Serum Biomarkers of Pathological Damage During Pancreas Disease (PD) in Atlantic Salmon (*Salmo salar*)

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Abstract

Atlantic salmon, *Salmo salar*, in aquaculture are susceptible to a number of infectious agents which cause significant economic impacts due to mortality and morbidity. However, the current diagnostic armamentarium available to industry has a number of limitations. More specifically histopathology remains the only way to diagnose pathological damage during disease requiring a high level of skill. Moreover its destructive nature makes it unsuitable for regular proactive health monitoring programmes. Therefore, there is significant demand for non-destructive markers which can indicate clinical disease specific tissue damage. Moreover, such tests can give valued information on the effects of disease management strategies, such as functional feeds, in alleviating the severity of disease. This thesis describes, through the use of a pancreas disease (PD) cohabitation model the discovery of a number of potential non-destructive serum markers of infection and clinical disease. In addition, the reduction in disease severity through the use of a functional feed is also described. Initial investigation demonstrated the ability of functional feed to reduce disease severity using histopathology lesion scores of tissues affected during PD (pancreas, heart, and red and white skeletal muscle) and serum activities of creatine kinase, alanine transaminase, and aspartate transaminase. However, given the ubiquitous nature of these enzymes the discovery of tissue specific markers of damage and infection was carried out using two dimensional gel electrophoresis and subsequent mass spectrometry identification of protein spots. This analysis identified a number of potential markers of PD associated pathological disease. For example, enolase and aldolase were identified as potential markers of white muscle pathology. Validation demonstrated that serum increase in enolase 3 is a biomarker for pathological damage to white muscle. A novel diagnostic test was also discovered based on a differential turbidity phenomenon which occurs when diseased sera is introduced to optimised buffer. This assay detected pathological damage to specific tissues when precipitated proteins were reconstituted and subjected to electrophoresis. In addition, reductions in disease severity during the trial in fish fed a functional feed were also detectible with this test. These biomarkers may have a significant impact in improving the ability to diagnose pathological damage (clinical disease) after infection and aid in fish health monitoring programmes.

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List of Abbreviations

α	Alpha
ß	Beta
γ	Gamma
µg	Micro gram
µl	Micro litre
µM	Micro molar
%	Percentage
€	Euro
°C	Degrees centigrade
1-DE	One dimensional separation
2-DE	Two dimensional electrophoresis
AFBINI	Agri-Food and Biosciences Institute of Northern Ireland
AGD	Amoebic gill disease
ALT	Alanine transaminase
AST	Aspartate transaminase
ANOVA	Analysis of variance
AU	Arbitary units
BKD	Bacterial kidney disease
BSA	Bovine serum albumin
C	Complement
CFH	Complement factor H
CHSE	Chinook salmon embryo
CK	Creatine kinase
CMS	Cardiomyopathy syndrome
Cp	ceruloplasmin
CV	Coefficient of variation
Cy	Cyanine
Δ_{340}	Delta change in optical density at 340 nanometers
DiGE	Difference gel electrophoresis
ELISA	Ezyme-linked immunosorbent assay
ENO	Anti-enolase antibody
ESI	Electrospray ionisation
FAO	Food and Agriculture Organization of the UN
FCR	feed conversion ratio
Fish in to fish out	FIFO
FM	Fish Meal
FO	Fish oil

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBP	Great British Pounds
GM	Genetically modified
H_2O_2	Hydrogen peroxide
HSMI	Heart and skeletal muscle inflammation
HIER	Heat-induced epitope retrieval
i.m.	Intra-muscular
i.p.	Intra-peritoneal
ID	Identification
IEF	Isoelectric focussing
IHC	Immunohistochemistry
IHN	Infectious hematopoietic necrosis
IHNV	Infectious hematopoietic necrosis virus
IPG	Immobilized pH Gradient
IPN	Infectious pancreatic necrosis
IPNV	Infectious pancreatic necrosis virus
ISA	Infectious salmon anaemia
ISAV	Infectious salmon anaemia virus
LPS	Lipopolysaccharide
M	Molarity
mAbs	Monoclonal antibodies
MAC	Membrane attack complex
min	Minutes
MOS	Mannoligosaccharide
MS	Mass spectrometry
Mw	Molecular weight
n	Sample number
NARA	Norwegian National Animal Research Authority
nm	Nanometer
NO	Nitric oxide
NOK	Norwegian Krone
NSAV	Norwegian salmonid alphavirus
OD	Optical density
OGE	OFFGEL electrophoreses
OIE	World organisation for animal health
p	Probability
PCR	Polymerase chain reaction
PD	Pancreas Disease
Pi	Isoelectric point
PMCV	Piscine Myocardiopathic Virus
ppm	Parts per million

PPD	Paraphenylenediamine
PPD	Plant protein
PRV	Piscine orthoreovirus
qPCR	Quantative poluymerase chain reaction
RT-PCR	Reverse transcription-polymerase chain reaction
SAT	Sodium acetate trihydrate
SAV	Salmonid alphavirus
scFOS	Short chain fructooligosaccharide
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SD	Sleeping disease
SDV	Sleeping disease virus
SE	Standard error
sec	Seconds
SEM	Standard error of mean
SESV	Southern elephant seal alphavirus
SPDV	Salmon pancreas disease virus
SPR	Serum precipitate reaction
Tf	Transferrin
VEE	Venezuelan equine encephalitis
VN	Virus neutralizing
VO	vegetable oil
w/v	Weight per volume
Wpc	Weeks post challenge

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Mark Braceland, September 2014

Author's Declaration

I declare that this thesis is the result of my own work unless otherwise stated where collaborative work is acknowledged. This thesis has not been submitted for any other degree at the University of Glasgow or any other institution.

Mark Braceland 17/09/2014.

Chapter 1

Literature Review

1. Literature Review

1.1 Aquaculture

Aquaculture, the rearing or cultivation of aquatic organisms for consumption is the fastest growing food production industry, expanding at an average annual rate of 8.4% since 1970 (Hall et al. 2011). This level of growth is attributed to not only the increasing intensity of production but also the extension of species within aquaculture. For example whilst worldwide production is dominated, in terms of tonnage, by the production of seaweed and carp species(FAO, 2012), extensive levels of other organisms such as salmonids, crustaceans, molluscs, and a number aquatic plant species are cultured. Global tonnage is dominated by China and the rest of Asia, attributing 61.5% and 29.5% respectively (in 2008) to global culture. This level of rapid growth has meant that aquaculture now accounts for over 50% of annual world fisheries production with expansion being driven by a number of factors including the reliance of an ever increasing world population on sustainable and reliable protein sources and dwindling wild fish populations due to the over exploitation of stocks (Jensen et al. 2014). Indeed finfish production in recent years has expanded so much that there are now over 310 species of teleostei produced globally with an estimated 154 million tonnes of fish being produced in 2011, overtaking production of wild catch fisheries (FAO, 2012). Whilst Europe only contributes 3.6% of worldwide tonnage its recent growth has largely been due to the cultivation of Atlantic salmon (*Salmo salar*) in North Western Europe.

1.2 Salmon Aquaculture

1.2.1 Background and Growth

Farming of Atlantic salmon, *Salmo salar*, was first successfully carried out in 1969 where smolts which were one generation removed from wild strains were introduced to marine cages, grown, and harvested in 1971 by Ove and Sivert Grøntvedt (Gjedrem et al. 1991). This began a surge of interest in the fish as an economically viable livestock with culture spreading, to varying levels of intensities, in subsequent years to (amongst others) Scotland, Ireland, the Faroe

Islands, the United States of America, Chile, and Australia (FAO, 2012). However, arguably it was not until 1990 that the cultivation of the species intensified dramatically, with the next 20 years until 2010 having an average tonnage increase exceeding by 9.2% per annum. The ability of the industry to undergo such rapid expansion has been multifactorial with biological and engineering developments increasing productivity (Asche, 2008) and reducing cost to the consumer. However, salmon aquaculture faces a number of challenges of sustainability for such growth. Indeed recent studies, such as by Asche et al. (2013), have shown that productivity in Norway is actually slowing and as such global growth has largely been due to the acquisition of new sites in Chile. Therefore, a focus on further increasing productivity, thus challenging sustainability issues, is a massive focus area of the salmon aquaculture industry.

1.2.2 Sustainability Issues

1.2.2.1 Sustainable Growth

Productivity and sustainability can be seen as two intrinsically linked concepts as without a high level of sustainability productivity cannot remain high. As such, there are a number of major sustainability issues that we must explore for the maintenance and growth of current salmon production. As mentioned previously one of the driving forces of recent increased production of the species by aquaculture has been the development of new farm sites. This is not as simple as simply placing nets/ cages in an area of salt water for the introduction of smolts for on growing and much thought must be carried out before deciding on a suitable site. For example, temperature has been shown to have an impact on the growth (Handeland et al. 2008) with optimal temperature for maximizing growth varying with weight. Moreover, temperature changes have been shown to have different effects when distinct sites are compared (Thyholdt, 2014). However, this study did not take into account feeding regimes, diet, or fish strain all of which may play a part in this complex relationship. In addition to temperature, other hydrographic conditions, such as salinity and currents must also be considered. Therefore, most production sites are located in inland sheltered waters (Asche et al. 2013) and while there is the possibility of expansion in existing areas such plans are often met with considerable

opposition from a number of groups, thus possibly making the availability of production sites, one of the most limiting factors to sustainable growth.

1.2.2 Sustainable Feed

Salmon nutrition and feed production is arguably the most complex area the aquaculture industry has had to face during its growth. However, it is an area where vast improvements have been made. Advancements in extruder technologies have facilitated the development of high energy diets which have dramatically reduced the feed conversion ratio (FCR) throughout the industry (Austreng, 1994; Einen and Roem, 1997; Naylor et al., 2009). In brief, FCR is the ratio between dry weight of feed fed to weight gain. FCR depends on specific diet formulations and there have been a huge improvements made throughout the industry with FCR ratios as low as 0.98–1.01 (Larsson et al., 2014) reported. Indeed salmon boast the lowest FCR of any livestock species (Naylor et al., 2009). This has dramatically increased the productivity of salmon farming. However, the availability of high quality fish meal and fish oil necessary for salmon feed is possibly the largest issue of sustainability the industry faces short and long term (Tacon and Meitan, 2008; Naylor et al., 2009).

In essence this concerns the amount of fish it takes to produce enough fish oil (FO) and fish meal (FM) to produce salmon feed and thus can be described as a ratio of fish in to fish out (FIFO) ratio (Tacon and Metian, 2009). Whilst this ratio has been significantly reduced from 7.5 to 3 between 1995 to 2011 (Bendiksen et al., 2011) there is considerable effort to reduce it to near to, or below, 1 due to the increase of economic cost of FM and FO, in addition to declining wild stock sources of these ingredients. The main research in reducing further this ratio has focused on the use of more abundantly available vegetable oil (VO) and plant protein (PP) (FAO, 2012), which have long been the sources of feed for herbivorous fish species, as partial or whole replacements for FO and FM in salmon feed. Indeed, recent work has shown that partial replacement, whilst altering gut morphology, does not appear to have any major negative effects on intestinal health of salmon (Moldal et al., 2014). In addition, a study by Liland et

al. (2013) demonstrated that 80% VO and 70% plant proteins resulted in a FIFO ratio of less than 1, with a net production of 1.3kg of Atlantic salmon per 1kg of FM protein. Flesh quality and growth are maintained at 75% VO without compromising flesh very long chain n-3 polyunsaturated fatty acid (VLCn-3 PUFA) content (Torstensen et al., 2005), thus indicating partial replacement of FO and FM may be not only be more sustainable but also have little impact on end product quality and productivity. However, such replacement has been shown to alter transcriptomic responses to infection (Tacchi et al., 2012) which may, or may not, have negative impacts on fish health during challenge. This is a much understudied area of FO and FM replacement research and it is unclear which replacement levels are suitable without compromising the ability of fish to effectively combat infection/ disease. In addition, this may prove to have a level of disease specific variation depending on which metabolic pathways may be altered. Given that disease poses one of the greatest challenges to the Atlantic salmon aquaculture industry (Kibenge et al., 2012) this information is paramount.

1.3 Major Diseases of Farmed Atlantic Salmon

1.3.1 General

Atlantic salmon, *Salmo salar*, are susceptible to a number of diseases of known and unknown aetiology, including a number of fungal, viral, bacterial, ectoparasitic, and amoebic pathogens which significantly reduce productivity, sustainability and profitability of the industry thus limiting its growth. Whilst disease in aquaculture, like in all livestock industries, is a natural challenge to production there is evidence that the relatively short time of separation of domesticated species from founding wild stock may mean that high stocking densities cause a chronic stress which provides an opportunity for pathogens which are asymptomatic under normal circumstances (Kibenge et al., 2012) to be associated with overt disease. Stocking density has also been shown to increase disease severity (Bowden et al., 2002). However, this is likely to be infection specific and there has been little research on the topic in a farm setting.

Whilst mortality and morbidity resulting from infection and disease is highly variable, and dependent on a number of factors (Yousaf et al., 2013), it represents a major sustainability issue to the industry (Kibenge et al., 2012). Moreover, a serious disease outbreak may have severe repercussions for production not only at a given site but for the whole industry in a given connected area. For instance, outbreaks of infectious salmon anaemia (ISA) in Scotland have had serious economic impacts on the industry, with the eradication programmes after the 1998-1999 outbreak costing in excess of £20 million (Hastings et al. 1999). Moreover, a more recent ISA outbreak in the country in Shetland (Murray et al. 2010) and that of in Chile between 2007 and 2009 highlights the very real danger infectious disease poses to the industry (Ashe et al., 2010).

1.3.2 Fungal Diseases

Fungal infections are serious primary and secondary stressors of Atlantic salmon in culture and in the wild (Meyer, 1991). Whilst there are several fungal species which affect salmon at various life history stages (Ramaiah, 2006) the main threat in aquaculture is that of *Saprolegnia* where outbreaks affect fish in fresh water stages of development (Theon et al., 2011). The reason this fungal species remains so economically damaging is that unlike other species it has proven difficult to eliminate from water pumped into hatcheries (in which it is ubiquitous) by standard treatments available to the industry, such as filtering and physical treatments (Ali et al., 2014). However, fungal infections are most prominent after some sort of lesion has been caused due to mechanical abrasion or ectoparasitic damage (Huntsman, 1918).

1.3.3 Ectoparasitic Diseases

Salmon are susceptible to multiple ectoparasitic infections which have a serious impact on productivity. Often, like in fungal infections, they are found in association with diseases of other aetiologies and as such can cause massive welfare and health issues.

One of the most topical ectoparasitic infections is *Neoparamoeba perurans* which is the aetological agent of amoebic gill disease (AGD) (Crosbie et al., 2012). Historically the disease was only a major problem to Atlantic salmon aquaculture in Tasmania but there has been a huge emergence of the disease in other geographical locations, now being present in every country where the species is farmed except Canada. The disease affects salmon post sea transfer and gross clinical signs include multifocal white patches on the gill surface and lamellar fusion caused by hyperplasia of epithelial and mucus cells (Nowak et al., 2014) thus causing reduced ability of the fish to respire. If left untreated the disease can become chronic causing variable levels of mortalities, therefore once diagnosed treatment is given using either freshwater or hydrogen peroxide (H_2O_2) baths (Adams et al., 2012).

Possibly one of the most highlighted infections of cultivated salmon is that of the ectoparasitic sea louse species *Lepeophtheirus salmonis* (Krøyer) and *Caligus elongatus* Nordmann. The lice feed on the mucus and tissue of the fish causing sores which can cause abbresions/ lesions for secondary infections, and physiological problems (Heuch et al., 2011; Skiftesvik et al., 2013) costing the industry hundreds of millions of Euros annually (Costello, 2009). This cost combined with public interest and association to other infections which may directly transmitted, be secondary, or increase in virulence due to stress (Oelckers et al., 2014) has meant that numbers of lice at a site are routinely assessed (Heuch et al., 2011). Indeed since 2012 in Chile a government mandate has meant all farms must report site numbers of lice weekly (Kristoffersen et al., 2013), with similar reports being made in Norway and Scotland (Heuch et al., 2005). Treatment of the infestation historically has been through application of

chemicals to delouse fish. However, these treatments have major drawbacks, including increasing parasite resistance (Sevatdal et al., 2005), environmental contamination (Davies et al., 2001; Heuch et al., 2005), detrimental effects to fish health (Mayor et al., 2008) and stress levels. This has led to alternative means for long term lice control in aquaculture, most notably through biological control: studies report reductions to as low as 1 louse per fish when ballan wrasse (*Labrus bergylta*) are introduced at a stocking density ratio of 5% to salmon stock (Skiftesvik et., 2013). Such research has been so successful that Marine Harvest Scotland has plans for widespread introduction of the wrasse species into production sites after successful trials (David Cockerill, personal communication).

1.3.4 Bacterial Diseases

Diseases of bacterial aetiology in general terms are not a major problem in Atlantic salmon aquaculture compared to other pathogens and parasites (Johansen et al., 2011). This is possibly in part due to their comparatively short viability outside of their host in the marine environment, successful implementation of vaccines, and ever increasing levels of disease prevention measures taken in both fresh and sea water sites (Bornø et al., 2010; Johansen et al., 2013). Indeed all salmonid fish are vaccinated against vibriosis (*Vibrio anguillarum*), cold water viriosis (*Vibrio salmonicida*), and furunculosis (*Aeromonas salmonicida*) in Norway. However, these infections, among others, can still cause significant economic impacts when outbreaks occur. Indeed, while great strides have been made in reducing the effects of bacterial kidney disease (BKD) caused by *R. Salmoninarumin* infection in Atlantic salmon aquaculture in Scotland (Murray et al. 2011) it is still notifiable throughout the UK. Moreover, growing concern regarding pathogen exchange between wild and farmed salmon has highlighted the potential ecological impact of bacterial infections (Johansen et al., 2011). For example, bacterial kidney disease (BKD (Bruno, 2004), is present in both wild and farmed salmon populations, less so in Scotland compared to other countries (Murray et al. 2011), and despite cases significantly dropping in the last 15 years it still has a major economic impact in fresh and sea water cultivation.

1.3.5 Viral Diseases

One of the biggest challenge to salmon aquaculture, in disease terms, is that of viral diseases (Robertsen, 2011; Kibenge et al., 2012). This is due to a number of factors including a lack of understanding of biology and disputed vaccine efficacy (Kibenge et al., 2012; Gudding and Van Muiswinkel, 2013). Moreover, compared with other pathogens, viruses can remain viable in the water column for a longer duration thus aiding their horizontal transfer and persistence within the marine environment. Indeed Atlantic salmon are susceptible to a number of viral infections causing significant economic costs due to mortality and morbidity. In addition, there is a growing interest in the economic and ecological impact of disease interactions between farmed and wild populations (Walker and Winton, 2010; Robertsen, 2011; Kibenge et al., 2012; Gudding and Van Muiswinkel, 2013; Madhun et al., 2014). The main viral diseases which challenge industrial profitability and the local and global scale culture of Atlantic salmon are detailed in table 1-1.

Table 1-1 Major viral diseases of Atlantic salmon. Table of the six major viral diseases of Atlantic salmon, *salmo salar*, aquaculture.

Disease	Aetological Agent	Family
Pancreas Disease (PD)	Togavirus: salmonid alphavirus (SAV)	Togaviridae
Infectious salmon anaemia (ISA)	Orthomyxovirus: infectious salmon anemia virus (ISAV)	Orthomyxoviridae
Infectious pancreatic necrosis (IPN)	Birnavirus: infectious pancreatic necrosis virus (IPNV)	Birnaviridae
Infectious hematopoietic necrosis (IHN)	Infectious hematopoietic necrosis virus (IHNV)	Rhabdoviridae
Heart and skeletal muscle inflammation (HSMI)	Piscine orthoreovirus (PRV)	Reoviridae
Cardiomyopathy syndrome (CMS)	Piscine Myocardiopathic Virus (PMCV)	Totiviridae

1.3.5.2 Infectious salmon anaemia (ISA)

Infectious salmon anemia (ISA) is caused by infection of the negative sense segmented ssRNA orthomyxovirus, infectious salmon anemia virus (ISAV) (Walker and Winton, 2010). This notifiable disease is characterised by anemia but other clinical signs and pathological damage can be variable (Aamelfot et al., 2014), with some affected fish exhibiting circulatory disturbances to a number of organs with widespread haemorrhaging in addition to changes in appearance and shape of the spleen, swim bladder, muscle and other tissues (Rimstad et al., 2011). However, whilst in most cases where severe anemia is observed there are necrotic lesions to the liver, gut and or kidney (Rimstad et al., 2011), some individuals may still not have any pathological damage at all. This variability is thought to be due to individual variance in the ability to cope with hypoxic shock (Aamelfot et al., 2014). There are also significant differences in ISAV isolates which may also explain the degree of variation in clinical and pathological changes observed in infected salmon (Markussen et al., 2008). Prevalence of the disease, compared to others, is relatively low (Robertsen, 2011): in 2012 only two cases were recorded in Norway (Johansen, 2012) with Canada being the only other country affected by the disease (OIE, 2012). In addition, mortality rates during ISA are epidemic dependent with a daily mortality rate from 0.05% to 0.1% (Aamelfot et al., 2014). Despite this, it is one of the most economically important diseases of Atlantic salmon in aquaculture as failure to diagnose the infection/ disease quickly and implement disease management strategies can result in mortality of >80% at a given site (Aamelfot et al., 2014). Such high mortalities in a localised area become an even greater problem when poor bio-security, such as movement of infected fish, causes an epidemic on a massive scale. This is typified by the ISA outbreak in Chile from 2007 to 2009 which caused a 60% reduction of production in the country (Kibenge et al., 2012). This outbreak was thought to be the result of importation of Norwegian broodstock which were infected with a virulent strain of ISA leading Vike et al. (2009) to stipulate that vertical transmission of the virus was possible. However, this evidence is anecdotal at best and it is just as parsimonious that these broodstock were infected via contamination of ovarian fluids (Aamelfot et al., 2014) which is also a form of horizontal transmission. The problem was almost certainly made worse by the introduction of more fish into the system in infected areas to try and offset losses. This outbreak is a classic example of how much of an impact poor biosecurity, and disease management can have on the industry.

1.3.5.2 Infectious pancreatic necrosis (IPN)

Infectious pancreatic necrosis (IPN) is a major problem in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon in both fresh and sea water environments (Kibenge et al., 2012; Johansen 2013). Its aetiological agent, infectious pancreatic necrosis virus (IPNV), is a non-enveloped double stranded RNA virus belonging to the *Birnaviridae* family (Crane and Hyatt, 2011) comprising a number of serotypes of varying virulence (Santi et al., 2004) belonging to two distinct serogroups (Kibenge et al., 2012). The virus has proven extremely difficult to eradicate, despite it being the most intensively studied of all fish viruses (Crane and Hyatt, 2011), due to its ability to withstand many treatments and tolerate high temperatures and varying pH levels (Kibenge et al., 2012). This, coupled with the wide array of hosts the virus is able to be establish itself within (Hill, 1982) and shedding from individuals recovered from clinical disease (Smail and Munro, 1985), makes the virus extremely difficult to eradicate from the environment. Indeed IPNV outbreaks are more common than those of any other viral infection in Norway (Robertsen, 2011; Johansen et al., 2013). However, fish possess a high level of immunity to IPNV (Robertsen, 2011) meaning that outbreak mortality rate is usually low. Culling and fallowing have been shown to be effective in reducing prevalence in Sweden and Scotland. However, such control measures are extremely expensive and the economic impact of the disease in the major Atlantic salmon producing countries is more usually addressed by vaccination of smolts prior to sea transfer.

1.3.5.3 Cardiomyopathy syndrome (CMS)

Primarily a disease of large adult fish (Kibenge et al., 2012; Johansen, 2012) cardiomyopathy syndrome (CMS) is characterised by necrosis of the heart where the atrium becomes enlarged and rupturing to the atrium occurs as well as filling of the pericardial sac with blood (Ferguson et al., 1990). There have also been reports of occasional pancreas and muscle lesions being observed in some individuals (Johansen, 2012). Despite being first described as a disease of farmed salmon in 1985 it was not until 2011 when its aetiological agent piscine myocardiopathic virus (PMCV) was first described by Haugland et al. (2011). However, little is known about the pathogenesis of the disease and, despite some reports of PMCV in wild salmon populations, whether there are any CMS disease interactions between farmed and wild fish (Johansen, 2012).

1.3.5.4 Heart and skeletal muscle inflammation (HSMI)

First identified in a production site in Norway in 1999 heart and skeletal muscle (HSMI) is a major disease of farmed Atlantic salmon, among others, in Norway, Scotland, and Chile (Kongtrop et al., 2004; Ferguson et al., 2005). Indeed HSMI is an emerging problem with the disease spreading to new areas causing case numbers to dramatically rise in number in recent years (Bornø et al., 2010; Robertsen, 2011; Johansen, 2012). This horizontally transmitted disease is caused by *Piscine orthoreovirus* (PRV) (Palacios et al., 2010) and usually affects Atlantic salmon five to nine months after sea transfer (Kongtrop et al., 2004). While morbidity is high with fish showing a number of histopathological changes to the heart, red muscle and other tissues, disease associated mortality is relatively low with external stressors having a major role in disease outcome. Interestingly despite a clear correlation in literature between PRV load and gross pathological damage (Johansen, 2012). It has also been shown that fish may be infected with PRV for a long period of time before any clinical signs are observed (Wiik-Nielsen et al., 2012) and that the viruses presence may not necessarily result in disease (Johansen, 2012).

1.3.5.5 Infectious hematopoietic necrosis (IHN)

Traditionally thought of as a disease of salmonid species belonging to the *Oncorhynchus* genus, such as sockeye salmon, *Oncorhynchus nerka*, Chinook salmon , *Oncorhynchus tshawytscha*, and cultured rainbow trout, *Oncorhynchus mykiss* (Traxler et al., 1993), infectious hematopoietic necrosis is caused by infection by the rhabdovirus infectious hematopoietic necrosis virus (IHNV) which is epizootic throughout western North America. The disease is spread horizontally in both fresh and saltwater production causing extensive haemorrhage and necrosis of a number of organs due to acute viraemia (Wolf, 1988). Infectious hematopoietic necrosis (IHN), due to a combination of factors has spread to a number of different species and regions (Crane and Hyatt, 2011). Introduction of Atlantic salmon in these regions has compromised production. Indeed this species (*Salmo salar*) has been shown to be extremely susceptible to the virus with high levels of mortality observed during outbreaks (Saksida, 2006). This increased virulence may be due to a lack of exposure historically to the pathogen with genetic factors cited as playing a role in disease severity (Miller et al., 2004). For instance, an epidemic from 2001-2003 caused a cumulative mortality, of direct mortality and culling for disease control, of over 12 million Atlantic salmon in British Columbia (Saksida, 2006) causing significant economic losses to the industry as a whole. Whilst IHN is not a disease problem in the major Atlantic salmon producing regions (Norway, Chile, Scotland, or Ireland), the observation of spread to European rainbow trout means that considerable caution is now taken. Vaccination has proved to have little or no protection during IHN outbreaks (Saksida, 2006) which is a growing concern not just in IHN but with other viral diseases.

1.4 Pancreas Disease (PD)

1.4.1 General

First discovered in 1976 and characterized by Munro *et al.* (1984) and McVicar *et al.* (1987), pancreas disease (PD) of Atlantic salmon, *Salmo salar*, is a major problem to the cultivation of the species in Norway, Scotland, and Ireland. In addition, the disease has been described in a number of other countries, such as North America (Kent and Elston, 1987), France, and Spain (Raynard *et al.,* 1992). Moreover, whilst the disease has not yet been described in Chile given the previous cases of disease spreading due to fish transfer from different countries there is significant monitoring for PD in the country. The disease is characterized by a number of behavioural and histopathological changes such as reduced appetite (McVicar, 1987) and a tendency for infected fish to stay close to cage corners, along with acute necrosis of the pancreatic acinar cells, lesions in the heart, and skeletal lesions (depending on the time period since infection). Occasionally lesions in the kidney, liver and brain can also be observed (McVicar, 1987; McLoughlin and Graham, 2007). This histopathological damage can, in some cases, be extremely severe causing long term morbidity effects such as runting and reduced fillet quality upon harvest due to the chronic effects of pathological damage and a reduced ability to uptake nutrients due to pancreas damage (Lerfall et al., 2012; Larsson et al., 2012). While direct economic effects of any disease are difficult to estimate with variability between years and sites (Johansen, 2012), PD is one of the most economically important diseases affecting the industry.

1.4.2 Economic Costs of Pancreas Disease

The economic losses attributed to PD are substantial. For instance, in Ireland during 2003-2004 around €12 million was lost due to the disease (Ruane et al., 2008). In addition, analysis of industrial records in Scotland from 1998 to 2009 by Kilburn et al. (2012) indicate that despite negligible impact of PD pre 2003 it accounted for the greatest level of biomass losses of any infectious disease in aquaculture. Arguably the country most affected by the effects of pancreas disease is Norway where direct costs of the disease for an average farm are estimated to be 14M Norwegian Krone (NOK) (Aunsmo et al., 2010). Indeed the

virus has been shown to be spreading to new areas and is still an emerging problem (Rimstad, 2011) highlighting the need for a better understanding of disease pathogenesis and the development of diagnostic tools for quick implementation of disease management strategies. Case numbers of the disease in Norway rose from 5 in 1995 to 109 in 2008, in part due to improvement of screening methods post identification of the aetiological agent by Weston et al (2002).

