reduced, but increased by the inclusion of three bio-indicators so the analysis was not pursued further.

The study described here is the first to illustrate changes in the hsp70, Ub and GAV response and to compare these changes with environmental factors in a commercial setting. The results show a high level of cross-correlations between all factors with no simplified patterns. Using the data generated from the ELISA it is now possible to investigate the significance of these factors regarding production outcomes at the farm level.

CHAPTER 7

FARM INVESTIGATION OF CHANGES IN HSP70, Ub AND GAV EXPRESSION IN *PENAEUS MONODON* WITH RESPECT TO PRODUCTIVITY.

7.1 Introduction

In addition to disease issues, rapid or dramatic changes in environmental factors can also have a major impact on production. A plethora of studies have been undertaken to determine the optimal environmental conditions for production factors similar to those investigated here (Staples and Heales, 1991; Allan and Maguire, 1992; Bray *et al.*, 1994; Chen *et al.*, 1995; Hall and de la Vega, 2003). Indeed these types of studies have provided the foundation of penaeid culture methods (DPI&F, 2006). Few studies have been undertaken to determine correlations between these factors. Most of these investigations have been performed under controlled conditions and relatively few factors were analysed simultaneously (Hall and de la Vega, 2003). Consequently, the underlying factors that explain the correlations between environmental and production factors in the culture environment remain poorly understood and losses in production due to these complex interactions are still considerable.

A range of physiological components of crustacea including shock proteins, blood glucose, ions, pH, osmolarity, lactate, osmolytes such as betaine, number/population of haemocytes, clotting time and others have been investigated in an attempt to find a set of bio-indicators that can be used to predict production outcomes (Lewis *et al.*, 1999; Hall and de la Vega, 2003). However the usefulness of each bio-indictor appears to be limited to controlled experimental systems or requires further research.

Our investigations (Chapter 6) have shown that changes in the bio-indicators, particularly hsp70 and GAV, correlate with changes in environmental factors under culture conditions. The results have given an insight into the prawns physiological response to environmental stressors commonly associated with penaeid prawn farming. However, it is not known how environmental factors, hsp70, Ub and GAV correlate with production factors and if they can be used to predict production outcomes.

This chapter aims to:

1) Apply ELISA to investigate correlations between levels of the bio-indicators (hsp70, Ub and GAV) and production factors in farmed *P. monodon* during growout.
2) Use data reduction methods to identify underlying factors that explain the correlations between the bio-indicators, environmental and production factors
3) Determine if the bio-indicators and environmental factors can be used to predict specified production outcomes.

7.2 Materials and Methods

In addition to the bio-indicators and environmental factors (see Section 6.2); average body weight (g), biomass (kg/pond), yield (tonnes/hectare), survival (%) and food conversion ratio (calculated as kilograms of food required to produce one kilogram of prawns) were calculated weekly by the farmer for each pond until harvest.

7.2.1 ELISA

The ELISA results described in Chapter 6 for hsp70, Ub and GAV were used in this investigation.

7.2.2 Statistical analysis

All statistical analysis was performed using the computer program SPSS 12.0.1 for Windows. Correlation analysis was used to determine significant associations between the three bio-indicators (i.e. hsp70, Ub and GAV) and production factors. Principal component analysis was used to identify the underlying factors explaining the correlations.

Production factors were separated into categories so that each category accounted for approximately half the ponds (Table 7.1). Using these categories, discriminant analysis was used to develop a predictive model based on bio-indicators and environmental factors verses production outcomes. All results are based on average weekly data.

Table 7.1: Summary of the production factors and categories used in the discriminant analysis. t/ha = tonnes per hectare

PRODUCTION FACTOR	CATEGORY
Survival	< or > 70%
Yield	< or > 7 t/ha
Biomass	< or > 6300 kg/pond
Average body weight	< or > 25 g

7.3 Results

7.3.1 Correlation analysis

Correlation analysis was performed for each individual pond, bio-indicator and production factor over grouped sample weeks. There were significant correlations between all factors (Table 7.2). The greatest number of significant correlations was associated with hsp70 and GAV (28 each). Of interest were average body weight, biomass and yield with 16, 17 and 17 significant correlations respectively, most pertaining to hsp70 and GAV. Food conversion ratio and survival resulted in 11 and five correlations respectively, split between the bio-indicators. In general, correlations between the bio-indicators hsp70 and GAV and production factors were positive. Survival was the only exception with negative correlations for hsp70 and GAV. Ub showed the least number of significant correlations (10) with positive correlations for biomass, survival and yield and both positive and negative correlations for average body weight and food conversion ratio.

Table 7.2: Significant correlation coefficients for hsp70, Ub and GAV with production factors for individual ponds (grouped weekly). Coloured numbers represent individual ponds. Bold numbers represent: 1) number of significant correlations, 2) positive and negative average correlation coefficients (calculated separately). Shaded cells indicate factors showing seven ponds or more with significant correlation coefficients.

	hsp70	Ubiquitin	GAV
Average body weight (g) **16 significant correlations**	**0.715, 0.802, 0.846, 0.812, 0.703, 0.818, 0.749** **7, 0.892**	**-0.835, 0.697** **2, 0.697, -0.835**	**0.919, 0.817, 0.880, 0.934, 0.783, 0.929, 0.927** **7, 0.884**
Biomass (kg/pond) **17 significant correlations**	**0.773, 0.738, 0.908, 0.791, 0.839, 0.917, 0.714, 0.831, 0.747** **9, 0.806**	**0.800, 0.700** **2, 0.750**	**0.923, 0.838, 0.894, 0.735, 0.911, 0.920** **6, 0.870**
Survival (%) **5 significant correlations**	**-0.719** **1, -0.719**	**0.815** **1, 0.815**	**-0.842, -0.908, -0.806** **3, -0.852**
Yield (tonnes/hectare) **17 significant correlations**	**0.764, 0.746, 0.910, 0.841, 0.903, 0.716, 0.836, 0.747** **8, 0.808**	**0.802, 0.702** **2, 0.752**	**0.909, 0.824, 0.890, 0.869, 0.735, 0.910, 0.919** **7, 0.865**
Food conversion ratio **11 significant correlations**	**-0.744, 0.780, 0.819** **3, -0.744, 0.800**	**-0.727, -0.860, 0.796** **3, 0.794, -0.694,**	**0.883, 0.903, 0.918, 0.747, 0.792** **5, 0.849**
TOTAL Correlation coefficients	**28**	**10**	**28**

Pond 1 (n=8)
Pond 2 (n=9)
Pond 3 (n=9)
Pond 4 (n=8)
Pond 5 (n=6)
Pond 6 (n=6)
Pond 7 (n=8)
Pond 8 (n=6)
Pond 9 (n=11)
Pond 10 (n=9)
Pond 11 (n=9)

Each pond showed a unique correlation pattern (Table 7.3). Ponds 1 and 2 showed significant correlations between production factors and hsp70 only, Pond 7 with GAV only, Pond 4 with hsp70 and Ub, Ponds 6 and 11 with hsp70 and GAV and Ponds 5, 8, 9 and 10 with all three bio-indicators. Pond 3 did not have any significant correlations.

The information used to calculate biomass and yield was similar and as such the correlation coefficient for these factors was greater than 0.998 for all ponds. Therefore biomass was not included in any further analysis.

Table 7.3: Summary of production factors with significant correlations for hsp70, Ub, GAV within individual ponds. ABW = average body weight, FCR = food conversion ratio

	hsp 70	Ubiquitin	GAV
Pond1	biomass survival yield		
Pond 2	ABW biomass yield		
Pond 3			
Pond 4	ABW, biomass yield FCR	biomass yield FCR	
Pond 5	biomass	ABW FCR	ABW biomass yield FCR
Pond 6	ABW biomass yield		ABW biomass survival yield FCR
Pond 7			ABW biomass yield
Pond 8	ABW biomass yield	survival	ABW survival yield FCR
Pond 9	ABW biomass yield	ABW biomass yield	ABW biomass yield
Pond 10	ABW biomass yield FCR	FCR	ABW biomass yield FCR
Pond 11	ABW biomass yield FCR		ABW biomass survival yield FCR

7.3.2 Principal component analysis

Production factors and the bio-indicators were subjected to principal component analysis. The Bartlett's test of sphericity was significant at $p<0.001$ and Kaiser-Meyer-Olkin measure of sampling adequacy was 0.591 (Appendix 9) indicating that principal component analysis was a suitable. To determine the minimum number of components that adequately represents the relationships between the factors, Kaiser's criterion was used. A total of two components with an Eigenvalue of greater than 1.0 were generated (Appendix 9).

Using the Varimax rotation method, a component matrix was generated. The output produced two components incorporating all factors (Appendix 9). Component one accounts for 44.4% of the total variance, followed by 17.3% for component two with a total cumulative variance explained of 61.7% (Table 7.4).

To facilitate interpretation of the analysis, factor loadings of less than 0.5 will not be discussed. Component one consisted of five factors, of which days in pond, average body weight, yield, hsp70 and food conversion ratio all contributed high factor loadings of 0.950, 0.902, 0.858, 0.706 and 0.586 respectively (Table 7.5). Component two consisted of two factors being survival and Ub with factor loadings of 0.853 and 0.665 respectively. GAV did not load strongly with either component, however it did load into component one with a factor loading of 0.440 (Appendix 9).

Table 7.4: Total variance explained from weekly data using principal component analysis for hsp70, Ub, GAV and production factors from two components (n=88 for each factor).

Total Variance Explained

	Rotation Sums of Squared Loadings		
Component	Total	% of Variance	Cumulative %
1	3.550	44.379	44.379
2	1.383	17.293	61.672

Extraction Method: Principal Component Analysis.

Table 7.5: Summary of the component matrix generated using principal component analysis for hsp70, Ub, GAV and production factors. Output produced two components: Component one consisting of days in pond (DIP), average body weight (ABW), yield, hsp70, food conversion ratio (FCR) and Component two consisting of survival and Ub. Only correlations above 0.5 are shown.

	Component	
	1	2
DIP	0.950	
ABW	0.902	
yield	0.858	
hsp70	0.706	
FCR	0.586	
survival		0.853
Ub		0.665
GAV		

7.3.3 Discriminant analysis

Production and environmental factors and the bio-indicators were subjected to discriminant analysis (all ponds and sample weeks grouped). All factors presenting Wilks' Lambda values of >= 0.999 in tests of equality of group means were excluded from the analysis. The assumptions for discriminant analysis for survival, yield and average body weight are detailed in Appendices 10, 11 and 12. Food conversion ratio data did not meet the assumptions for discriminant analysis so was not included. Due to a correlation coefficient of greater than 0.9 for salinity am and pm across most ponds the latter was excluded from this analysis (Table 7.6).

Discriminant analysis of survival verses environmental factors and bio-indicators showed 94.3% of the original ponds were accurately classified and 89.8% of cross-validated ponds were correctly classified (Table 7.6). A total of 11 factors were used resulting in 80.6% of the variance explained. The standardized canonical discriminant function coefficients showed that three factors had coefficients greater than 0.5 (Table 7.7).

Table 7.6: Summary of the results of discriminant analysis of production factors verses bio-indicators and environmental factors (am and pm grouped) (n=88 for each factor) for all ponds over all weeks. In cross-validation, each pond is classified by the functions derived from all ponds other than that pond. Temp = water temperature, ABW = average body weight, DIP = days in pond, DO = dissolved oxygen.

	Survival	Yield	ABW
Category	< or > 70%	< or > 7 t/ha	< or > 25 g
Eigenvalue	1.858	0.614	0.285
Canonical correlation (shown as % variance explained)	0.806 (80.6)	0.617 (61.7)	0.471 (47.1)
Factors included in analysis (Wilks' Lambda values of < 0.999 in tests of equality of group means)	hsp70 GAV DIP temp am, pm salinity am pH am, pm DO am, pm secchi pm	hsp70 GAV Ub DIP pH pm DO am, pm secchi pm	GAV Ub temp am, pm salinity am pH am, pm secchi am, pm
Factors excluded from analysis	Ub secchi am salinity pm	temp am, pm salinity am, pm pH am secchi am	hsp70 DIP DO am, pm salinity pm
Ponds correctly classified	94.3%	76.1%	68.2%
Cross-validated ponds correctly classified	89.8%	68.2%	63.6%

Days in pond, hsp70 and salinity am had large coefficients of 2.421, -1.375 and 1.000 respectively.

For yield verses environmental factors and bio-indicators showed 76.1% of the original ponds were accurately classified and 68.2% of cross-validated ponds were correctly classified (Table 7.6). A total of eight factors were used resulting in 61.7% of the variance explained. The standardized canonical discriminant function coefficients showed that three factors had coefficients greater than 0.5 (Table 7.7). Days in pond showed the larges coefficient of 1.356, followed by hsp70 and DO am with coefficients of -0.932 and 0.669 respectively.

Although the results for average body weight showed greater than 60% of cross-validated ponds correctly classified, the Eigenvalue is very low (0.286) and the total variance explained is 47.2% (Table 7.6). These values were considered unacceptable and further analysis of this factor was not undertaken.

To determine if correlations between am and pm data had an effect on the output and to reduce the number of factors, discriminant analysis was also performed for survival and yield using am and pm data separately.
Results from am and pm data for survival verses environmental factors and bio-indicators showed 96.6% (am) and 93.2% (pm) of the original ponds were accurately classified and 87.5% (am and pm) of cross-validated ponds were correctly classified (Table 7.8). A total of seven (am) and eight (pm) factors were used resulting in 79% and 79.6% respectively of the variance explained. The standardized canonical discriminant function coefficients showed that the same three factors for both am and pm had coefficients greater than 0.5 (Table 7.9). Days in pond, hsp70 and salinity had coefficients of 2.320 (am), 2.258 (pm), -1.422 (am), -1.373 (pm) and 0.915 (am), 0.939 (pm) respectively.

Table 7.7: Standardized canonical discriminant function coefficients generated by discriminant analysis of survival and yield verses bio-indicators and environmental factors. Factors with coefficients greater than 0.5 in bold. Temp = water temperature, DIP = days in pond, DO = dissolved oxygen.

Survival		Yield	
< or > 70%		< or > 7 t/ha	
Factor	**Coefficients**	**Factor**	**Coefficients**
DIP	***2.421***	***DIP***	***1.356***
hsp70	***-1.375***	***hsp70***	***-0.932***
salinity am	***1.000***	***DO am***	***0.669***
secchi pm	0.367	Ub	0.441
pH pm	0.230	GAV	-0.441
GAV	-0.212	DO pm	0.440
pH am	0.173	Secchi pm	0.096
DO pm	0.145	pH pm	-0.072
DO am	0.128		
temp pm	0.110		
temp am	0.000		

Results from am and pm data for yield verses environmental factors and bio-indicators showed 71.6% (am) and 72.7% (pm) of the original ponds were accurately classified and 71.6% (am) and 69.3% (pm) of cross-validated ponds were correctly classified (Table 7.8). A total of five (am) and seven (pm) factors were used resulting in 58.4% and 54.3% respectively of the variance explained. The standardized canonical discriminant function coefficients showed that three (am) and two (pm) factors had coefficients greater than 0.5 (Table 7.9). Days in pond and hsp70 had coefficients of 1.435 (am), 1.226 (pm) and -1.027 (am), -1.017 (pm) respectively and dissolved oxygen had a coefficient of 0.598 (am) (Table 7.9).

Table 7.8: Summary of the results of discriminant analysis of production factors verses bio-indicators and environmental factors (am and pm separate) (n=88 for each factor) for all ponds over all weeks. In cross-validation, each pond is classified by the functions derived from all ponds other than that pond. Temp = water temperature, ABW = average body weight, DIP = days in pond, DO = dissolved oxygen.

	Survival		**Yield**	
Category	< or > 70%		< or > 7 t/ha	
	AM	PM	AM	PM
Eigenvalue	1.663	1.728	0.517	0.417
Canonical correlation (shown as % variance explained)	0.790 (79%)	0.796 (79.6%)	0.584 (58.4%)	0.543 (54.3%)
Factors included in analysis (Wilks' Lambda values of < 0.999 in tests of equality of group means)	hsp70 GAV DIP salinity temp pH DO	hsp70 GAV DIP salinity temp pH DO secchi	hsp70 GAV Ub DIP DO	hsp70 GAV Ub DIP pH DO secchi
Factors excluded from analysis	Ub secchi	Ub	salinity temp pH secchi	temp salinity
Ponds correctly classified	96.6%	93.2%	71.6%	72.7%
Cross-validated ponds correctly classified	87.5%	87.5%	71.6%	69.3%

Due to the similarities between am and pm results and to reduce the number of factors, pm data will not be included in further analyses.

Table 7.9: Standardized canonical discriminant function coefficients generated by discriminant analysis of survival and yield verses bio-indicators and environmental factors (am and pm). Factors with coefficients greater than 0.5 in bold. Temp = water temperature, DIP = days in pond, DO = dissolved oxygen.

Survival			Yield		
< or > 70%			< or > 7 t/ha		
Factor	Coefficients		Factor	Coefficients	
	AM	PM		AM	PM
DIP	***2.320***	***2.258***	***DIP***	***1.435***	***1.226***
hsp70	***-1.422***	***-1.373***	***hsp70***	***-1.027***	***-1.017***
salinity	***0.915***	***0.939***	***DO***	***0.598***	0.354
secchi		0.365	GAV	-0.362	-0.462
pH	0.325	0.339	Ub	0.360	0.456
GAV	-0.205	-0.236	pH		0.060
temp	0.065	0.063	secchi		-0.005
DO	0.036	0.129			

7.3.4 Predictive ability of discriminant analyses

To determine the predictive significance of these results, discriminant analysis was conducted for each production factor for all ponds (grouped) weekly including all weeks from one week prior to harvest to the first sample week, then two weeks prior to harvest to the first sample week, then three weeks prior to harvest etc to the first sample week. Analysis was stopped when the conditions for the analysis could no longer be met.