1.4.3 *Alphavirus*es

The aetiological agent of pancreas disease, *salmonid alphavirus* (SAV), was first described by Weston et al. (2002) and has also been shown to cause sleeping disease (SD) in trout (Crane and Hyatt, 2011).SAV belongs to the genus *Alphavirus* (family *Togaviridae*), known to consist of 26 species which in turn consists of numerous strains or subtypes (Strauss and Strauss, 1994) which are closely related in terms of molecular characteristics and structure (Strauss and Strauss, 1994; Powers et al. 2001). All *Alphaviruses* share a minimum amino acid sequence identity of around 45% in structural proteins (which are more divergent) and about 60% in non-structural proteins (Strauss and Strauss, 1994). Within species, diversity seems to be directly linked with the dispersal potential of the host species, for example Venezuelan equine encephalitis (VEE) complex viruses which primarily use rodent hosts and *Culex* mosquito species as vectors (which themselves have limited dispersal) tend to show high levels of diversity (Powers *et al.,* 2001) when compared to *Alphavirus* species which use birds as hosts (Sammels *et al.,* 1999).

Alphaviruses are small and spherical in structure with a genome consisting of a single strand of positive sense RNA of approximately 11.7Kb long which possesses multiple copies of a single species of capsid protein of approximately 30kDa in weight (Powers *et al.,* 2001; Straus and Straus, 1994). The virion envelope found in all *Alphaviruses* consists of a lipid bilayer (assembled using lipids derived from the plasma membrane of the host cell) with multiple copies of two encoded glycoproteins, E1 and E2, embedded within this bilayer (Harrison, 1986). Non-

structural protein genes are encoded in the 5’ two thirds of the genome, which are labelled nSP1 to nSP4 in Figure 1.1, while structural proteins are translated from subgenomic mRNA at the 3’ terminal third of the genome.

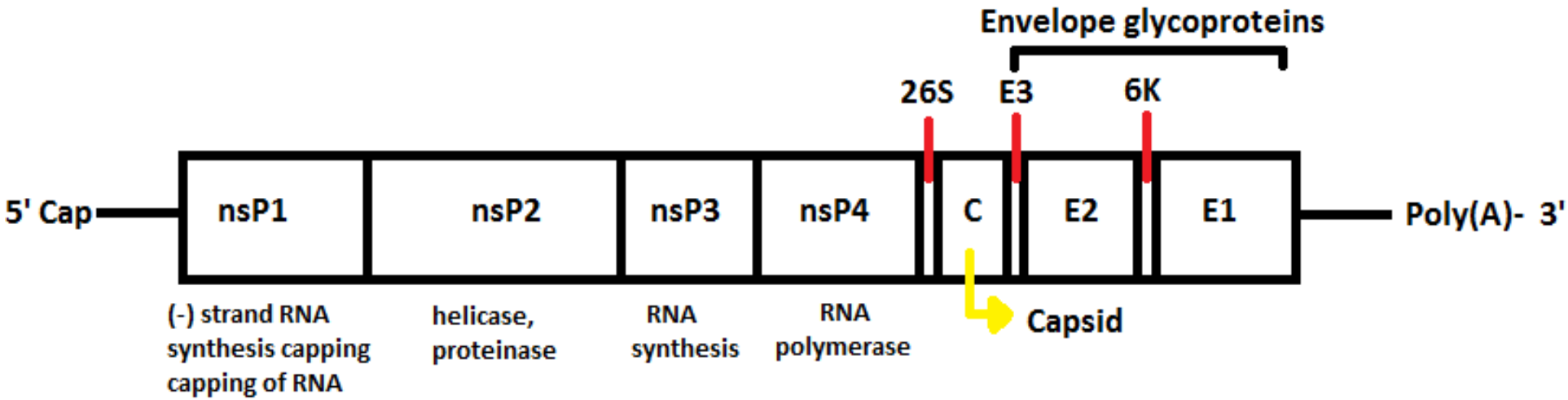

Figure 1.1: Alphavirus genes and functions. Simple diagram of Alphavirus genome with gene functions and associated functions indicated. Taken and altered from Powers *et al.*, 2001.

Traditionally it has been thought that the genus arose around several thousand years ago (Weaver, 1993; Weaver et al, 1993). However, a more recent phylogenetic study carried out by Powers et al, (2001) has clearly shown that this estimate is far too recent, whilst re-demonstrating that the viruses most probably arose as insect borne or insect viruses. Interestingly this work has shown that *salmonid alphavirus* (SAV) and the recently discovered (La Linn *et al.,* 2001) southern elephant seal alphavirus (SESV) do not fit into the widely accepted hypothesis that the genus that we see today originated in the New World from a an insect-borne plant virus (Levinson *et al.,* 1990; Weaver *et al.,* 1993; Weaver, 1995) and instead neither this hypothesis nor one in which the genus would have originated in the Old World as it would be equally parsimonious for either origin for these two species.

The way in which Alphaviruses replicate is also of great interest as although they can replicate within the cytoplasm with virions becoming mature by budding through the plasma membrane where the glycoproteins E1 and E2 are assimilated of both their arthropod vectors and their vertebrate hosts (Strauss and Strauss, 1994; Powers *et al.,* 2001). Lifelong infection can be observed can be observed in arthropods where infected cells survive and continue to produce virus at low levels. In contrast cytolytic infection is produced in vertebrate cells. Numerous cell culture experiments have shown in great detail the conditions in which Alphaviruses thrive and have highlighted that in vector enucleated cells virus replication is extremely low (Erwin and Brown, 1983), but when vertebrate enucleated cells are infected virus replication is almost normal. This seems to demonstrate that nucleated cells are not pivotal in virus proliferation within the terminal host, however, surprisingly when a virus is within such a cell around half of nsP2 produced after infection is transported to the nucleus (Peranen *et al.,* 1990). As stated previously all Alphavirus species have been shown to be arthropod- borne where in typical terrestrial systems various mosquito species are used by the viruses to reach their vertebrate host (Strauss and Strauss, 1994; Powers *et al.,* 2001; Weston *et al.,* 2002). However, to date no SAV intermediate host has been identified and instead its ability to remain viable in the water

column for long periods of time (Graham et al., 2007) is thought to aid in wide dispersal and horizontal transfer (Viljurgen et al., 2009).

1.4.4 History of *Salmonid alphavirus* Taxonomy

Before 2008 it was thought that *salmonid alphavirus* species consisted of three subtypes (Hodneland *et al.,* 2005; Weston *et al.,* 2005; Hodenland and Endresen, 2006; Karlsen *et al.,* 2006; Graham *et al.,* 2007), SAV1, SAV2 and SAV3. SAV Subtype 1 (SAV1) is the causal agent of pancreas disease in Atlantic salmon, *Salmo salar* L., farmed in Scotland and Ireland and was the first SAV to be isolated (Nelson *et al.,* 1995). Sleeping disease in trout species, primarily rainbow trout, *Oncorhynchus mykiss*, was shown to be caused by infection with subtype named SAV2 or SDV (sleeping disease virus) (Villoing *et al.,* 2000; Graham *et al.,* 2007). In contrast, Norwegian isolates belonged to a third distinct subtype named SAV3 or Norwegian salmonid alphavirus (Weston *et al.,* 2005; Hodneland *et al.,* 2005). It is important to note that both SAV1 and SAV3 may be referred to as SPDV, salmon pancreas disease virus.

A short time before the isolation of the causal agent of pancreas disease or sleeping disease it had been demonstrated that histological lesions of the pancreas, muscle and heart in their respective hosts were common features (Boucher and Baudin-Laurencin, 1996). However, it was not until isolates had been taken of these aetiological agents in 1995 (Nelson *et al.,* 1995) and 1997 (Castric *et al.,* 1997) respectively that further analyses could be carried out. Welsh et al. (2000) demonstrated in a comprehensive study, which followed on from initial work which identified SPDV as the first fish alphavirus found (Weston *et al.,* 1999), shared many of the characteristics of animal alphaviruses in terms of its RNA composition and protein. Meanwhile, Villoing *et al*. (2000) characterized SDV as an alphavirus. A comparative study was then carried out using the complete genomes of SPDV and SDV which demonstrated that they shared 91.1% complete sequence identity, with 95% amino acid identities over their non-structural proteins and 93.6% at their structural proteins (Weston *et al.,* 2002). In addition, Weston et al. (2002) also showed by viral neutralization

tests with sera from artificially infected salmon indicated that SPDV and SDV were closely related isolates of the same species and the name *salmonid alphavirus* was proposed. It was not until 2005 that a distinct SPDV strain was isolated from *Salmo salar* in Norway (Hodneland *et al.,* 2005) and named Norwegian salmonid alphavirus (NSAV) or SAV3 due to its possession of 91.6 and 92.9% nucleotide sequence identity to SAV1 and SAV2 respectively, host species and the pathological changes caused. However, more recently Fringuelli et al. (2008) demonstrated through phylogenetic and molecular epidemiological work on SAV which was based upon partial E2 and nsP3 gene sequences that there exists six distinct SAV subtypes which vary in geographical location (see Table 1-2). Isolates were obtained for the study (Fringuelli el al. 2008) from clinical samples of either heart or serum (either from associates' archived or new samples), except for three strains which were CHSE-214 cell culture. This was in order to minimize the risk of false results due to substitutions in the genomic sequences which has been shown to occur (Karlsen *et al.,* 2006) during SAV passage through cell culture (the three strains, French strains EE37 and VF03 and the Scottish strain F02-85 which were developed in cell culture were examined after two, four and five passages to check for substitutions). Phylogenetic analyses were carried out by unrooted neighbour joining trees and parsimony methods created using nsP3 and E2 gene fragment amino acid sequences independently and resulted in a high degree of agreement between the two with the classification of each strain remaining constant within both clusters (Fringuelli *et al.,* 2008). The three new identified subtypes (SAV4, SAV5, and SAV6) have significantly added to our understanding of *salmonid alphavirus* species. SAV4 was identified in samples from both Ireland and Scotland, and interestingly two isolates of this subclass (91-115 and 91-116) came from salmon raised in a farm in Donegal (Ireland) where SAV1 was also isolated (from 1993 samples) showing the presence of two subclasses in one farm in as many years suggesting two separate introductions of infection. SAV5 was described as endemic to Scotland as only samples from Scottish farms contained the subclass, whilst only one isolate (F/1045/96) fell into the SAV6 subclass which came from a PD outbreak in Connemara (Ireland), 1996.

1.4.5 SAV Subtypes: Geographical Location and Differences

These six identified SAV isoforms differ not only in their genetic sequence but also in their geographical location, the species which they infect and disease they cause (McLoughlin and Graham, 2007; Graham et al., 2011; Hjortaas et al., 2012). Table 1-2 shows this information of all isotypes which affect Atlantic salmon and trout. It is important to note that SAV 2 has also been shown to infect freshwater brown trout, *Salmo trutta*, causing sleeping disease (SD) but the main species affected, in economic terms, is rainbow trout (McLoughlin and Graham, 2007).

Table 1-2: The six subtypes of SAV. Table showing the six distinct isoforms of salmonid alphavirus (SAV) that affect farmed salmonids with the environment and location in which they occur as well as the species affected and the disease caused in these.

Isotype	Environment	Species Affected	Disease	Geographical Location
SAV1	Sea Water	Atlantic Salmon	PD	Ireland, Scotland
	Fresh Water	Rainbow trout	PD	Scotland
SAV2	Sea Water	Atlantic Salmon	PD	Scotland, Norway
	Fresh Water	Trout	SD	UK, France, Spain, France Italy, Germany, Croatia
SAV3	Sea Water	Atlantic Salmon	PD	Norway
	Sea Water	Rainbow trout	PD	Norway
SAV4	Sea Water	Atlantic Salmon	PD	Ireland, Scotland
SAV5	Sea Water	Atlantic Salmon	PD	Scotland
SAV6	Sea Water	Atlantic Salmon	PD	Ireland

The distribution of the different SAV strains is interesting in that they not only differ in the countries in which they are distributed but also there is considerable separation between regions within a country. For instance, in a comprehensive study of historical data of PD and SD infections in Scotland and Ireland by Graham et al. (2012) there was shown to be a regional separation in the most prominent subtype. This was most apparent in Scottish data with SAV1 being the most prevalent isotype in Argyll and Bute, SAV2 the main aetiological agent of PD in Shetland and Orkney, and SAV4 and SAV5 prevailing in the southern and northern Western Isles respectively.

For a long time SAV3 had thought to be the only SAV strain present in Norway causing PD in marine reared Atlantic salmon and rainbow trout, being endemic to the country. However, SAV2 was recently identified in the north-western part of the country (Hjortaas et al., 2013) and, despite being initially thought not to be widespread in Norwegian aquaculture, has now been identified at a number of new sites in the past year leading to the establishment of an SAV2 endemic zone (Jansen et al., 2014). This zone is just north of the long established SAV3 endemic region and interestingly there appears to be a clear separation in geographical location of observations of marine SAV2 and SAV3 with SAV3 occurring more in the South, although there does appear to be a spreading of SAV2 and given that many sites in the SAV3 endemic region do not subtype isolates it is possible SAV2 is becoming ever more prevalent. This analysis also demonstrated temporal differences in prevalence, with marine SAV2 being most prominent in October and November in contrast to SAV3 which is routinely identified between February and July, where its identification peaks, but is almost absent later in the year, thus indicating some variation in the properties of SAV subtypes.

Indeed, Jensen et al. (2014) identified lower associated mortality and lower severity of clinical signs when PD was caused by SAV2 infection when compared to SAV3. This finding is supported by a previous experimental trial by Weston et al. (2002) reported that, Atlantic salmon were infected with SDV (now known to be SAV2), they had less severe lesions in a number of tissues compared to when

they were infected with SPDV (it is unclear which specific subtypes were used). Moreover, in a comprehensive infection challenge trial carried out by Graham et al. (2011) the effects of infection with the six known SAV subtypes were recorded. This study identified differing levels of PD associated pathological damage to tissues with SAV1 and SAV3 causing the most severe, followed by subtypes 4 and 5, but SAV2 and SAV6 causing mild histopathological changes. Similarly, viral loads in the heart tissue, determined by RT-PCR, found that subtypes 1 and 3 had the highest load, SAV4 and SAV5 had possessed intermediate levels with SAV2 and SAV6 the lowest. These results indicate carrying levels of virulence in SAV strains and also found varying rates of horizontal transfer within treatment groups.

1.4.6 Virus Transmission and Biophysical Properties

The *salmonid alphavirus* species is a typical alphavirus in terms of its structure and genome (see figure 1-1) (Powers *et al.,* 2001; Weston *et al.,* 2002), however, subtle differences have been highlighted between SAV and other described alphaviruses. The study in which the species name was proposed (Weston *et al.,* 2002) discovered that subtypes SAV1 (SPDV) and SAV2 (SDV) when compared to other alphaviruses share only 41.7 to 43.6% sequence identity within their non-structural polyprotein and just 31.3 to 33.7% in their structural polyprotein. These values are much less than those observed when amino acid identities between the mammalian alphaviruses are compared. Despite this, SAV sequences do possess most or all of the conserved sequence elements which are believed to play an important role in virus replication and life cycle (Weston *et al.,* 2002). Possibly the most significant difference between the *salmonid alphavirus* species (and its subclasses) and other alphaviruses is that, until now, the intermediate host which it uses has not been agreed (Powers *et al.,* 2001; Snow *et al.,* 2010).

Two invertebrate species which have been studied in order to identify the intermediate host the bivalve mollusc *mytilus edulis* (mussel) and the arthropod *Lepidophthirus salmonis* (sea louse). Experimental infection of mussels was

carried out in the University College of Cork (Graham *et al.,* 2008) and tissue samples taken from individuals over a 20 day period for RT-PCR SAV screening to test if the mussels were virus positive. The results from this study showed that whilst 40% of gills and 60% of hepatopancreas samples were positive at day zero (immediately after infection); by day five post infection no tissue samples were positive. Such a short duration of positivity of SAV in mussel tissues shows that the alphavirus is unable to establish an infection within the host to proliferate, thus positive results at days zero, two and three were due to bioaccumulation and contamination meaning that they pose no threat to salmonoids in terms of harbouring the virus. It is also plausible sea lice are an intermediate host of the virus as Atlantic salmon that show the clinical/ behavioural signs of pancreas disease often have a higher than average sea lice burden (Rodger and Mitchell, 2007). In addition, another recently discovered aquatic alphavirus SESV (Southern Elephant Seal Virus) has been shown to be transmitted to the seal via the seal louse *Lepidophthirus macrohini* (Linn *et al.,* 2001). Despite this, the literature is not fully in agreement on whether sea lice are the intermediate host of SAV or even if an intermediate host is involved in SAV transmission. For instance, in Norway SAV3 has been reported in lice using the RT-PCR method of identification (Petterson *et al.,* 2009), whilst Weston et al. (2005) and Karlsen et al. (2006) have also described sea lice as a possible vector. Rodger and Mitchell (2007) studied the link between PD and sea lice infestation in Ireland in which they found a significant link from data collected in 2003, which may suggest lice are a vector of SAV. However, the study also used data from 2004 in which no significance was found. Therefore, given that evidence for a sea lice vector of SAV transmission is minimal it is not generally thought to be the major transmission route, if at all.

The possibility of vertical transmission, from broodstock and eggs and fry has also been studied in response to the reporting of SAV2 vertical transmission (cited as personal communication by J. Castric in McLoughlin and Graham, 2007; Kongtorp *et al.,* 2010) and SAV3 infection discovered at a smolt producing site in Norway (Nylund *et al.,* 2003). However, natural outbreaks of PD have only been reported in the seawater phase (McLoughlin and Graham, 2007) and information regarding vertical transmission of freshwater SAV2 in rainbow trout has not been

published. In light of this, it would seem that if vertical transmission does occur its impact would be negligible with horizontal transmission being by far the most important cause of viruses spread (Rodger & Mitchell, 2007; Fringuelli *et al.,* 2008; Kristoffersen *et al.,* 2009; Bratland and Nylund, 2009). It has recently been shown that shedding of SAV into the environment is directly linked with viraemia (Andersen *et al.,* 2010). Moreover, natural outbreaks of PD in Atlantic salmon have only been reported in the seawater phase of production. In light of this, it would seem that if vertical transmission does occur its impact would be negligible, with horizontal transmission being by far the most important means of virus spread with shedding of mucus and faeces being recently described as transmission routes for SAV.

It has been shown that SAV can be spread directly from fish to fish (primary host to primary host) through shedding and in faeces (McLoughlin *et al.,* 1996; Andersen *et al.,* 2010). Furthermore, it has recently been shown that virus is still present in organ tissues many months after an individual is infected with SAV (Graham *et al.,* 2010) which could hypothetically be transmitted. This may not only explain why susceptibility to and severity of PD increases with higher stocking densities of cages and farms (Tri Nations Report, 2005) but may also point to wild fish stocks acting as a reservoir. This hypothesis of a wild reservoir has also been concluded from evidence that SPDV tends to recur in successive generations of *Oncorhynchus mykiss* when they are introduced into areas where SAV has previously been discovered, even if thorough management practices have been implemented, such as fallowing (McLoughlin and Graham, 2007). It is of course the possible that SAV-like species are naturally found in marine and freshwater environments (which is the recent ancestor of the species described in aquaculture), but is also possible that fish farms have led to the establishment of potential reservoirs of re-infection, with wild infected fish passing the virus on to farmed stock via shedding and in faeces. However, despite SAV not yet being isolated from wild salmonids (Graham, 2005) Snow et al. (2010) has recently detected alphavirus RNA in wild marine fish. In this study screened fish were caught close to the Shetland Isles (Scotland), with screening carried out using RT-PCR assay targeting a region of the SAV nsP1 gene. Screening of SAV presence in tissues by qPCR has shown that the virus is able to persist for long

periods of time in kidney and heart tissue of Atlantic salmon (Graham et al., 2010). Positive SAV presence was also found in flatfish species; long rough dab *Hippoglossoides platessoides*, common dab *Limanda limanda*, and plaice *Pleuronectes platessa*. Following these results sampling was extended to Stonehaven bay (Scotland) where heart tissue samples tested positive from common dab. Analysis of the nucleotide sequences obtained (from further sequencing of the E2 gene region) has revealed minor differences between the strains found in the study and previously sequenced subclasses of SAV found in farmed salmon and trout, with SAV isolates found in wild fish most closely related to SAV subtype 5. However, it is at present unclear if there are any PD or SD disease interactions between a wild fish reservoir and farmed salmonids.

Interestingly Graham *et al*. (2007) has shown that SAV1 is able to survive in sterile sea water for up to two months at lower temperatures. This information suggests that in favourable conditions the virus could spread between cages or even farm to farm without any animal host. In light of this the first statistical model on the spread of PD in salmon farming to incorporate hydrodynamics as a causal agent (by carrying the virus) was developed by Voljurgein et al. (2009). Despite the possession of a limited dataset, it was demonstrated that SAV in the marine environment is transmitted via water currents by passive drift and thus could be used, with knowledge of local water contact relationships, to predict and thus implement targeted control measures to limit the impact caused. More recent analysis of hydrodynamics and transmission dynamics cites water movement as the largest contributor to transmission of the virus over contact networks (i.e. distance and sharing of equipment) (Stene et al., 2014). Therefore, while little can be done to prevent spread from farm to farm, which are connected in terms of water movement, elevating the detrimental effects of PD, and indeed any other disease, is reliant on both rapid detection of the virus/ disease and the implementation of disease management strategies.

1.5 Diagnostic tools and Disease Management Strategies

1.5.1 General

The armamentarium for diagnosing infection and disease in finfish aquaculture has grown significantly in recent years (Adams and Thompson, 2014). However, many novel methodologies are not implemented widely for a number of reasons such as being too expensive and the need for a high level of skill. Therefore, the industry, in the main, relies on a select few diagnostic tools to diagnose infection and disease. In terms of salmonid aquaculture the industry relies on virus isolation, PCR, serology, gross pathology, and histopathology for diagnosing viral disease and infection (McLoughlin and Graham, 2007; Jansen et al., 2010; Adams and Thompson, 2011; Adams and Thompson, 2014). These diagnostic tools have increased in use as the salmon aquaculture industry moves away from reliance on behavioural signs of disease to a more proactive approach. However, despite all of their advantages each of these approaches has limitations, out with technical drawbacks such as sensitivities and risk of false positives, which shall be discussed.

1.5.2 Virus Isolation

Virus isolation in cell culture is a well-established tool for identifying viral infection in salmonids where the aetiological agent is isolated traditionally from tissues (Christie at al., 1998; Graham et al., 2003). Following initial isolation a number of downstream applications can be used for identifying viral particles such as immunostaining techniques with virus specific monoclonal antibodies (mAbs) (Todd et al., 2001) for visualization of the virus in culture. However, such immunostaining is qualitative or at best semi-quantitative for viral load and relies on the aetiological agent being well defined. Moreover, it relies on the availability of specific mAbs to the virus which if not commercially available are both expensive and time consuming to produce. Viral load can be determined at a quantitative level by viral titre tests which are common place using most commonly cytopathic effect (CPE) (Knüsel et al., 2007) or alternatives such as heterologous interface (Desvignes et al., 2002). However, during cell culture there can be a number of contamination factors and relies on standardized

procedures between labs for trusted industrial data. Moreover, virus isolation from tissues is destructive, meaning that the nature of sampling causes a reduction in benefit of early diagnosis due to economic costs of the technique. This limitation is somewhat overcome by the use of non-destructive virus isolation through the use of blood derived sera (Jewhurst et al., 2004). Despite this, there is a comparatively short time window when viruses can be detected in the humoral system (viremic stage) requiring early sampling after an infection event. Furthermore, tests such as virus isolation from either tissue or sera can only indicate infection which may or may not necessarily result in clinical disease (Johansen, 2012).

1.5.3 Polymerase Chain Reaction (PCR)

By far the most common molecular method for determining the presence of a given virus is polymerase reaction (PCR) (Adams, 2009; Adams and Thompson, 2011; Adams and Thompson; 2012). Reverse transcription-polymerase chain reaction (RT-PCR) is well established as a diagnostic tool for a number of infections in salmonids demonstrating a higher sensitivity than direct virus isolation (Knüsel et al., 2007). There have, for example, been numerous conventional RT-PCR tests used to detect SAV which amplify various regions of virus RNA (McLoughlin and Graham, 2007) with conventional methods being qualitative (Villoing *et al.,* 2000) whilst more recently developed methods allow for quantitative results of viral load in an individual (Christie *et al.,* 2007). RNA extraction for this methodology is most commonly carried out using tissues with the heart the best source of this when testing for presence of SAV as it can remain positive for the longest period of time, at least until 140 days post infection (Christie et al., 2007). Such sampling is of course destructive and thus other sampling methods have been investigated. Successful SAV RNA isolation is possible from serum, although similarly to virus isolation can only be detected for a short period of time post-infection. Interestingly another non-destructive source of viral RNA can be found in faeces and mucus (Graham et al., 2011). Despite the study only testing for a positive signal in these fluids in groups infected with isotypes 1, 3 and 6 it would be expected in infections with the others as these are thought to be transfer routes. However, persistence did not

seem to be longer than in previous studies which tested serum (Christie et al., 2007). The use of specific probes for amplification also has positive aspects in that specific typing can be carried out (subtype specific) but is limited by the need for aetiological agent being clearly defined, thus for diseases of unknown aetiology may not be applicable.

1.5.4 Serology

While virus isolation and PCR tests are diagnostic tools for direct pathogen detection, serology is an alternative methodology of identifying infection by identifying specific antibodies in serum (Adams and Thompson, 2011). This methodology is not well established in finfish aquaculture except for in PD where the presence or absence of specific virus neutralizing (VN) antibodies is tested (McLoughlin et al., 1996) and has advantages over virus isolation and molecular methods as immunoglobulins persist in the humoral system much longer. However, there appears to be variation in the time taken for seroconversion (presence of VN antibodies) depending on the infectious isotype (Graham et al., 2011), thus meaning that clinical disease may already have occurred before identifying a sample positive for antibody. In addition, serological tests cannot distinguish presence of antibody between infected and vaccinated fish (Adams and Thompson, 2011). Given that vaccination against PD is commonplace in Atlantic salmon aquaculture means this is a potential problem. Moreover, as with virus isolation and PCR tests previously discussed, serology requires aetiology to be well defined and presence of VN antibodies due to infection may not necessarily indicate clinical disease.

1.5.5 Gross Pathology

Gross pathological signs of disease are those that can be identified through macroscopic assessment of a tissue or body cavity. Such assessment is an important on site means of assessing general fish health and is carried out routinely. Possibly one of the best examples of this is the non-destructive assessment of gill health for the diagnosis and monitoring of AGD. This approach

has been shown to be reasonably accurate and consistent with histopathological changes which are occurring at the time (Adams et al., 2004) with a number of semi quantitative scoring systems being used throughout the industry. However, while some non-destructive gross pathological signs for viral diseases are identifiable most significant manifestations of the disease occur in internal organs and tissues (see sections 1.3 and 1.4) meaning that pathology identification is destructive. Moreover, it has been shown that these are highly varied between individual and may or may not present themselves at a given stage of disease. Therefore, histopathology is the gold standard in diagnosing clinical disease.

1.5.5 Histopathology

By far the most well established methodology for disease diagnosis in salmon aquaculture is histopathology, where sectioned tissue samples (after fixation, waxing, cutting, and staining) are examined under a microscope for clinical manifestations of disease. In this way damage to specific tissues can be determined in both a qualitative and semi-quantitative manner thus describing the nature of clinical disease and also is an important tool for investigating diseases of unknown aetiology. In addition, when the pathogen is suspected, immunohistochemistry (IHC) can also be carried out on sections to stain, using specific antibodies, as in virus isolation, virus in the tissue. Despite this, histopathology is a laborious and time consuming process which takes a high level of skill for proper assessment. In addition, direct costs are added to significantly, as with all destructive diagnostic tools, by the effect of fish being removed from the population therefore not going to harvest. Therefore, it is not economically viable for proactive monitoring of health at a population level in this way and it is usually not until a problem is suspected by another means it is used. In addition, due to the sequential pathology of many diseases, such as PD, all disease indicators may not be visible at the same time point (McLoughlin *et al.,* 2002) meaning that repeat sampling may be needed over a period of time again adding to cost. Thus there is a significant demand within the industry for non-destructive means of health assessment which are capable of diagnosing clinical disease.

1.6.1 Disease Prevention and Management

For the limitation of the negative effects of infectious disease to the industry there are a number of general and specific preventative measures which are carried out. For example, general bio-security is becoming more rigorous in all stages of production from hatchery to slaughter (Munro et al., 2003; Delabbio et al., 2004) with disinfection procedures, quick removal of dead fish, pre-entry disease testing of new stock, and record keeping of a number of analytes all being highly important in preventing outbreaks (Delabbio et al., 2004). By far one of the most successful preventative measures against infectious disease to date has been vaccination with considerable success in giving protection against a number of infectious agents (Sommerset et al., 2005; Hølvold et al., 2014). However, there remain a number of issues of vaccine efficacy (Kibenge et al., 2012; Gudding and Van Muiswinkel, 2013), with many vaccines available in salmon aquaculture against viral infections, e.g. SAV, which may lessen disease severity but do not fully protect fish (Hølvold et al., 2014). Therefore, it is paramount that a high level of biosecurity and regular monitoring of fish stocks is implemented. Moreover, when infection and or disease is identified, disease management strategies are put in place not only to lower the effects of the specific outbreak but also to lessen future impacts in new cycles.