Due to the volume of data generated by this analysis, only the week showing greater than 75% of correctly classified ponds for survival and yield will be discussed (Appendix 13 and 14).

Analysis of survival verses environmental factors and bio-indicators including all weeks up to three weeks prior to harvest showed 96.4% of the original ponds were accurately classified and 89.3% of cross-validated ponds were correctly classified (Table 7.10).

A total of seven factors were used resulting in 83.2% of the variance explained. The standardized canonical discriminant function coefficients showed that two factors had coefficients greater than 0.5 (Table 7.11). Days in pond had the largest coefficient of 2.062 followed by hsp70 with a coefficient of -1.617.

Table 7.10: Summary of the results of discriminant analysis for survival and yield verses bio-indicators and environmental factors (am data only) (n=56 for each factor) using data including all weeks up to three weeks before harvest. In cross-validation, each pond is classified by the functions derived from all ponds other than that pond. Temp = water temperature, DIP = days in pond, DO = dissolved oxygen.

	Survival	**Yield**
Category	< or > 70%	< or > 7 t/ha
Eigenvalue	2.254	1.365
Canonical correlation (shown as % variance explained)	0.832 (83.2)	0.760 (76.0)
Factors included in analysis (Wilks' Lambda values of < 0.999 in tests of equality of group means)	hsp70 Ub DIP temp am salinity am DO am secchi am	hsp70 GAV Ub DIP pH am DO am temp am salinity am secchi am
Factors excluded from analysis	GAV pH am	
Ponds correctly classified	96.4%	89.3%
Cross-validated ponds correctly classified	89.3%	78.6%

Analysis of yield verses environmental factors and bio-indicators including all weeks up to three weeks prior to harvest showed 89.3% of the original ponds were accurately classified and 78.6% of cross-validated ponds were correctly classified (Table 7.10). A total of nine factors were used resulting in 76% of the variance explained. The standardized canonical discriminant function coefficients showed that three factors had coefficients greater than 0.5 (Table 7.11). Days in pond, hsp70 and salinity am showed large coefficients of 1.895, -1.211 and 0.860 respectively.

Table 7.11: Standardized canonical discriminant function coefficients generated by discriminant analysis of survival and yield verses bio-indicators and environmental factors (am data only) (n=56 for each factor) using data including all weeks up to three weeks before harvest. Factors with coefficients greater than 0.5 in bold. Temp = water temperature, DIP = days in pond, DO = dissolved oxygen.

Survival		**Yield**	
< or > 70%		< or > 7 t/ha	
Factor	**Coefficients**	**Factor**	**Coefficients**
DIP	***2.062***	***DIP***	***1.895***
hsp70	***-1.617***	***hsp70***	***-1.211***
salinity am	0.471	***salinity am***	***0.860***
DO am	-0.184	secchi am	0.463
secchi am	0.171	Ub	0.398
temp am	0.157	DO am	0.325
Ub	-0.005	GAV	0.108
		temp am	-0.093
		pH pm	-0.060

7.4 Discussion

The aim of this investigation was to determine if a subset of factors, most of which are routinely measured at the farm, could be used to predict production outcomes. To date, this type of research has not been attempted on the scale presented here or using data obtained under commercial conditions.

The study described here is also the first to illustrate changes in the hsp70, Ub and GAV response and to compare these changes with production factors in a commercial setting.

Our investigations demonstrate that the majority of ponds showed high correlations between the bio-indicators (hsp70 and GAV) and production factors particularly average body weight and yield. However individual ponds displayed unique correlation patterns. This was not unexpected due to the complex nature of the system investigated. Many other factors such as disease status (Allan and Maguire, 1992; Le Moullac and Haffner, 2000; Cheng *et al.*, 2003b), pond sediment type/condition (Smith, 1996; Peterson, 1999; Peterson *et al.*, 2001), type of phytoplankton (D'Souza and Kelly, 2000), liming and partial harvest regime to name a few, potentially have an effect on production factors so interpretation of the results is difficult. However, in general the correlation coefficients for these production factors were positive suggesting that with increases in average body weight and subsequent increases in yield over prolonged culture in this type of system, there was a correlated increase in hsp70 and GAV.

To better understand the underlying factors that explain the correlations, principal component analysis was performed. This was successful in identifying factors that were closely related by grouping them into two components. Days in ponds, average body weight, yield, hsp70 and to a lesser extent food conversion ratio all displayed strong positive factor loadings in component one. This is to be expected as the longer the prawns remain in the culture system, the higher the average body weight and the yield. Thus, the commonalities in component one between the first three factors are characterised by growth or production. Interestingly hsp70 is included in this component suggesting that it may be a useful indicator relating to production. However, it is suggested that factors routinely measured by the farmer such as average body weight and yield are significantly easier and more cost effective to measure than hsp70.

To date no publications investigating correlations between hsp70 and growth in invertebrates could be found. However, a number of studies have shown a correlation between hsp70 and tumour growth in human cancer cells (Rohde *et al.,* 2005) and human prostate cell lines (Wang *et al.,* 2004).

Survival and Ub displayed strong positive factor loadings in component two suggesting that this component may be measuring survival/health. However, due to the low correlations relating Ub to environmental and production factors, it is unlikely that it is a useful indicator of survival and the results should be interpreted with caution. Interestingly, GAV does not load strongly into either component, yet at a factor loading of greater than 0.4, it does load into component one suggesting that GAV may be associated with production factors rather than survival/health factors. Munro *et al.,* (2008) investigated the relationship between GAV and production of *P. monodon* at farm level using molecular techniques. Their results showed that ponds with no obvious GAV outbreak (i.e. no mass mortalities) but low production had a high increase in viral load and a moderate increase in prevalence but suffered chronic low level mortalities. It is suggested that changes in the levels of GAV described in the present investigation did not correlate strongly with survival as prawns heavily infected with GAV die and so are removed from sampling thereby truncating the linear correlation coefficients. In the case of low level chronic infection within the pond, these mortalities may go unnoticed.

Discriminant analysis was performed to determine 1) if the bio-indicators and environmental factors could be used to distinguish between specified production outcomes and 2) which factors contribute most to these outcomes. The first analysis using all the environmental data revealed that for this unique data set and specified category (< or > 25g), the bio-indicators and environmental factors could not be used to accurately classify ponds on the basis of average body weight. Considering the high correlations for average body weight, it is suggested that the mutually exclusive categories chosen were not appropriate for the data used.

To reduce the number of factors to be measured, analysis investigating survival and yield using am and pm data independently was undertaken. The results showed that using am data only reduced the number of factors required to correctly classify greater than 70% of the ponds for both survival and yield to seven and five respectively. Of these, the factors most important in distinguishing between the categories were days in pond, hsp70, salinity and to a lesser extent, dissolved oxygen. Changes in salinity have been shown to have a significant effect on both survival and growth of prawns under laboratory conditions (Kumlu *et al.*, 2000; Villarreal *et al.*, 2003; Tantulo and Fotedar, 2006; Joseph and Philip, 2007). The significance of the results presented here are that based on the length of time the prawns are in the pond (or prawn age) and changes in hsp70 and salinity, the statistical model described here can predict which ponds will be classified into which production category.

This however, is taking into account all data (am only) up to the day of harvest and it would be beneficial to the farmer if a prediction could be made as early in the crop as possible to optimise profit. This can be achieved by harvesting early if production was likely to falter thus reducing costly input resources (e.g. food, wages, and electricity). Using the model described above and the available information supplied by the farmers for the environmental factors, survival and yield, we have shown that it is possible to predict production outcome three weeks prior to harvest thus providing the farmer with management choices. To date only three studies could be found relevant to this present research and two of these studies were conducted by the same investigators. By means of an artificial neural network using survival, growth, price seasonality and labour force constraints, Yu *et al.,* (2006a; 2006b) developed a production schedule based on these factors and showed how the model could benefit the farmer by improving profitability. It must be noted however, that the data used in this investigation and the investigation described by Yu *et al.,* (2006a; 2006b) accounted for only a portion of the crop season; was limited to one commercial farm and so may not be robust enough across factors to enable generalized conclusions.

A more robust study was performed by Jackson and Wang (1998) who used the Gompertz growth model to predict *P. monodon* growth rates under varing environmental conditions. The environmnetal data used was collected by farm staff (similarily to this present study) from 48 ponds over 12 crops from a commercial prawn farm in north-eastern Queensland. The results showed a significant effect on growth rate due to days in pond, temperature and mortality. A significant effect was also noted for pH and DO however, due to the amount of missing data for these factors they were excluded from further analysis. The effects of salinity on growth rates were also considered to be very small however, the investigators did acknowledge that these environmental factors may have a greater effect on growth rates than could be determined in their study.

In the investigation described here, the environmental factors were measured twice daily; however only average weekly data was used. How variations in the change of these factors over a week, for example maximum or minimum weekly water temperatures, may affect the results is unknown. Furthermore, the categories used here were chosen so as to have approximately half the ponds in each category to allow analysis and did not differentiate between high and low productivity or profit. How changes in the categories may affect the results is unknown. Only by increasing the size of the data set using considerable resources could these questions be partially answered.

CHAPTER 8

USE OF CLASSIFICATION AND REGRESSION TREE (CART) ANALYSIS TO PREDICT PRODUCTION OUTCOMES IN CULTURED *PENAEUS MONODON* WITH RESPECT TO ENVIRONMENTAL FACTORS, HSP70, Ub AND GAV.

8.1 Introduction

Using discriminant analysis and the information supplied by the farmer for the environmental factors, survival and yield, we have shown that it is possible to predict production outcome three weeks prior to harvest thus providing the farmer with management choices (Chapter 7). However, the dependent production factors were separated into mutually exclusive categories to allow analysis, not to differentiate between high and low productivity. The results gained from this analysis showed what factors were important to production but did not provide a point at which their role changes with respect to outcome. This makes interpretation of the results difficult for those with limited statistical knowledge. A statistical method well suited to ecological data that provides more interpretable results is classification and regression tree (CART) analysis.

Classification and regression tree analysis, or binary recursive partitioning as it is technically known, is a non-parametric statistical method used to identify patterns or relationships in complex data sets that may sometimes be hidden (Clark and Pregibon, 1993; De'ath and Fabricius, 2000; Lewis, 2000). This method is used not only for exploration of data sets but also for the explanation, classification and prediction of patterns and processes within the data. By producing binary trees, the CART model often makes interpretation of the results easier when compared to other regression models.

Classification and regression tree analysis has been used in variety of applications since the mid 1980's and the research utilising this technology is increasing. To date, CART analysis has been successfully applied in ecology (De'ath and Fabricius, 2000), image processing (Gerger *et al.*, 2003; Herold *et al.*, 2003), taxonomy (Saraswati and Sabnis, 2006), meteorology (Burrows *et al.*, 1995), fire risk assessment (Amatulli *et al.*, 2006), water contamination (Gregor *et al.*, 2002), genomics (Davuluri *et al.*, 2000; Dyer *et al.*, 2007) and to predict clinical outcomes (Tafeit *et al.*, 2000; Hennessy and Rotz, 2004; Toschke *et al.*, 2005; Robledo *et al.*, 2007).

Classification and regression tree analysis consists of four basic steps with a set of associated rules. These steps have been explained in detail elsewhere (Clark and Pregibon, 1993; De'ath and Fabricius, 2000). However, in brief they consists of 1) the building or development of a binary tree by the selection of a splitting variable and recursively splitting of the data into two exclusive branches or nodes, 2) pruning to reduce the size of the tree until the optimum tree size is achieved and 3) assignment of a predictive value at each terminal branch.

Tree development begins with the production of a parent branch based on the dependant variable and all the independent variables. The CART software achieves this by using sums of squares about the group means (similar to least squares linear models) as the splitting criteria and a brute strength search checking all possible independent variables and their values. The variable that minimises the most prediction error within the data set is chosen as the parent branch. Then, the parent branch is given a value or class and is split into two child branches (De'ath and Fabricius, 2000; Lewis, 2000). For example, using survival as the dependent variable and a range of environmental factors as independent variables, the CART software may determine that temperature is the best variable with a split of < or > 37°C. Thus the parent branch is split into two mutually exclusive child branches. This process of splitting branches is repeated for each branch and continued recursively until no more splits are possible.

As mentioned above, the tree continues to grow until it cannot grow anymore resulting in a very complex tree. To determine the optimal tree size a method of ‘cross-validated error rate-complexity’ pruning is used. Basically, when graphed this method shows the relationship between tree complexity (represented by the number of terminal branches) and the cross-validated error rate for the dataset. The optimal size tree is usually the one that shows the lowest cross-validated error rate. Cross-validation can be undertaken in two ways depending on the size of the dataset. For large datasets, a random subset of data is selected and using only this data, a sequence of nested trees are built. Treating each tree individually, the response of the remaining data is predicted and the error from the prediction and the observed values are calculated. The tree showing the lowest predicted error is selected. For smaller data sets an *N*-fold cross-validation method is used. Briefly, the data is divided into *N* mutually exclusive subsets (usually 10) of approximately equal size. Each subset is then removed and a tree is grown from the remaining subsets. This tree is used to predict the responses for the excluded subset. The estimated error (i.e. sum of squared differences of the observations and predictions) for each subset is calculated and the sum over all the subsets is determined. This is repeated for each size tree and the tree with the lowest estimated error rate is chosen. To further reduce the complexity of the tree whilst conserving accuracy or predictability, the one standard error rule can be used. This means that the tree within one standard error from the tree yielding the lowest cross-validated error rate is chosen. For predictive models, the final or terminal branches will be assigned a predictive value relating to the dependant variable chosen.

This chapter aims to use CART analysis to develop individual decision trees for production factors to provide the farmer with predictive models for production outcomes.

8.2 Materials and Methods

Data obtained for the bio-indicators (Section 6.3.2), environmental factors (Section 6.2.2) and production factors (Section 7.2) were used in the analysis.

8.2.1 Statistical analysis

Classification and regression tree analysis was performed using R statistical software and the mvpart package. The 10-fold cross-validation method was used for all trees. Tree size (represented by the number of terminal branches) was selected by choosing the tree with the lowest cross-validated error rate and the highest R-squared value thus maximising the models predictive performance. To determine the optimal tree size and predictive performance two plots where created for each analysis: 1) a plot of cross-validated errors versus the complexity 2) a plot of the R-squared value verses the number of splits (i.e. tree size). Due to the loss of decision branches useful to the farmer, trees derived from the one standard error rule, will not be discussed.

Trees were developed for the prediction of average body weight (g), survival (%), food conversion ratio, yield (t/ha), biomass (kg/pond) and growth. Box plots of the terminal branches for each tree were produced showing the distribution around the mean (Appendices 15-20).

All results are based on the following independent variables: 1) average weekly data for the environmental factors (am and pm), 2) maximum and minimum weekly data for the environmental factors (max (am and pm) and min (am and pm)) and 3) weekly data for hsp70, Ub and GAV.

8.3 Results

8.3.1 CART analysis for average body weight

CART analysis was performed using average body weight as the dependant variable. The plot of cross-validated errors versus the complexity parameter shows the tree with the lowest cross-validated error has four terminal branches (Appendix 15). The plot of the R-squared value verses the number of splits shows that a tree size of 4 produces the highest R-squared and cross-validated R-squared values (R^2 = 0.73 and R^2cv = 0.55 respectively). These values equate to 73% and 55% of the variance explained (Figure 8.1). A box plot of the terminal branches indicates that each branch is normally distributed confirming that mean predictions at each terminal branch are appropriate (Appendix 15).

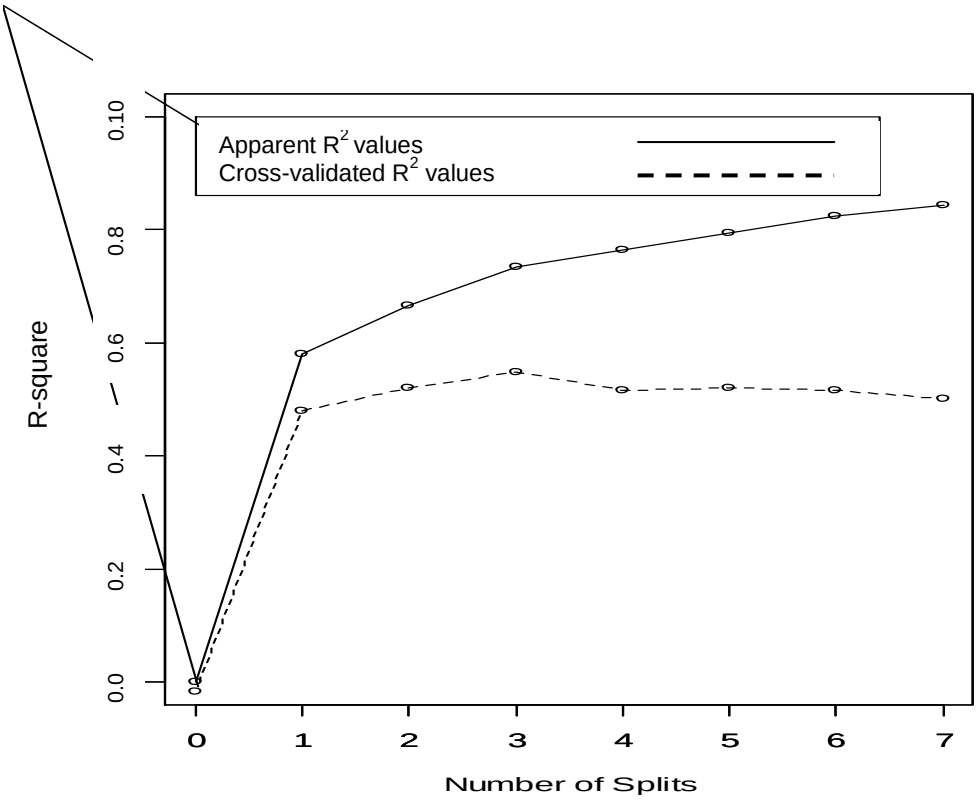


Figure 8.1: Plot of apparent and cross-validated R-squared values verses number of splits (tree size) for average body weight.