Disease management strategies are numerous and to an extent similar to preventative steps as they aim not only to reduce morbidity and mortality of the given outbreak but also to prevent the spread of disease to other sites/ populations. Moreover, they are not, except for in cases where specific treatments can be utilized, necessarily disease specific. For instance, there are currently no antiviral treatments available for treatment of salmon when infected with a viral pathogen. Therefore, many of the management/ prevention tools set out in PD literature may be extremely useful in dealing with other viral disease outbreaks: avoidance of movement of equipment or livestock between infected and uninfected areas; separate diving staff for each site; sites which have had a history of PD when using S0 should consider switching to S1 stock; reducing stocking numbers in cages when PD is present; smolts entering a site where there has been a history of PD should be injected with PD vaccine; sites

or bays with a history of smolt strain susceptibility should consider not using certain genetic lineages; and farm sites with a history of severe PD should consider both reducing or completely ceasing livestock exportation to other sites, and fallowing of the water body (mandatory in Norway) (McLoughlin *et al.,* 2003; Jansen *et al.,* 2010). Despite this, a combination of factors including horizontal transmission, the biophysical properties of viruses, and the role of hydrodynamics in viral spread we do not seem to be suppressing the effects of viral diseases in Atlantic salmon (Robertsen, 2011) at present. Therefore, a number of advanced techniques are being investigated to try and lower the impacts of infectious disease, for example, breeding for resistance to varying levels of success. However, one of the most promising disease management tools for lowering the negative impacts of infectious disease is the use of functional/ protective feeds.

1.6.2 Functional Feeds

Nutrition is pivotal to the production of all livestock where specific nutritional requirements must be met at specific points in time for the production of adequate product for the consumer. Indeed it is paramount for the health and welfare of fish in aquaculture, where great strides have been made improving productivity and the product itself (Trichet, 2010; Tacchi et al., 20012). Moreover, there has in recent years been a realisation that altering the diet composition of feed during infection and disease can have major benefits to health, reducing morbidity during disease or even preventing it (Trichet, 2010). The usefulness in this form of clinical nutrition is well established and is achieved either by supplementation or reduction in specific compounds to supplement or modulate the immune system to give a favourable outcome (Tacchi et al., 2012). A number of different compounds have been shown to have such effect in aquaculture including among others numerous fatty acids, free amino acids, free nucleotides, antioxidants, carotenoids, minerals, carbohydrates, and ß-glucans (reviewed in Trichet, 2010). Moreover, the application of these functional feeds is becoming ever more common in Atlantic salmon disease. For example, their use has been shown to reduce sea lice burden in an experimental challenge trial by Provan et al. (2013), where

epidermal mucus proteome was found to be significantly altered thus indicating an increase in synthesis of proteins important in lice detachment. In addition, the severity of inflammation during both CMS and HSMI has been shown to be reduced significantly when fish were fed diets with lower, compared to standard core diet, dietary lipid and fatty acid composition which were shown to have significant modulation effects on the immune system (Martinez-Rubio et al., 2012; Martinez-Rubio et al., 2014). However, while the severity of pancreas disease in Atlantic salmon has been shown to be linked to dietary levels of vitamin E and polyunsaturated fatty acids (Raynard et al. 1991) there is little recent published evidence of how nutrition can aid in reducing morbidity of the disease.

1.7 Aims of Study

Despite the clear advancements in Atlantic salmonaquaculture since its inception it is clear that the industry still faces a number of challenges which threaten its expansion and sustainability. For instance, infectious disease has serious economic impacts through resulting mortality and morbidity. Such impact can be limited through identification of any issue as quickly as possible for the implementation of any disease management strategies or treatment available. However, while current non-destructive means of health monitoring they rely on the identification of aetological agents. This, from a disease monitoring standpoint, can cause problems for the industry as infection may or may not result in clinical manifestations of disease which is a crucial consideration for disease management tools, such as administering functional feeds. Therefore, this study aims to identify serum markers of disease for the non-destructive assessment of fish health.

Using a pancreas disease (PD) cohabitation disease trial fish are exposed to salmonid alphavirus subtype 3 with serum and tissue samples for histopathological assessment taken at a number of time points. Moroever, the effects of nutrition on disease severity will be examined with two treatment groups with one receiving a commercial diet and another enriched with Vitamin C, vitamin E, schort chain fructooligosaccharide, mono oligosaccharides, and nucleotides. Inital investigation of markers of

pathological damage shall be through the use of established and novel (to Atlantic salmon aquaculture) biochemical assays of analytes. Subsequently the study aims to examine the changing serum proteome during the disease using a gel based proteomics approach to identify both markers of the immune response to infection and markers of specific tissue pathologies. Validation of results will be carried out for targets using other techniques such as western blotting and assays. The development of such assays also aims to make novel tools of assessing pathological damage for use in industry.

Chapter 2

Disease Trial, Histopathology and Blood Biochemistries

2.1 Introduction

2.1.1 Infectious Disease Trials

Horizontally transmitted diseases in Atlantic salmon, *salmo salar*, aquaculture are some of the most common and economically significant problems we face in striving for higher sustainability. While we can increase our understanding of these diseases through investigation of natural outbreaks, there are a number of limiting factors in doing so. Namely these include, but are not limited to, occurrence of multiple infections and or diseases, and the lack of control over other potentially mitigating factors, such as age and size of fish, and epidemiology at a given site between outbreaks. Therefore, the development of experimental infection/ disease challenge models has enabled for the control of these factors. Within this methodology there exist a number of different challenge methods (Nordmo and Ramstad, 1997) such as intra-peritoneal (i.p.) and muscular (i.m.) injection, bath treatment, and cohabitation protocols. Arguably the most readily standardized methodology of investigating disease is by either via intra-peritoneal or intra-muscular injection, where a titre of aetiological agent is injected into each fish for the investigation of a given outcome(s). This protocol not only allows for the standardization of magnitude of exposure each individual but also benefits in terms of comparison of different groups and individual variance as all individuals are exposed at the same time. However, this does not replicate true infection of infectious disease in salmon thus omitting initial defences of salmon to bacterial and viral particles, such as mucosa in the gills and body surface, which not only act as a physical barrier to pathogens but comprise a number of anti-infectious components (Sansonetti et al, 2004). Bath treatments, where fish are exposed to pathogens suspended in water for a given time, and cohabitation models, where naive population is exposed to diseased/ infected individuals, by definition allow for a simulation of natural outbreaks. However, bath treatments have proven difficult to administer as dosage must be high to ensure the successful infection of fish (Nordmo et al, 1997). Indeed the number of particles needed for bath protocols far exceeds the actual exposure dosage as one must ensure contact between pathogen and host which not only makes the methodology difficult to standardise and control 'infectious dosage' but also may exceed the culture capacity of a laboratory. In

addition, where the aetiology is not clearly defined or agent not easily cultured this methodology is unsuitable. Therefore, cohabitation disease trials have become an increasingly popular means of investigating infectious disease. This protocol has been carried out, when aetiology was not defined, for both CMS (Bruno and Noguero, 2009) and HSMI (Kongtrop et al, 2004) using tissue homogenates from farmed salmon diagnosed with the given disease to successfully establish the disease, not only in those fish subjected to i.p. injection but also cohabitation fish. However, when aetiology is defined it is possible to culture viral particles using cells for the downstream administering of a given titre of the agent. For instance, Chinook salmon embryo (CHSE-214) monolayers are widely used for the cultivation of SAV (Hjortaas et al, 2013), and as such have been utilized in cohabitation PD cohabitation trials previously. For example, Graham et al. (2011) have previously carried out a comparative study of all six known SAV (SAV 1-6) subtypes using a cohabitation trial where Trojan fish, were exposed to 10^3 $TCID_{50}$ of SAV (one out of the six) were introduced to naïve individuals. This procedure successfully simulated what is observed in aquaculture sites thus validating the methodology in investigating PD.

2.1.2 Application of Clinical Serum Biochemistry

The protein content of blood sera or plasma can give valued information on the disease state of an individual at a specific point in time and in a non-destructive manner. Biochemical analysis of sera (serum biochemistry) exploits the characteristics or function of proteins to infer a given proteins concentration. Such an approach is termed clinical pathology and has the ability to aid in the identification of a number of conditions such as pathological damage when a targets level is above or below homeostatic levels. Moreover, certain enzymes, with variable sensitivity and specificity, are linked with specific pathologies and conditions.

Indeed clinical pathology is routine in domesticated animals (Kaneko et al, 2008) and is used in conjunction with other methodologies to diagnose a multitude of diseases. Often in terms of livestock this form of investigating pathological diseases due to the economic worth of the animal at an individual level is

advantageous as it avoids unnecessary slaughter which also has an animal welfare advantage. Despite this, the application of clinical pathology has been severely limited in aquaculture potentially due to a number of factors such as, the sheer number of stock on a site and comparative worth (to other livestock). Despite this, some investigations have used serum activities of enzymes to investigate disease severity and progression with creatine kinase (CK) activity being the most common. For instance, Rodger et al (1991) and McLoughlin et al (1994) showed that during pancreas disease CK levels in the sera were significantly raised due to associated pathological damage. In addition, more recently Yousaff and Powell (2012) showed significant increases in CK activities during HSMI and PD. Moreover, there has been a surge of interest in the use of CK as a non-destructive marker of pathological damage to tissues within the industry with a number of institutions and companies now offering this as a service. Despite this, the usefulness of this enzyme and others (such as alanine aminotransferase and aspirate transaminase) has not been fully established and requires further investigation and validation.

2.1.3 Aims of Study

- Simulate natural pancreas disease outbreak through the use of a pancreas disease challenge model
- Assess its efficiency by standard diagnostic tools including histopathology, serology, RT-PCR, and virus isolation
- Investigate the usefulness of serum biochemistry in identifying pathological damage by non-destructive means
- Compare disease severity, using histopathology and serum biochemistry data, in fish fed different diets

N.B. Results of RT-PCR, serology, histopathology, and virus isolation in this chapter are published in Braceland et al. (2013).

2.2 Methods

2.2.1 Cohabitation Disease Trial

The following experimental procedure was approved by the Norwegian National Animal Research Authority (NARA) prior to the trial commencing and was carried out at Veso Vikan (Norway). The trial was carried out in 2010 prior to this PhD project starting.

Atlantic salmon (*Salmo salar*) parr of mean weight 30g (<15% CV) were randomly distributed into duplicate 1m^3 tanks (=1400 total fish). Following a six week acclimatisation period, the fish were fed to a target of 1.5% body weight per day. A commercial formulated feed (CPK 2mm; 3mm, with oil/ protein at 22/48, BioMar AS, Denmark) was used as the base diet for enrichment and was offered to fish throughout the experiment as a control diet. See Table 2.1 for information on diet enrichment compounds used. Water temperature was maintained at 12-14 ±1ºC, water flow 0.8l/kg min, and light / dark regime 12:12 hr. After 42 days, 60 fish from each duplicate tank were transferred into triplicate (three tanks fed one diet ie diet triplicates)0.6 m3 tanks and water temperature was increased to 14±1ºC. Additional fish from the duplicate tanks were maintained separately to be used as Trojan shedders i.e. challenge tanks all containing 120 fish (with Trojan fish to be added later). Naïve fish to be used as Trojans were marked by clipping their adipose fin and injected with SAV 3 infected CHSE cell culture supernatant at ca. 10^5 TCID/fish into their intraperitoneal cavity. Thirty inoculated Trojans were added to each of the challenge tanks 6 days after their assembly. The challenge and time course of sampling was staggered between replicate tanks over three consecutive days and kept constant at each sampling. Cohabitant fish were sampled at 0, 2, 3, 4, 5, 6, 8, 10 and 12 weeks post challenge (wpc). At each time point 9 fish per tank were killed by lethal overdose of anaesthetic benzocaine chloride (Apotekproduksjon AS, Oslo, Norway), 1g/10L water for 5 minutes being used and blood collected in non-heparinised vacutainers for analysis of serum biochemistry and serological and virological analysis. From 6 of these fish, pyloric caecae and pancreas (hereafter referred to as pancreas), heart and skeletal muscle tissue were

processed from standardised locations for histology. RT-PCR was also carried out using heart samples for SAV3 detection. In addition, a small section of liver from each fish was placed in RNA later for genomic analysis. Fish sampled at time point 0 were removed from the tanks before the addition of Trojan shedders. Figure 2.1 shows a simplified schematic diagram of this disease trial.

Table 2-1: Diet enrichment information.

Diet	Enrichment
A	N/A (control)
B	Vitamin C (1000ppm), Vitamin E (400ppm), scFOS, MOS, neucleotides

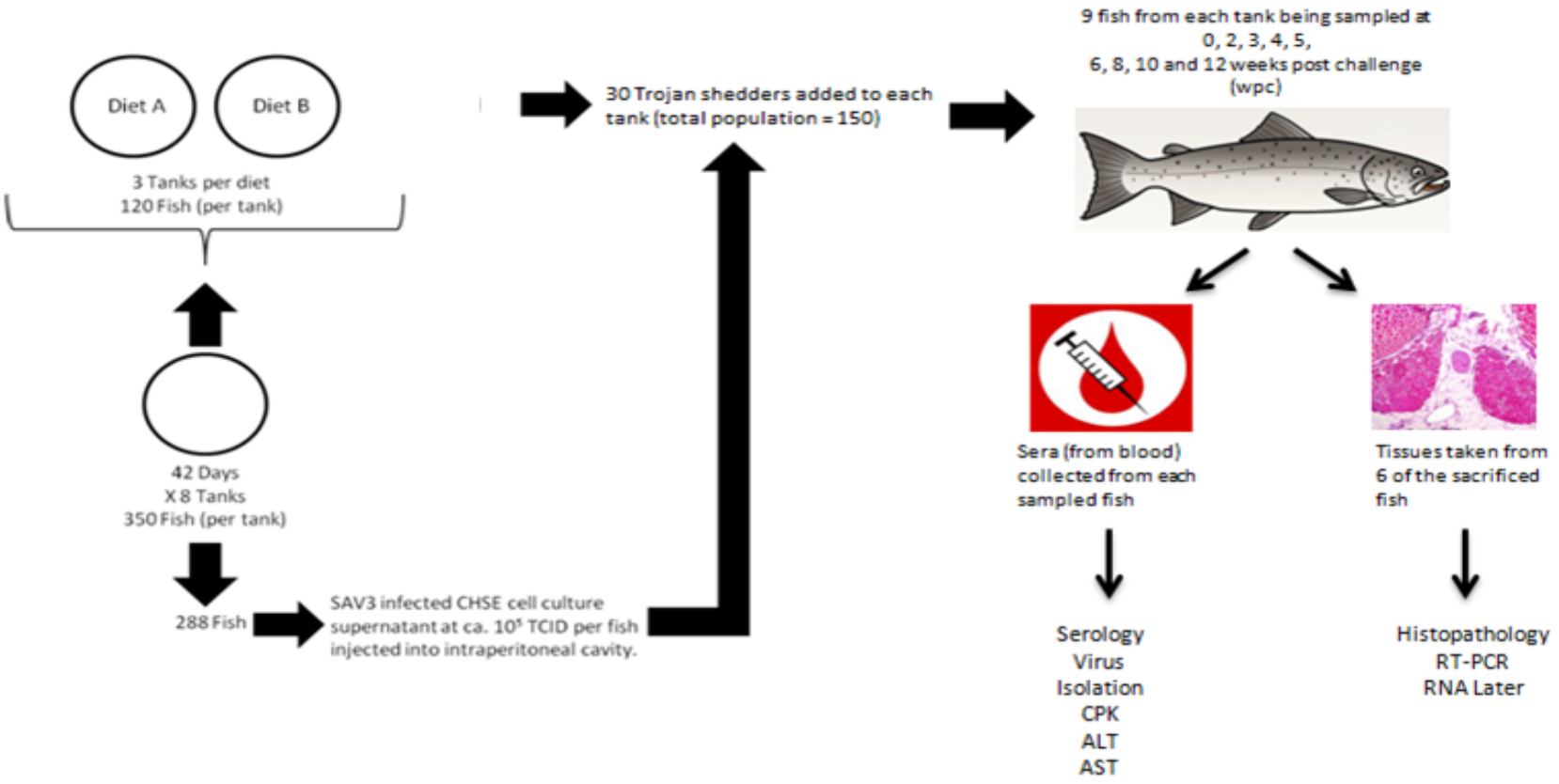

Figure 2.1: Simplified schematic diagram of cohabitation pancreas disease trial, sampling information, and initial analysis of both tissues and sera that were carried out and contained in this chapter.

2.2.2 Virological, Serological and RT-PCR Testing

The following tests were carried out at Agri-Food and Biosciences Institute (AFBI) by staff of the Fish Diseases Unit as described in Graham et al. (2011).

Detection of SAV3 infection was carried out by attempting to isolate SAV and detection of virus neutralizing antibodies from the sera of fish at 0, 4, and 5 Wpc. To confirm that fish were SAV free at the start of the experiment the viraemia of the serum was investigated. Reverse transcriptase polymerase chain reaction (RT-PCR) was carried out with heart tissue from sacrificed fish from W2pc to W5pc to investigate the horizontal spread of infection.

2.2.3 Histopathology

Histopathological scoring of tissues was carried out by Marian McLoughlin (Aquatic Vet Services, Belfast, UK).

Tissues for histology from the salmon were immediately fixed in 3.5% v/v formaldehyde in buffered saline pH 7.0 (4.0g NaH2PO4.2H2O, 6.5g Na2HPO4.2H2O) prior to further processing by standard paraffin wax techniques, sectioned and stained with haematoxylin and eosin (H & E). The tissue sections were examined by an experienced pathologist and a scoring system was used to semi-quantify the distribution and severity of the tissue lesions in the pancreas, heart and skeletal muscle as used in previous studies (McLoughlin et al., 2006; Christie et al., 2007). Table 2-2 illustrates the differential scoring system used in more detail.

Table 2-2: Semi-quantitative scoring system for histopathological assessment of tissue lesions.

Tissue	Score	Description
Pancreas	0	Normal
	1	Focal pancreatic acinar cell necrosis
	2	Multifocal necrosis/atrophy of pancreatic acinar tissue, some remaining
	3	Total absence of pancreatic acinar tissue
Heart	0	Normal
	1	Focal myocardial degeneration and inflammation <50 fibres affected
	2	Multifocal myocardial degeneration and inflammation 50-100 fibres affected
	3	Severe diffuse myocardial degeneration and inflammation >100 fibres affected
Skeletal muscle	0	Normal
	1	Focal myocytic degeneration and inflammation
	2	Multifocal myocytic degeneration and inflammation
	3	Severe diffuse myocytic degeneration
	R	Repair

2.2.4 Serum Biochemistries

The serum concentrations of creatine kinase (CK), ALT and AST were assayed by AFBI (Belfast, UK) using an Olympus (model au640) auto-analyzer (Middlesex, UK).

Total serum CK activity was assayed using a standardised methodology in which the colour change of reaction mix was monitored at an absorbance of 340/660 nm using Olympus reagent to manufacturer instructions (OSR6279). Increase of absorbance is due to the formation of NADPH which directly proportional to the activity of CK in the sample. Activity of ALT of serum samples from this cohabitation disease trial were assayed using Olympus reagent number OSR6507. In short, consumption of NADH was monitored by a decreasing absorbance at a wavelength of 340nm; this is proportional to ALT abundance. The consumption of NADH was measured at a wavelength of 340nm, with a reduction of absorbance being correlated to AST activity. Reagents were purchased from Olympus (cat # OSR6509).

2.2.5 Statistical Analyses

Statistical analysis of the data provided by the cohabitational trial was undertaken as part of this thesis. A mixed model analysis of variance was used to analyse the data using the mixed procedure of SAS (SAS Institute, Cary, N. Carolina). Due to the repeated measurements, a first order anti-dependence covariance structure was specified for the residuals. The model fitted the effects of diet and time of sampling. The dependent variables were serum activity of the analytes or histopathological scores of tissues (separately). This was carried out to investigate the relationship of histopathology and serum biochemistry results against the diet fed to fish. In this way any significant correlations between results of the trial could be identified, for example between histopathology and analyte activity or diet and histopathology or analyte activity etc. In addition, any significant dietry difference was given according to time point and also overall effect.

3. Results

2.3.1 Virology, Serology and RT-PCR

Cohabitant fish were SAV free before introduction of Trojan shedders, as determined by virus isolation, virus neutralization and RT-PCR (Table 2-3). Post introduction there was a subsequent horizontal spread of the viral infection with the majority of sampled fish being infected by week 4.

Table 2-3: Percentage of positive RT-PCR, SAV isolation and virus neutralization samples throughout trial. The percentage of samples from salmon sampled at each time point that gave positive results for SAV infection detected by reverse transcriptase polymerase chain reaction (RT-PCR), virus identification (SAV) and virus neutralisation (VN) are detailed. '–' indicates that testing was not carried out

Sampling time point	**Percent of Positive Samples (%)**		
	RT-PCR	**SAV**	**VN**
W0	-	0	0
W2	75	-	-
W3	87	-	-
W4	97	13	27
W5	88	2	67
W6	-	-	-
W8	-	-	-
W10	-	-	-
W12	-	-	-

2.3.2 Histopathology of Pancreas Disease

Negligible mortality was observed during or pre-and post-trial. The development of lesions over time is illustrated in Figure 2.3 which shows mean lesion scores for each tissue at each sampling point (See appendices for individual fish information). Since this study was based upon a cohabitation model individuals were likely to be at different stages in the disease process at each sampling point due to variation in the time of infection. The pancreas was the first tissue to develop lesions at week 2 and was also the slowest to recover, with a minority of samples still not fully recovered by week 12. Conversely, the heart demonstrates an extremely quick recovery, with a peak in lesion severity in fish sampled in week 4 and then a rapid recovery. The histopathological damage to red and white muscle was more delayed with the peak damage occurring at 6 and 8 weeks respectively.

Histopathological assessment scores from each tissue (heart, pancreas, white muscle, and red muscle) from fish fed both diet A and B were compared statistically at each time point by mixed procedure GLM. Fish fed diet B had significantly lower lesion scores at specific time points during the trial (figure 2.3), as follows, in heart, pancreas and red muscle at W4pc ($p = 0.073$; $p = 0.0206$; $p = 0.0006$ respectively) and W6pc ($p = 0.0288$; $p = 0.014$; $p = 0.049$). There was also a significant reduction in lesion severity of the white muscle at W6pc ($p = 0.0331$). No other significant differences were observed between diets.

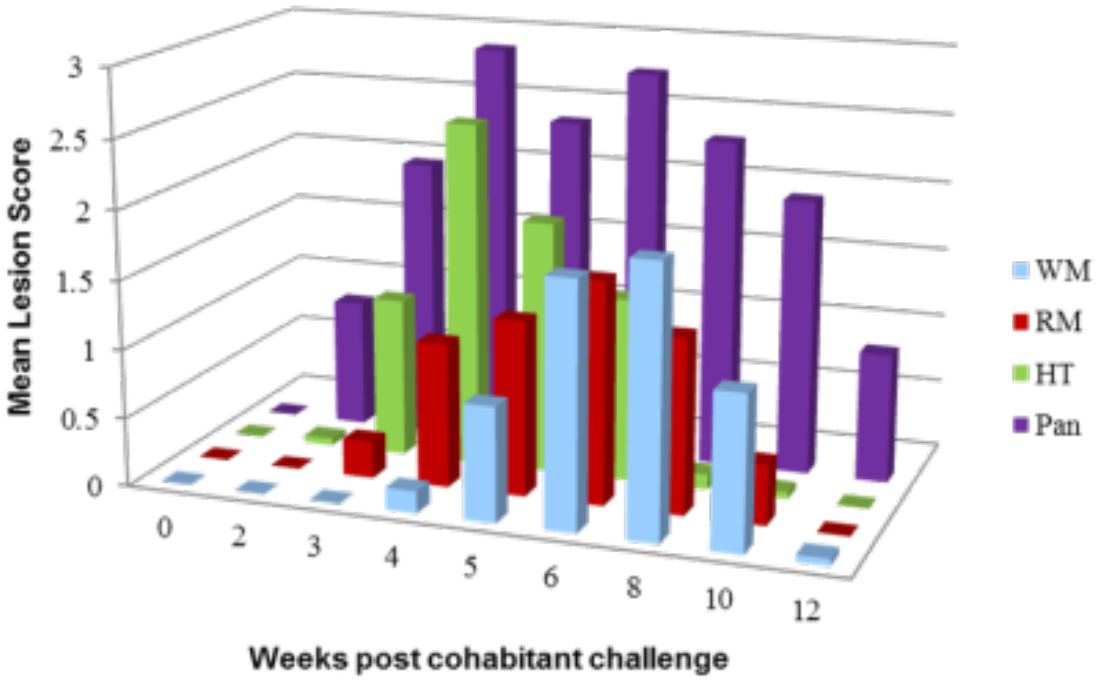

Figure 2.2: Mean histopathological scores of tissues during trial.
Histopathological scoring of mean lesion scores in relation to week post challenge, of all samples irrespective of diet fed, with SAV in pancreas (Pan), heart (HT), red muscle (RM) and white muscle (WM) of salmon (n=72 per time point).

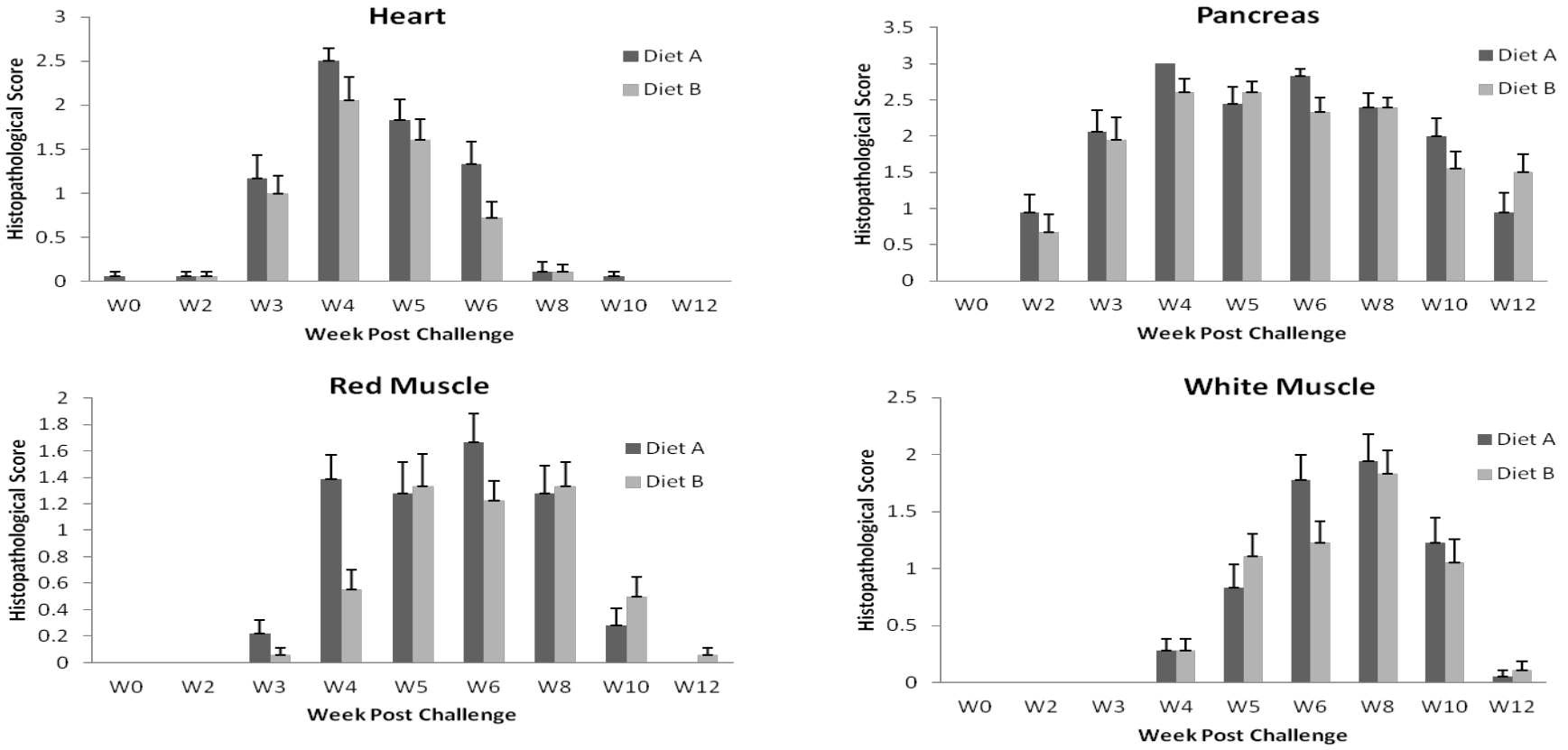

Figure 2.3: Dietary differences in histopathological damage to tissues. Dietary difference in mean histopathological score for each sampled tissue, from top left clockwise, heart, pancreas, white muscle, and red muscle at each sampling time point (weeks post challenge). Error bars indicate the standard error of the mean(SEM).

2.3.3 Serum Biochemistries

The serum activities of three analytes, creatine kinase (CK), aspartate transaminase (AST), and alanine transaminase (ALT), were assayed and their respective activities compared with; wpc, histopathological scores of fish, and diet effect investigated by sampling time point. As previously mentioned diets C and D were omitted from this analysis.

The mean serum activity values of fish fed diet A and diet B at each sampling time point (or wpc) with SE of the mean (in international activity units per litre: U/L) for CK, AST and ALT can be seen in FiguresFigures 2.4, 2.5 and 2.6 respectively. There was significantly lower serum activities in fish fed diet B at: W4pc and W6pc for both CK ($p = 0.011$; $p = 0.0076$ respectively) and ALT ($p = 0.0013$; $p = 0.0017$ respectively) (see Figures 2.4 and 2.6). In addition, significantly lower AST activities were observed in fish fed diet B at W6pc ($p = <0.001$) (Figure 2.5).

All three of the analytes correlated significantly with the histopathology results of lesions scores to skeletal muscle (white muscle correlation: $p = 0.027$; red muscle correlation $p = 0.009$) and heart ($p = 0.038$). However, analyte activities did not correlate with pancreas lesion scores therefore indicating that changes in serum activities of CK, ALT, and AST are not in due to a single tissue specific pathology rather appear to be influenced by pathological damage to skeletal muscle and the heart.

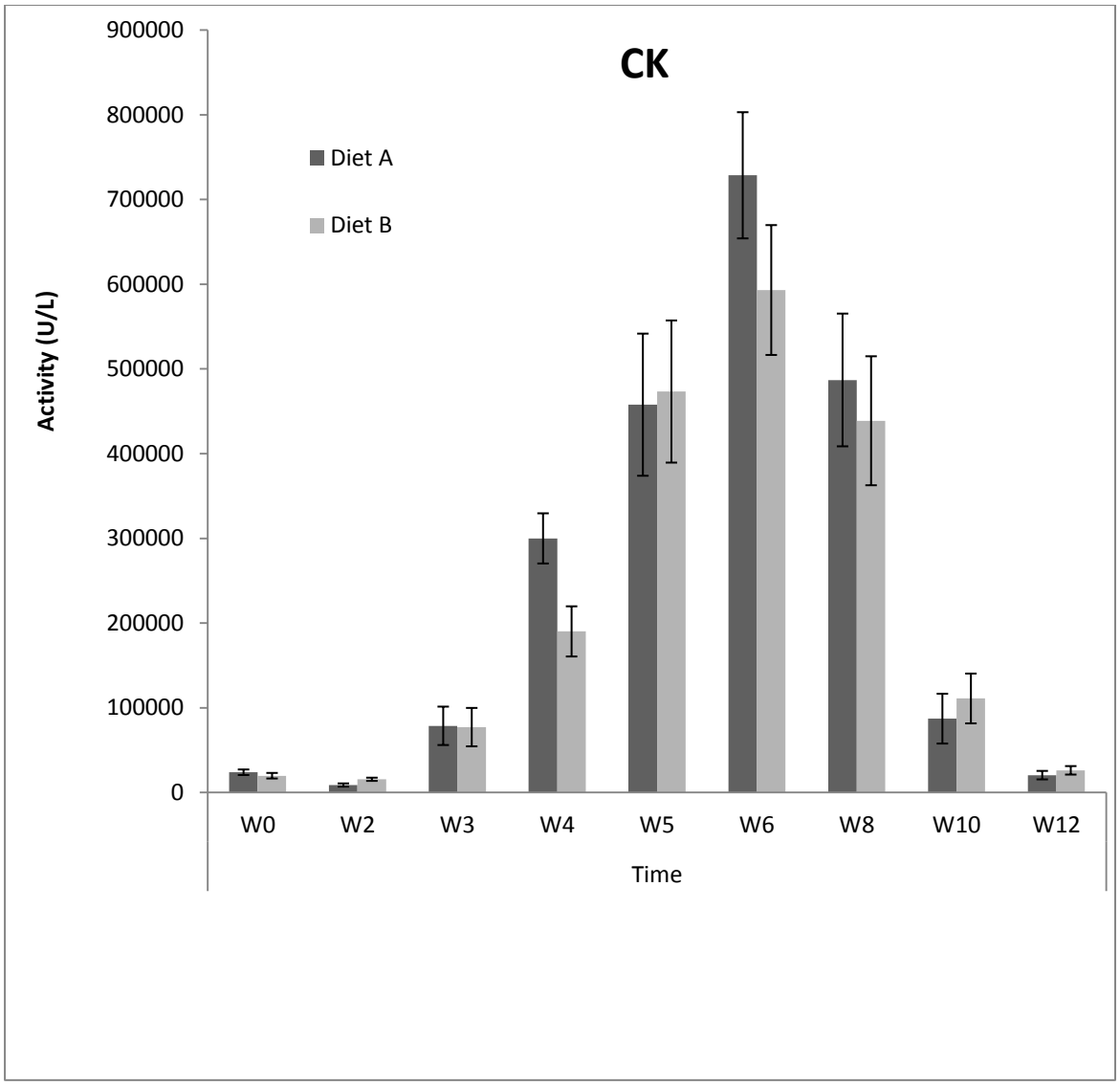

Figure 2.4: Serum creatine kinase (CK) activities. Mean serum activity of creatine kinase (CK) at sampling time points week 0 to week 12 of fish fed diet A and B separately. SE error of the mean is indicated by error bars.

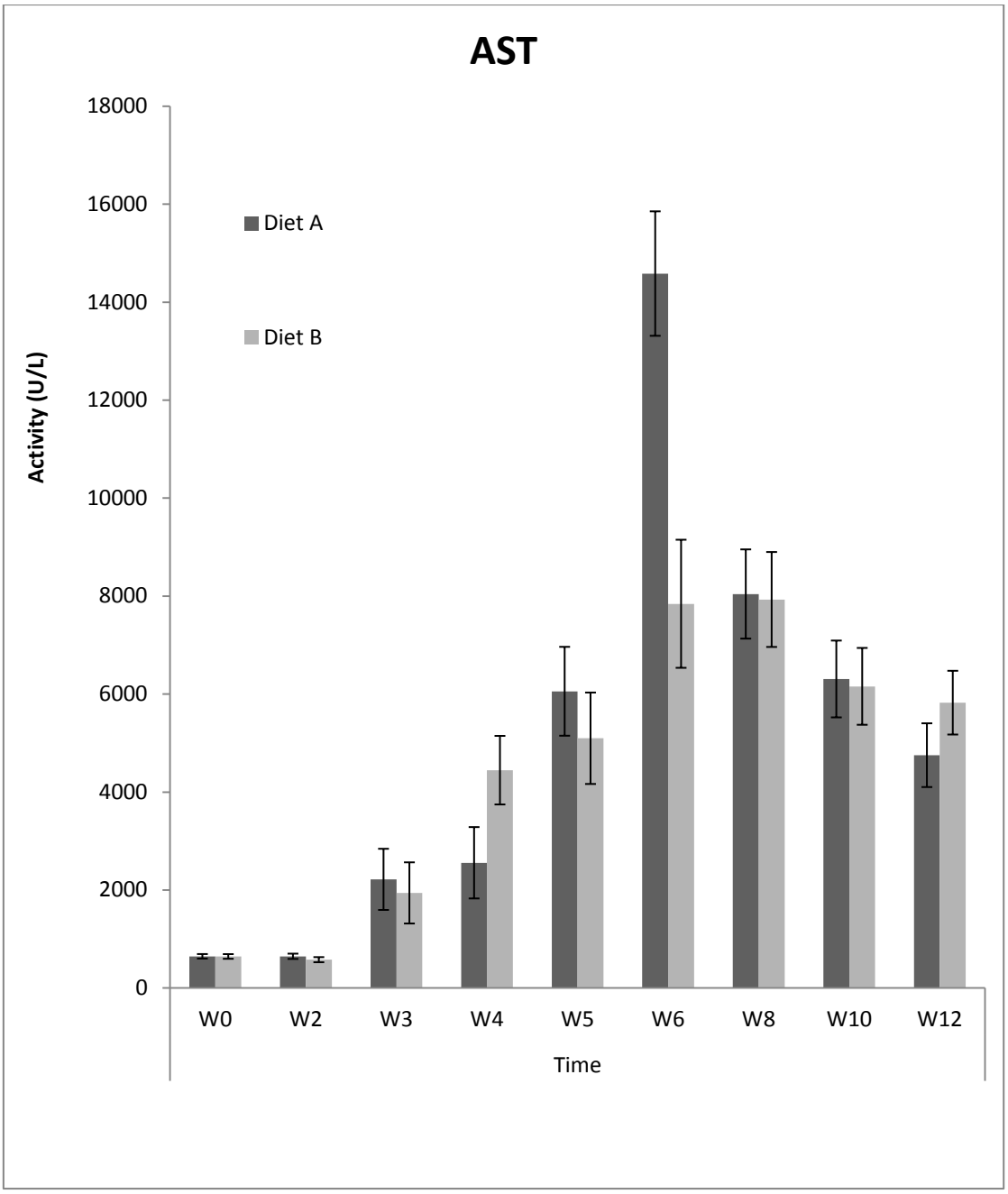

Figure 2.5: Serum Aspartate transaminase (AST) activities. Mean serum activity of aspartate transaminase (AST) at sampling time points week 0 to week 12 of fish fed diet A and B separately. SE error of the mean is indicated by error bars.

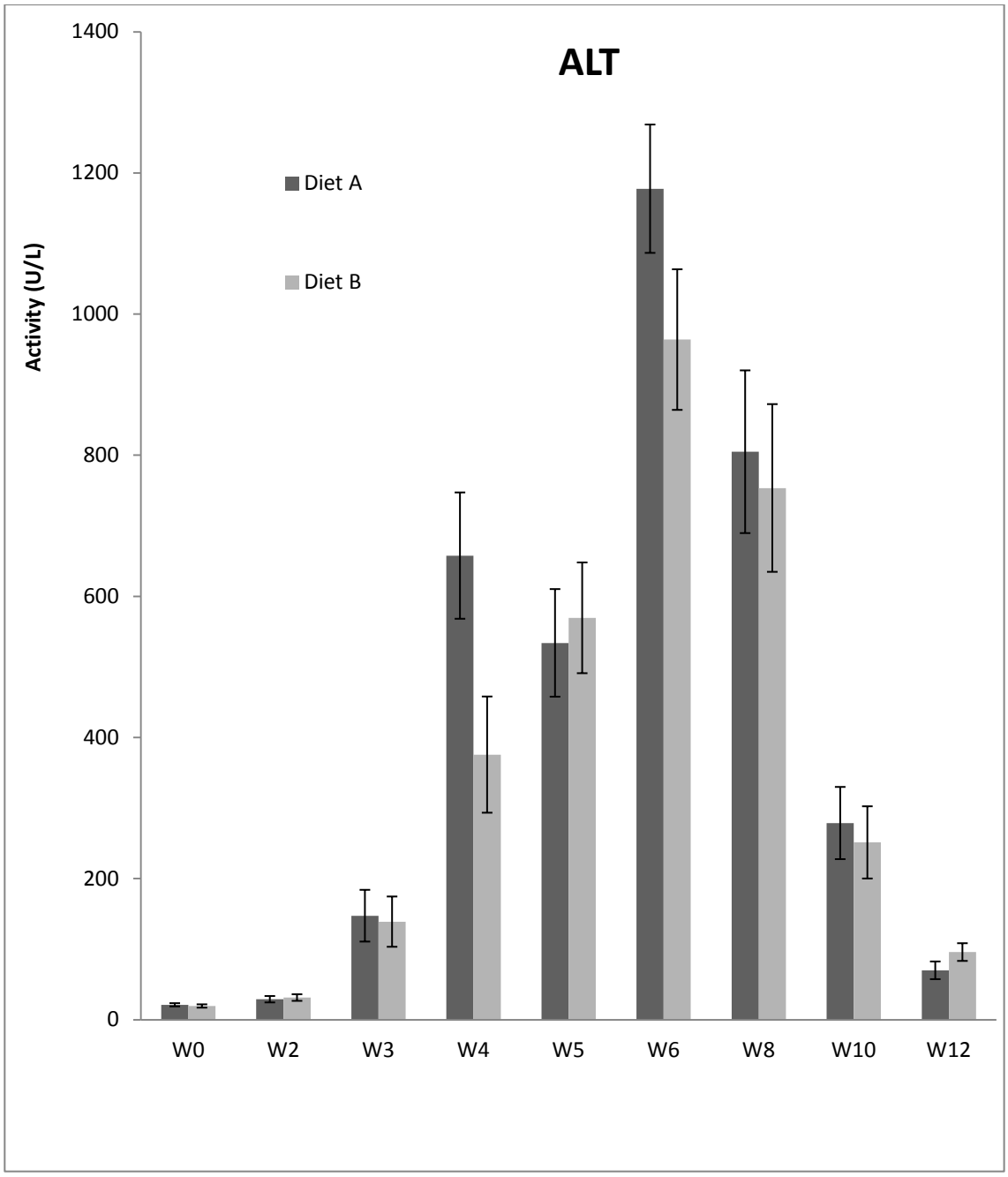

Figure 2.6: Serum alanine transaminase (ALT) activities. Mean serum activity of alanine transaminase (ALT) at sampling time points week 0 to week 12 of fish fed diet A and B separately. SE error of the mean is indicated by error bars.

2.3.4 Discussion

Creatine kinase, aspartate transaminase and alanine transaminase serum activities were assayed to establish their usefulness as markers of pathogenesis during PD. All three of these analytes (irrespective of diet) rose to a peak at W6pc. CK and ALT mean concentrations then fell to near basal levels by W12pc. However, whilst AST activities fell after W6 this decline was much less pronounced and levels remained relatively high even at W12pc. These profiles did not correlate with a specific tissue's pathology; rather activities appeared to be influenced by heart, red and white muscle damage. Conversely there was no significant correlation with pancreas histopathology scores. Thus indicating that these analytes may be useful as general health markers as a prognostic tool but would not be suitable in terms of diagnosing a specific infection, disease, or pathogenesis. In addition, it was found from statistical analysis of raw data provided that there was a significant overall reduction in ALT activity in fish fed a functional feed (B). Whilst no significant reduction in overall CK and AST serum activities was observed it is interesting to note that fish fed diet B had significantly lower values of CK at W4pc and W6pc (which is the same time points where there was a significant difference in ALT values) and lower AST activities at W6pc. This is supported by the observation of lower severity in pathological damage to tissues at these time points (W4 and W6pc) in fish fed diet B which is no doubt the cause for these lower analyte activities at these time points. In addition, it is possible that the lack of no significant overall reduction of severity is an artefact of the nature of statistical analysis of such data, where healthy scores are included as fish fed the control diet are also in good health at the beginning of the trial, but we observe that when pathological damage is at its worst functional feed reduces severity.

The functional feed in this study was enriched with a number of components, with levels of vitamin C, vitamin E, MOS, scFOS, and nucleotides increased compared to their dosage given in the control diet. All of these ingredients have in some way been shown to have an effect on fish in a positive way and as such

must be discussed in further detail. For example, increasing dietary levels of ascorbic acid in the form of vitamin C has been shown to stimulate growth and give protection against *Aeromonas hydrophila* in Asain catfish, *Clarias batrachus* (Kumari and Sahoo, 2005). Similarly enrichment of the vitamin to high levels has been shown to increase the resistance of channel catfish, Ictalurus punctatus, to *Edwardsiella ictaluri* infection (Liu et al., 1989). In addition, increased vitamin C has been shown in the salmonid species rainbow trout, *O. mykiss*, to stimulate the alternative complement pathway, and when used in conjunction with yeast derived glucans, to increase specific immune response following vaccination against enteric redmouth disease (Verlhac et al., 1996). Moreover, the vitamin has been shown in Atlantic salmon to have an effect on lysozyme, complement components and iron levels during bacterial challenge (WaagbØ et al., 1993). Therefore, enrichment of the vitamin is thought to be beneficial in fighting infection and disease having some sort of immune stimulant function. Vitamin E levels in feed have also been shown to have a major influence during disease of fish with deficiency in the vitamin shown to lower survival and lower stress tolerance, which is an important consideration in aquaculture, in gilthead seabreem, *sparus aurata*, juveniles (Montero et al., 2001). Raynard et al. (1991) also found that during challenge with pancreas disease fish that were fed inadequate amounts of vitamin E had more severe pathological damage than those who were fed adequate levels. One of the most rapidly growing areas of fish nutrition is the use of prebiotic and probiotics (Merrifield et al., 2010; Mohapatra et al., 2012; Song et al., 2014). This form of intervention is where microbes (probiotics) or stimulants for bacterial growth (prebiotics) are used to manipulate gut microbiota to give a health benefit to the fish, with a multitude of these being shown to give beneficial effects under a number of conditions. This study used a functional feed which was also enriched with the prebiotics mannoligosaccharide (MOS) and short chain fructooligosaccharide (scFOS) which are the two main compounds currently used as prebiotics in salmonid aquaculture (Tacchi et al., 2012). Mannoligosaccharide (MOS) have a profound effect on the immune system through the action of binding mannose receptor on the endothelium, which is a receptor for antigen receptor and presenting cells, and mannose binding lectins which activate the complement pathway (Tsutsui et al, 2006; Russell et al., 2009). Moreover, its use has been shown to have positive

effects on innate immune parameters of rainbow trout (Staykov et al., 2007) indicating its use may aid in fighting infection. Short chain fructooligosaccharides (scFOS) is well established in human and terrestrial species promoting growth and immunoregulation but is by comparison understudied in important aquaculture species with conflicting evidence of its effects (Song et al., 2014). Despite this, it has recently been shown to induce a number of innate immune responses in red swamp crayfish, *Procambarus clarkii*, (Dong and Wang, 2013). The addition of supplementary nucleotides has also been shown to have a range of effects in aquaculture species such as increasing total gut area (Burrels et al., 2001a) presumably aiding in the uptake of nutrients which would aid FCR and growth. Moreover, the inclusion of free nucleotides has been shown to increase resistance to bacterial, viral, and ectoparasitic infections (Burrels et al., 2001b). While it is not possible to explain the precise mechanisms by which the functional feed has given this reduced severity of tissue lesions and resulting serum enzyme activities it has added to our understanding of how pivotal nutrition can be in this capacity. Moreover whilst this formulation has previously been shown, by Tacchi et al. (2012), to alter the transcriptome of Atlantic salmon compared to those fed the control diet, this is the first evidence of its protective effects during a disease challenge.

It is important to note that whilst serum ALT and AST activity have not been widely investigated as a marker of pathogenesis in salmon disease, a number of studies have investigated CK in this manner. Ferguson et al. (1986) and Rodger at al. (1991) showed that levels in fish affected with PD to be higher than in healthy fish. However, these studies did not investigate the relationship between histopathology and the sera analyte levels. In addition, in a study by Yousaff and Powell (2012) on the effect of heart and skeletal muscle inflammation and cardiomyopathy syndrome (CMS) CK and lactate dehydrogenase (LDH) activities, it was shown that CK correlated with histopathological damage during HSMI (but not CMS). Despite this, there are a number of issues with using CK (and from this study it would also indicate the same issues may be apparent in using ALT and AST), as a diagnostic/ prognostic

tool in the field. First, the range of concentrations in healthy sera (W0 fish in this study) (due to a high and variable activity) is large as described in the literature (Ferguson et al., 1986; Roger et al., 1991; Yousaff & Powell 2012) thus making it hard to identify subtle changes in concentrations on which to base health management decisions at a given time point. This is especially a problem when a fish farm site has an outbreak of HSMI where changes may be quite small and fall within this range. Indeed, it would appear that values (U/L) of CK are extremely high when fish are affected with PD compared to HSMI. In addition, to this the literature has only investigated total CK concentrations, which is ubiquitous, thus not indicating a specific pathology. These two issues are much more of an issue given the nature of sampling at an aquaculture site, where there is a low probability that a fish will be sampled multiple times over a given period, meaning that individual variability can be easily accounted for. Therefore, it would be useful in terms of diagnostics to have other non-destructive targets that may add to information attained from serum analysis and may be more sensitive, accurate, and reliable in diagnosing pathogenesis.

Despite this, as aquaculture strives to become more sustainable through maximizing output, non-destructive means of health assessment and disease identification must be further developed. Without doubt interest in this area is growing with potential targets of specific pathologies beginning to be identified. In addition, they offer an interesting application of quantitative pathological assessment of fish where we can examine the fish's relative health or disease severity as a whole rather than at a given place in a tissue with histopathological assessment. In addition, the reduction in histopathological lesion scores and serum activities at certain points in this trial has shown the protective nature of this functional feed's formulation. Moreover, these tools may have further utility as supportive evidence for the benefits of the utilization of other functional feed formulations.

Chapter 3

Proteomic investigation of changing proteome during pancreas disease

3.1 Introduction

3.1.1 Proteomics

Proteomics is a well-established post-genomic tool which allows investigation of biological systems through the study of protein composition. Essentially, it is the study of the entire or part of the protein content (proteome) of a given cell, organ, or organism (or its humoral system) at a given moment in time. Such investigation has a number of applications such as biomarker detection of infection and disease, and the study of protein-protein interactions, protein expression, protein turnover, alternative splicing, and post translational modifications, applied to the understanding of conditions in an extensive range of species, from primates to the single celled organisms.

Livestock production has also benefited greatly from the use of proteomics which is increasingly being used to understand the biology of infection and markers of quality. For instance our understanding of meat and milk quality, gut health, infection and disease, neurodegenerative disorders, reproductive biology, and even obesity have all benefited from proteomics (reviewed in Bendixen et al. 2011). Interestingly despite proteomics being a relatively old 'omics' approach recently the use of the approach has grown rapidly due to improvements in sensitivity and reliability of technical approaches, mass spectrometry (MS), and advances in analytical software (Abdallah et al. 2012).

3.1.2 Proteomic Methodologies

Proteomic methodologies rely on the separation of peptides according to physiochemical properties (such as molecular weight and isoelectric point) and/ or affinity interactions for identifying proteome components of a complex sample. Many recent developments in proteomics have been in gel free approaches which unlike gel based proteomics do not utilize gel electrophoresis to separate proteins prior to MS analysis (Abdallah et al. 2012). Despite this most studies apply some sort of protein separation such as liquid chromatography,

centrifugal filtration, or OFFGEL electrophoreses (OGE) utilized for the reduction of complexity of sample where proteins of at a given size or isoelectric point (pI) range are investigated. In addition, gel free approaches have also proven useful in quantifying a given peptide/ proteins concentration in a sample. This 'MS-based quantitation' is a bottom up process where specific peptides are quantified in a sample by either prior labelling or by the somewhat cheaper methodologies of quantification of the relationship of ion peaks and thus its abundance.

Currently, gel based separation remains the most established approach prior to MS protein identification. One dimensional separation (1-DE) by either molecular weight (Mw) or isoelectric focusing (IEF) to separate proteins by isoelectric point (pI) is the simplest method of proteomic investigation where bands are visualised by staining and then excised for MS identification. A greater level of separation is gained by two dimensional electrophoresis (2-DE) where proteins are separated by both pI and Mw. Since the first description of this procedure by O'Farell (1975) significant improvements have been made in the resolution and broad and narrow pH ranges for pI focusing are commonly used. Such separation means that each protein spot usually but not always consist of one protein but multiple spots may consist of modified forms of the same protein (O'Donnel et al. 2004). After separation in 'classical' 2-DE studies proteins are stained before analysis with one of a number of possible dyes that bind protein for visualization of proteome. One of the main criticisms of this methodology has in the past been poor reproducibility of gels (Lilley et al. 2002). This led to the development of another 2-DE technique to be developed called difference gel electrophoresis (DiGE) (Ünlü et al. 1997) which uses three fluorescent cyanine (Cy) dyes (Cy2, Cy3, Cy5) to investigate directly the difference in proteome between two given samples. However, this significantly limits the number of samples that can be directly compared thus making it not ideal for investigating the changing proteome over a long period of time or with many conditions. Alternatively and with correct technical care and the use of multiple replicates one can minimize variability. Through the use of advancing gel image analytical software it is possible to ensure that a given spot's profile is significant and

reproducible by power and analysis of variance (ANOVA) analysis. In addition a gel based proteomics approach can be favoured over a gel free approach in order to visualize isoforms of proteins which may give valuable information on possible tissue specific forms of an enzyme or alternate splicing during disease.

3.1.3 Proteomics in Fin Fish

The application of proteomics in teleostei species has been relatively limited (in comparison with other livestock). However; its use is growing year on year (Rodrigues et al. 2012) with recent studies investigating a number of biological questions in many species, from model organisms such as zebra fish (De Wit et al. 2010) to leopard sharks (Dowd et al. 2010). However, it is in species used in aquaculture that much recent emphasis has been placed as an increase in efficiency in livestock production through a better understanding of biological processes. For instance, Kolditz et al. (2008) used transcriptomics and proteomics to investigate the role of liver metabolism on fat allocation and its effect on flesh quality of rainbow trout (*Oncorhynchus mykiss*) and the effect of variable fat content diets fed to the fish. The liver proteome has been examined to investigate the impact of using genetically modified (GM) soy in Atlantic salmon, *Salmo salar*, feed identifying minor differences in liver protein composition in fish fed GM soy compared to non-GM (Sissner et al. 2009). The effect of nutrition has also been studied using proteomics in relation to infection and disease by a number of groups (Rodrigues et al. 2012). For instance, a recent study by Provan et al. (2013) identified a significant effect on the protein composition of Atlantic salmon mucus between control and diets enriched with immunostimulants during sea lice infection. The proteome of humoral systems, such as blood derived serum and plasma, has also been investigated as a means of assessing fish health and welfare. One example of this is Russell et al. (2006) who identified through the use of 2D-PAGE a number of potential markers of health during the acute phase response (APR) of rainbow trout to *Aeromonas salmonicida* lipopolysaccharide infection. However, only one study has directly investigated the effects of pathology during disease on the serum proteome. Stentiford et al. (2005) in a multidisciplinary study investigated the proteomic and metabolomic change in relation to the pathogenesis of liver tumours in wild

dab (*Limanda limanda*). However, to date little proteomic investigation has been carried out to discover humoral markers of infection and pathogenesis during disease in Atlantic salmon.

3.1.4 Aims of Study

- To discover potential serum markers of infection and pathogenesis of PD in Atlantic salmon by 2D-PAGE
- MS/MS fingerprinting to identify proteins found to alter in abundance through the twelve weeks of sampling.
- Determine association between histopathological data of pathological damage to tissues during the disease and the serum proteome.

N.B. The findings of this chapter have been published in Braceland et al (2013).

3.2 Materials and methods

3.2.1 Sample Preparation and Two-Dimensional Electrophoresis (2-DE)

In order to keep the 2-DE investigation to a manageable size, 1 µl of each serum sample collected from each fish sampled at a particular time point was pooled according to week to create samples for the analysis of changing protein composition throughout the time course. Total protein concentration of the pooled samples was determined by Bradford assay, using Bradford Reagent (Sigma-Aldrich, Poole UK), in accordance with the manufacturer's protocol. Samples were dilutef in loading buffer (BioRad Hemel Hempstead, UK), to an equal protein loading (of 208 µg) for 2-DE protein separation by isoelectric focusing based on isoelectric point (pI) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) based on molecular weight (Mw). Three replicate 2-DE gels were run of the serum pool of samples from each time point to allow for technical replication. Separation by pI was carried out using 11cm immobilized pH Gradient (IPG) strip with a pH range of 3 to 10 (BioRad, Hemel Hempstead, UK). After protein loading of the IPG strips, with serum diluted in a rehydration buffer (8M Urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte®)

(BioRad, Hemel Hempstead, UK) and covered in 500µl of mineral oil, a combined rehydration and focusing step was carried out over 17 h with a total of 35000 V-hr. The IPG strips were removed, oil drained and then treated with two equilibration buffers both made from a stock solution comprised of 6M urea, 0.375 M Tris-HCl, pH 8.8, 2% (w/v) SDS, 20% (v/v) glycerol, the first of these containing 2% (w/v) dithiothreitol (Sigma-Aldrich, Poole, UK) to reduce the proteins and subsequently the alkylating agent iodoacetamide at 2.5% (w/v) (Sigma-Aldrich, Poole, UK). IPG strips were then placed onto Criterion SDS-PAGE gels and submerged in XT Mops running buffer and subjected to electrophoresis at 200V for one hour. (Bio-Rad, Hemel Hempstead UK) Subsequently gels were stained in Coomassie brilliant blue G-250 dye 0.1% (w/v) in de-stain solution for 1 hour and then de-stained using a solution of methanol: water: acetic acid, (4:5:1) overnight, scanned and saved in 16-bit grey TIFF format images for gel image analysis.

3.2.2 Gel Image Analysis

Images were uploaded onto 'Nonlinear Progenesis SameSpots 2D gel image analysis software (Nonlinear Dynamics, Newcastle, UK) which was used to identify protein spots that were differentially expressed through time (inferred by the programme by normalised spot intensities). Initial results were filtered using the programme's statistical analysis function, with only those with a power value of >80% and ANOVA significance score of <0.05 between groups of replicate gels, being chosen for protein identification. Samespots analysis was performed in the laboratory of Dr P Cash, University of Aberdeen. To investigate the relationship among different time-points and different proteins with respect to spot intensity and the dataset was analysed using cluster analysis. ArrayStar software (DNASTAR, Madison, WI, USA) was the programme used to perform Hierarchical Cluster Analysis based on Euclidean distance with the results being illustrated in the form of a heat map.

Spot information (profiles) were also used in a general linear model procedure in SAS version 9.3 (SAS Institute, Cary, N. Carolina) for regression analysis. Each spot was regressed on the mean value of each tissue's histopathological score at

each sampling time point in a separate model. Therefore, the probability that a protein spot increased or decreased in intensity in association with tissue damage as determined by histopathology was assessed.