The tree produced shows an initial split of the parent branch on salinity.min.am (< or >= 38.15 ppt) (Figure 8.2). If the minimum am salinity over a week is >= 38.15 ppt the parent branch is split into two child branches on days in pond (< or >= 129.5) which is further split into two predictive values. If days in pond is < 129.5 then the average body weight of the prawns is predicted to be 13.4g. Alternatively if days in pond is >= 129.5 then the average body weight is predicted to be 17.1g. At a minimum am salinity over a week of < 38.15 ppt the parent branch is split into two child branches on pH.max.am (< or >= 7.755) which is further split into two predictive values. If the maximum am pH over a week is >= 7.755 then the average body weight is predicted to be 20.4g. The highest average body weight of 23.9g is predicted if the maximum am pH over a week is < 7.755.

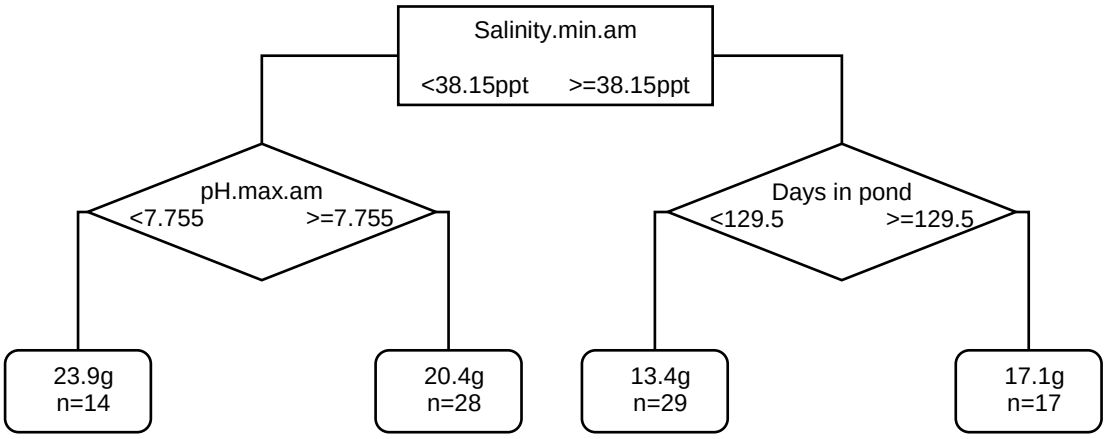


Figure 8.2: Tree yielding the minimum cross-validated error rate for predicting average body weight.

8.3.2 CART analysis for survival

CART analysis was performed using survival as the dependant variable. The plot of cross-validated errors versus the complexity parameter shows the tree with the lowest cross-validated error has five terminal branches (Appendix 16).

The plot of the R-squared value verses the number of splits (or tree size) shows that a tree size of five produces the highest R-squared and cross-validated R-squared values (R^2 = 0.592 and R^2cv = 0.31) (Figure 8.3). These values equate to 60% and 31% of the variance explained.

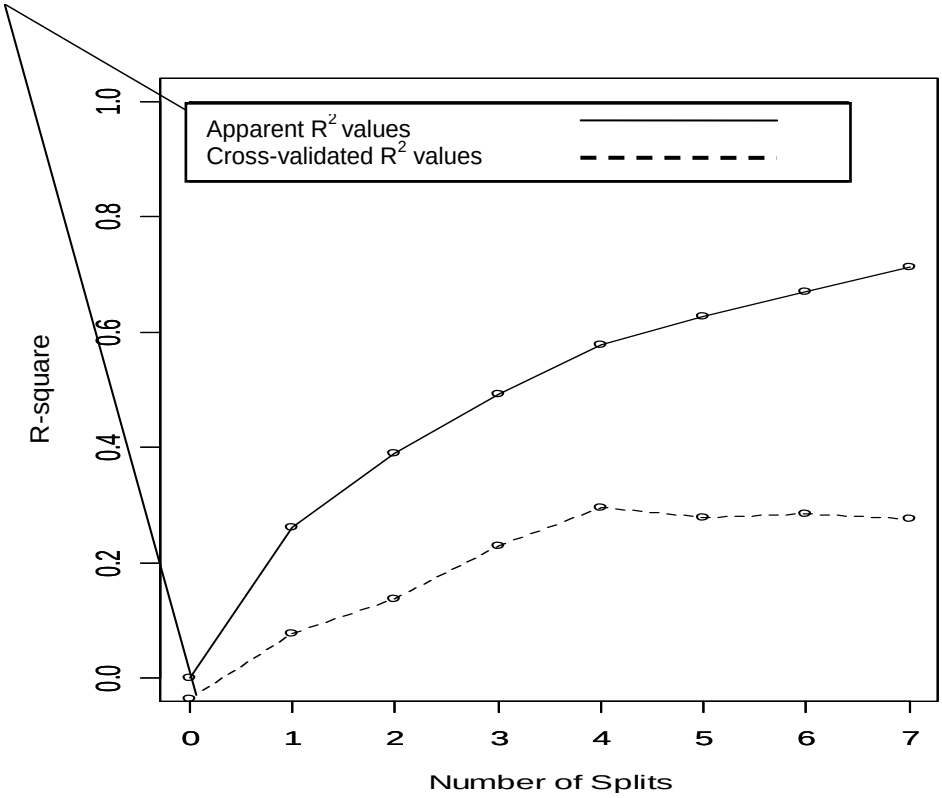


Figure 8.3: Plot of apparent and cross-validated R-squared values verses number of splits (tree size) for survival.

A box plot of the terminal branches indicates that each branch is normally distributed confirming that mean predictions at each terminal branch are appropriate (Appendix 16).

A tree is produced which shows an initial split of the parent branch on DO am (< or >= 4.44 ppm) (Figure 8.4). At a DO am level of < 4.44 ppm the parent branch is split into two child branches on salinity.min.pm (< or >= 32.8 ppt). If the minimum pm salinity over a week is < 32.8 ppt the predicted survival is 50.8%. At a minimum am salinity over a week of >= 32.8 ppt the branch is split into two more child branches on water temperature.min.pm (< or >= 28.75°C).

If the minimum pm water temperature over a week is >= 28.75°C the predicted survival is 57.4%, and if the minimum pm water temperature over a week is < 28.75°C the predicted survival is 73.3%.

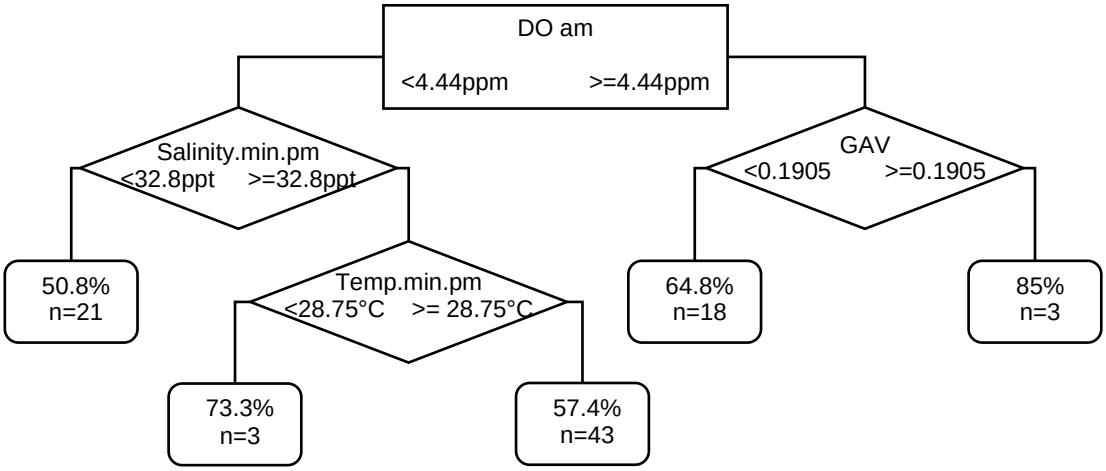


Figure 8.4: Tree yielding the minimum cross-validated error rate for predicting survival.

At a DO am level of >= 4.44 ppm the parent branch is split into two child branches on GAV (< or >= 0.1905) which is further split into two predictive values. At GAV < 0.1905 the survival is predicted to be 64.8%. The highest survival of 85% is predicted if GAV is >= 0.1905.

8.3.3 CART analysis for FCR

CART analysis was performed using FCR as the dependant variable. The plot of cross-validated errors versus the complexity parameter shows the tree with the lowest cross-validated error has four terminal branches (Appendix 17). The plot of the R-squared value verses the number of splits (or tree size) shows that a tree size of four produces the highest R-squared and cross-validated R-squared values (R^2 = 0.617 and R^2cv = 0.387) (Figure 8.5). These values equate to 62% and 38% of the variance explained.

A box plot of the terminal branches indicates that each branch is normally distributed confirming that mean predictions at each terminal branch is appropriate (Appendix 17).

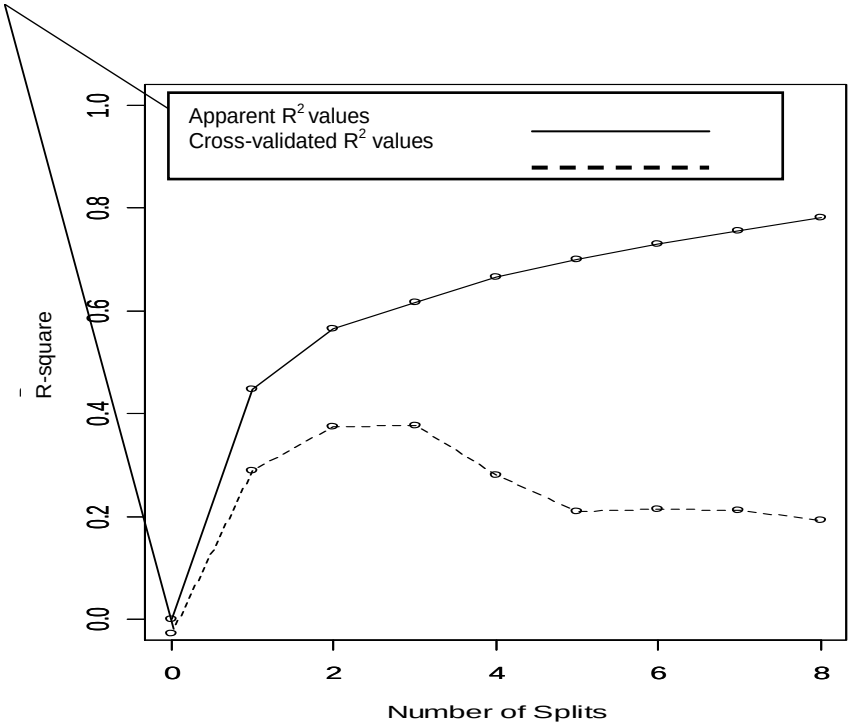


Figure 8.5: Plot of apparent and cross-validated R-squared values verses number of splits (tree size) for food conversion ratio.

A tree is produced which shows an initial split of the parent branch on days in pond (< or >= 113.5) (Figure 8.6). At >= 113.5 days in pond the parent branch is split into two child branches on days in pond (< or >= 136.5). If days in pond is < 136.5 the branch is split into two more child branches on pH am (< or >= 7.95). At a pH am of < 7.95 the predicted FCR is 1.64 and at a pH am >= 7.95 the predicted FCR is 1.8. If days in pond is >= 136.5 the predicted FCR is 1.77. At < 113.5 days in pond the predicted FCR is 1.43, representing the best result.

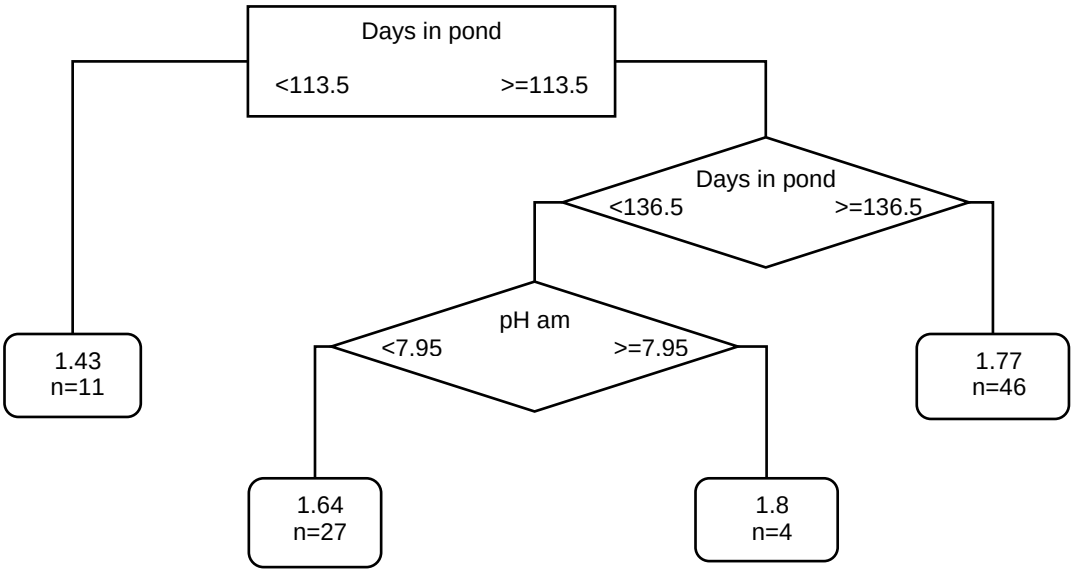


Figure 8.6: Tree yielding the minimum cross-validated error rate for predicting food conversion ratio.

8.3.4 CART analysis for yield

CART analysis was performed using yield as the dependant variable. The plot of cross-validated errors versus the complexity parameter shows the tree with the lowest cross-validated error has six terminal branches (Appendix 18). The plot of the R-squared value verses the number of splits (or tree size) shows that a tree size of six produces the highest R-squared and cross-validated R-squared values (R^2 = 0.8 and R^2cv = 0.412) (Figure 8.7). These values equate to 80% and 41% of the variance explained. A box plot of the terminal branches indicates that each branch is normally distributed confirming that mean predictions at each terminal branch are appropriate (Appendix 18).

A tree is produced which shows an initial split of the parent branch on days in pond (< or >= 145.5) (Figure 8.8). At < 145.5 days in pond the parent branch is split into two child branches on days in pond (< or >= 119). If days in pond is >= 119 the branch is split into two more child branches on GAV (< or >= 0.1905) which is further split into two predictive values of 4.7 and 6.9 t/ha (respectively). At < 119 days in pond the predicted yield is 3.11 t/ha.

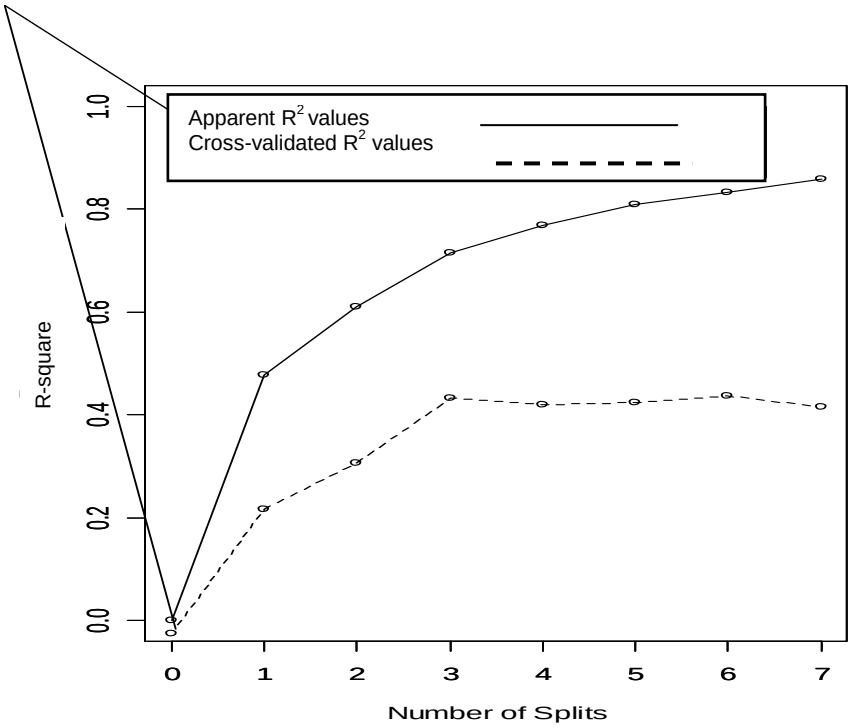


Figure 8.7: Plot of apparent and cross-validated R-squared values verses number of splits (tree size) for yield.