3.2.3 Spot Preparation and Mass-Spectrometry

Chosen protein spots were excised manually by scalpel and placed in individual vials to be subjected to in-gel digestion for protein extraction prior to identification via mass spectrometry analysis. Gel pieces were washed with 100mM NH_4HCO_3 for 30 minutes and then for a further hour with 100 mM NH_4HCO_3 in 50% (v/v) acetonitrile. After each wash all solvent was discarded. Gel plugs were then dehydrated with 100% acetonitrile for 10 minutes prior to solvent being removed and dried completely by vacuum centrifugation. Dry gel pieces were then rehydrated with 10 µl trypsin at a concentration of 20 ng/µl in 25 mM NH4HCO3 (Cat No. V5111, Promega, Madison, WI, USA) and proteins allowed to digest overnight at 37˚C. This liquid was transferred to a fresh tube, and gel pieces washed for 10 min with 10 µl of 50% acetonitrile. This wash was pooled with the first extract, and the tryptic peptides dried to completion. Tryptic peptides were solubilized in 0.5 % (v/v) formic acid and fractionated on a nanoflow uHPLC system (Thermo RSLCnano) before analysis by electrospray ionisation (ESI) mass spectrometry on an Amazon ion trap MS/MS (Bruker Daltonics). Peptide separation was performed on a Pepmap C18 reversed phase column (LC Packings), using a 5 - 85% v/v acetonitrile gradient (in 0.5% v/v formic acid) run over 45 min. at a flow rate of 0.2 ml / min. Mass spectrometric (MS) analysis was performed using a continuous duty cycle of survey MS scan followed by up to five MS/MS analyses of the most abundant peptides, choosing the most intense multiply-charged ions with dynamic exclusion for 120s. MS data were processed using Data Analysis software (Bruker) and the automated Matrix Science Mascot Daemon server (v2.1.06). Protein identifications were assigned using the Mascot search engine to interrogate protein sequences in the NCBI databases restricting the search to teleostei, allowing a mass tolerance of 0.4 Da for both MS and MS/MS analyses. In addition, the search consisted of a carbamidomethyl fixed modification and a variable oxidation.

3.2.4 Statistical Analysis

The relationship between disease pathology and protein abundance, given by spot intensities, was examined by multilinear regression general linear model (GLM) analysis to investigate the statistical correlation between individual spots intensities against tissue specific pathologies. For this every spots intensity profile from W0pc to W12pc was regressed against individual (ie white muscle, red muscle, heart, and pancreas individually) mean tissue histopathology scores over the same period.

3.3 Results

3.3.1 Profiling Changes in The Serum Proteome

There were a number of clearly visible differences in the serum proteome over the 12 week period of the trial, illustrated in Figure 3.1 where representative gels from each of the nine sampling time points are shown. To quantify and identify changes, scanned 2D-PAGE gel images from each sampling time point were compared using 'SameSpots' software to identify protein spots which were differentially expressed in the serum as a result of PD. In total, 894 spots were identified by the SameSpot software of which 72 spots were found to differ significantly over the course of infection (Figure 3.2). These were excised for peptide mass fingerprinting from the gel where they showed greatest intensity. Protein identification following DEAMON /MASCOT searching is given in Table 3.1. Spot intensities at each time-point were analysed using Hierarchical Cluster Analysis to more clearly identify whether an association with disease progression over time could be identified (Figure 3.3) and to group proteins which possessed similar expression profiles. Figure 3.3 also lists spot numbers and their corresponding identities obtained by ion trap mass spectrometry analysis of excised spots.

The dendrogram at the top of Figure 3.3 shows the relationship of the spot intensities at the nine time points illustrating progression of the infection.

There was a clear separation between those recorded from 0 – 4 wpc and those recorded from 5 wpc onwards. Comparison of the proteome at 12 wpc with those prior to 4 wpc indicated a return to homeostasis. The overall fold increase or decrease in spot intensities from basal level (week 0) for up regulated and down regulated proteins respectively is shown in Table 3.1. Whilst most proteins and enzymes increased in their abundance there were also a number of proteins that declined in abundance (e.g. albumin).

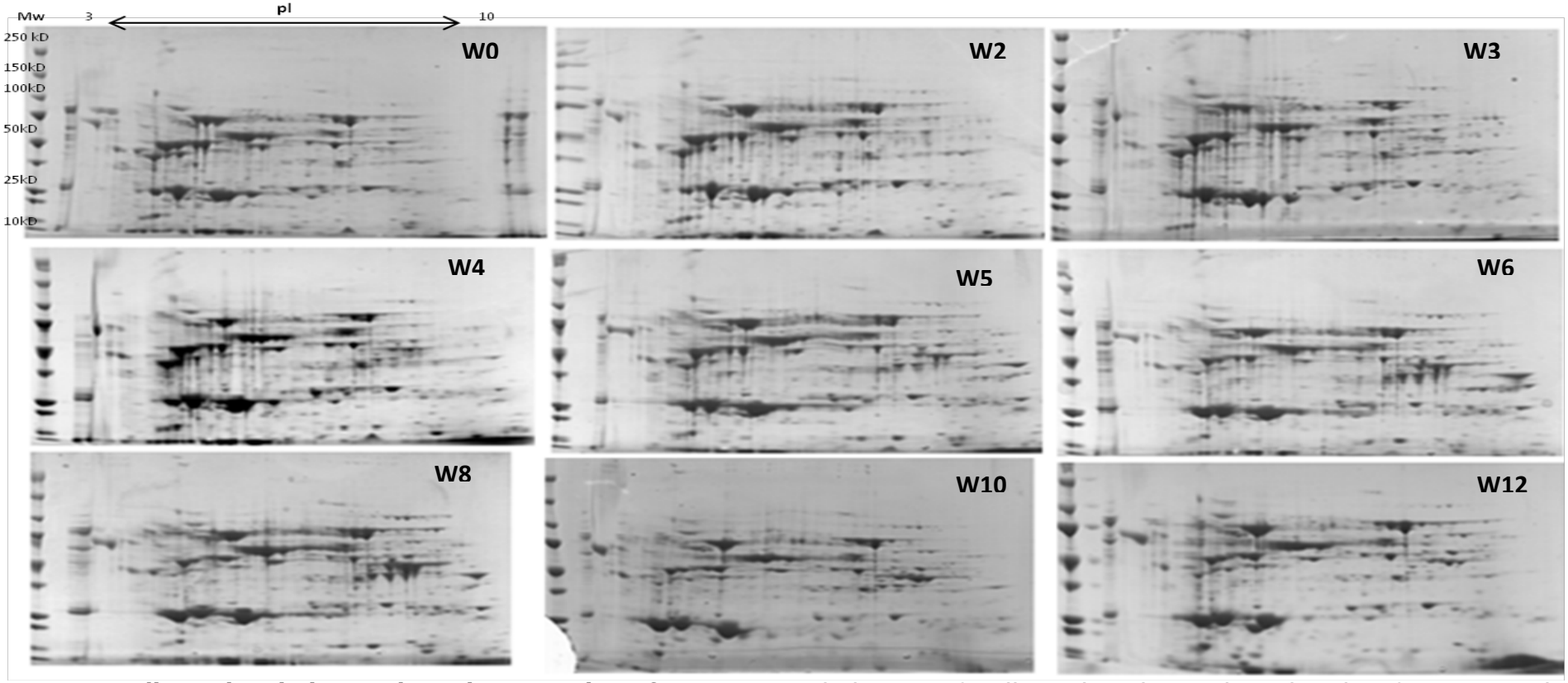

Figure 3.1 Two dimensional electrophoresis separation of serum. Sample images of 2-dimension electrophoresis gels, after coomassie staining and subsequent de-staining, from all sampling time points from week 0 to 12 post challenge. From left to right: Top row = Week 0, 2 and 3 images. Middle row = Week 4, 5 and 6 images. Bottom row = Week 8, 10 and 12 images. Molecular weight (Mw) and isolectric point (pI) range are indicated on the gel at the top left (week 0).

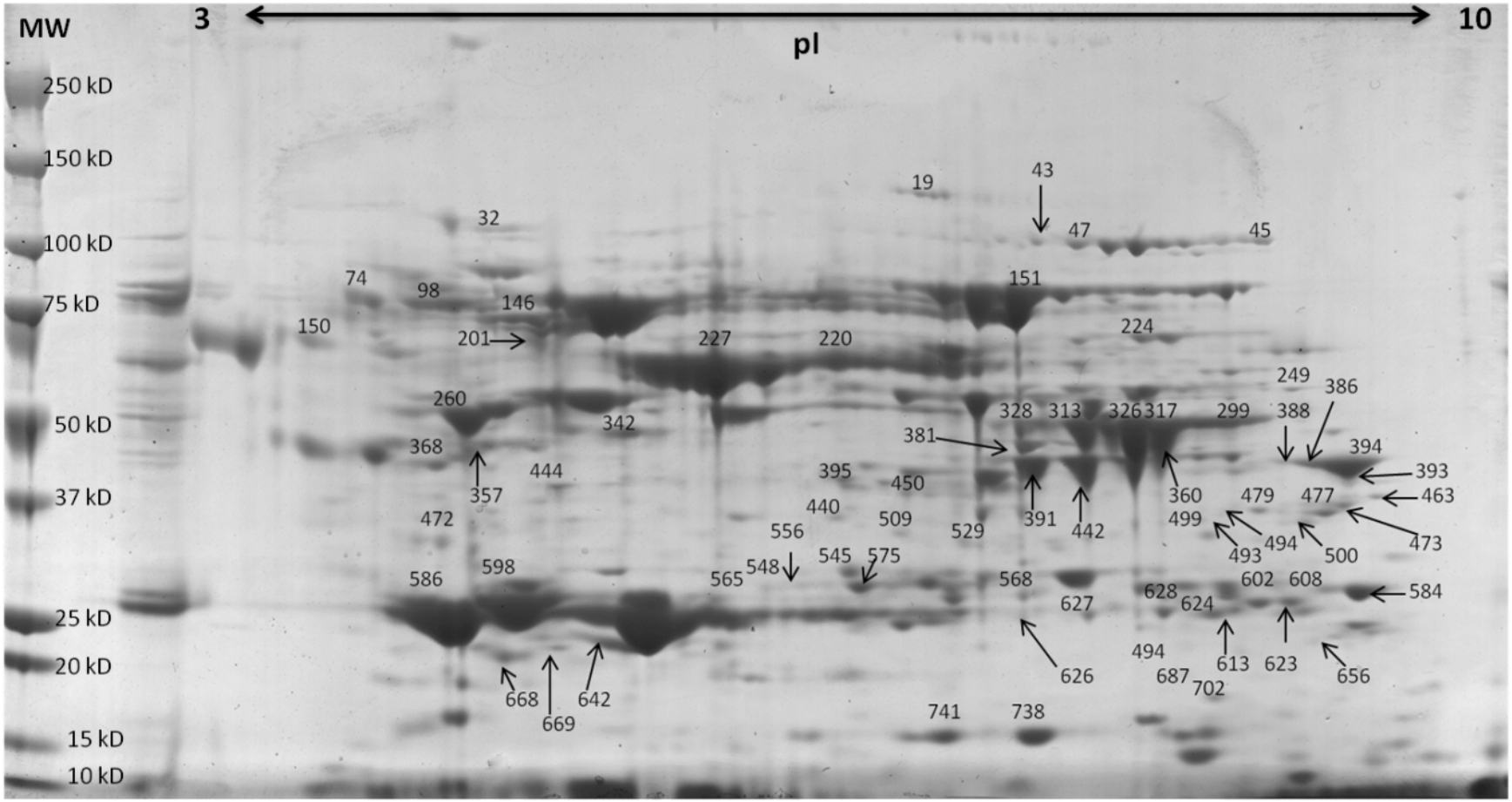

Figure 3.2: Protein spots subjected to mass spectrometry. Protein spots identified as being differentially expressed following infection on a 2-dimension electrophoresis

Table 3-1 Protein spot identifications. Protein spots (n=72) showing significant change in spot intensity during the 12 weeks post infection, where negative symbol (-) indicates a fold decrease in spot intensity, compared to pre-infection on week 0.

Spot Number	Identification (Uniprot Reference)	Max. Fold Change	Estimated pI	Estimated MW (kD)	MOWSE Score	Peptide Matches	% coverage
19	Serotransferrin II + Ig heavy chain C region (TRF2SALSA + A46533)	3.1	6.91	112	821, 65	43, 2	38, 5
32	C1 inhibitor (Q70W32)	5.4	5.21	97	137	6	14
43	Serotransferrin II (TRF2SALSA)	4.5	7.36	91	527	14	19
45	Complement factor H (Q2L4Q6)	10.4	8.37	91	79	2	3
47	Alpha-2 enolase-1 + Complement factor H (Q9DDG6 + Q2L4Q6)	3.2	7.52	90	111, 94	3, 3	9, 3
74	No significant identification	4.4	4.33	81			
98	Apolipoprotein A-I precursor (JH0472)	2.6	4.96	76	113	5	23
146	Complement component C9 (Q4QZ25)	2.2	5.2	70	226	7	17
150	Hemopexin-like protein (P79825)	9.6	4.22	68	64	3	9
151	Transferrin + Ig heavy chain C region (T11749 + A46533)	1.5	7.27	68	967, 139	32, 4	35, 12
201	Antithrombin + Complement C9(Q9PTA8+ Q4QZ25)	-2.8	5.43	59	156,196	8, 6	14, 21

Spot Number	Identification (Uniprot Reference)	Max. Fold Change	Estimated pI	Estimated MW (kD)	MOWSE Score	Peptide Matches	% coverage
220	Hemopexin-like protein + Beta-actin (P79825 + Q4U1U5)	2.5	6.57	57	166, 107	3, 3	8, 12
224	Hemopexin-like protein (P79825)	2.2	7.5	56	150	7	16
227	Hemopexin-like protein (P79825)	1.5	6.06	56	128	11	14
249	Serotransferrin II (TRF2SALSA)	5.4	8.36	51	101	3	7
260	Serum albumin 2 + Serum albumin 1 (ABONS2 + ABONS1)	-1.7	4.77	50	364, 320	14, 13	24, 20
299	Enolase (Q7ZZM5)	5	8.03	47	308	14	25
313	Pyruvatekinase (Q8QGU8)	3.5	7.58	46	155	6	11
317	Alpha-2 enolase-1 (Q9DDG6)	7.6	7.94	45	648	37	53
326	Alpha-2 enolase-1 (Q9DDG6)	5.6	7.78	45	560	23	42
328	Alpha-2 enolase-1 (Q9DDG6)	5.2	7.27	44	292	15	39
342	Serum albumin 2 (ABONS2)	-11.1	5.54	44	666	32	31
357	Serum albumin 2 + Complement factorB/C2-B (ABONS2 + Q9DEC8)	2.4	5.2	41	302, 49	13, 3	18, 3
360	Alpha-2 enolase-1 (Q9DDG6)	15.3	7.77	41	624	28	44

Spot Number	Identification (Uniprot Reference)	Max. Fold Change	Estimated pI	Estimated MW (kD)	MOWSE Score	Peptide Matches	% coverage
368	Serum albumin 2 + Serum albumin 1(ABONS2 + ABONS1)	-2.8	4.88	40	318, 295	15, 13	19, 16
381	Alpha-2 enolase-1 + Creatine kinase (Q9DDG6 + Q8JH38)	2.8	7.28	38	704, 101	30, 5	28, 14
386	Aldolase (Q804Y1)	13.8	8.72	38	108	3	23
388	Aldolase (Q804Y1)	8.1	8.62	38	94	1	9
391	Muscle-type creatine kinase CKM1 (Q8JH39)	5.1	7.35	38	252	9	17
393	Glyceraldehyde3-phosphate dehydrogenase (O42259)	15.7	8.79	38	102	9	36
394	Aldolase A (Q8JH72)	11.8	8.82	38	231	11	20
395	Serotransferrin II (TRF2SALSA)	2.2	6.56	38	210	5	9
440	Alpha-2 enolase-1(ABONS1)	4.5	6.64	36	164	3	9
442	Creatine kinase (Q98SS7)	3	7.61	36	218	6	17
444	Complement C3-1 + Cystein proteinase inhibitor protein (P98093 + Q70SU8)	-3.1	5.4	36	156, 36	10, 2	6, 12
450	Serotransferrin II precursor (TRF2SALSA)	-2.6	6.79	36	444	15	23
463	Glyceraldehyde3-phosphate dehydrogenase (Q90ZF1)	10.6	8.98	34	247	18	41

Spot Number	Identification (Uniprot Reference)	Max. Fold Change	Estimated pI	Estimated MW (kD)	MOWSE Score	Peptide Matches	% coverage
472	Serum albumin 2 (Q98SS7)	-3.7	4.79	34	323	12	19
473	Fructose-bisphosphate aldolase (Q4RVI9)	16.9	8.81	34	98	1	3
477	Aldolase A (Q8JH72)	10.1	8.7	33	130	5	18
479	Glyceraldehyde 3-phosphate dehydrogenase (O42259)	7.1	8.43	33	190	20	34
493	Glyceraldehyde 3-phosphate dehydrogenase (O42259)	11.6	8.18	33	147	13	12
494	Glyceraldehyde 3-phosphate dehydrogenase (Q90ZF1)	15.3	8.2	33	280	13	45
499	Proteasome (Q7ZVP5)	8.2	8	32	35	1	1
500	Aldolase A (Q8JH72)	15.4	8.58	32	110	4	17
509	Serotransferrin II (TRF2SALSA)	-2.6	6.78	31	250	11	18
529	Alpha-2 enolase-1 (Q9DDG6)	5	7.07	30	80	3	11
545	Serotransferrin II (TRF2SALSA)	-5.7	6.57	28	64	3	5
548	Serum albumin 1 (P21848)	-7	6.29	27	122	3	7
556	Serotransferrin 2 + Immunoglobulin light chain (TRF2 + AAG18369)	-5.3	6.36	27	404, 150	18, 7	16, 33
565	Hemopexin-like protein (P79825)	7.8	6.26	26	51	4	8
568	Transferrin (Q8AYG2)	-3.6	7.36	26	285	12	36

Spot Number	Identification (Uniprot Reference)	Max. Fold Change	Estimated pI	Estimated MW (kD)	MOWSE Score	Peptide Matches	% coverage
575	Apolipoprotein A-I + Ig light chain precursor (JH0472 + AAG18369)	1.8	6.62	26	188, 76	8, 4	28, 24
584	Malatedehydrogenase1a (B8JMZ0)	5.8	8.88	25	70	1	4
586	Apolipoprotein A-I (JH0472)	1.9	4.76	25	262	8	27
598	Apolipoprotein A-I (JH0472)	1.5	5.15	24	257	19	42
602	No significant identification.	3.6	8.63	24			
608	Triosephosphate isomerase (Q70I40)	2.8	8.5	24	170	6	22
613	Serum albumin 2 (ABONS2)	2.4	8.1	24	488	19	23
623	Triosephosphate isomerase + Ig light chain (Q70I40 + AAG18369)	3.4	8.57	23	305, 109	10, 5	49, 19
624	Triosephosphate isomerase (Q70I40)	2.4	8.1	23	194	11	50
626	Ig light chain (AAG18369)	2.4	7.26	23	190	8	24
627	Triosephosphate isomerase + Ig light chain constant region (Q70I40 + AAN40739)	2.6	7.53	23	131, 87	5, 4	22, 25
628	Triosephosphate isomerase (Q70I40)	3.9	7.85	23	432	19	65
642	Apolipoprotein A-I (JH0472)	3.8	5.57	21	435	27	37

Spot Number	Identification (Uniprot Reference)	Max. Fold Change	Estimated pI	Estimated MW (kD)	MOWSE Score	Peptide Matches	% coverage
656	Serotransferrin II (TRF2SALSA)	-5.3	8.63	20	251	6	10
668	Apolipoprotein A-I (JH0472)	-4.8	5.17	19	325	19	34
669	Apolipoprotein A-1 (JH0472)	-4.1	5.42	19	221	14	32
687	Serotransferrin II (TRF2SALSA)	4.6	7.93	18	199	7	12
702	Prostaglandin D synthase (Q9DFD7)	-3.3	8.16	17	82	1	9
738	Putative oncoprotein nm 23 (Q2L4Q6)	7.2	7.3	14	58	11	22
741	Nucleoside diphosphate kinase (Q7ZZQ7)	4.6	6.9	14	58	8	22

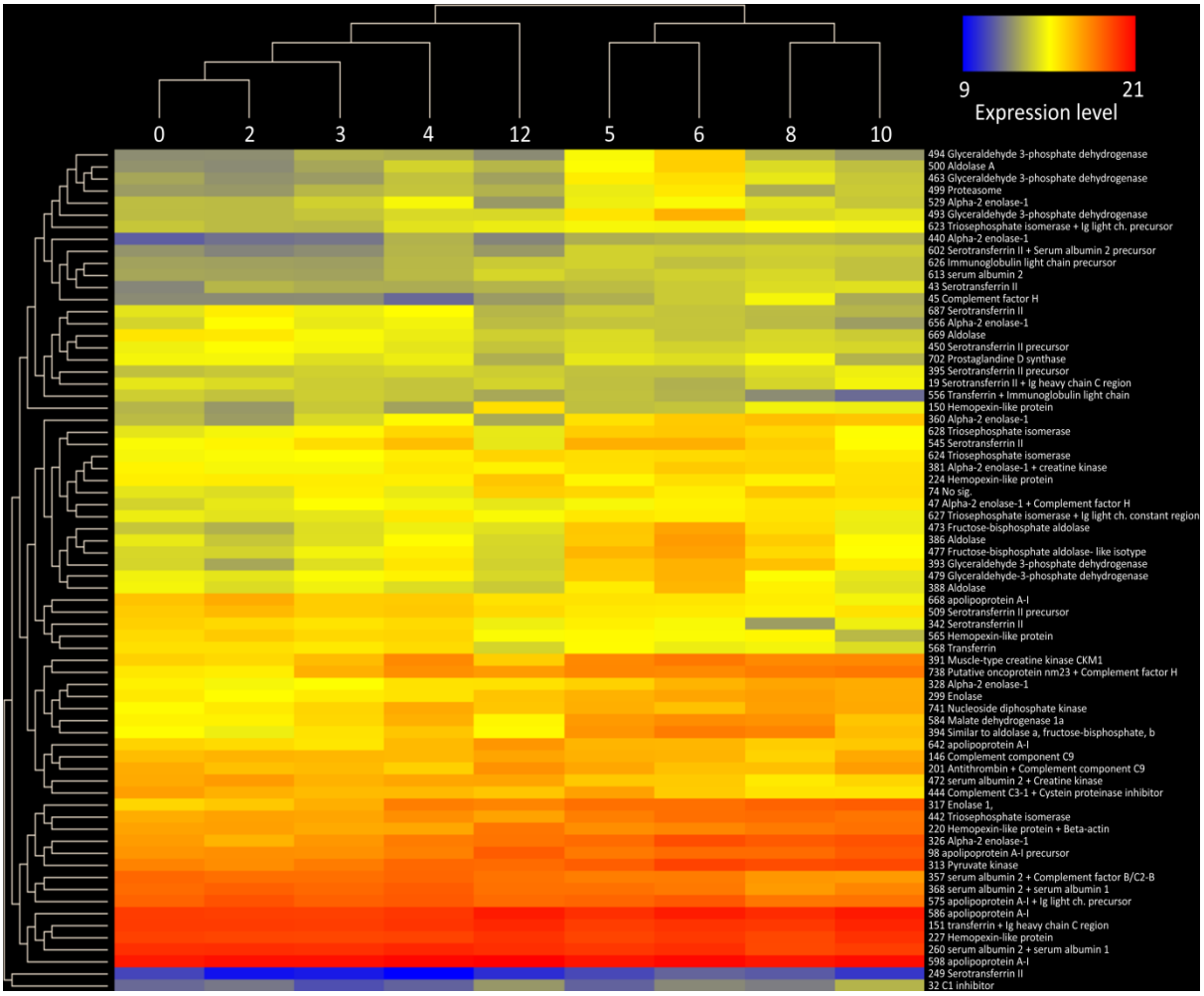

Figure 3.3: Arraystar heat map representing the results of Hierarchical clustering of spot intensities. The relationships among different sampling time-points are illustrated as a dendrogram at the top of the diagram. The right-hand side gives spot number and corresponding identity from MS/MS which are grouped by profile similarity with a dendrogram showing the relationships on left-hand side. The heat map is on a colour scale where low protein abundance is represented in blue and high abundance in red.

3.3.4 The Relationship Between Tissue Pathology and The Serum Proteome

The relationship between disease pathology and protein abundance, given by spot intensities, was examined by multilinear regression general linear model (GLM) analysis of mean pathological scoring and all mean spot intensities at each sampling time point. Table 3.2, which lists spot numbers and their corresponding protein identity, also indicates the probability (Pr > F) that a given spot expression profile is linked with a particular tissue pathology, with values <0.05 being regarded as significant. However, it is important to note that significance in this case is approached with cautrion due to the high number of comparisons and the use of a p = 0.05 threshold. Moreover, this demonstrates the merits of validating any perceived significance correlation by other methods (chapter 3) for promising results. In addition, the comparison of a continuos measurement such as spot intensity against a semi quantitative histopathological scoring system may increase false positives/ untrue correlation thus again increasing the need for further validation of said results.

In addition, expression profiles were plotted on a graph for each protein against the mean pathology pattern of each tissue sampled. As an example Figure 3.4 illustrates the relationship between mean white muscle histopathology results and the spot intensity of protein spot 313, identified as pyruvate kinase between weeks 2 and 12. Week 0 was removed from this analysis as white muscle showed no lesions at this time point. Graphs for other proteins are given as extra material in the Appendix.

The alterations in serum proteins as a result of PD fell into two categories. The first category included proteins which demonstrated a change in serum abundance (spot intensity) associated with damage to a particular tissue or tissues where an increase in intensity was significantly related to the damage ($p<0.05$). In contrast there were proteins for which the abundance change was

not associated with tissue damage and were possibly present in serum as humoral components of host defence (Table 3-2). Among the group of proteins, the concentration of which was associated with tissue pathology, were a number of enzymes described by ontology as being involved in intracellular pathways. These include creatine kinase, enolases, triosephosphate isomerase, and malate dehydrogenase 1a. The second group of proteins, alteration of which were not related to tissue damage , included a number of well defined (in other systems) humoral constituents of the immune response such as a number of complement components, hemopexin, transferrin, and apolipoprotein.

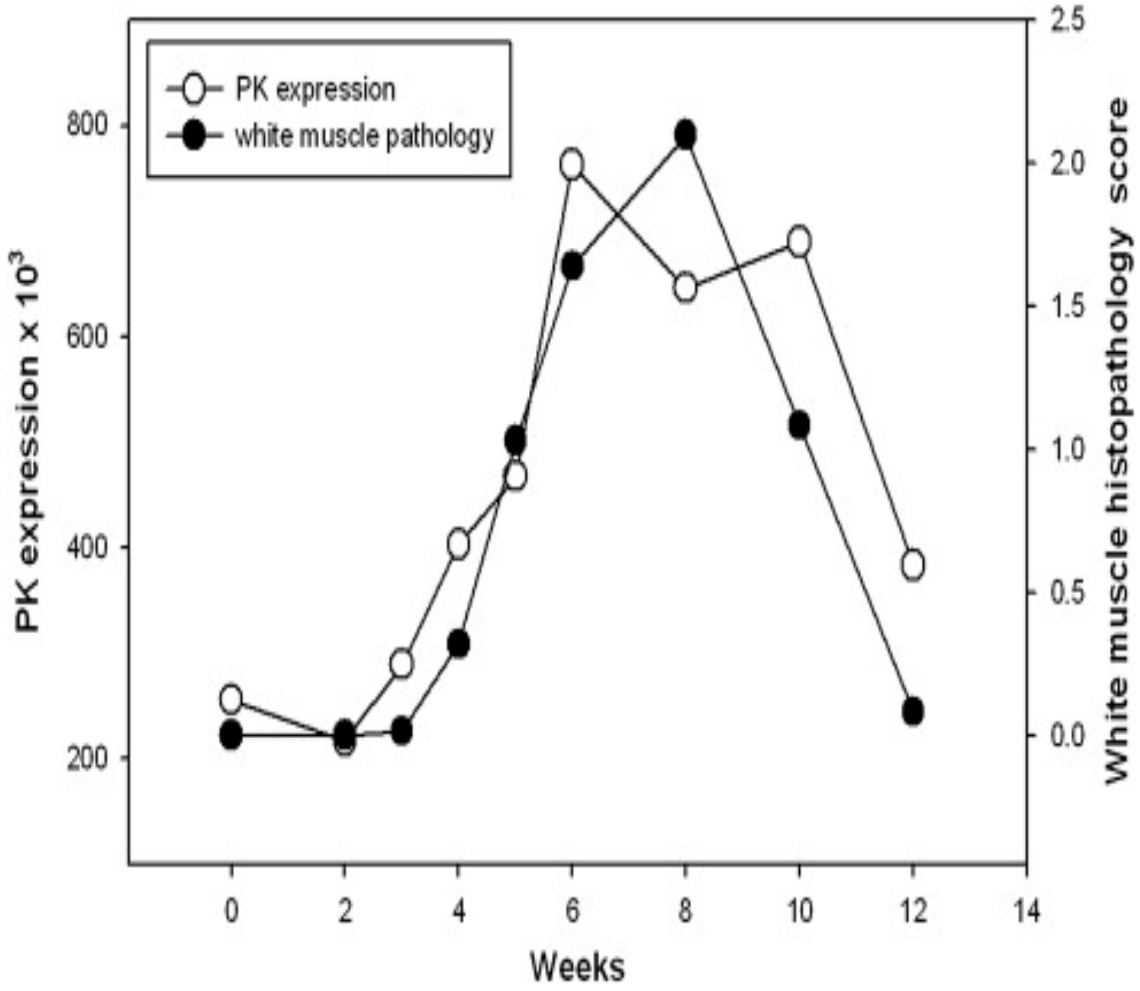

Figure 3.4: Spot profile v histopathological score. Plotting spot 313 (pyruvate kinase) mean intensity and mean histopathological scoring of white muscle against sampling time points. Open circles plot histopathology scores and filled circles plot spot intensity at a given time point.