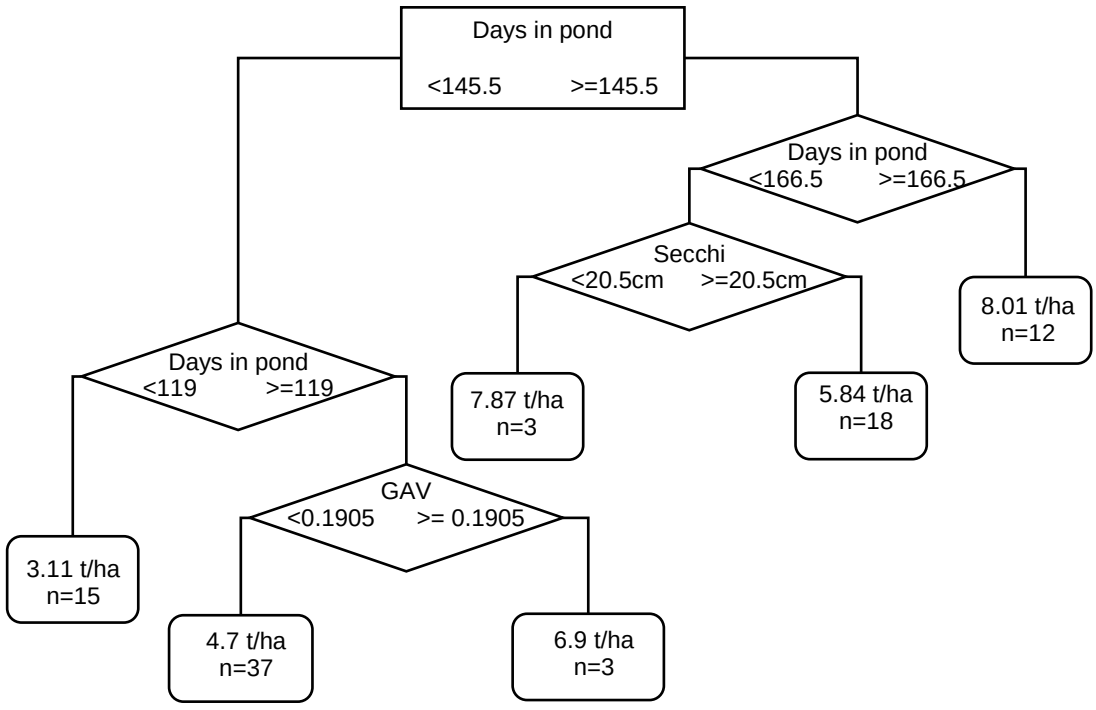


Figure 8.8: Tree yielding the minimum cross-validated error rate for predicting yield.

At >= 145.5 days in pond the parent branch is split into two child branches on days in pond (< or >= 166.5). If days in pond is <166.5 the branch is split into two more child branches on secchi pm (< or >= 20.5) which is further split into two predictive values of 5.84 and 7.87 t/ha (respectively). At >= 166.5 days in pond the predicted yield is 8.01 t/ha representing the highest outcome.

8.3.5 CART analysis for biomass

CART analysis was performed using biomass as the dependant variable. The plot of cross-validated errors versus the complexity parameter shows the tree with the lowest cross-validated error has five terminal branches (Appendix 19). The plot of the R-squared value verses the number of splits (or tree size) shows that a tree size of five produces the highest R-squared and cross-validated R-squared values (R^2 = 0.785 and R^2cv = 0.532) (Figure 8.9). These values equate to 79% and 53% of the variance explained. A box plot of the terminal branches indicates that each branch is normally distributed confirming that mean predictions at each terminal branch is appropriate (Appendix 19).

A tree is produced which shows an initial split of the parent branch on salinity am (< or >= 40.05 ppt) (Figure 8.10). At a salinity am of >= 40.05 ppt the parent branch is split into two child branches on salinity am (< or >= 41.45) which are then split into two predictive values of 2740 kg/pond and 3830 kg/pond (respectively). At a salinity am of < 40.05 ppt the parent branch is split into two child branches on days in pond (< or >= 168). If days in pond is <168 the branch is split into two more child branches on pH.min.pm (< or >= 7.605) which is further split into two predictive values of 4220 kg/pond and 5120 kg/pond (respectively). At >= 168 days in pond the predicted biomass is 6650 kg/pond representing the highest outcome.

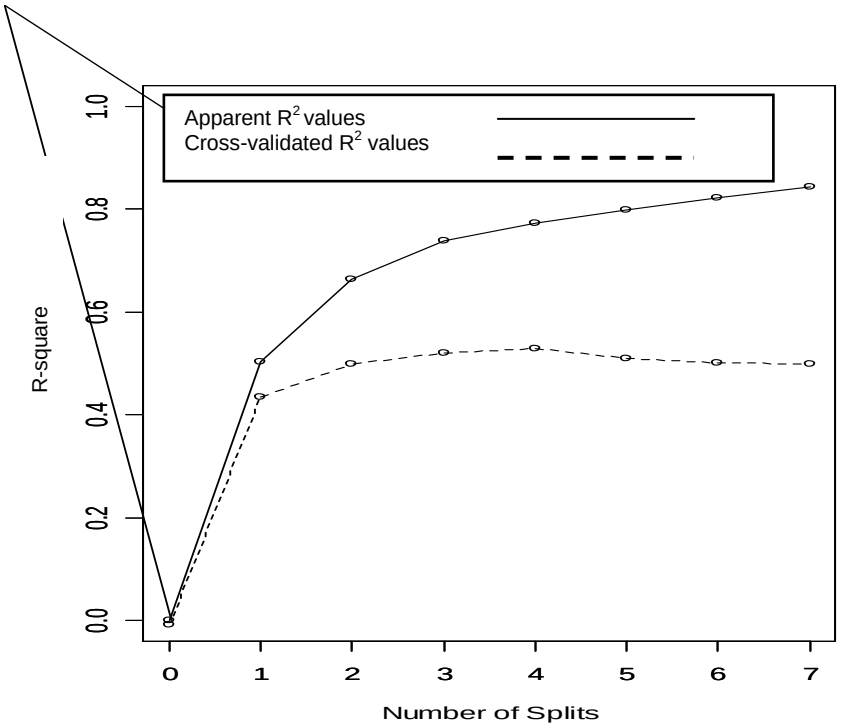


Figure 8.9: Plot of apparent and cross-validated R-squared values verses number of splits (tree size) for biomass.

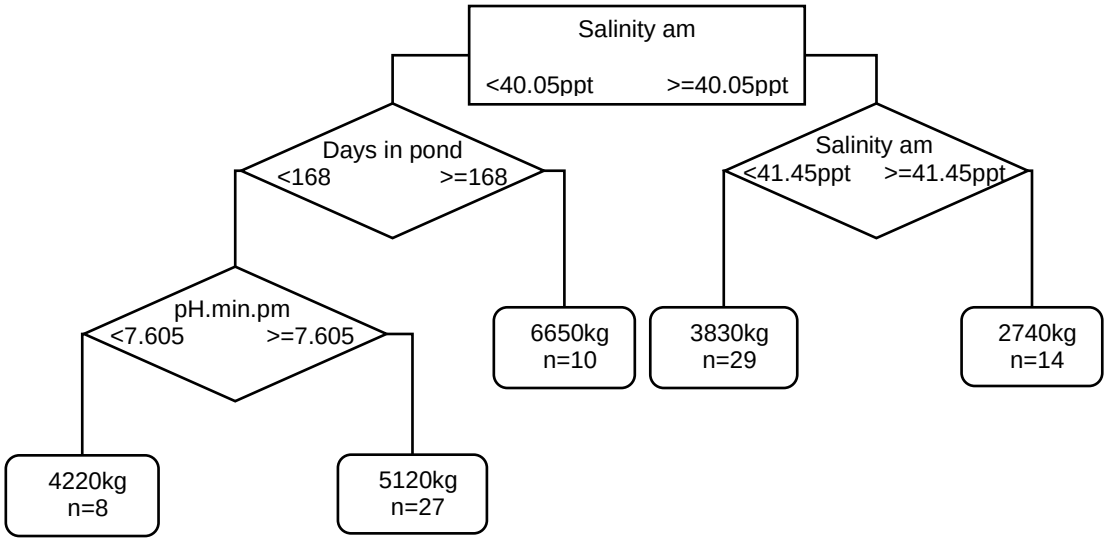


Figure 8.10: Tree yielding the minimum cross-validated error rate for predicting biomass.

8.3.6 CART analysis for growth

CART analysis for growth was unsuitable for producing a functional tree. The cross-validated error rate verses the complexity plot and the R-squared value verses tree size plot show an increase in error and decrease in predictive performance with an increase in tree size (Appendix 20).

8.4 Discussion

Classification and regression tree analysis is a powerful statistical method for analysing complex ecological data and providing predictive outcomes. This method has significant potential for the aquaculture as; 1) it is non-parametric so can deal with categorical variables as well as skewed data, 2) it can deal with missing variables, which often occurs with environmental data measured at the farm and 3) the trees produced are relatively simple for nonstatisticians to interpret. It does have a weakness in that it is a linear method and therefore data without linear components are not well analysed by the method.

In this investigation CART analysis was undertaken to develop individual decision trees for average body weight, survival, FCR, yield and biomass using environmental factors, hsp70, Ub and GAV as predictor variables. Each production factor had a unique tree however, there were some general similarities.

For average body weight and biomass the highest predicted outcome for average body weight was associated with minimum morning salinity less than 38 ppt and a maximum morning pH of less than 7.8 (23.9 g). Whilst for biomass, morning salinity less than 40 ppt, and greater than 168 days in pond produced the highest outcome (6650 kg/pond). In addition to the salinity and days in pond, a minimum afternoon pH of greater than 7.6 also produced a high biomass (5120 kg/pond).

The optimal salinity and pH for the culture of *P. monodon* is reported to be 15-25 ppt and 7.8 respectively (DPI&F, 2006). The results presented here are in accordance with these optimal levels and it is suggested that the use of CART analysis for predicting average body weight and biomass provides the farmer with an interpretable and accurate management tree.

For food conversion ratio and yield the initial branch was split based on days in pond. The best predicted FCR was associated with prawns that had been in ponds less than 113 days (1.43). No published literature could be found describing changes in the FCR due to prawn age or days in pond. The few investigations found regarding FCR were concerning the relationship with diet type/nutrition (Smith *et al.*, 2007; Venero *et al.*, 2007), stocking densities (Arnold *et al.*, 2006) and water quality (Hari *et al.*, 2006). In prawn aquaculture of the kind investigated here, a FCR of 2 or less is considered to be acceptable, therefore a predicted FCR of 1.8 at greater than 136 days in pond (the highest for this investigation) is within a suitable range. To determine the usefulness of this information regarding profit (i.e. food is very costly so a reduced FCR will increase profit), further studies using a greater sample number is required and additional components, such as feed type, should be included in the analysis.

Prawns that had been in ponds greater than 167 days produced the highest yield (8 t/ha) and those at less than 116 days in pond produced the lowest (3 t/ha). This is to be expected as the longer the prawns are in the pond with favourable conditions, the higher the yield is likely to be. The results from the CART analysis did show other factors in addition to the days in pond that have an effect on yield. Between 119 and 145 days in pond GAV plays a role. During this time if GAV is kept at an OD of above 0.19 then the predicted yield is 6.9 t/ha. This suggests that monitoring GAV over the latter stages of the crop may be beneficial in predicting when to harvest. For example, if the prawns have been in the pond for between 119 and 145 days and the OD for GAV is less than 0.19 the yield will be low (4.7 t/ha).

This anomaly probably represents the few ponds (6.9 t/ha) that continue to thrive before emergengy harvest or epizootics reduce production (4.7 t/ha) and the GAV loads drop as infected prawns die.

The tree produced for survival was split based on morning dissolved oxygen. The best predicted survival (85%) was associated with a morning DO of greater than 4.4 ppm and an OD for GAV of greater than 0.19. This again is probably due to the scenario outlined in the paragraph above. A survival of 73% was predicted if the morning DO was less than 4.4 ppm, the minimum afternoon salinity was greater than 33 ppt and the minimum afternoon water temperature was less than 29°C. The optimal DO, salinity and water temperature for the culture of *P. monodon* is reported to be 4 ppm, 15-25 ppt and 24-34°C respectively (DPI&F, 2006). However, these optima are rarely achieved or maintained on farms, particularly in north Queensland where salinities of greater than 40 ppt are common and monsoonal rains can elicit significant salinity and temperature drops in less than 24 hours. The results presented here show that even at relatively low DO and marine seawater salinities, if the water temperature is less than 29°C a survival rate of 73% is possible. It must be noted however that the predicted survivals of 85% and 73% were produced with case sizes of three so must be viewed with caution. However, the box plots were narrow suggesting a good fit of the model and an acceptable result.

Of interest was hsp70 which was not evident as an important component associated with production outcomes. This is probably due to hsp70 being a response variable itself rather than a predictor variable using environmental factors. However if larger trees were produced, the association between hsp70 and production outcomes would be revealed but larger trees did not optimse the variability explained.

In conclusion, the use of CART analysis resulted in the successful development of predictive decision trees for average body weight, survival, food conversion ratio, yield and biomass for the unique pond set investigated.

Gill-associated virus proved to be an important predictor for survival and yield. However, the results shown here suggest that high yield and survival is associated with high levels of GAV. At first glance these results may seem contradictory to the ecology of infectious disease. The nature of GAV is such that viral load typically increases with days in pond until those prawns with lethal levels die leaving only prawns with low to moderate viral load (Munro *et al.*, 2008). The level of GAV increases again until mortalities and the cycle continues until harvest. These cyclical low level mortalities effect survival and yield, therefore if prawns with high viral loads can survive those ponds will have high survival and yield. Thus, the results from the CART analysis showed that the few ponds (n=3) with prawns displaying relatively high GAV had high survival and yield. This truncated distribution (demonstrated in Figure 6.3) is difficult for commonly used statistical methods to deal with and this should be considered when interpreting these results. Munro and Owens (2007) investigated a range of tissue types to determine the most appropriate for the detection of GAV in *P. monodon.* Using an ELISA they showed that gill tissue produced significantly higher OD than pleopod. Thus, it is suggested that the use of GAV as a bio-indicator requires further research using gill tissue rather than pleopod.

The results presented here may be unique to the pond set investigated and broad generalisations outside the scope of this investigation will not be postulated. Nevertheless, it is suggested that using CART analysis on more farm data can provide farmers with an interpretable statistical means to predict production outcomes based on environmental factors routinely measured at the farm.

CHAPTER 9

GENERAL DISCUSSION AND CONCLUSIONS

Prawn aquaculture has been defined as being “a high-risk, capital-intensive industry that is site-specific and requires technical expertise” (Lobegeiger and Wingfield, 2007). To be successful in this industry, the farmer must have a firm knowledge of production finances/profit, marketing, risk factors and management. The industry is largely driven by profit which can be significantly affected by factors such as production outcomes. The quality of postlarvae from hatcheries (Munro *et al.*, 2008), disease and environmental factors (including water quality) all play an intrinsic role in production. However on prawn farms, many of these factors can not be controlled or can change rapidly resulting in decreased growth rates (Bray *et al.*, 1994) and at worst, significant economic losses due to resultant viral epizootics (Flegel and Pasharawipas, 1998). So it is important that a system be developed whereby farmers are able to maximise profit by identifying environmental and production risk factors and making informed management decisions.

While the use of bio-indictors to assess stress in cultured crustaceans is slowly increasing, most studies have been undertaken under controlled laboratory conditions (Hall and de la Vega, 2003) the results of which have little relevance to the culture environment. With the exception of assay development, it seems ineffectual to pursue further research in the laboratory unless it is tested at the farm level which is indeed what is reported here.

Shock protein 70, ubiquitin and gill-associated virus were chosen as bio-indicators in an attempt to determine if they could be used to predict production outcomes.

To investigate the response of these bio-indictors with respect to changes in environmental factors, a cost-effective assay able to investigate large numbers of samples quickly using non-lethal methods was required. The assay believed best suited to this undertaking was an ELISA and indeed ELISAs for a shock protein (Cimino *et al.*, 2002) and GAV (Munro and Owens, 2007) in *P. monodon* were developed. Using changes in salinity as a stressor, an ELISA for the detection of changes in the Ub response was developed and modifications to optimise the hsp70 ELISA were undertaken in the early component of this investigation.

Considering the number of factors impacting on the health and production of cultured prawns, it was not known if the ELISAs would be sensitive enough to detect changes in the hsp70 and Ub responses under farm conditions. So to 1) determine the utility of the ELISAs with respect to farm conditions; 2) determine the appropriate sample number and 3) optimise sampling techniques and preparation, prawns were sampled from two ponds at a farm. One pond was considered to be ‘healthy’ and high yield and the second was considered ‘moribund’ and low yield. The results showed that prawns from the high yield pond had a significantly lower hsp70 and Ub response when compared to prawns from the low yield pond. The significance of these results are that the ELISAs can detect changes in the hsp70 and Ub response at farm level between ponds experiencing opposing production outcomes indicating that a large scale farm investigation was feasible. In addition, it was shown that the transportation of prawns also resulted in significant increases in hsp70 and Ub suggesting a possibility for the use of these bio-indicators in the live crustacean transport industry. Further research using conditions more indicative of the commercial transport industry are required to fully elucidate this.

A farm trial was commenced to answer the question ‘Can changes in hsp70, Ub, GAV and environmental factors be used to predict production outcomes?’

A large prawn farm in northern Queensland agreed to participate in the trial and supplied all the environmental and production data for the trial period and in addition to hsp70 and Ub, GAV was included as an indicator of disease. The data generated by this investigation was enormous with a high number of non-orthogonal correlations across all factors. The first step was to determine patterns across ponds between the bio-indicators. This was very difficult as each pond showed a unique stress pattern (Appendix 6).

Correlation analysis was performed on the bio-indicators verses the environmental and production factors. A high number of correlations across all factors was shown with each pond presenting unique correlation patterns that made interpretation difficult. Of particular interest was days in pond, average body weight and yield with the majority of ponds analysed having highly significant correlations with hsp70 and GAV (Figures 6.2 and 7.2). This provides strong evidence that prolonged culture in this type of system results in increased levels of stress and viral load.

In an attempt to determine the biological significance of these interactions, correlation analysis was conducted for each bio-indicator and environmental factor from six days prior to sampling up to and including the day of sampling. Of particular interest was pH and salinity. The results presented here for pH indicated that levels of hsp70 and Ub decreased one, two and three days following liming (i.e. six, five and four days prior to sampling) after which there was no significant correlation. It was determined that this result was associated with the farm’s liming regime. These findings were reported to the collaborating farm with the recommendation that liming of ponds be performed twice weekly to ensure pH levels remain stable. The farm indeed changed their liming regime as suggested, however the impact of this change is currently unknown and further investigations are required.

Salinity was also of interest as it was correlated to all bio-indicators at all days. This finding is of importance as it shows that changes in salinity at farm level affect the prawn's physiology significantly and may consequently affect growth, health and production. As it is almost impossible for the farmer to control the salinity in ponds particularly during the monsoon season, it is proposed that the addition of an osmolyte such as betaine in the feed may have a significant impact on reducing the stress associated with changes in salinity.

The bio-indicators, environmental and production factors were subject to principal component analysis in an attempt to better understand the underlying factors that explain the correlations and to reduce the data necessary for farmers to monitor.

For the environmental factors, days in pond, salinity, hsp70 and GAV all displayed strong factor loadings into one component. These four factors were grouped together due to the commonalities, and in addition to the results from the correlation analysis, it evident that they are intrinsically related.
For the production factors, days in pond, average body weight, yield, hsp70 and food conversion ratio all displayed strong positive factor loading into one component. The commonalities among these factors are characterised by growth or production suggesting that hsp70 may be a useful indicator relating to production. However, it is more practical for the farmer to use factors routinely measured, such as average body weight and yield, than to measure changes in the hsp70 response. Under this scenario, bio-indicators, environmental and production factors were placed into components based on commonalities. However, the interpretation of the results is complex and no substantive conclusions could be reached from the analysis. Furthermore the amount of factors needed to be measured by the farmer was not reduced, but increased by the inclusion of three bio-indicators, so the analysis was not pursued further.

The ultimate aim of this investigation was to determine if the bio-indicators and environmental factors could be used to predict production outcomes thus providing the farmer with a means of ultimately improving profitability. Using discriminant analysis and the available environmental and production information supplied by the farm we have shown that it is possible to accurately predict which ponds will produce < or > 70% survival and < or > 7 tonnes per hectare (yield) three weeks prior to harvest using morning environmental data only and hsp70. Profitability can be improved by harvesting early if production was likely to falter thus reducing costly input resources (e.g. food, wages, and electricity). This analysis is however, complex and difficult to interpret without a thorough statistical knowledge. The results are also based on set production categories (e.g. < or > 7 t/ha) that may not represent an appropriate division of the data to achieve the most accurate predictive outcome.

The use of CART analysis resulted in the successful development of predictive decision trees for average body weight, survival, food conversion ratio, yield and biomass for the unique data set investigated. With this complex data set, trees were grown on specific values from environmental measurements. Of the factors measured, salinity and days in pond were important predictors for average body weight, biomass, FCR and yield and to a lesser extent, pH and GAV were also involved. Dissolved oxygen, GAV, salinity and water temperature were important in predicting survival. Although the optimal levels for environmental and water quality factors are well known for *P. monodon* it is very difficult for factors, particularly salinity and temperature, to be controlled at farm level. So the development of a tree showing a discrete number for the factor that has the most impact on production outcome is crucial. With this information the farmer has a prediction of the outcome and so can make an informed management decision. It is suggested that the use of an osmolyte in the feed may prove beneficial in reducing the stress response of the prawn to salinity changes.

Of interest was hsp70 which was not evident as an important component associated with production outcomes by CART analysis as indicated by discriminant analysis (Chapter 7). This is probably due to hsp70 being a response variable itself rather than a predictor variable with regard to environmental data. However if larger trees were produced, the association between hsp70 and production outcomes would be revealed though larger trees did not optimse the variability explained.

Using principal component analysis days in pond, salinity, hsp70 and GAV were grouped into one component due to commonalities so it is suggested that days in pond and salinity may be stronger predictors of production outcome than hsp70. If these factors were removed from the analysis it is suggested that hsp70 may replace them. This requires further research with a larger data set.

So can hsp70, Ub and GAV be used for predicting production outcomes? This study has shown that the transportation of prawns resulted in significant increases in hsp70 and Ub suggesting a possibility for the use of these bio-indicators in the live transport industry and changes in the hsp70 response was useful for indicating stress associated with fluctuations in pH due to the farms liming regime. However, Ub was not highly correlated to any of the environmental or production factors, did not load highly in principal component or discriminant analysis and did not show as a predictor in CART analysis. This may be due to the cyclical nature of the Ub monomer (Section 2.7). It is suggested that measuring changes in the polyubiquitin response may prove to be more useful.

The utility of hsp70 as a predictive bio-indicator of production outcome appears to be limited due to its high correlation with days in pond and salinity as it was not shown as a predictor by CART analysis. Gill-associated virus on the other hand was shown to be a predictor for survival and yield and may be a useful bio-indicator for these production factors.

In conclusion, it must be noted that the investigation discussed here is of an exploratory nature and there are a number of limitations on the interpretation of the results. The data used here accounted for only a portion of the total cropping season; was limited to one commercial farm, did not take into consideration other factors (i.e. disease status, pond sediment type/condition, type of phytoplankton, and partial harvest regime) and so may not be robust enough across factors to enable a generalised conclusion. The environmental factors were measured twice daily, however only average weekly data was used in most analyses except the CART analysis. Furthermore, the categories in the discriminant analysis were chosen so as to have approximately half the ponds in each category to allow analysis not to differentiate between high and low productivity or profit. How changes in the categories may affect the results is unknown and only a larger data set can answer those questions.

Using CART analysis it has been possible to investigate the complex ecology of *P. monodon* culture with respect to production outcomes, and predictive environmental factors have been determined using actual farm records. The results from this analysis are exciting and it is proposed that this method has significant potential in the aquaculture industry. Many farms have several cropping seasons of environmental and production records. It is recommended that further research using all the available data, possibly on an individual pond basis, be used in CART analysis. It is proposed that the results gained with the increase in sample size and additional relevant factors will provide accurate, interpretable and industry based prediction trees enabling the farmer to increase productivity and profitability.

The significant outcomes of this research are:

An ELISA suitable for the detection and quantification of ubiquitin was developed and the previously developed ELISAs for the detection of hsp70 and GAV in *P. monodon* were optimised. Using these assays it is now possible to test a large number of samples quickly, easily and at relatively low cost. It also provides a means to screen farmed and wild populations for changes in hsp70 and Ub levels and may serve as a basis for possible health management strategies particulary relating to transport stress.

Correlation and principal component analyses of the bio-indicators, environmental and production factors showed complex interactions between all factors. Monitoring changes in the hsp70 response associated with pH was effective at providing the farmer with useful information relating to the farm's liming regime.

Using discriminant analysis a model was produced making it possible to predict ponds by the categories of < or > 70% survival and < or > 7 tonnes per hectare (yield) three weeks prior to harvest using relatively fewer factors than currently being measured by the farmer thus providing the farmer with management choices.

The use of CART analysis resulted in the successful development of predictive decision trees for average body weight, survival, food conversion ratio, yield and biomass for the unique data set investigated. Of the factors measured, salinity and days in pond were important predictors for average body weight, biomass, FCR and yield and to a lesser extent, pH and GAV were also involved. Dissolved oxygen, GAV, salinity and water temperature were important in predicting survival. Using CART analysis it has been possible to investigate the complex ecology of *P. monodon* culture with respect to production outcomes, and predictive environmental factors have been determined using actual farm records.

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APPENDIX 1: Major stress protein families of multicellular eukaryotes and their function and cellular location (adapted from Feder and Hofmann, 1999; http://www.tulane.edu/~biochem/med/sp.htm and Parsell and Lindquist, 1993).

STRESS PROTEIN	COHORTS	SIZE RANGE (KDa)	LOCATION	STRUCTURE	FUNCTION
100 (Clp Proteins)	?	75-100	Class I Cytoplasm Class II Plastid		ATPase; dissociates aggregates, facilitates proteolysis; essential in yeast for acquired thermotolerance
90 (HtpG)	Immunophilins hsp70, others	80-90	Cytoplasm, Nucleus, Chloroplast, Mitochondria Endoplasmic retuculum		Stabilises proteins prior to complete folding or activation; forms stable complexes with inactive glucocorticoid receptor and other transcription factors; most abundant non-ribosomal protein (cytosolic version); most abundant protein in endoplasmic reticulum (ER version)
70 (Dna K)	hsp40	60-90	Cytoplasm, Mitochondria Endoplasmic Retuculum Plastid	Consist of two interdependent domains, N-terminal contains a high affinity ATP-binding site, C-terminal is responsible for binding substrate proteins and polypeptides	ATPase; stabilises proteins prior to complete folding, transport across membranes and proteolysis; found associated with misfolded and unassembled proteins
60 Chaperonins	hsp10	50-70	Type I Mitochondria Chloroplast Type II Cytoplasm	Most share a common oligomeric structure, a double doughnut of two seven-membered rings, mitochondrial sp60 forms a single seven-membered ring	ATPase; promotes efficient folding; only in mitochondria and chloroplast of eukaryotes; distant homolog in cytosol is specialised for folding actin and tubulin; *a.k.a., chaperonin*
40 (Dna J or J Domain)		17-277 (average 40)	Cytoplasm, Nucleus, Chloroplast, Mitochondria Endoplasmic retuculum Peroxisome Secretory pathway Plasmid membrane		
Small stress proteins	?	12-30	Class I Cytoplasm Class II Cytoplasm Class III Chloroplast Class IV Encomembrane	Has a conserved hydrophobic sequence element in the C-terminal region, a distinct oligomeric structure that sediments on sucrose gradients	Blocks aggregation; involved in regulation of actin assembly/disassembly; most abundant in *Artemia* cysts.
Heat Shock Factor (HSF)		28-55	Class A and C Cytoplasm, Nucleus Class B Nucleus		

APPENDIX 2: Major stress protein families and their expression in aquatic animals during times of environmental stress.

STRESS PROTEIN	ORGANISM	METHOD OF DETECTION	STRESS INVESTIGATED		REFERENCE
26kDa	*Artemia* cysts	Western blotting	Anoxia	*In vivo*	Clegg *et al.*, (2000a)
	Poeciliopsis lucida (desert fish)	Southern and Northern Blotting, Flourography	Heat	*In vitro*	Norris *et al.*, (1997b)
STRESS PROTEIN	**ORGANISM**	**METHOD OF DETECTION**	**STRESS INVESTIGATED**		**REFERENCE**
60kDa	*Poeciliopsis* sp. (desert fish)	Western blotting	Heat	*In vivo* *In vitro*	White *et al.*, (1994)
	Procambarus clarkii (crayfish glia)	Western blotting	Heat, sodium arsenite	*In vitro*	Rochelle *et al.*, (1991)
STRESS PROTEIN	**ORGANISM**	**METHOD OF DETECTION**	**STRESS INVESTIGATED**		**REFERENCE**
70kDa	*Suberites domuncula Olivi (sponge)*	Western blotting	PCB	*In vivo*	Schroder *et al.*, (1999)
	Cassostrea virginica (oyster) haemocytes	Western blotting	Heat and cold	*In vitro*	Tirard *et al.*, (1995)
	Oyster haemocytes	Radioactively labelled proteins	Hyper/hypoosmotic	*In vitro*	Tirard *et al.*, (1996)
	Cassostrea gigas (oyster) gill	Western blotting	Heat	*In vitro*	Clegg *et al.*, (1998)
	Mytilus edulis L. (bivalve) gill	Western blotting	Heat	*In vivo*	Chapple *et al.*, (1998)
	Mytilus trossulus (mussel) gill	Western blotting	Heat	*In vivo*	Hofmann and Somero (1995)
	Mytilus galloprovincialis (haemocytes)	Western Blotting	Magnetic field	*In vivo*	Malagoli *et al.*, (2004)
	Tegula sp (marine snail)	Fluorography	Heat	*In vitro*	Tomanek and Somero (1999; 2000)

APPENDIX 2: Major stress protein families and their expression in aquatic animals during times of environmental stress.

STRESS PROTEIN	ORGANISM	METHOD OF DETECTION	STRESS INVESTIGATED		REFERENCE
70kDa	Crayfish CNS	Western blotting	Heat	*In vitro*	Rochelle *et al.*, (1991)
		Radioactively labelled proteins	Crush injury	*In vivo*	Xue and Grossfeld (1993)
	Procambarus clarkii (crayfish) glia	Western blotting	Heat	*In vitro* *In vivo*	Sheller *et al.*, (1998)
	Cherax quadricarinatus (crayfish) pleopod	ELISA, Western blotting	Heat	*In vivo*	Cimino *et al.*, (2002)
	Penaeus monodon (prawn) pleopod	ELISA, Western blotting	Heat and hypoosmotic	*In vivo*	Cimino *et al.*, (2002)
	Rimicaris exoculata (shrimp)	Western Blotting	Heat	*In vivo*	Ravaux *et al.*, (2003)
	Artemia (crustacean)	Western blotting	Heat	*In vivo*	Clegg *et al.*, (2000b), Frankenberg *et al.*, (2000)
		Western blotting	Heat, osmotic, DDT and lindane	*In vivo*	Dunlap and Matsumura (1997)
	Bullfrog tadpole	Western blotting	PCB and toxaphene	*In vivo*	Dunlap and Matsumura (1997)
	Poeciliopsis sp. (desert fish)		Heat		
		Western blotting		*In vivo*	White *et al.*, (1994)
			Heat	*In vitro*	
	Fathead minnow	Southern and Northern blotting		*In vitro*	Norris *et al.*, (1997b)
			Heat		
	Chinook salmon	Western blotting		*In vivo*	Dunlap and Matsumura (1997)
	Salmo salar (salmon) gill and liver	Western Blotting	Heat		
		Fluorography	Heat and hyperosmotic	*In vivo* *In vivo*	Dunlap and Matsumura (1997) DuBeau *et al.*, (1998)
	Flounder renal tubule		Heat		
		Western blotting		*In vitro*	Brown *et al.*, (1992)

APPENDIX 2: Major stress protein families and their expression in aquatic animals during times of environmental stress.

STRESS PROTEIN	ORGANISM	METHOD OF DETECTION	STRESS INVESTIGATED		REFERENCE
70kDa	*Sparus sarba* (teleost) hepatic tissue	Northern blotting and PCR	Hormonal effects	*In vivo*	Deane *et al.*, (1999)
	Rainbow trout epidermal cultures	Immunocytochemical Polyclonal Rabbit hsp70	2,4-dichloroaniline	*In vitro*	Kilemade and Mothersill (2001)

STRESS PROTEIN	ORGANISM	METHOD OF DETECTION	STRESS INVESTIGATED		REFERENCE
90 kDa	*Dendronephthya klunzingeri* (octocoral)	Fluorography	Heat	*In vitro*	Tomanek and Somero (1999; 2000)
	Tegula sp. (marine snail gill)	Northern and Western blotting	Heat, cadmium and both combined	*In vivo*	Wiens *et al.* (2000)
	Mytilus galloprovincialis (haemocytes)	Western Blotting	Magnetic field	*In vivo*	Malagoli *et al.*, (2004)
	Lobster midgut gland	Northern blotting & RT-PCR	Heat & hormonal	*In vivo*	Chang *et al.* (1999)
	Crayfish (CNS)	Radioactively labelled proteins	Crush injury, heat sodium arsenite	*In vivo*	Xue and Grossfeld (1993) Rochelle *et al.* (1991)
	Poeciliopsis lucida (desert fish)	Southern and Northern blotting	Heat	*In vitro*	Norris *et al.* (1997b)
	Flounder (renal tubule)	Western blotting	Heat	*In vitro*	Brown *et al.* (1992)

APPENDIX 3: Aerial view of penaeid culture using a semi-intensive system. Individual ponds are usually 1.5 m deep, one hectare, earthen ponds with several paddle wheel aerators or water movers. Prawns are cultured using ‘green water’ methods (i.e. phytoplankton growth is encouraged). The different colour between ponds is often due to different phytoplankton species.

APPENDIX 4: ELISA protocol for the detection of hsp70 and ubiquitin in *Penaeus monodon.*

1. Dilute samples in PBS to a final concentration of 30μg total protein per 50μl (i.e.0.6μg/μl).
2. Place diluted samples into a 90°C water bath for five mins then cool.
3. Mix all samples 1:1 with carbonate/bicarbonate buffer (TropBio, Townsville) (i.e. 50μl sample plus 50μl buffer).
4. Add 100μl of diluted sample and controls to each well of a 96 well, flat bottom microtitre plate.
5. Cover plate with a single sheet of paper towel and place into a 37°C incubator overnight. Ensure all wells are completely dry before proceeding.
6. Add 125 μL of blocking buffer (TropBio, Townsville) to each well and incubate for one hour at 27°C.
7. Flick off excess blocking buffer and dry plate for one hour at 37°C.
8. Dilute hsp70 and Ub MAbs in TEN-TC buffer (TropBio, Townsville) to a dilution of 1/5000 and 1/2000 respectively. Add 100 μL of diluted MAb to each well and incubate for one hour at 27°C.
9. Flick off excess MAb and rinse wells twice with TEN-TW buffer (TropBio, Townsville).
10. Dilute goat anti-mouse HRPO conjugated antibody in TEN-TC buffer to a dilution of 1/1000. Add 100 μL of diluted MAb conjugate to each well and incubate for one hour at 27°C.
11. Flick off excess MAb solution and rinse all wells six times with TEN-TW buffer.
12. Add 100 μL of ABTS to each well and incubate in the dark at room temperature for six hours.
13. Measure optical density (OD) at a dual absorbance of 414nm and 492nm.