Table 3-2 Significant correlation analysis of spot profiles and tissue lesion scores. Probability (P) of relation between change in protein spot intensity and histopathology of tissues in salmon infected with SAV. Significant correlations ($p < 0.05$) are in bold.

		Probability (P)			
Spot Number	**Identification**	**Pancreas**	**Heart**	**Red Muscle**	**White Muscle**
19	Serotransferrin II + Igheavy chain C region (TRF2SALSA+A46533)	0.4775	0.7044	0.642	0.8544
32	C1 inhibitor(Q70W32)	0.542	0.3536	0.9656	0.3992
43	Serotransferrin II (TRF2SALSA)	0.0904	0.8329	0.204	**0.0166**
45	Complement factor H (Q2L4Q6)	0.3102	0.5645	0.1132	**0.0061**
47	Alpha-2 enolase-1 + Complement factor H (Q9DDG6+Q2L4Q6)	**0.0241**	0.7643	0.0663	**0.0059**
74	No significant identification	0.2794	0.5719	0.4255	0.1125
98	Apolipoprotein A-1 Iprecursor(JH0472)	0.2179	0.5856	0.474	0.1619
146	Complement component C9(Q4QZ25)	0.1032	0.6175	0.8862	0.9013
150	Hemopexin-like protein(P79825)	0.872	0.3269	0.7833	0.804
151	Transferrin + Ig heavy chain C region(T11749+A46533)	0.0786	0.8847	0.5434	0.3814
201	Antithrombin + Complement component C9 (Q9PTA8+Q4QZ25)	0.5868	0.5314	0.5774	0.9455
220	Hemopexin-like protein + Beta-actin (P79825+Q4U1U5)	0.2798	0.4227	0.5775	0.1481
224	Hemopexin-like protein(P79825)	0.2104	0.9728	0.857	0.7601
227	Hemopexin-like protein (P79825)	0.077	0.8458	0.5829	0.4513
249	Serotransferrin II(TRF2SALSA)	0.2894	0.5577	0.1483	**0.0339**

		Probability (P)			
Spot Number	**Identification**	**Pancreas**	**Heart**	**Red Muscle**	**White Muscle**
299	Enolase(Q7ZZM5)	0.1996	0.5102	0.1088	**0.0032**
313	Pyruvatekinase(Q8QGU8)	0.0806	0.8639	0.0559	**0.0039**
317	Enolase1,(Alpha)(Q6GMI7)	0.1688	0.6918	0.0963	**0.0042**
326	Alpha-2enolase-1(Q9DDG6)	0.1489	0.6904	0.0853	**0.0061**
328	Alpha-2enolase-1(Q9DDG6)	0.2178	0.5131	0.1028	**0.0013**
342	Serum albumin 2 (ABONS2)	0.371	0.1541	0.8433	0.3281
357	Serum albumin 2 + Complement factorB/C2-B (ABONS2+Q9DEC8)	0.1225	0.1539	0.71	0.6958
360	Alpha-2enolase-1(Q9DDG6)	0.1997	0.7136	**0.047**	**0.0002**
368	Serum albumin 2 + Serum albumin 1(ABONS2+ABONS1)	0.0766	0.0806	0.7414	0.6659
381	Alpha-2 enolase-1 + Creatine kinase (Q9DDG6+Q8JH38)	0.0618	0.9464	**0.0272**	**0.0041**
386	Aldolase(Q804Y1)	0.1753	0.911	**0.0229**	**0.0204**
388	Aldolase(Q804Y1)	0.153	0.7466	0.0532	0.0752
391	Muscle-typecreatinekinaseCKM1(Q8JH39)	**0.0462**	0.7088	**0.004**	**0.0009**
393	Glyceraldehyde3-phosphate dehydrogenase(O42259)	0.1131	0.9435	**0.0028**	**0.0002**
394	AldolaseA(Q8JH72)	0.1324	0.9747	**0.0067**	**0.0003**
395	Serotransferrin II (TRF2SALSA)	0.0732	0.7892	0.4174	0.1811

		Probability (P)			
Spot Number	**Identification**	**Pancreas**	**Heart**	**Red Muscle**	**White Muscle**
440	Alpha-2enolase-1(ABONS1)	0.0534	0.8544	0.0097	0.0006
444	Complement C3-1 + Cysteinproteinase inhibitor protein (P98093 +Q70SU8)	0.3808	0.3852	0.8427	0.4569
450	Serotransferrinv II precursor(TRF2SALSA)	0.0744	0.1147	0.8475	0.8393
463	Glyceraldehyde3-phosphatedehydrogenase(Q90ZF1)	0.1712	0.8982	**0.0287**	**0.0277**
472	Serum albumin 2 + Creatine kinase(ABONS2+Q98SS7)	0.2774	0.2544	0.7464	0.3222
473	Fructose-bisphosphate aldolase(Q4RVI9)	0.1771	0.8644	**0.0292**	**0.0336**
477	Aldolase A (Q8JH72)	0.1181	0.7005	**0.0143**	**0.0255**
479	Glyceraldehyde-3-phosphatedehydrogenase(O42259)	0.0972	0.4788	**0.0307**	0.1032
493	Glyceraldehyde3-phosphatedehydrogenase(O42259)	0.2358	0.7868	0.0975	0.1501
494	Glyceraldehyde3-phosphate dehydrogenase(Q90ZF1)	0.271	0.8078	0.132	0.2332
499	Proteasome (Q7ZVP5)	0.1454	0.6439	0.0706	0.1262
500	AldolaseA(Q8JH72)	0.1836	0.7625	**0.039**	0.0693
509	Serotransferrin II (TRF2SALSA)	0.1302	0.1569	0.9378	0.5525
529	Alpha-2enolase-1(Q9DDG6)	**0.0105**	0.1837	**0.0004**	**0.0232**
545	SerotransferrinII(TRF2SALSA)	**0.01**	0.1764	**0.0002**	**0.0215**

		Probability (P)			
Spot Number	**Identification**	**Pancreas**	**Heart**	**Red Muscle**	**White Muscle**
548	serumalbumin1(P21848)	0.381	0.1562	0.7586	0.2887
556	Transferrin+Immunoglobulinlightchain(Q8AUU0+AAG18369)	0.1617	0.063	0.7479	0.5452
565	Hemopexin-likeprotein(P79825)	0.1514	0.0657	0.7578	0.6317
575	Apolipoprotein A-I + Ig lightchain precursor (JH0472+AAG18369)	**0.0124**	0.115	0.3685	0.7495
584	Malatedehydrogenase1a(B8JMZ0)	0.0555	0.7354	**0.0006**	**0.0001**
586	ApolipoproteinA-I (JH0472)	0.1272	0.781	0.3957	0.1807
598	Apolipoprotein A-I (JH0472)	**0.018**	0.3991	0.3503	0.4654
602	No significant identification	0.0931	0.8462	**0.0318**	**0.0005**
608	Triosephosphate isomerase(Q70I40)	**0.0134**	0.6271	**0.0079**	**0.0029**
613	Serum albumin 2 (ABONS2)	0.1367	0.6988	0.1639	0.0516
623	Triosephosphateisomerase+ Ig light chain(Q70I40+AAG18369)	0.1508	0.5987	0.0971	**0.0068**
624	Triosephosphate isomerase(Q70I40)	0.0893	0.8886	0.1385	0.0628
626	Ig light chain (AAG18369)	0.1144	0.7554	0.1929	0.0606
627	Triosephosphate isomerase + Ig light chain constant region(Q70I40+AAN40739)	**0.0396**	0.6455	**0.0066**	**0.0115**

		Probability (P)			
Spot Number	**Identification**	**Pancreas**	**Heart**	**Red Muscle**	**White Muscle**
628	Triosephosphateisomerase(Q70I40)	**0.0082**	0.3003	**0.0001**	**0.0047**
642	ApolipoproteinA-I(JH0472)	0.4075	0.7725	0.675	0.6736
656	SerotransferrinII(TRF2SALSA)	0.0929	**0.0296**	0.7203	0.6526
668	ApolipoproteinA-I(JH0472)	0.2306	0.1865	0.8973	0.4736
669	Apolipoprotein A-1 (JH0472)	0.5808	0.4595	0.5278	0.3262
687	SerotransferrinII(TRF2SALSA)	0.1577	0.0552	0.8437	0.5952
702	Prostaglandin D synthase(Q9DFD7)	0.1102	0.356	0.2959	0.4512
738	Putative oncoprotein nm 23 (Q2L4Q6)	0.1136	0.9017	0.0695	**0.0056**
741	Nucleoside diphosphate kinase(Q7ZZQ7)	0.0736	0.8809	**0.0467**	**0.007**

3.4 Discussion

3.4.1 Monitoring Pancreas Disease Via Proteomics

The analysis of spot profiles at sampling points demonstrated that the serum proteome of salmon was altered markedly in response to SAV3. The heat map of proteome responses following the 2-DE showed a distinct change in the serum proteome between pre and post week 4 (Figure 3.3), with the exception of week 12, which clustered with samples collected between weeks 1 and 4; thus, indicating the near return of homeostasis. These results corresponded with the histopathological results (see chapter 2 Figure 2.3) indicating that 2-DE could be used as a useful investigative approach to monitor PD. Furthermore, using both histopathology and proteomic approaches allowed proteomic results to be separated into proteins which rise or fall in association with tissue damage and those which were likely to be part of the host response to SAV3 (Table 3.2) as their alteration in intensity was unrelated to the histopathology score. The use of hierarchical clustering to analyse spot intensities effectively highlighted the common responses within groups of protein spots and clearly illustrated a temporal trend in the dataset. It is important to note that this analysis was carried out using pooled samples, determined by time point. Whilst, there is an argument for using biological replicates using individual fish it was considered that pooling samples prior to electrophoresis was the optimal approach as resource constraints limited the study to 3 gels per time point. As this study was a cohabitation trial there are, due to infection dynamics, fish at various disease stages which would, when using an individual fish approach, potentially require many replicates and pooling of samples was thus required to make the study feasible. Moreover, as this experimental methodology simulates conditions during an outbreak of PD at an aquaculture site it was decided that pooling would allow for the analysis of changes at a site as a whole.

3.4.2 Biomarkers of Tissue Pathologies

The use of histopathology in conjunction with a 2-DE proteomic approach allowed statistical analysis to be carried out to test the hypothesis that the expression profile of specific proteins was correlated with the pathology of examined tissues. Analysis of this relationship allowed the identification of the tissue source of the identified serum proteins.

The abundance of only one spot significantly correlated to the histopathology results of heart damage. This was spot 656 and was identified by MS as the Serotransferrin II. The expression profile of this protein was significantly related to the pathological damage to the heart. The lack of additional biomarkers of heart damage was most likely because the abundances of other potential specific protein biomarkers were not raised sufficiently in the serum to be detected by the proteomic methodology used in this study

The pathological damage to the pancreas as well as the white and red skeletal muscle was much more pronounced and longer lasting than that observed in the heart (chapter 2 Figure 2.3), and there were more protein spots correlated with histopathology lesion scores of this tissue (Table 3.2). Six spots were found to be correlated with damage to pancreas as well as correlating to damage to white and red muscle. Three of the spots were identified as triosephosphate isomerase (spots 608, 627, and 628) and were in close proximity on 2-DE gels, whilst the remaining three spots were identified as creatine kinase, alpha-2 enolase-1, and serotransferrin (spots 391, 529 and 545 respectively). Enolase and creatine kinase are enzymes of glycolysis and were presumably derived from the damage to muscle. Transferrins are found in the fibroblasts of the pancreas and skeletal muscles thus it was possible that this serotransferrin was membrane bound/ intracellular transferrin isoform that leaked into the circulation due to tissue damage. Only three spots were exclusively correlated with damage to the pancreas (Table 3.2). One was identified as albumin (spot 260) while the other two spots contained apolipoprotein (spots 575 and 598). However, as the

apolipoprotein spot intensities during PD infection only increased by <2 fold they were unlikely candidates as biomarkers of pancreas damage.

The two types of skeletal muscle studied in this investigation were white and red muscle. These muscle fibres are differentiated by two functional characteristics, specifically contractile speed and metabolic activity. White fibres (fast) possess a higher action potential due to the quicker generation of ATP by glycolysis compared to red (slow) fibres which in general terms generate ATP by oxidative (aerobic) processes. However, glycolysis does occur in red muscle fibres, which explains the finding that many glycolytic enzymes were found to rise in serum spot intensities at the peak of PD pathological damage to both muscle types (Table 3.2). Many of these glycolytic enzymes have been observed and studied in both muscle types in salmon, and have been found to possess higher activity levels in white muscles than in red (Johnston 1977; Martinez et al. 2004). Only two spots, identified as Glyceraldehyde-3-phosphatedehydrogenase and aldolase A (479 and 500 respectively), were exclusively identified as related to red muscle pathology. Rather than highlighting the metabolic differences between these two types of Salmo salar skeletal muscle these differences may indicate that red and white muscle fibres display a differential expression of multiple isozymes of these enzymes.

Conversely this study identified a number of possible unique biomarkers of white muscle damage due to SAV3 as spots 43, 45, 249, 299, 313, 326, 328, and 738 (for protein identification see Table 3.1 or Table 3.2) were related to histopathological change in white muscle. An explanation of this observation is that in Atlantic salmon the white muscle mass is much greater than red. Complement factor H (CFH) was one of the proteins found in this study to be a possible biomarker of white muscle damage; in fact all three spots identified as this protein (45, 47 and 738) possess expression profiles that correlated significantly with white muscle pathology. This glycoprotein is an important component of the innate immune system with a number of known functions related to it being a regulator of the complement system alternative pathway

(Meri and Pangburn, 1990) and acting to reduce local concentrations of toxic products of inflammation (Jozsi et al. 2004). The expression profile of all three spots which contained CFH was that of a continuous rise in intensity until a peak at week 8wpc and then a sharp fall to near basal intensities in week 12.

3.4.3 Humoral Components of The Serum Response During Pancreas Disease

Complement is a vital component of the immune system of all animals. However, fish are unique in that their complement components exhibit a greater diversity than that of those observed in the mammalian system (Holland and Lambris 2002). In addition to complement Factor H described above, other Complement components were identified by 2-DE and found to change following SAV3 challenge in salmon but without a correlation to histopathology. Thus complement components C3, C9, complement factor B, and the complement inhibitor C1 (spots 444, 146, 357 and 32 respectively), which have been previously characterised as part of the fish innate immune system, were identified in protein spots on 2-DE. Interestingly the complement membrane attack complex (MAC), of which C9 is a pivotal component, damages the envelope of enveloped viruses (Nakao et al. 2011). Moreover, it has been shown that salmonid antibodies are dependent on the presence of complement to neutralize (VHSV) and infectious hematopoietic necrosis virus (IHNV) both of which are enveloped rhabdoviruses (Holland and Lambris 2002). Given that SAV is also an enveloped virus it is possible that complement also plays a role in its neutralization by Atlantic salmon antibodies *in vivo*. The expression profiles of C3 and complement factor B fell significantly at 5wpc whilst at the same time fractions of immunoglobulins rose sharply, (for example spot 626), which may have indicated that as immunoglobulins were synthesised to combat SAV, complement components declined in their serum concentration. In contrast, the expression profile of C1 inhibitor was substantially different from these previously discussed complement components. The late peak in serum abundance of this protein at 10wpc may indicate that in the latter stages of disease recovery it becomes advantageous to inhibit complement activation due

to the harmful effects of the alternative pathway and MAC can have on host tissues (Lorenzen and Lapatra 1999).

Hemopexin-like protein (spots 150, 220, 224, 227 and 565) was found to be significantly altered during SAV3 infection. In mammalian species hemopexin is an acute phase protein (APP), synthesised in hepatocytes and extra-hepatocytic sites to be secreted into circulation, and possesses a high affinity for free circulating haem thus facilitating its clearance (Wicher and Fries 2010). Hemopexin in teleostei is usually referred to as hemopexin-like protein and has been studied at a genetic level by a number of groups. Its expression is highly up regulated during bacterial infections of Atlantic salmon (Tsoi et al. 2004) and rainbow trout (Bayne et al. 2001) with the proposal that hemopexin-like protein is up regulated in order to clear free haem from the circulation which would be detrimental to the proliferation of bacteria. In this study the expression profiles of the hemopexin-like protein spots was also found to change following infection. The first four of these spots were in close proximity and exhibited very similar expression profiles remaining near basal week 0 levels until at 5wpc they increased sharply and reached their peak intensity at 10wpc and started to fall again by 12wpc. This may have indicated an increase in serum haem concentration during PD with higher levels of hemopexin being synthesised to aid its clearance. In other teleost species two isoforms of hemopexin-like protein have been identified. For instance, medaka (Oryzias latipes) possesses two hemopexin-like proteins which differ not only in their tissue expression locations but also in their ability to bind haem (Hirayama et al. 2004). Interestingly, spot 565 showed the opposite from these previously discussed spots in terms of expression profile. Despite also displaying early stable expression levels it fell sharply in intensity at 5wpc and continued to fall until 10wpc before starting to rise. Therefore, it is possible that Atlantic salmon also possess two isoforms of hemopexin-like protein with the first contained in spots 150, 220, 224 and 227 and the second located in spot 565. However it is possible that alterations in the hemopexin-like protein spots, especially for the lower molecular weight spot 565, could be due to unspecific degradation of proteins of higher molecular weight. It was notable that the matched peptides profiles were similar between

the hemopexin-like spots so that the differences in these protein spots could be due to post translational modification or degradation. However it is possible that there could be two homologous isoforms thus more work is required to ascertain if, as in other fish (Hirayama et al. 2004), salmon possess multiple hemopexin isoforms.

A total of 13 protein spots, differentially expressed during PD, were identified as transferrin (Tf). This glycoprotein is one of several humoral proteins with an affinity to iron. Iron binding by Tf facilitates the transport of iron from the circulation to cells (Bayne and Gerwick 2001) thus preventing a potentially toxic iron build up, although other functions are known (Stafford and Belosevic 2003). The intensities of 4 of the 13 Tf spots had a significant relationship with tissue damage (pancreas and skeletal muscle) and have been previously discussed. However, the change in expression of nine Tf spots did not exhibit such a relationship to tissue damage, and were thus deemed components of the immune system response to SAV infection. Of these Tf spots number 151 was by far the largest (in terms of area), migrated with the highest Mr (~68kD) and remained at a relatively constant expression level until between 8 and 10wpc at which point its expression increased sharply only to fall slightly by 12wpc. This spot was presumably the main protein constituent of Tf in the serum and is the full length isoform. Other spots identified as Tf were smaller in size with lower Mr than spot 151. These could have been degradation products, although Stafford et al (2001) has shown that certain transferrin fragments may not be just simply degradation products to be eliminated from the circulation, but play a significant role in the innate immune system of fish. These studies demonstrated the ability of transferrin fragments (but not full length transferrin) of goldfish (Carassius auratus) to induce nitric oxide (NO) activation of macrophages which is vital in viral disease recovery since NO is a potent antiviral-agent and immune system modulator (Akaike and Maeda 2000). This observation of multiple transferrin fragments with different expression profiles may also help explain the considerable variability in the reporting of transferrin as either a positive or negative APP (Bayne and Gerwick 2001) in the fish immune system as certain approaches in studies may or may not detect these

fragments in terms of total transferrin. However, given the large number of spots (13 in total) which contained the protein it cannot be overlooked that, as with other proteins, many of these changes in concentration could be due to unspecific degradation.

Interestingly the S-nitrosylation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) within cells can initiate a cascade ultimately ending in apotosis of cells (Hara et al. 2005). However, this study not only found GAPDH spots associated with tissue pathologies, but also two spots (493 and 494) which rose dramatically in intensity at 5wpc reaching their peak expression at 6wpc before falling to near basal intensities at 8wpc independently of tissue damage. Since GADPH is regarded as an intracellular enzyme involved in glycolysis this at first may be regarded as a surprising observation. However, endogenous authentic GADPH has previously been shown to be secreted outside of cells without causing cell lysis by Yamaji et al (2005) leading to the hypothesis that it may possess a role in defence against pathogens. Therefore, GADPH may be classed as an example of a moonlight protein where a primarily intracellular protein is secreted by cells and exhibits very different functions extracellularly. Another intracellular enzyme found by 2-DE that may possess 'moonlighting' functions extracellularly was the cytoplasmic glycolytic enzyme aldolase. This study found two spots of aldolase that showed no relationship with histopathology (Table 3.2) though whether there is a moonlighting function of this enzyme is currently unknown.

Other proteins found in this study which increased in serum concentration were the apolipoproteins. Apolipoprotein A-1 together with apolipoprotein A-II constitute the most abundant circulating protein observed in teleosts. Its primary function is the binding and transportation of lipids. However, other antiviral, antimicrobial and anti-inflammatory defensive functions are known (Villaroel et al. 2007; Whyte 2007). This study identified four protein spots containing apolipoprotein which did not exhibit a relationship between expression profile and histology. Interestingly, despite apolipoprotein A-I being widely defined as negative acute phase proteins in mammals all, except spot

668, exhibited a steady increase in intensity with a peak at approximately 6 or 8wpc confirming previous results which found apolipoprotein to be up regulated during fish disease (Magnadottir 2006; Russell et al. 2006). This finding demonstrated a major difference in the expression of this protein during disease in fish and may indicate additional functions of the protein in fish that do not exist in mammalian apolipoprotein. Despite this, as with transferrin, the possibility of this expression profile being due to nonspecific degradation of protein cannot be overlooked.

Protein synthesis can also fall during disease as a shift due to a preferential synthesis of specific proteins. This is the widely accepted hypothesis of why albumin (an extremely abundant serum protein in all animals) is observed to act as a negative acute phase protein in most instances. This study also observed albumin to be down regulated following SAV3 challenge with the protein spots containing it showing a steady decline in expression. Albumin hepatic expression has also been shown to be reduced in other fish species during a number of diseases (Gerwick et al. 2006). Whilst albumin is a well-known negative acute phase protein, two other proteins which declined in serum abundance during PD are not as well documented. These are antithrombin (spot 201) and prostaglandin-D synthase (spot 702). Antithrombin inactivates several enzymes involved in the coagulation system and thus down regulation may allow the benefits of coagulation during disease. Prostaglandin-D synthase in serum has not been studied to any extent in fish or other species immunity, although inhibition of the protein has been shown to correlate with muscular necrosis (Mohri et al. 2009) which may have significance in PD and other viral diseases that cause necrosis of muscle fibres. It is therefore probable that these proteins are being down regulated for sufficient nucleotides to be available for the synthesis of afore up regulated proteins previously discussed. Moreover, giving us an insight into the pathways and components of the immune system which are pivotal for fighting SAV infection, for example, complement system and acute phase proteins.

This histopathological and proteomic study of PD in Atlantic salmon, *Salmo salar*, has identified numerous serum proteins which are altered in abundance during the disease. Giving an insight, at the protein level into pathways and components of the immune system which are pivotal for fighting SAV infection, for example, complement system and acute phase proteins as well as potential markers of pathological damage during PD. A correlation between pathology and changes in spot intensity of intracellular proteins and enzymes was established, including variations in a number of tissue specific isozymes. Furthermore alterations, not associated with histopathology, were identified for components of humoral immunity which were presumably involved in both limiting the establishment of PD and aiding the return to homeostasis. A number of proteins, including complement components, apolipoprotein, hemopexin, and transferrin were identified as increasing in serum concentration, whilst albumin and antithrombin levels appeared to decrease during PD. Prior to their use as biomarkers of either tissue damage or humoral response in PD, the diagnostic value of these proteins should be validated by complementary approaches,such as western blotting, immunohistochemistry and ELISA. The proteomics approach described here could be used to investigate other disease challenge models to look for points of similarity and difference between diseases of importance in aquaculture. This would help identify protein changes most associated with morbidity and death, and to deliver insights into disease aetiology and identify mitigation strategies.

Chapter 4

Validation and Development of Potential Markers of Pathology

4. Introduction

4.1.1 The Biomarker Pipeline

Post genomic tools such as proteomics have had great success in identifying novel biological markers of a number of diseases and traits in many species (Moore et al., 2007). For an identified potential target to have an impact on the armamentarium available for clinicians in both human and animal health there are a number of steps which must be carried out from identification to field use. Rifai et al. (2006) describe this process as a biomarker pipeline where potential targets are identified from a small number of samples before qualification, verification, and validation by clinical assay development with ever increasing sample numbers. However, to date the impact of many 'omic' studies in general terms to real world application has been poor. This lack of impact has been due to a number of failings and remains one of the greatest challenges in animal health. Indeed this transition from potential marker identification to assay development is more challenging in understudied species, such as salmon due to a lack of suitable tools such as suitable antibodies.

In brief, for a biomarker to be a useful tool in diagnostics it must be accurate and sensitive for disease state, have low variation within a population undergoing the same stimuli and be reproducible and consistent over time and circumstance (Moore et al., 2007). If normally present in biological fluid or tissue under homeostatic conditions a 'healthy range' of analyte concentration must be established so a problem may be identified swiftly when out with this range. In addition, any diagnostic test target must also be consistent and accurate with robustness to biological and analytical variation.

4.1.2 Biomarkers of Pathological Damage

Biological markers to assess fish health fall into a number of categories. For instance, markers of infection such as acute phase proteins (Eckersall and Bell, 2010) can be used to give an indication that there is an infectious agent present at a given time. These can be extremely useful as non-destructive health monitoring tools and have advantages over specific tests of virus presence and antibody presence commonly used in salmon aquaculture (Adams and Thompson, 2011). Acute phase proteins are detected in fish serum much earlier than antibody and can also be used as markers when the aetiological agent is not known. However, it is widely established that aetiological agents can be present at a given site for a period of time before, if any, clinical disease is observed (Kristofferson et al., 2009; Jansen et al., 2010). This, in terms of disease management strategies, creates a massive problem as one cannot be certain when a clinical outbreak occurs, using the current armamentarium available, unless histopathological analysis is routinely carried out thus further reducing output of fish to harvest and at significant sampling cost. Whilst creatine kinase (CK) is being used ever more routinely in salmon aquaculture as a prognostic tool and a general biomarker of pathological damage this assay has a number of limitations with more sensitive and tissue specific markers in high industrial demand for accurate diagnosis of clinical disease.

4.1.3 Skeletal Muscle Damage

Atlantic salmon aquaculture, like any industry, relies on the production of an end product which is profitable. In fish culture the main aim, therefore, is to produce flesh, comprised of skeletal muscle and fat, which is desirable in the market place. Quality of flesh is assessed in a number of ways (Larsson et al., 2012) and is the result of a number of complex genetic and nutritional processes. Upon harvest of fish the quality of flesh is the major determinant of price and thus profitability of the cycle. Whilst downgrading of fillet can be caused by nutritional factors, toxic myopathies, and muscle breakdown due to excessive exertion (Larsson et al., 2012), one of the most important causes is infectious diseases which cause skeletal muscle myopathy.

Pancreas disease (PD) has been shown to have significant effects on fillet quality even in fish clinically healthy following recovery from disease, most probably due to chronic effects of pathological damage to skeletal muscle, and pancreas damage resulting in reduced ability to uptake nutrients (Lerfall et al., 2011; Larsson et al., 2012). However, whilst there are a number of non-destructive tools to detect the aetiological agent SAV and presence of antibody to the virus, there are not any tissue specific biomarkers currently available to diagnose associated pathologies. Therefore, a biomarker which could be used as a diagnostic tool for skeletal muscle myopathy would have great application in the field not only in diagnosing PD associated muscle myopathy but pathological damage due to other stressors.

4.1.4 Validating Proteomic Results

Given that the major constituent of skeletal muscle in salmon is white muscle any biomarker of pathological damage to this tissue would have potential at not only diagnosing the pathology in terms of disease but also as a marker of flesh quality. Therefore leading on from proteomic analysis of serum proteome changes during PD, enolase and aldolase were selected for further evaluation of their potential as markers of white muscle myopathy (section 3.4.2). In addition to this, glyceraldehyde 3-phosphate dehydrogenase was chosen due to its surprising spot profiles in proteomic work. This ubiquitous enzyme has been previously shown to increase in serum abundance in other systems due to tissue pathology although it did not correlate with pathological damage during PD. Therefore, it was investigated to demonstrate if this apparent lack of correlation (between serum abundance and tissue pathology) was due to limitations of the gel based proteomic approach used.

4.1.5 Enolase

Classically this enzyme is defined as a glycolytic enzyme catalyzing the conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP) in the ninth and penultimate step of glycolysis (Pancholi, 2001). However, it has been shown in numerous studies to have a number of other functions leading to it being regarded as a "moonlighting" enzyme (Avilan et al., 2011). Enolase distribution and isoform status in mammalian species is clearly defined with three paralogs being enolase 1 (α enolase), 2 (γ enolase) and 3 (ß enolase) (Tracy and Hedges, 2000). Whilst these can form heterodimers, in the main they are separated by their tissue distribution with enolase 2 being expressed in neurons, enolase 3 in the muscle, and enolase 1 in all other tissue types. Indeed these isoforms have previously been shown to be biomarkers of a number of conditions in human medicine: enolase 1 serum concentrations are significantly higher in patients with hepatic fibrosis compared to healthy individuals (Zhang et al., 2013), raised enolase 2 levels have been shown to indicate ischemic stroke (Singh et al., 2013), and the presence of enolase 3 has been shown to correlate with progressive muscular dystrophy status (Mokuno et al., 1984).

Enolase enzyme and its distribution is poorly defined in teleost species and little is known of its pathophysiology in salmonids. Landrey et al. (1978) investigated the enzyme in coho salmon, *Oncorhynchus kisutch*, examining both enolase activity in different tissues by enzymatic activity and isoforms distribution by cellulose acetate strip electrophoresis. In this study he stipulates there to be at least six isoenzymes with clear tissue separation of some of these forms stipulating that polyploidy may contribute to complexity and non-allelic forms arising through gene duplication. In some way this is supported by more recent work with two enolase 1 gene homologs in brown trout, *Salmo trutta*, being identified by Tracy and Hedges (2000) thus indicating there may be an added complexity in salmonid enolase due to gene duplication. Moreover, increased enolase gene family complexity may be found in other teleost species with a novel gene homologue being observed in sea bream, *Sparus aurata*, which was found to increase in expression 48 hours post bacterial lipopolysaccharide (LPS) infection thus indicating this isoform has some acute phase reaction (APR)

function (Ribas et al., 2004). Nakagawa and Nakagawa (1991) demonstrated differential enzyme activity in muscle enolase in red sea bream (*Pagellus bogaraveo*), Pacific mackerel (*Scomber australasicus*), and carp (*Cyprinus carpio*). However, serum concentrations of the enzyme have never been assayed in any fish species and thus its use as a non-destructive marker of muscle damage remains unknown /undetermined.

4.1.6 Aldolase

Aldolase is a ubiquitous enzyme involved in glycolysis which catalyses the reversible cleavage of fructose 1,6-bisphospahate to glyceraldehydes 3-phosphate and dihydroxyacetone phosphate (Meyerhof and Lohman, 1934). However, like enolase and several other glycolytic enzymes, aldolase has been shown to possess numerous non glycolytic functions (Lorenzatto et al., 2012) thus being categorised as a moonlighting protein. Three genetically distinct isoforms of the enzyme exist in mammalian species (Lebherz and Rutter, 1969) of which expression is tissue and life stage dependent (Numazaki et al., 1984; Llewellyn et al., 1998). Aldolase A is expressed in the muscle and most other tissue types, aldolase B is the predominant isotype in the liver and kidney, and aldolase C expressed in the central nervous system (Haimoto et al., 1989) but has been found in other tissue types (Haimoto and Kato, 1986). Serum presence of these isoforms has been reported numerous times in association with pathological disorders in humans.

Teleost aldolase, by comparison, is much understudied. However, enzyme activities have been shown to alter in tissues of the green sunfish (*Lepomis cyanellus*) and walking catfish (*Clarias batrachus*) in response to temperature changes and pesticides respectively (Shaklee et al., 1977; Tandon and Dubey, 1983). Aldolase B has also been cloned and expression levels during smoltification studied in Atlantic salmon (Llewellyn et al., 1998). Despite this, as with enolase, its potential as a serum marker of pathological damage has not been investigated.

4.1.7 Glyceraldehyde 3-phosphate (GAPDH)

Another glycolytic enzyme which has been shown to possess moonlighting functions is glyceraldehyde 3-phosphate (GAPDH) (Tristan et al., 2011). The enzyme's diagnostic potential has previously been shown in human sera as a marker of myocardial infarction (Karliner et al., 1971) and liver cirrhosis (Shibuya and Ikewaki, 2002). In fish little is known about the enzyme but two isoforms have been identified in gilthead sea bream (*Sparus aurata*) and the European sea bass (*Dicentrarchus labrax*) (Sarropoulou et al., 2011).

4.1.6 Aims of Study

- To investigate the suitability of enolase, alsolase A, and GAPDH as biomarkers of skeletal muscle damage
- Validate proteomic results and subsequent statistical analysis by other means
- To develop an ELISA for the most suitable candidate for quantification and establish suitability as a diagnostic tool.

N.B. Work presented in this chapter is published in Braceland et al. (2014).

4.2 Materials and methods

4.2.1 Fish husbandry and challenge and histopathology

Fish serum samples (unless otherwise stated) and histopathological data were derived from the disease trial explained in section 2.2.1.

4.2.2 Histopathology

Tissue sections were examined by an experienced pathologist and a scoring system was used to semi-quantify the distribution and severity of the tissue lesions in the pancreas, heart and skeletal muscle as shown in section 2.2.3.

4.2.3 Preparation of tissue lysates From Healthy Fish

Whole skeletal muscle was collected from healthy adult fish, independent of the trial described above, with an average weight of 3kg from a commercial fish farm (Scotland, UK). Fish were killed using a lethal overdose of anaesthetic (MS-222, Pharmaq), tissues removed and frozen in dry ice and then stored until use at -80°C. Protein was extracted into lysate from tissue samples by grinding one gram of tissue with a mortar and pestle kept frozen by the periodic addition of liquid nitrogen. Once a fine powder was formed it was transferred to another mortar and 10ml of cell lysis buffer (20mM Tris-HCL, pH 7.5) added and the tissue buffer mix ground for a further five minutes. The resulting extract was then transferred into a 15ml universal tube and centrifuged at 10,000xg at 4°C for ten minutes and the supernatant removed, transferred into another tube and the centrifugation repeated. The solid residue was discarded after each centrifugation. Finally the supernatant was passed through a 0.45µm syringe driven filter and stored at -80°C.

4.2.4 Protein Concentration Determination

Protein concentrations were determined for tissue lysates and serum samples via the Bradford assay (Sigma-Aldrich, Poole, UK) with protein concentrations being determined using a bovine serum albumin (BSA) protein standard. Serum samples were pooled according to week post challenge (Wpc).

4.2.5 Western Blotting

The suitability of enolase, aldolase and GAPDH as markers of PD associated pathologies was initially investigated by western blotting. Aldolase (aldolase A) was probed using a rabbit anti N-terminal region sequence polyclonal (Aviva systems biology, San Diego, USA) at an optimized concentration of 1:2000 (diluted in 5% skimmed milk TTBS). A mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) monoclonal antibody (Millipore, Darmstadt, Germany) raised against GAPDH immunogen from rabbit muscle was used at a dilution of 1:500.

Three separate commercially available rabbit polyclonal primary antibodies were used to investigate serum enolase in Atlantic salmon sera by western blot analysis. The first of these was produced against a peptide sequence designed using human enolase 1 sequence (Aviva systems biology, San Diego, USA) and is referred to as ENO1a herein. The others were purchased from GeneTex (Hsinchu City, Taiwan) and were produced using peptides based on zebrafish enolase 1 and enolase 3 sequences, and are herein referred to as ENO1b and ENO3 antibodies respectively. These were found to cross react with salmon enolase through western blotting using optimized concentrations of: 1:2000 (in 5% skimmed milk TTBS) for ENO1a and ENO1b antibodies and 1:5000 for ENO3 antibody. Samples for Western blotting were pools of sera derived by pooling 1µl aliquots from each fish (n = 108) from the same week post challenge, thus resulting in nine pools being created from week 0 to week 12 post challenge.

Samples were prepared in sample buffer (Biorad, Hemel Hempstead, UK) and XT reducing agent (Biorad, Hemel Hempstead, UK) and then diluted in water until the final protein loading was 8 µg per sample. The samples were then heated on a heating block for 5 minutes at 95°C. Proteins were separated by one dimensional electrophoresis (1-DE) using Criterion XT precast gels 4-12% Bis-Tris (Biorad, Hemel Hempstead, UK). Gels were run at 200V in a 3-(N-morpholino) propanesulfonic acid (MOPS) running buffer (Biorad, Hemel Hempstead, UK) for one hour, with one well containing 8µl of the protein molecular weight standard 'all blue' (Biorad, Hemel Hempstead, UK). Gels were then removed and protein transferred to a 0.2 µm pore size nitrocellulose membrane using the iBlot® dry blotting system (Novex life technologies, Paisley, UK). Nitrocellulose was then placed into Ponceau S stain for ten minutes to ensure the transfer of protein to the membrane was successful and the global protein profile intensity was comparable for each sample. The stain was subsequently washed off using TTBS buffer which comprised of 50 mM Tris, 150 mM NaCl, and 1% Polyethylene glycol sorbitan monolaurate. The membrane was then blocked for one hour in 10% w/v powdered skimmed milk (dissolved into TTBS) and washed three times in TTBS. Primary antibody was diluted in TTBS containing 5% w/v powdered milk and left rocking overnight at 4°C. Antibody was then removed and the membrane washed three times before treatment with HRP linked donkey polyclonal secondary antibody to rabbit IgG (Abcam, Cambridge, UK) at a 1:10,000 dilution (in TTBS containing 5% w/v powdered milk) for one hour at room temperature. After washing the blot was then developed using Pierce enhanced chemiluminescence (ECL) Western Blotting Substrate (Thermo scientific, Rockford, IL USA). This ECL substrate binds HRP on the membrane facilitating band detection after exposure to a photographic emulsion coated film, in this case Hyperfilm™ Film (GE Healthcare, Buckinghamshire, UK) and development with a X-ray film developer. Exposure times were optimized and films scanned (Umax Poerlook III, Umax, Taiwan) and saved in TIFF format for analysis of band intensities was carried out using Image J (http://rsbweb.nih.gov/ij/) as described in Gassmann et al (2009).

Western blots of the same samples were run and separately stained with a rabbit polyclonal antisera to aldolase at 1:2000 dilution (Aviva systems biology, San Diego, USA) and a monoclonal antibody to 3-phosphoglyceraldyhyde dehydrogenase at a 1:250 dilution (Millipore, Massachusetts, USA). Donkey polyclonal secondary antibody (HRP linked) at a 1:10,000 dilution and a donkey polyclonal antibody to mouse IgG (Abcam, Cambridge, UK) at 1:5000 were used to detect antigen bound aldolase and GAPDH antbodies respectively.

Western blots were also carried out using anti enolase 3antibody (ENO3) following two dimensional electrophoresis (2D-E) and subsequent transfer, to examine the specificity of the antibody. In brief, 2-DE was carried out as in Braceland et al. (2013) with pooled W0pc and W8pc serum to see if binding of antibody was consistent with previous proteomic discovery of the enzyme in terms of molecular weight and isolectric point (pI) and changed in serum abundance as indicated by 1-DE western blots and previous proteomic information.

4.2.6 Enzyme-linked Immunosorbent Assay for Enolase (ELISA)

For ELISA determination of enolase concentrations the primary antibodies used were ENO1a and ENO3 at dilutions (in TTBS) of 1:500 and 1:1000 respectively, chosen on the basis of western blot results and optimised for ELISA.

Purified salmon enolase was not available to use as a calibrator, therefore, a pool of whole muscle lysate, known to possess a high concentration of the enzyme, was used as a standard by serial dilution. Arbitary units (AU) were given to this standard using serial dilutions from 100AU down to 10AU in ELISA coating buffer (0.2M sodium bicarbonate pH9.4). Accuracy was determined by parallel curves of dilutions of serum sample with a high enolase content versus the calibrator. Precision was established by calculating the intra and inter

coefficients of variance by calculating variation of optical density within and between runs respectively. The limit of detection was assessed as the amount in AU of enolase detectable at 3SD from the mean of blank. Specificity was demonstrated bythe antibody cross reactivity on western blot.

To each well 100µl of either diluted sample, standard or blank (buffer alone, unless otherwise stated) was added and the plate left overnight at 4°C. Wells were emptied then washed 3 times using 250 µl TTBS, then blocked with 10% w/v (in TTBS) powdered skimmed milk and left on a shaker for one hour at room temperature. The plate was washed again three times and then 100µl of primary antibody at appropriate dilution in TTBS added to each well and the plate left on a shaker for one hour. Another series of washes were carried out and then 100µl of a HRP linked donkey polyclonal secondary antibody to rabbit IgG (Abcam, Cambridge, UK) at a 1:10,000 dilution added for one hour with the plate on a shaker. Finally wells were washed with TTBS three times and then developed using 100µl (per well) of a 3, 3', 5, 5' - tetramethylbenzidine (TMB) Microwell Peroxidase Substrate Kit (Insight biotechnology, Middlesex, UK) which by peroxidase reaction catalysed by HRP forms a blue byproduct. After incubation, on a shaker, of 10 minutes the reaction was stopped by the addition of 50µl of 1M hydrochloric acid. The addition of HCl causes the colour of solution to change to yellow thus enabling accurate measurement of the intensity at 450 nm using a plate reader (FLUOstar OPTIMA, BMG Labtech, Ortenberg, Germany). The ELISA was validated by assessment of performance characteristics.

Two sample sets were analysed by ELISA. First, enolase content of the pooled samples used as for western blots was determined. Second, sera from fifty individual fish (see Table 1) from the PD trial were selected on the basis of skeletal muscle pathology due to the significant correlation of enolase abundance with skeletal muscle myopathy (see results). This was carried out to investigate the relation of the histopathological scoring system to this non destructive biomarker and by examining serum concentrations from fish with a range of spectrum of muscle pathology severity.

4.2.7 Statistical analysis

Any correlation between enolase concentrations and histopathological lesion scores was examined through statistical analysis applying a general linear model procedure in SAS version 9.3 (SAS Institute, Cary, N. Carolina).

4.2.8 Immunohistochemistry

Tissues for histology, from healthy salmon independent of the trial (4.2.3), were immediately fixed in 3.5% v/v formaldehyde in buffered saline pH 7.0 (4.0g $NaH_2PO_4.2H_2O$, 6.5g $Na_2HPO_4.2H_2O$) prior to further processing by standard paraffin wax techniques and then sectioned in preparation for IHC. In the first instance sections were stained with haematoxylin and eosin (H & E). IHC was carried out using muscle sections which were subjected Heat-induced epitope retrieval (HIER) using Menarini Access Retrieval Unit using in a sodium citrate (pH 6) buffer at 125ºC with full pressure for 1 minute and 40 seconds. Slides were then loaded on to a Dako autostainer and rinsed with a tris-tween (pH 7.5) buffer (herein buffer A) and then treated with Dako Real™ peroxidase blocking solution for five minutes before being rinsed twice with buffer A for a further five minutes. Primary antibodies were diluted to 1:1000 (a number of concentrations were initially tested for optimization) using Dako universal diluent and incubated for 30 minutes. Sections were then rinsed again with buffer A twice for five minutes before treatment with an anti-rabbit IgG HRP labelled polymer (Dako, Cambridgeshire, UK) secondary antibody at a dilution of 1:10,000 for 30 minutes. Post washing (buffer A 5 minutes twice) development was carried out using Dako K5007 DAB and then rinsed with distilled water, counterstained with Gills Haematoxylin for 30 seconds, and washed in water again. Finally sections were dehydrated by treatment with 70% meth spirits then absolute alcohol, cleared with xylene and then mounted in DPx mounting media (Cell Path cat # SEA-0302-00A)

4.3 Results

4.3.1 Histopathology

The mean histopathological pathology scores, using the semi-quantitative scoring system previously described (Table 2-2) can be seen in Figure 4-1. The scores were used for statistical comparison between pathology and enolase levels determined by ELISA (see section 4.3.4).

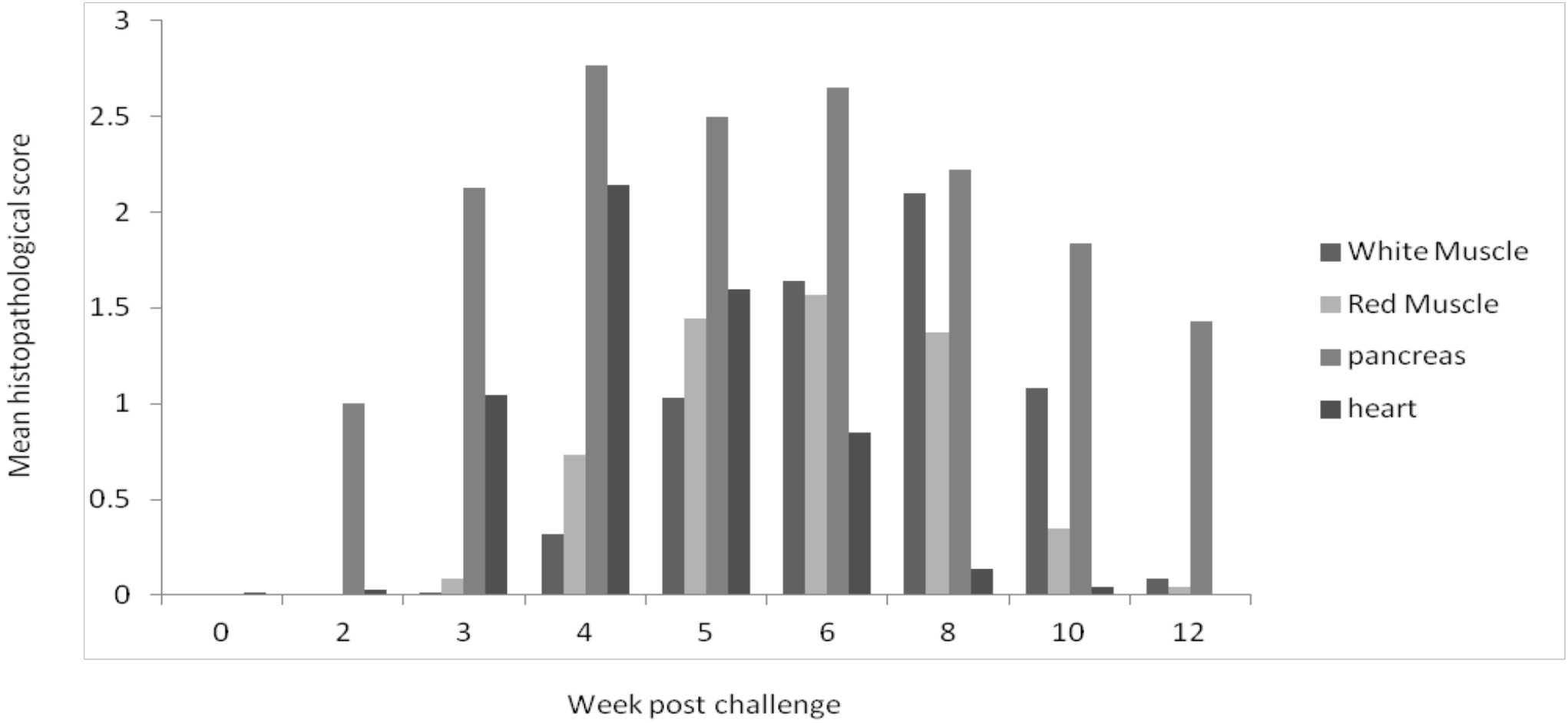

Figure 4.1: Mean tissue histopathology lesion scores for analysis. Mean histopathological score (using a semi quantitative scoring system) of each tissue at the nine sampling time points from week 0 to week 12 post challenge. (n per time point = 108)

4.3.2 1-DE Western blotting

Western blots were carried out using all three commercially available antibodies to enolase: anti ENO1a (Figure 4-2a), anti ENO1b (Figure 4-2b) and anti ENO3 (Figure 4-2c). All of these antisera detected a dominant protein with a molecular weight (Mw) of just under 50KDa with some lesser abundant proteins apparent with ENO1b and ENO3. These could be due to nonspecific cross reactions or in the case of protein with low Mw, could be degradation product. Semi quantification of band intensities of the Western blots with antibody to enolase was carried out using image J (Figures 4-1a, 42b and 4-2c) with similar band profiles being seen in all three blots. Some variation in the specificity and reactivity was observed between these antibodies which may account for a failure to detect enolase in W3 with ENO3 (at a concentration above the sensitivity of the test). Strong signals were detected in weeks 4-10pc samples with weaker signals detected in weeks 3 and 12. These profiles agreed, in the main, with pathology scores of white muscle (Figure 4-1) with a higher band intensity being observed where there is an increase in lesion severity. In addition, the observation of a band at the same Mw and of high intensity when using muscle lysate indicated further that pathogenesis of PD may be influencing sera concentrations. Blots using all three antibodies gave a higher intensity in band when using W6 serum compared to W8 serum at which time point mean white muscle pathology was at its highest.

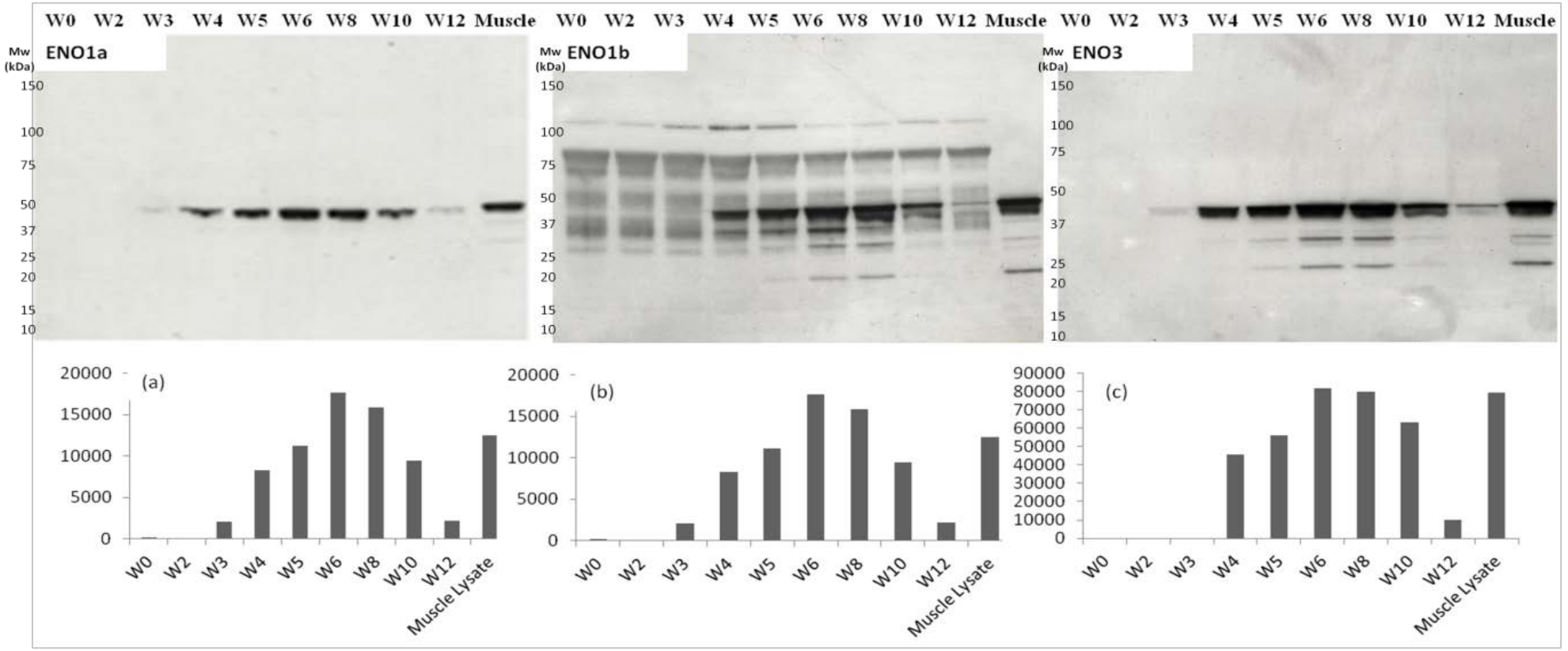

Figure 4.2: Western blots using enolase antibodies. Western blots using: (a) anti ENO1a (Aviva systems biology) antibody at 1:2000 concentration. (b) ENO1b (GeneTex) antibody at 1:2000 (c) anti ENO3 (GeneTex) antibody at 1:5000. Sample identifications for each lane can be found at the top of each with 'W' indicating week post introduction and 'Muscle' indicating which lane a muscle lysate was used. Band intensities from image J analysis of western blot are given below respected blots.

Therefore, an immunological assay in the form of an ELISA was developed out using ENO1a and ENO3 antibodies (ENO1b omitted due to previously discussed background staining) to investigate in a more quantitative manner the relationship between serum enolase concentrations and the pathogenesis of PD.

Western blot with antiserum to aldolase (Figure 4-3a) and to GAPDH (Figure 4-3b) confirmed that these enzymes were also raised in serum during the PD infection. In comparison to the western blot with antibody to enolase, the aldolase blot showed more background staining suggestive of some non-specific interaction while the blot with antibody to GAPDH revealed a shorter time window in the infection where the protein could be detected.

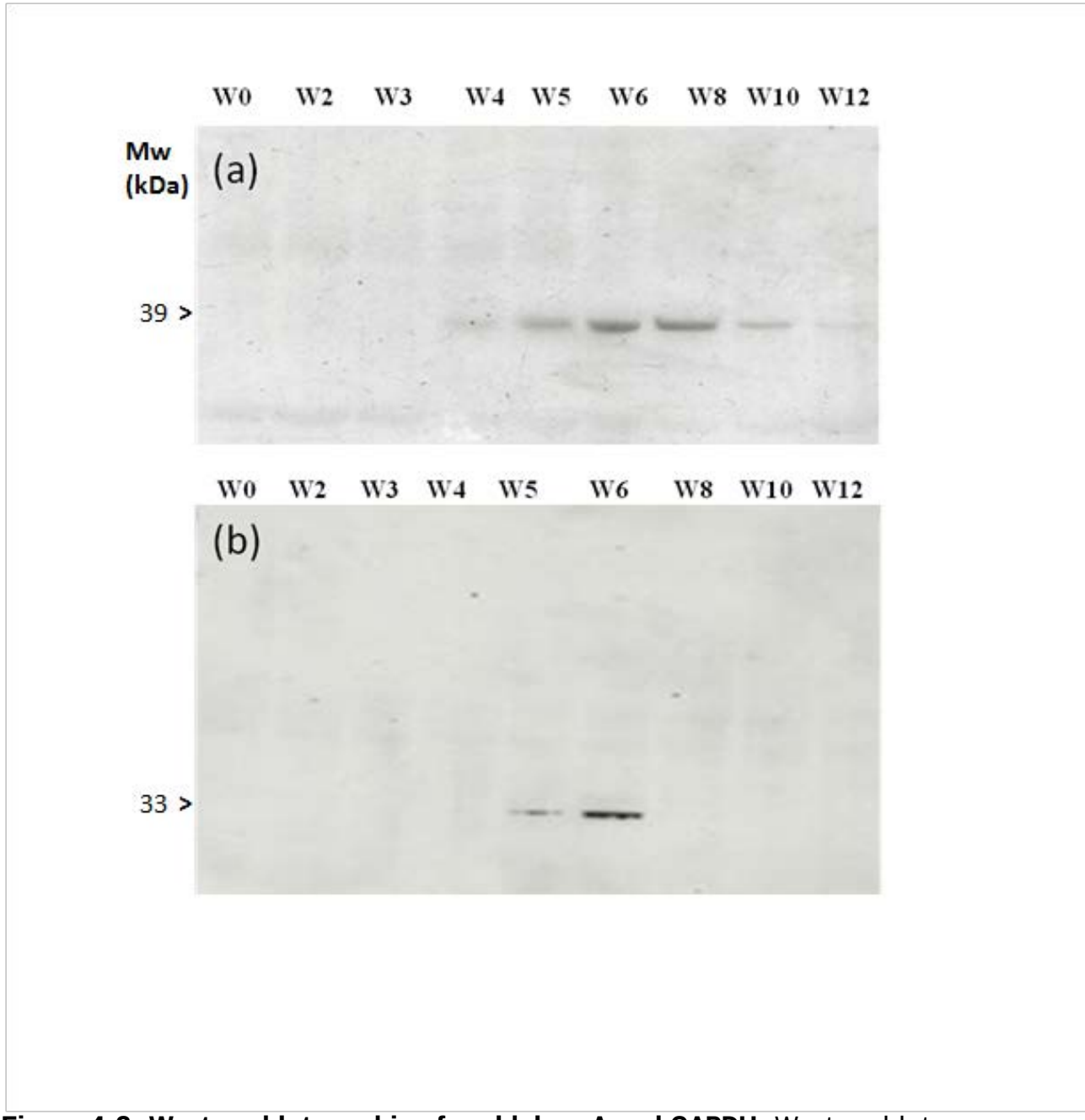

Figure 4.3: Western blots probing for aldolase A and GAPDH. Western blot using: (a) anti Aldolase (Aviva systems biology) antibody at 1:2000 concentration. (b) anti GAPDH antibody at 1:250 concentration. Sample identifications for each lane can be found at the top of each with 'W' indicating week post introduction.

4.3.3 2-DE Western Blotting

Figure 4-4 shows the specificity of ENO3 with major binding at the appropriate Mw (~48kD) and pI (7 to 8). Whilst there is some binding in other areas this is most likely to be albumin and apolipoprotein which are, based on previous investigations, the most abundant proteins in the salmon serum proterome. This non-specific binding does not appear to alter depending on disease state, having similar intensity in both W0pc and W8pc blots. In addition, there appears to be an absence of any specific ENO3 binding in W0pc with high intensity with W8pc, thus in agreement with 1-DE western blots.