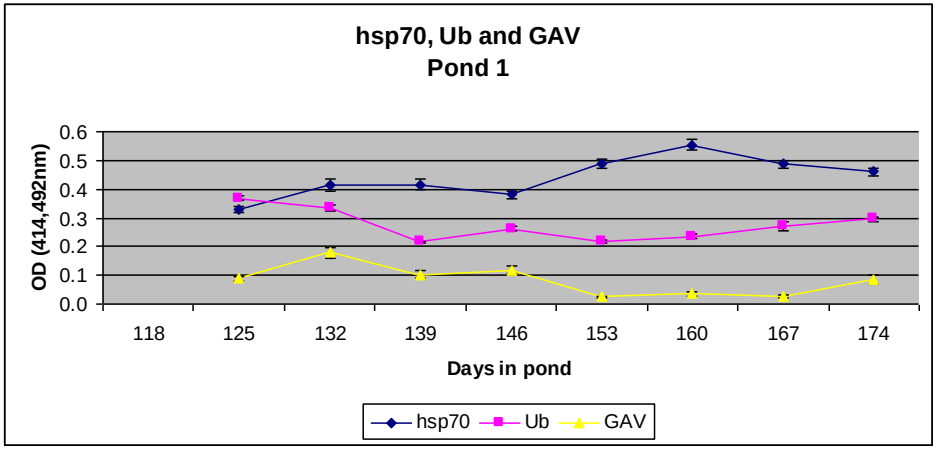
NOTE: Positive control used was pooled leg from hypoosmotically stressed *P. monodon,* negative control was FBS. Blank control was PBS.

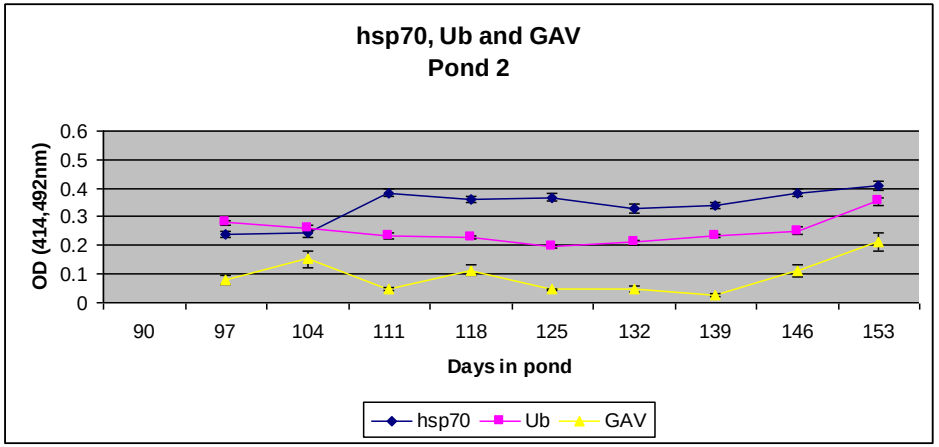
APPENDIX 5: ELISA protocol for the detection of gill-associated virus in *Penaeus monodon*.

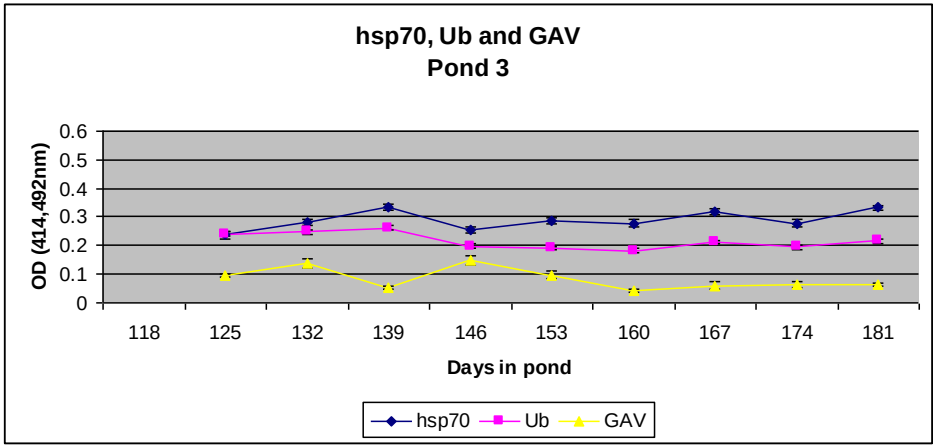
1. Bring diluents, buffers and ABTS to room temperature before use.
2. Dilute PAb 1/80 in carbonate buffer (TropBio, Townsville) and add 50μl to each well of a 96 well, flat bottom microtitre plate.
3. Cover plates to reduce evaporation and incubate overnight at room temperature.
4. Flick off excess PAb and wash once with TEN-TW buffer (TropBio, Townsville).
5. Add 100μl blocking buffer (TropBio, Townsville) to each well and incubate at 27°C for two hours.
6. Flick off blocking buffer and dry plates at 37°C for one hour.
7. Dilute samples and controls in TEN-TC buffer (TropBio, Townsville).
8. Add 50μl of diluted sample and controls per well and incubate for one hour at 27°C.
9. Flick off excess sample and wash three times with TEN-TW buffer.
10. Dilute GAV MAb in TEN-TC buffer to 1/1000. Add 50 μL to each well and incubate for one hour at 27°C.
11. Flick off MAb and wash three times with TEN-TW buffer.
12. Dilute goat anti-mouse HRPO conjugated antibody in TEN-TC buffer to a dilution of 1/1000. Add 50 μL to each well and incubate for one hour at 27°C.
13. Remove excess MAb solution and rinse all wells six times with TEN-TW.
14. Add 50 μL of ABTS to each well and incubate in the dark at room temperature for one hour.
15. Measure optical density (OD) at a dual absorbance of 414nm and 492nm.

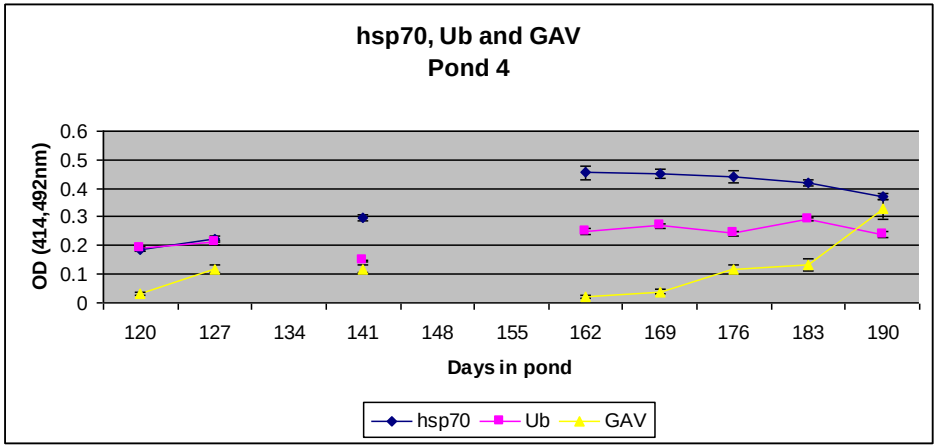
NOTE: Positive control used was pooled leg from hypoosmotically stressed *P. monodon.* Negative control was gill tissue from GAV negative *P. merguiensis*. Blank control was TEN-TC buffer.

APPENDIX 6: Line graphs for hsp70, ubiquitin and gill-associated virus.
Comparison of average hsp70, ubiquitin (Ub) and gill-associated virus (GAV) optical density in pleopods of *P. monodon* as determined by ELISA. Each graph represents an individual pond. Days in pond represents the number of days prawns had been in each pond on the day of sampling. Missing values indicate no sample analysis. Sample points n=26-30 (+/- SE).

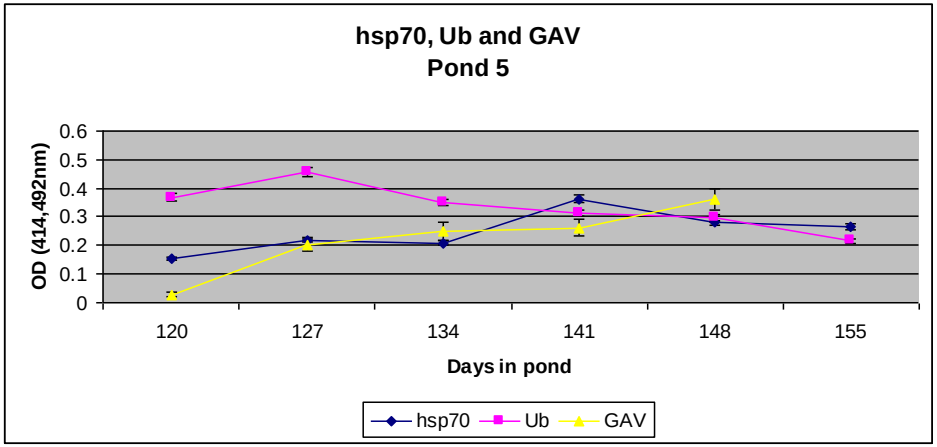


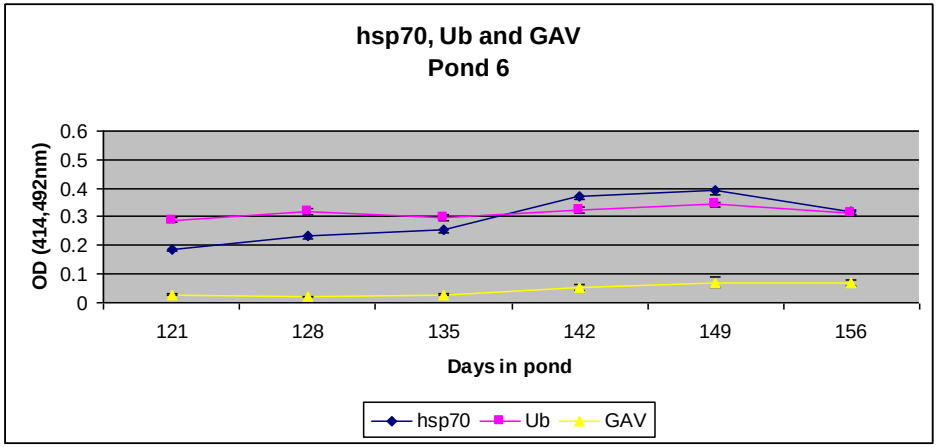


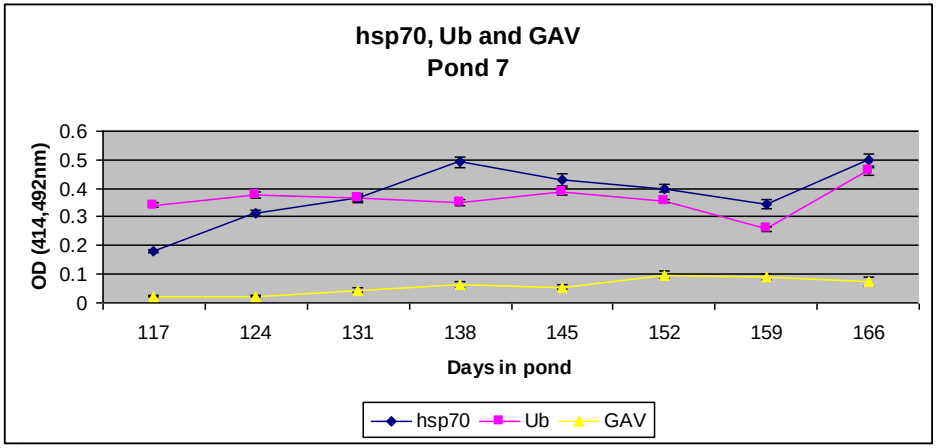


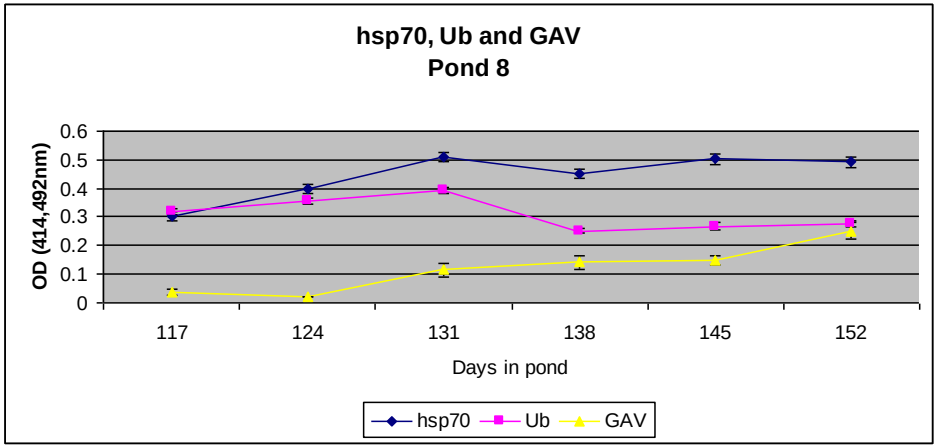


APPENDIX 6: Line graphs for hsp70, ubiquitin and gill-associated virus.
Comparison of average hsp70, ubiquitin (Ub) and gill-associated virus (GAV) optical density in pleopods of *P. monodon* as determined by ELISA. Each graph represents an individual pond. Days in pond represents the number of days prawns had been in each pond on the day of sampling. Missing values indicate no sample analysis. Sample points n=26-30 (+/- SE).









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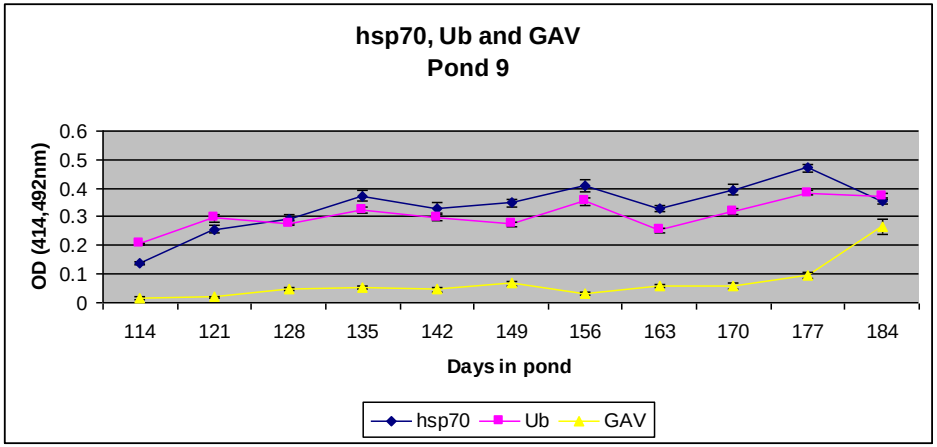
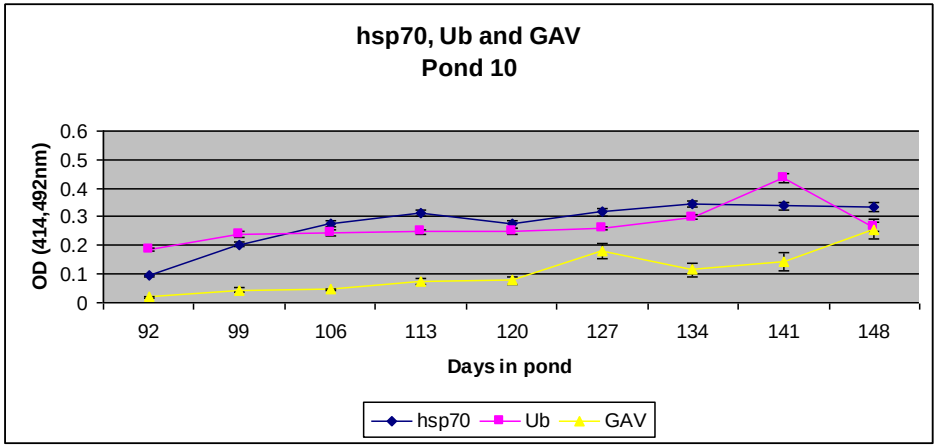
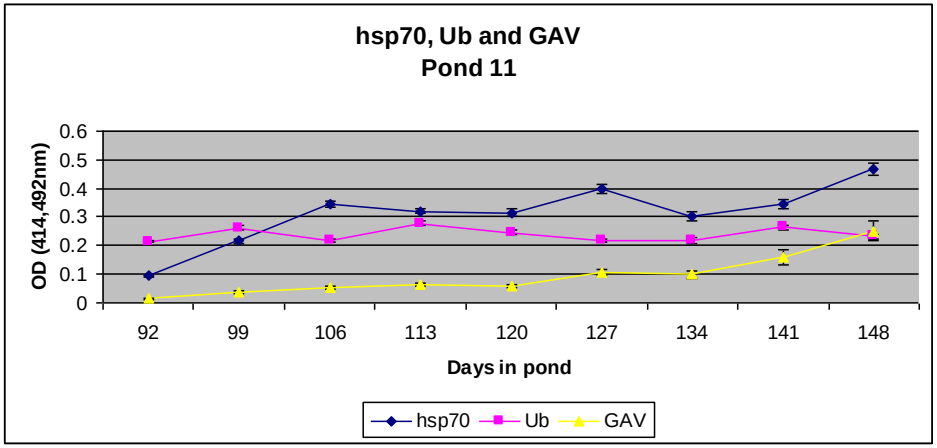






APPENDIX 7: Significant correlation coefficients.
Average significant correlation coefficients for hsp70, Ub and GAV with environmental factors for all ponds (grouped) daily from six days previous to sampling up to and including the day of sampling. DOS = day of sampling. Temp = water temperature. DO = dissolved oxygen. MSLP = mean sea level pressure. Black = hsp70, red = Ub, blue = GAV

	Days Previous To Sampling						
	DOS	**1**	**2**	**3**	**4**	**5**	**6**
Temp am	0.908	0.784, 0.823			0.729, 0.699		
Temp am	-0.729, -0.641	-0.809	-0.877, -0.929	-0.848, -0.802	-0.987		-0.888
Temp pm		0.802	0.885	0.714, 0.822			
Temp pm		-0.881	-0.983	-0.998	-0.780	-0.894, -0.675	-0.783, -0.806
Salinity am	0.821	0.836	0.830	0.714			
Salinity am	-0.758, -0.753, -0.883	-0.836, -0.765, -0.902	-0.806, -0.789, -0.904	-0.800, 0.790, -0.831	-0.737, -0.863, -0.832	-0.782, -0.804, -0.885	-0.721, 0.754, 0.921
Salinity pm	0.821						
Salinity pm	-0.797, -0.717, -0.827	-0.796, -0.748, -0.855	-0.840, -0.767, -0.845	-0.783, -0.794, -0.825	-0.786, -0.828	-0.775, -0.788, -0.856	-0.754, -0.847
pH am					0.737		
pH am	-0.828	-0.823	-0.782	-0.938, -0.945	-0.771, -0.819	-0.896	-0.894, -0.851
pH pm					0.762		
pH pm			-0.998	-0.819		-0.889, -0.833	-0.913
DO am		0.777	0.708, 0.854	0.906	0.837		0.706
DO am	-0.877, -0.838, -0.668		-0.867, -0.890	-0.747		-0.764, -0.841	
DO pm	0.806	0.807	0.760			0.907	0.706
DO pm		-0.796, -0.751	-0.977	-0.747			
Secchi am			0.948			0.778	
Secchi am	-0.662, -0.926			-0.815, -0.876	-0.671, -0.762		-0.682, -0.809
Secchi pm							0.716
Secchi pm		-0.757	-0.981, -0.817			-0.879	-0.853, -0.602
MSLP	0.730		0.896	0.856		0.787	0.713, 0.728
MSLP	-0.730			-0.776		-0.785	-0.693

APPENDIX 8: Principal component analysis: Environment.
Correlation Matrix generated using principal component analysis for hsp70, Ub, GAV and environmental factors excluding salinity pm. DIP = days in pond, Temp = water temperature, DO = dissolved oxygen.

Correlation Matrix

	hsp70	Ub	GAV	DIP	Temp am	Temp pm	Salinity am	Salinity pm	pH am	pH pm	DO am	DO pm	Secchi am	Secchi pm
hsp70	1.000	0.228	0.162	0.580	-0.139	-0.219	-0.418	-0.401	-0.254	-0.100	-0.216	-0.069	-0.005	-0.155
Ub		1.000	0.119	0.097	0.060	0.016	-0.008	0.007	0.014	0.040	-0.018	-0.189	-0.089	-0.095
GAV			1.000	0.290	-0.034	-0.119	-0.174	-0.142	-0.103	-0.004	-0.107	0.222	-0.061	-0.121
DIP				1.000	-0.103	-0.217	-0.725	-0.695	-0.328	-0.081	-0.337	0.150	-0.030	-0.176
Temp am					1.000	0.861	0.104	0.126	-0.142	-0.198	-0.300	-0.019	0.121	0.082
Temp pm						1.000	0.270	0.288	0.061	-0.071	-0.223	-0.005	0.006	0.004
Salinity am							1.000	**0.993**	0.304	0.153	0.283	0.096	-0.172	-0.120
Salinity pm								1.000	0.314	0.183	0.249	0.093	-0.183	-0.152
pH am									1.000	0.685	0.381	-0.056	-0.247	-0.057
pH pm										1.000	0.253	0.141	-0.385	-0.347
DO am											1.000	-0.123	-0.182	-0.122
DO pm												1.000	-0.185	-0.155
Secchi am													1.000	0.844

APPENDIX 8: Principal component analysis: Enviromnent.
Correlation Matrix, Kaiser-Meyer-Olkin (KMO), Bartlett's Test, Total Variance Explained and Rotated Component Matrix generated using principal component analysis for for hsp70, Ub, GAV and environmental factors excluding salinity pm.

KMO and Bartlett's Test

Kaiser-Meyer-Olkin Measure of Sampling Adequacy.		.542
Bartlett's Test of Sphericity	Approx. Chi-Square	525.832
	df	78
	Sig.	.000

Total Variance Explained

	Initial Eigenvalues			Extraction Sums of Squared Loadings		
Component	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	2.942	22.631	22.631	2.942	22.631	22.631
2	2.525	19.420	42.051	2.525	19.420	42.051
3	1.864	14.335	56.386	1.864	14.335	56.386
4	1.295	9.960	66.346	1.295	9.960	66.346
5	1.036	7.973	74.319	1.036	7.973	74.319
6	.947	7.284	81.602			
7	.683	5.257	86.859			
8	.536	4.120	90.979			
9	.511	3.932	94.911			
10	.274	2.106	97.017			
11	.196	1.507	98.523			
12	.102	.784	99.307			
13	.090	.693	100.000			

Extraction Method: Principal Component Analysis.

Rotated Component Matrix[a]

	Component				
	1	2	3	4	5
DIP	.885			-.135	
Salinityam	-.766	.164	-.234	.135	-.101
hsp70	.665	-.145	-.142	-.177	.341
GAV	.505		-.103	.110	-.252
DOam	-.489	-.458	-.154	.281	.144
Tempam		.933		-.117	
Temppm	-.208	.927			
Secchipm			.952		
Secchiam			.915	-.195	
pHam	-.271			.890	
pHpm			-.265	.871	
DOpm	.182		-.225		-.794
Ub	.210	.140	-.175	.123	.671

Extraction Method: Principal Component Analysis.
Rotation Method: Varimax with Kaiser Normalization.

a. Rotation converged in 5 iterations.

APPENDIX 9: Principal component analysis: Production.
Correlation Matrix, Kaiser-Meyer-Olkin (KMO), Bartlett's Test,Total Variance Explained and Rotated Component Matrix generated using principal component analysis for hsp70, Ub, GAV and production factors excluding salinity pm. DIP = days in pond, ABW = average body weight, FCR = food conversion ratio

Correlation Matrix

	hsp70	Ub	GAV	DIP	Survival	Yield	ABW	FCR
hsp70	1.000	0.222	0.162	0.590	-0.193	0.479	0.559	0.306
Ub		1.000	0.119	0.106	0.249	0.087	0.038	0.120
GAV			1.000	0.290	0.080	0.366	0.358	0.177
DIP				1.000	-0.167	0.863	0.829	0.579
Survival					1.000	0.096	-0.375	0.057
Yield						1.000	0.750	0.347
ABW							1.000	0.392

KMO and Bartlett's Test

Kaiser-Meyer-Olkin Measure of Sampling Adequacy.		.591
Bartlett's Test of Sphericity	Approx. Chi-Square	408.447
	df	28
	Sig.	.000

Total Variance Explained

	Initial Eigenvalues			Extraction Sums of Squared Loadings		
Component	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	3.551	44.391	44.391	3.551	44.391	44.391
2	1.382	17.281	61.672	1.382	17.281	61.672
3	.931	11.640	73.312			
4	.833	10.419	83.731			
5	.661	8.268	91.999			
6	.459	5.738	97.737			
7	.132	1.648	99.385			
8	.049	.615	100.000			

Extraction Method: Principal Component Analysis.

Rotated Component Matrix[a]

	Component	
	1	2
DIP	.950	
ABWg	.902	-.238
YeildTHa	.858	.143
hsp70	.706	
FCR	.586	.207
GAV	.440	.300
Survival	-.189	.853
Ub	.162	.665

Extraction Method: Principal Component Analysis.
Rotation Method: Varimax with Kaiser Normalization.
a. Rotation converged in 3 iterations.

APPENDIX 10: Discriminant analysis: Survival.
Summary of canonical discriminant functions generated using discriminant analysis for survival including eigenvalues, Wilks' Lambda, standardized canonical discriminant function coefficients and classification results excluding salinity pm. (n = 88.)

Eigenvalues

Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	1.858[a]	100.0	100.0	.806

a. First 1 canonical discriminant functions were used in the analysis.

Wilks' Lambda

Test of Function(s)	Wilks' Lambda	Chi-square	df	Sig.
1	.350	84.522	11	.000

Standardized Canonical Discriminant Function Coefficients

	Function
	1
hsp70	-1.375
GAV	-.212
DIP	2.421
Tempam	.000
Temppm	.110
Salinityam	1.000
pHam	.173
pHpm	.230
DOam	.128
DOpm	.145
Secchipm	.367

Classification Results[b,c]

			Predicted Group Membership		
		Survivalgroup	> 70% survival	< 70% survival	Total
Original	Count	> 70% survival	37	2	39
		< 70% survival	3	46	49
	%	> 70% survival	94.9	5.1	100.0
		< 70% survival	6.1	93.9	100.0
Cross-validated[a]	Count	> 70% survival	36	3	39
		< 70% survival	6	43	49
	%	> 70% survival	92.3	7.7	100.0
		< 70% survival	12.2	87.8	100.0

a. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

b. 94.3% of original grouped cases correctly classified.

c. 89.8% of cross-validated grouped cases correctly classified.

APPENDIX 11 Discriminant analysis: Yield.
Summary of canonical discriminant functions generated using discriminant analysis for yield including eigenvalues, Wilks' Lambda, standardized canonical discriminant function coefficients and classification results excluding salinity pm. (n = 88)

Eigenvalues

Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	.614[a]	100.0	100.0	.617

a. First 1 canonical discriminant functions were used in the analysis.

Wilks' Lambda

Test of Function(s)	Wilks' Lambda	Chi-square	df	Sig.
1	.620	39.250	8	.000

tandardized Canonical Discriminant Function Coefficient

	Function
	1
hsp70	-.932
GAV	-.441
Ub	.441
DIP	1.356
pHpm	-.072
DOam	.669
DOpm	.440
Secchipm	.096

Classification Results[b,c]

			Predicted Group Membership		
		Yieldgroup	> 7T	< 7T	Total
Original	Count	> 7T	31	9	40
		< 7T	12	36	48
	%	> 7T	77.5	22.5	100.0
		< 7T	25.0	75.0	100.0
Cross-validated[a]	Count	> 7T	27	13	40
		< 7T	15	33	48
	%	> 7T	67.5	32.5	100.0
		< 7T	31.3	68.8	100.0

a. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

b. 76.1% of original grouped cases correctly classified.

c. 68.2% of cross-validated grouped cases correctly classified.

APPENDIX 12: Discriminant analysis: Average body weight.
Summary of canonical discriminant functions generated using discriminant analysis for average body weight including eigenvalues, Wilks' Lambda, standardized canonical discriminant function coefficients and classification results excluding salinity pm. (n = 88).

Eigenvalues

Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	.285[a]	100.0	100.0	.471

a. First 1 canonical discriminant functions were used in the analysis.

Wilks' Lambda

Test of Function(s)	Wilks' Lambda	Chi-square	df	Sig.
1	.778	20.468	9	.015

Standardized Canonical Discriminant Function Coefficients

	Function 1
Ub	.732
GAV	.331
Tempam	-.277
Temppm	.010
Salinityam	.213
pHam	.855
pHpm	-.567
Secchiam	.146
Secchipm	-.330

Classification Results[b,c]

		ABWgroup	Predicted Group Membership: > 25g	Predicted Group Membership: < 25g	Total
Original	Count	> 25g	33	13	46
		< 25g	15	27	42
	%	> 25g	71.7	28.3	100.0
		< 25g	35.7	64.3	100.0
Cross-validated[a]	Count	> 25g	30	16	46
		< 25g	16	26	42
	%	> 25g	65.2	34.8	100.0
		< 25g	38.1	61.9	100.0

a. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

b. 68.2% of original grouped cases correctly classified.

c. 63.6% of cross-validated grouped cases correctly classified.

APPENDIX 13: Discriminant analysis: Survival 3 weeks prior to harvest. Summary of canonical discriminant functions generated using the bio-indicators and environmental factors (am data only) for survival 3 weeks prior to harvest including eigenvalues, Wilks' Lambda, standardized canonical discriminant function coefficients and classification results excluding salinity pm. (n = 56)

Eigenvalues

Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	2.254[a]	100.0	100.0	.832

a. First 1 canonical discriminant functions were used in the analysis.

Wilks' Lambda

Test of Function(s)	Wilks' Lambda	Chi-square	df	Sig.
1	.307	59.591	7	.000

Standardized Canonical Discriminant Function Coefficient

	Function
	1
hsp70	-1.617
Ub	-.005
DIP	2.062
Tempam	.157
Salinityam	.471
DOam	-.184
Secchiam	.171

Classification Results[b,c]

			Predicted Group Membership		
		Survivalgroup	> 70% survival	< 70% survival	Total
Original	Count	> 70% survival	25	0	25
		< 70% survival	2	29	31
	%	> 70% survival	100.0	.0	100.0
		< 70% survival	6.5	93.5	100.0
Cross-validated[a]	Count	> 70% survival	24	1	25
		< 70% survival	5	26	31
	%	> 70% survival	96.0	4.0	100.0
		< 70% survival	16.1	83.9	100.0

a. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

b. 96.4% of original grouped cases correctly classified.

c. 89.3% of cross-validated grouped cases correctly classified.

APPENDIX 14: Discriminant analysis: Yield 3 weeks prior to harvest. Summary of canonical discriminant functions generated using the bio-indicators and environmental factors (am data only) for yield 3 weeks prior to harvest including eigenvalues, Wilks' Lambda, standardized canonical discriminant function coefficients and classification results excluding salinity pm. (n = 56)

Eigenvalues

Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	1.365[a]	100.0	100.0	.760

a. First 1 canonical discriminant functions were used in the analysis.

Wilks' Lambda

Test of Function(s)	Wilks' Lambda	Chi-square	df	Sig.
1	.423	42.612	9	.000

Standardized Canonical Discriminant Function Coefficients

	Function
	1
hsp70	-1.211
Ub	.398
DIP	1.895
Tempam	-.093
Salinityam	.860
DOam	.325
Secchiam	.463
GAV	.108
pHam	-.060

Classification Results[b,c]

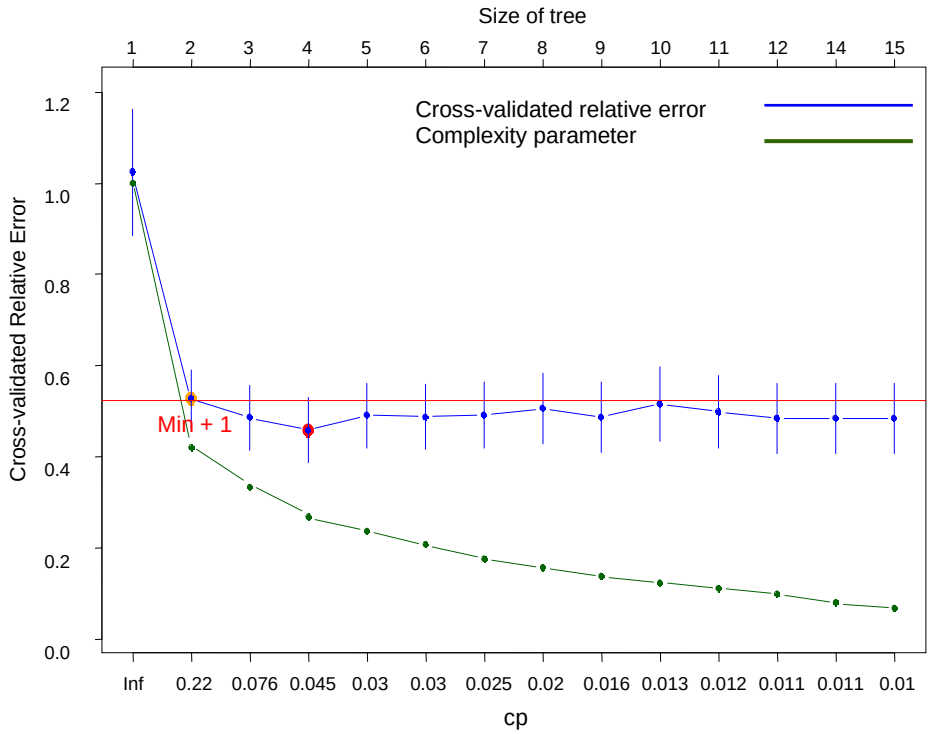
			Predicted Group Membership		
		Yieldgroup	> 7T	< 7T	Total
Original	Count	> 7T	25	1	26
		< 7T	5	25	30
	%	> 7T	96.2	3.8	100.0
		< 7T	16.7	83.3	100.0
Cross-validated[a]	Count	> 7T	23	3	26
		< 7T	9	21	30
	%	> 7T	88.5	11.5	100.0
		< 7T	30.0	70.0	100.0

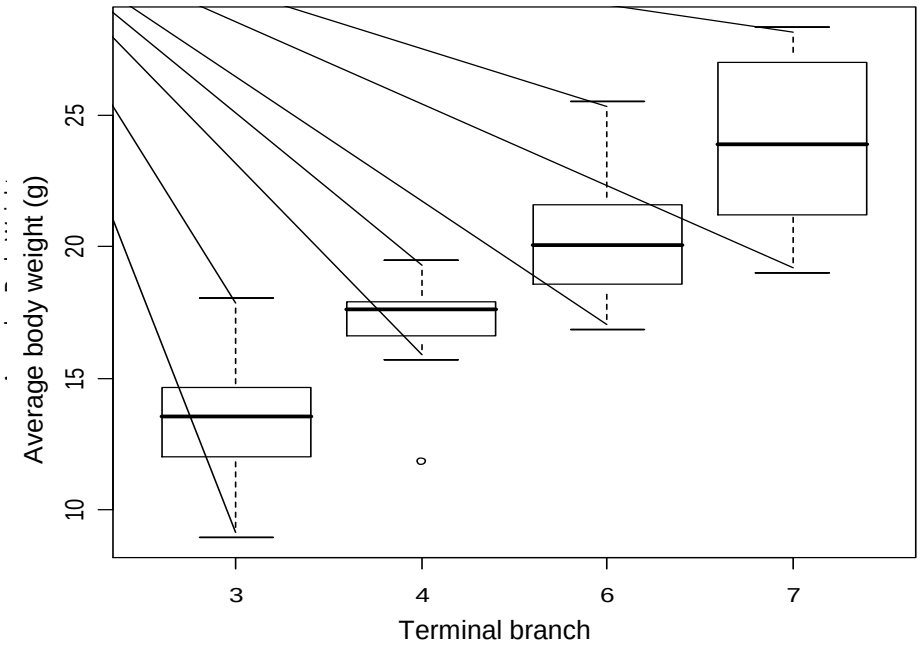
a. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

b. 89.3% of original grouped cases correctly classified.

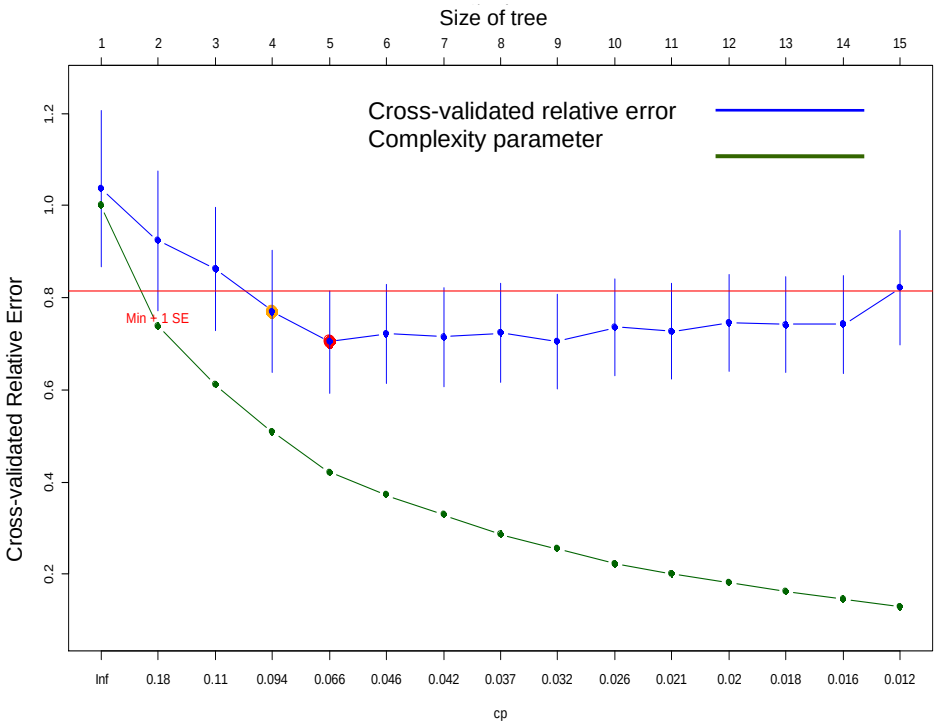
c. 78.6% of cross-validated grouped cases correctly classified.

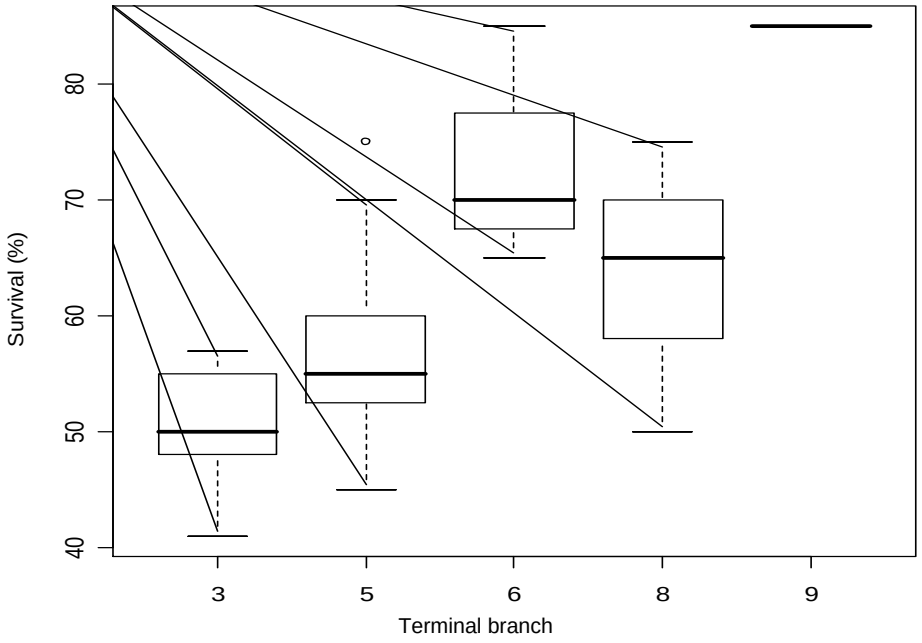
APPENDIX 15: CART analysis: Average body weight.
Plot of cross-validated errors for all trees verses the complexity parameter value and box plot of terminal branches of the tree yielding the minimum cross-validated error.



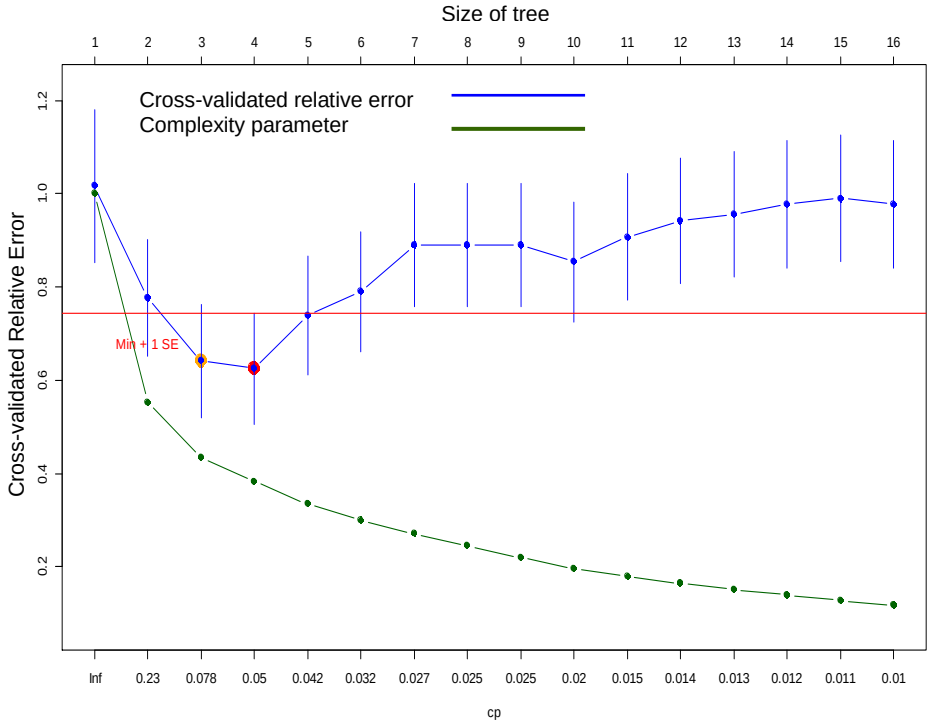


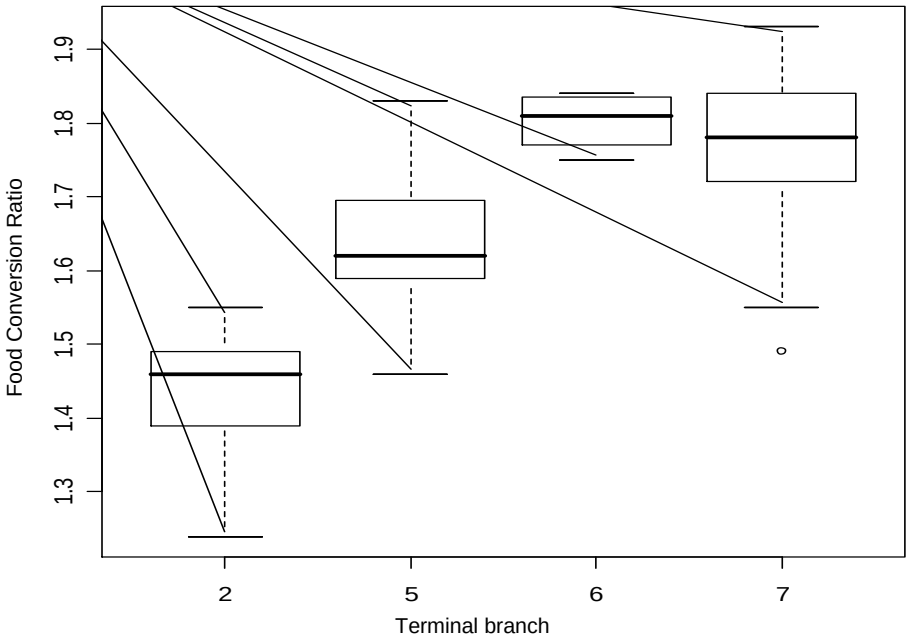
APPENDIX 16: CART analysis: Survival.
Plot of cross-validated errors for all trees verses the complexity parameter value and box plot of terminal branches of the tree yielding the minimum cross-validated error.



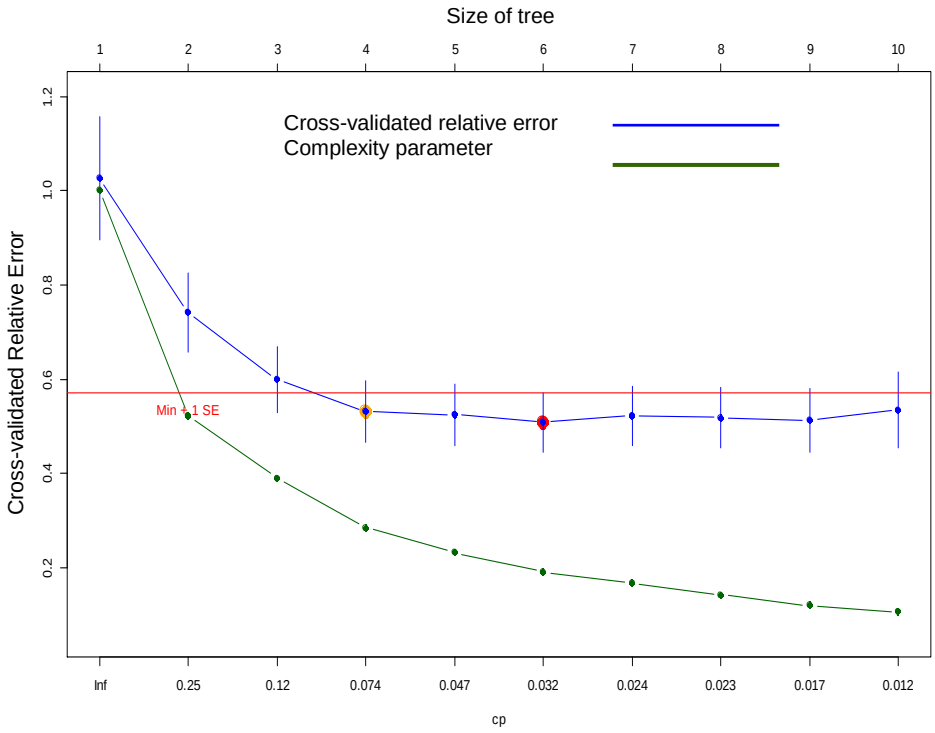


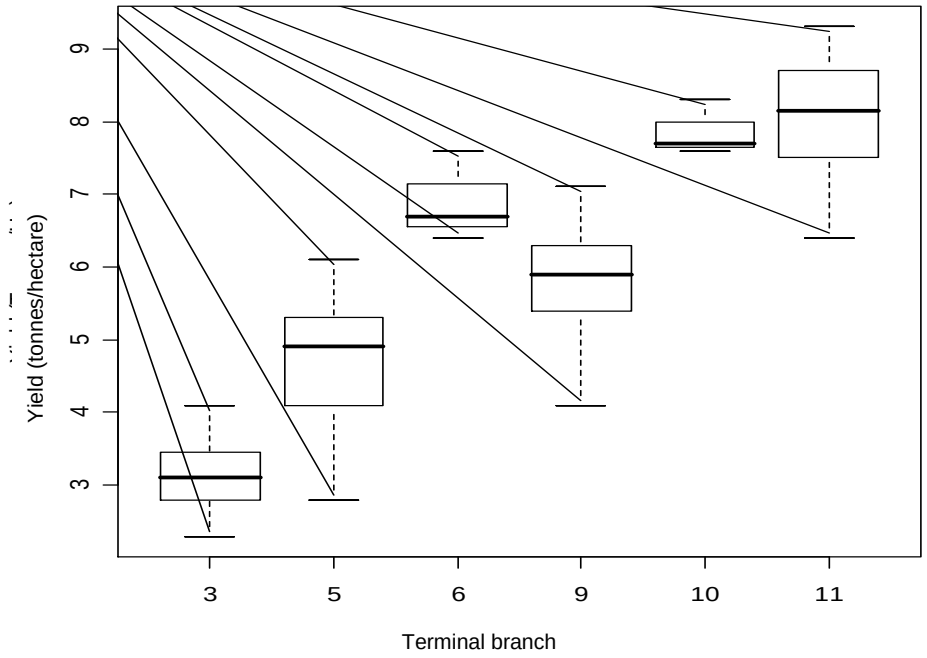
APPENDIX 17: CART analysis: Food conversion ratio.
Plot of cross-validated errors for all trees verses the complexity parameter value and box plot of terminal branches of the tree yielding the minimum cross-validated error.



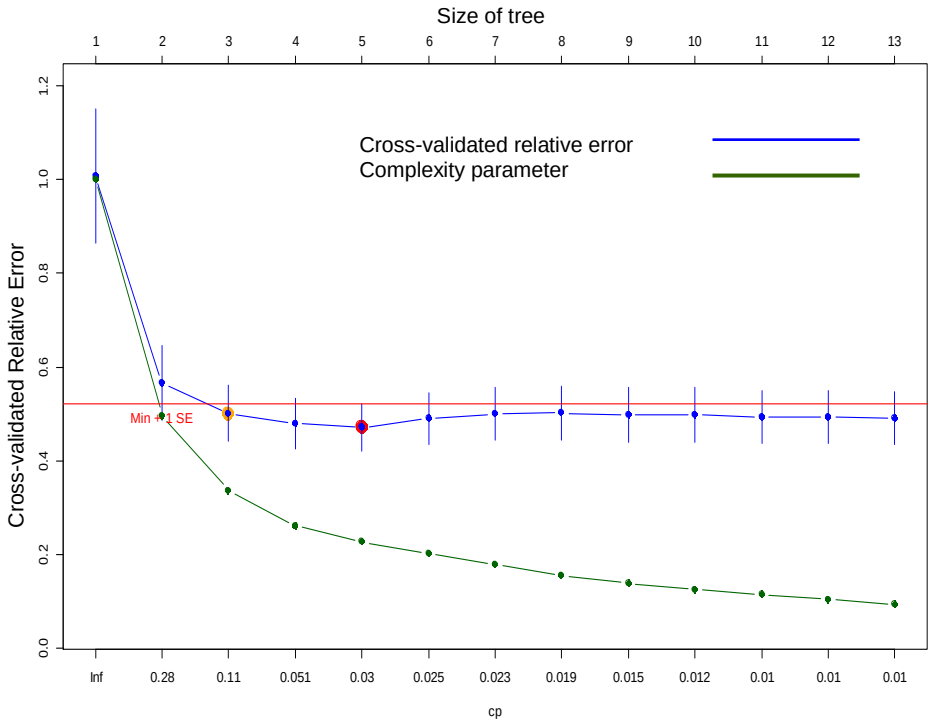


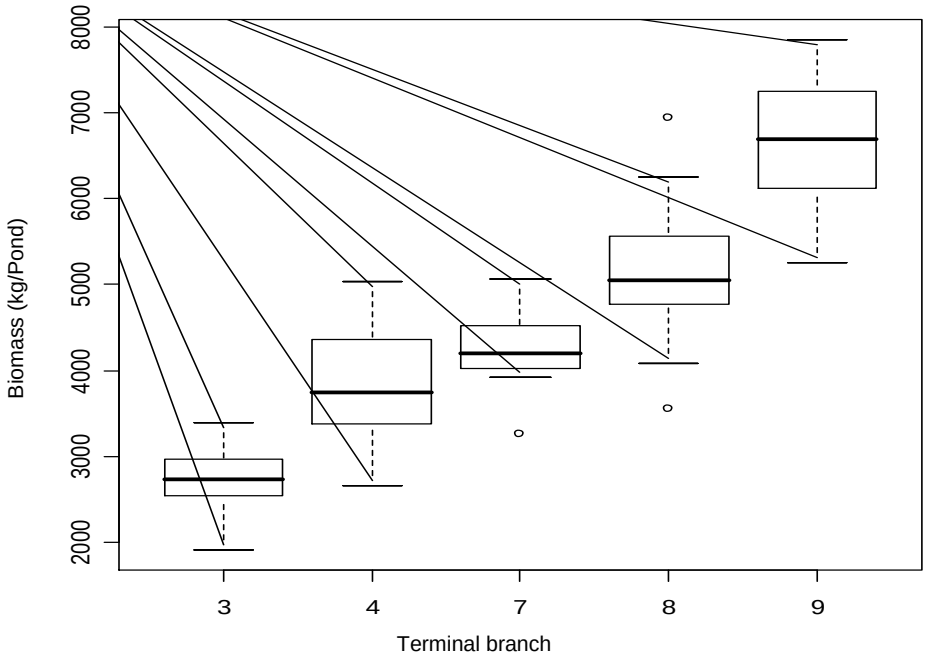
APPENDIX 18: CART analysis: Yield.
Plot of cross-validated errors for all trees verses the complexity parameter value and box plot of terminal branches of the tree yielding the minimum cross-validated error.





APPENDIX 19: CART analysis: Biomass.
Plot of cross-validated errors for all trees verses the complexity parameter value and box plot of terminal branches of the tree yielding the minimum cross-validated error.





APPENDIX 20: CART analysis: Growth.
Plot of cross-validated errors for all trees verses the complexity parameter value and plot of the R-squared value verses the tree size.