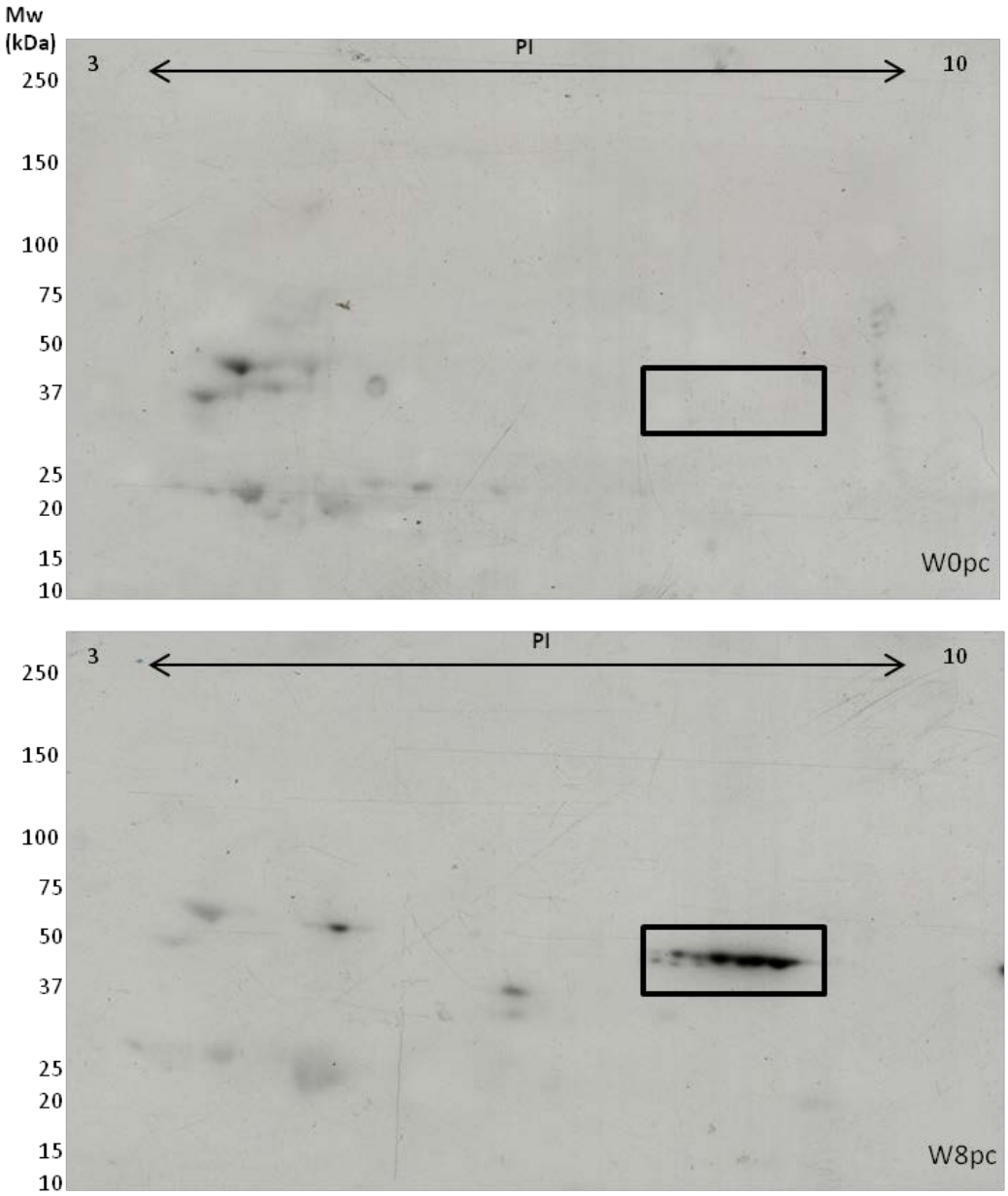

Figure 4.4: Two dimensional western blot probing for enolase. Two dimensional western blot using anti ENO3 (GeneTex) antibody at 1:5000. The top image shows the staining post development when week 0 post challenge pool was used and the bottom shows week 8 post challenge. A black box is around the area we hypothesise to be specific ENO3 staining at the correct Mw and pI.

4.3.4 ELISA

Discordance between band intensities and proteomic and histopathology data could be due to the higher sensitivity of western blots compared to the gel based proteomic investigation or possible image J saturation effects of high sample concentration of enolase. Therefore, an ELISA was developed to quantify serum concentrations of enolase with the use of ENO1a and ENO3 antibodies being compared to more accurately determine levels of target.

Average intra assay %CV using ENO1a antibody was 3.4% and ENO3 3.5% (n= 98). Inter assay %CV of low standard (W4pc pool) was 10.8%, and high standard (W8pc) = 6% when using antibody ENO1a, whilst ENO3 antibody gave inter assay %CV results of 12.4% and 4.3% for low and high standards respectively (n= 30). Limit of detection was found to be 2AU (arbitrary units) for ENO1a and 2.1AU when the ELISA was carried out using ENO3 antibody. When using W0pc sera as a blank the limit of detection was found to be 4.5 AU (ENO1a) and 4.7 AU (ENO3). The same pooled sera used in western blots were used in an indirect ELISA which used a whole muscle lysate 'standard' to give a sample concentration in arbitrary units. Optical density (OD) values were corrected against a fetal calf serum (FCS) blank. Quantification of serum enolase using both ENO1a and ENO3 antibodies by ELISA resulted in a similar profile of change after infection and validated western blot results (Figure 4-6). In addition, the concentration of enolase 1 and enolase 3 concentrations significantly correlated with mean white muscle histopathology scores (Table 1) with pearson coefficient values of 0.9639 and 0.945 respectively. Moreover, concentrations of enolase did not correlate significantly with the pathology of any other tissue (red muscle, heart, and pancreas) in salmon with PD.

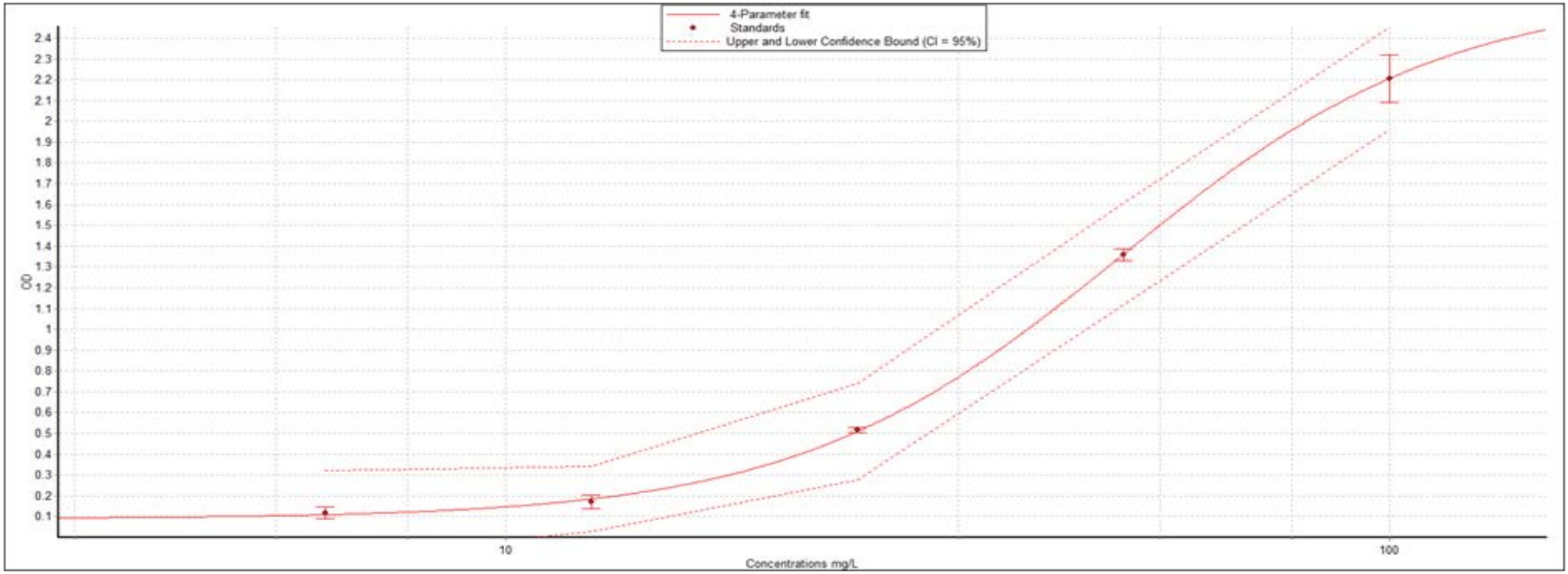

Figure 4.5: Standard curve of ELISA. Standard curve of skeletal muscle standard with arbitrary units on x axis and optical densities on the y axis.

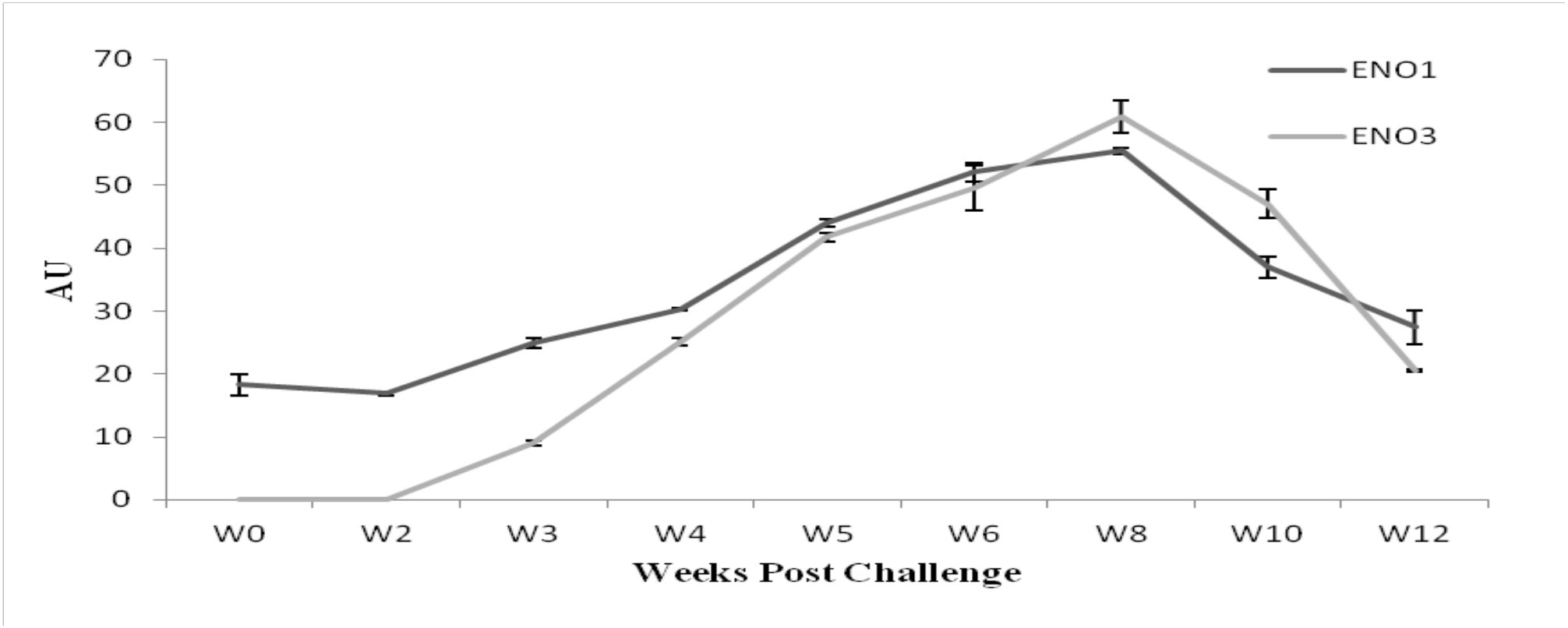

Figure 4.6: Enolase concentrations. Enolase concentrations of pooled sera from the nine sampling time points (x axis) post challenge. Arbitrary units are given as sera optical densities (ODs) were read against the OD of whole skeletal muscle lysate using varying dilutions.

Table 4-1 Mean white muscle pathology against enolase 1 and enolase 3 concentrations. Mean white muscle histopathological score for each time point of sampling and corresponding enolase 1 and enolase 3 concentrations.

Week	Average White Muscle Histopathology Scores	Enolase 1 Conc. (Au)	Enolase 3 Conc. (Au)
0	0	18.325	0
2	0	16.915	0
3	0.0139	24.967	9.062
4	0.3194	30.258	25.051
5	1.0278	43.966	41.763
6	1.6389	52.093	49.6
8	2.0972	55.499	60.905
10	1.0833	37.024	47.042
12	0.0833	27.42	20.607

Fifty fish from the trial were selected to give a range of histopathological scores (Table 4-2) and their enolase 3 concentrations assayed. In a modification the ELISA pooled W0 sera was used as the blank so concentrations could be corrected against those of a 'healthy fish' thus giving more actual relevance. These concentrations were then compared to all tissue pathological scores by linear regression procedure GLM. Results verified those previously with the only significant correlation being found with white muscle pathology $p = <0.0001$ and 38% variation explained. Figure 4-7 illustrates this relationship with the enolase 3 concentrations of specific fish plotted against their corresponding histopathological score of white muscle damage. A clear trend of higher sera enolase concentrations can be seen in fish with more severe histopathological damage.

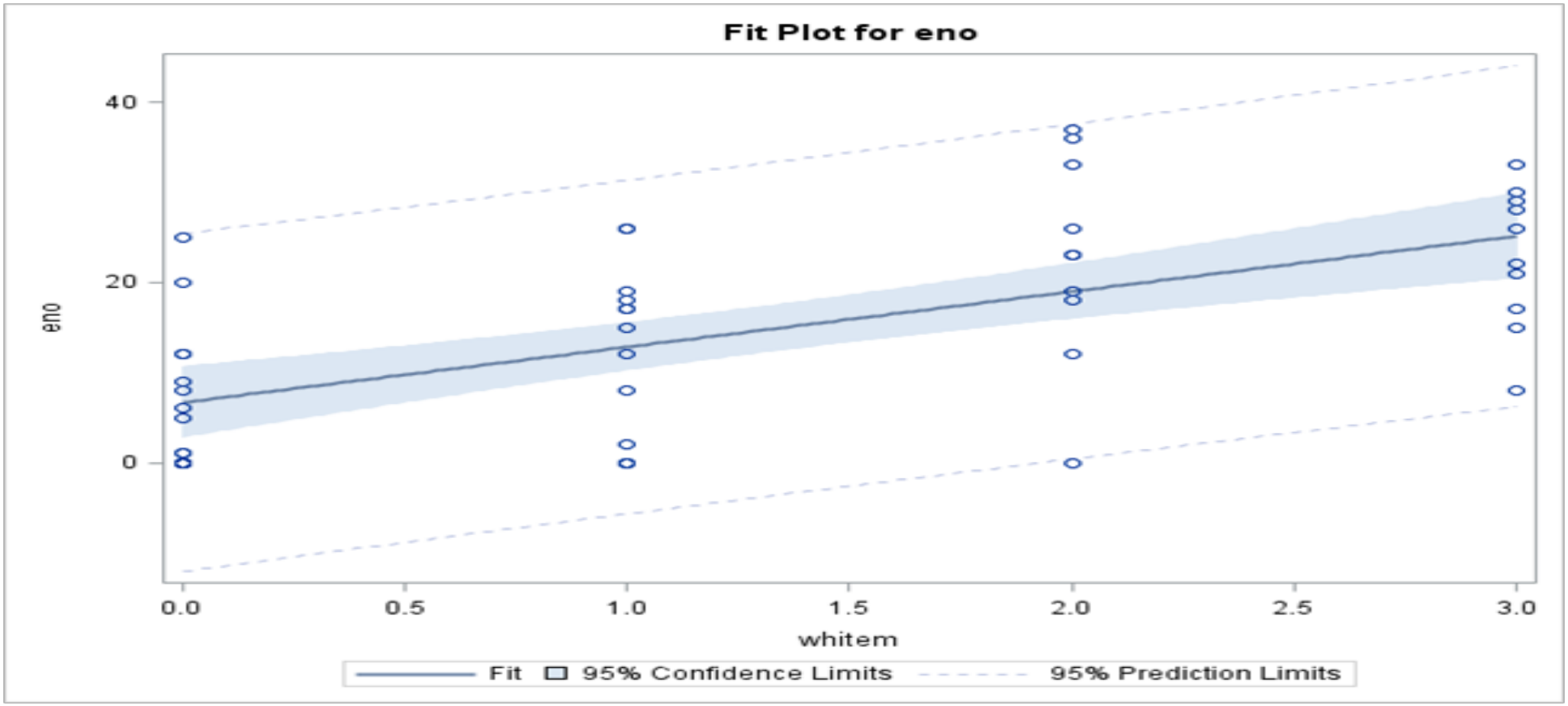

Figure 4.7: Plot of white muscle pathology v enolase concentration. SAS output of Individual fish enolase 3 concentrations plotted against corresponding white muscle histopathological score.

Table 4-2 Enolase concentrations of 50 fish against tissue lesion scores. Samples used with their identification (ID), histopathological damage scores of all tissues examined (heart, pancreas, red and white muscle) and enolase concentration in arbitrary units (AU).

ID (tank.fish.week)	Heart	Pancreas	Red Muscle	White Muscle	ENO 3 CONC. (AU)
1.1.0	0	0	0	0	5
8.6.12	0	2	0	0	0
3.3.0	0	0	0	0	0
4.4.5	2	3	0	0	12
12.1.12	0	3	0	0	0
2.3.3	2	3	1	0	0
1.5.3	3	3	1	0	0
4.2.6	2	3	1	0	25
6.6.3	2	3	1	0	1
11.6.3	2	3	1	0	12
4.3.4	1	3	2	0	8
2.1.4	3	3	2	0	1
2.2.4	2	3	2	0	6
7.4.5	3	3	2	0	20
1.3.4	3	3	2	0	0
1.6.4	2	3	3	0	9
10.2.5	1	3	3	1	18
4.5.5	3	3	3	1	12
8.2.6	1	3	3	1	26
1.6.6	2	3	3	1	17
3.5.10	0	1	0	1	19
3.2.8	0	2	0	1	0
7.1.5	3	2	0	1	0
9.4.12	0	3	0	1	2
11.5.4	2	3	0	1	0
1.6.10	1	3	0	2	33
5.3.10	0	2	0	2	12
6.2.8	1	3	0	2	23

ID	Heart	Pancreas	Red Muscle	White Muscle	ENO 3 CONC. (AU)
(tank.fish.week)					
7.4.10	0	2	0	2	0
8.2.8	0	2	0	3	30
9.1.8	0	3	0	3	29
10.4.8	0	1	0	3	33
9.5.10	0	3	0	3	8
11.4.10	0	3	0	3	28
11.6.5	1	3	3	3	21
6.5.5	0	3	3	3	15
2.2.6	2	3	3	3	17
11.3.6	0	3	3	3	22
8.1.8	0	3	3	3	26
2.6.10	0	2	2	2	37
6.6.10	0	2	2	2	26
1.6.5	2	2	2	2	23
3.6.8	0	2	2	2	19
1.6.8	0	3	2	2	36
5.5.4	2	3	1	1	8
3.6.4	2	3	1	1	15
11.2.12	0	3	1	1	26
H3.6.6	0	2	1	1	12
6.1.10	0	2	1	1	17

4.3.5 Immunohistochemical Staining

Immunohistochemistry (IHC) was carried out using the antibodies utilised for western blot and ELISA results. Figure 4-8 illustrates IHC staining of a muscle section comprising both red and white muscle fibres using ENO3 antibody. A clear differential staining pattern can be observed, with little or no staining seen in red muscle indicating a low concentration or absence of antigen but with clear staining in the white muscle.

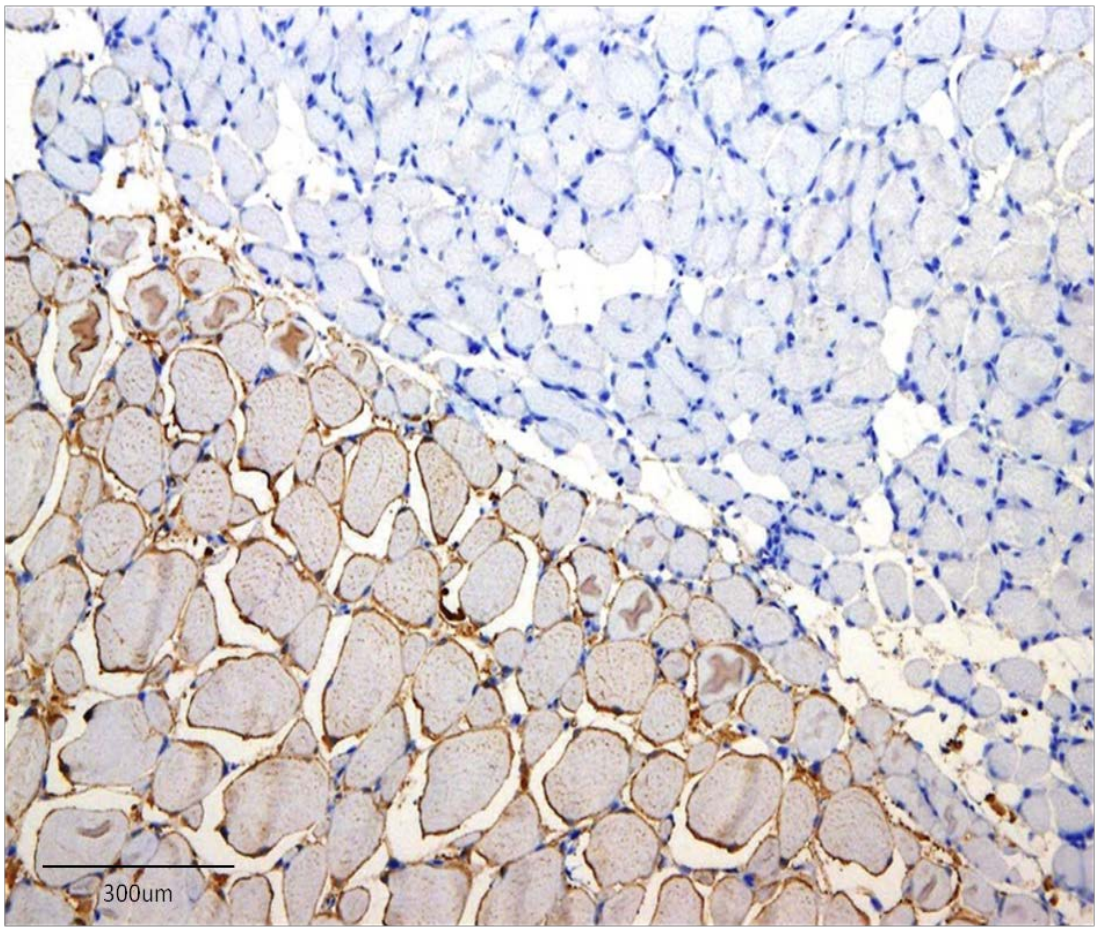

Figure 4-8 Immunohistochemical staining of muscle probing enolase. Immunohistochemical staining of enolase using ENO3 antibody and subsequent staining with Haematoxylin. Presence of antigen is indicated by brown staining.

4.5 Discussion

This study has provided validation for biomarker discoveries using proteomics previously described in chapter 3. Western blotting demonstrated that aldolase and enolase shared very similar profiles, whilst GAPDH abundance shows a sharp rise at W5pc, peaking at W6pc and then falling rapidly as described in Braceland et al., (2013). Enolase, using antibody ENO3 was chosen as the most appropriate marker of PD pathogenesis, rather than aldolase or GAPDH, due to lower background binding, a greater dynamic range and being its detection for longer in the sera. Through the use of histopathology and serum protein analysis a significant relationship between white muscle pathology and serum enolase content has been demonstrated. A serum enolase biomarker may be useful as a non-destructive and cost effective health monitoring tool in Atlantic salmon as it may detect myopathy in apparently normal fish and predict flesh quality issues such as bleaching of fillet and melanisation at harvest. This would allow for the implementation of means of reducing financial impacts incurred through downgrading by implementing management strategies or postponing harvest until these problems are rectified.

In addition, enolase also holds promise in assessing the sensitivity of histopathological scoring systems. Semi-quantitative scoring systems such as the one used in this study (McLoughlin et al., 2006; Christie et al., 2007), whilst extremely useful in describing the extent or degree of pathology, are limited in a number of ways. First, due to the categorical nature of any semi-quantitative scoring system there will always be a level of subjectivity, as to where one sample is placed, and given that there will in nature always be heterogeneity this can lead to the reduction of accuracy. For instance, whilst there was a significant correlation of white muscle histopathological scores and enolase 1 and 3 sera abundance, the level of variance between concentrations of fish placed in the same category (or score) was reasonably high. This was reflected in the fact that whilst the probability of a correlation being at $p = <0.0001$ the amount of variance explained was only 38%. Whilst this is still a reasonably high degree of variance being explained it is probable that the development of a less categorical histopathological scoring system would increase sensitivity and thus

accuracy. However, the use of such a system would take longer to carry out using more resource and require a high level of skill. In contrast, development of a continuous quantitative non-destructive serum based biomarker based system allows for the non-destructive diagnosis of white muscle pathology and assessment of its severity. In addition, humoral non-destructive markers can give an indication of the extent of pathological damage in terms of the whole organism rather than just in the small area sampled for histopathological analysis.

It is important to note that currently, in salmon disease, other non-destructive markers of skeletal muscle myopathy are used, with the most common being creatine kinase (CK) (Ferguson et al., 1986; Roger et al., 1991; Yousaff and Powell 2012). However, the assay relies on the enzyme activity of CK which whilst a reliable and well established method has some disadvantages over the enolase ELISA described here. For example, the range of concentrations in healthy sera (due to a high and variable activity) is large (Ferguson et al., 1986; Roger et al., 1991; Yousaff and Powell 2012) thus making it hard to identify subtle disease associated changes on which to base health management decisions. This is especially a problem when a site has an outbreak of HSMI and CMS where changes may be quite small and fall within the healthy range. Moreover, the literature thus far has only investigated total CK concentrations meaning the assay lacks tissue specificity as the enzyme is ubiquitous and while there is a muscle specific isoforms identified which can specifically be assayed for the issues of enzymatic activity tests still remain.

Unless a biomarker is truly tissue specific, histopathology remains the most reliable means of identifying specific pathogenesis. Enolase in vertebrates is characterized as possessing three paralogs; enolase 1 (α enolase), 2 (γ enolase) and 3 (ß enolase) (Tracy and Hedges, 2000). Enolase 2 is regarded as neuron specific, thus enolase 1 and enolase 3 were chosen in this study for immunoassay development, with Enolase 3 being laterally chosen as the best marker due to low interassay CV and reliability of ELISA. Both correlated exclusively with white muscle pathology in PD affected fish as determined by histopathology. Whilst

this was expected in the case of enolase 3, this specific correlation was interesting in terms of enolase 1 as enolase activity (via an enzyme kinetics assay) has been shown in heart lysate from coho salmon, *Oncorhynchus kisutch* (Landrey et al., 1978). Moreover, the isoform has been proposed as an early indicator of myocardial infarction in humans (Mair, 1997). An explanation as to why enolase concentrations were not altered in serum of fish sampled with low muscle but high heart pathology could be that the amount of enolase being released passively by pathological damage into the humoral system is below the sensitivity of western blot and ELISA using this antibody. Despite this, even when mean pathological damage is low (0.0139) at week 3 post challenge there is a detectable increase from previous sampling points in enolase concentration. However, this may be due to the quantity of muscle when compared to heart. A similar issue was found by Yousaff and Powell (2012) who reported during CMS affected fish no significant correlation of pathological damage to the heart on serum creatine kinase (CK) and lactate dehydrogenase (LDH) activities while in the same study heart pathology in fish with HSMI significantly correlated with CK and LDH levels. However, this is possibly an artefact of sampling times where sampling has missed the effect of pathology on the proteome, with the enzymes leaked already being turned over. Despite this, the lack of correlation (between heart pathology and enolase concentration) in this current study is interesting and future work will aim to identify the distribution of specific isoforms of enolase in salmon. This would be of great use as if isoforms are found, as in mammals, to be tissue specific they may be used to differentiate between pathologies of different tissues in a non-destructive manner as is routinely carried out in human medicine and for other livestock species. In fact studies where the direct effect of specific pathogenic mechanisms on the humoral proteome or enzyme concentrations is distinctly limited in salmon infectious disease. Creatine kinase has also been studied in terms of sera levels in fish with PD (Ferguson et al., 1986; Rodger at al., 1991). However, the relationship between sera levels and histopathology was not directly investigated.

In conclusion, this study has identified, through the use of commercial antibodies, significant correlations of enolase 1 and 3 serum concentrations with muscle pathology in PD. The enolase ELISA has been shown to have potential use

as a non-destructive health monitoring tool for this and possibly other diseases involving myopathy in salmon and other teleostei species. Such markers, currently lacking in aquaculture, may be beneficial to the industry by being an early stage indicator of a problem developing at a given site. Monitoring in such a way, may have economic benefits as early diagnosis of a disease problem may permit implementation of other disease management strategies such as functional feeds at the most optimal time. In addition, this marker may also be useful, due to its correlation with white muscle pathology, a means of assessing the sensitivity of histopathological scoring systems such as the one used in this study. Monitoring could also help lower the ecological impact of the industry in terms of farmed to wild stock disease interactions as earlier identification of clinical disease may lead to quicker recovery through swifter action. Future work on this enzyme aims to examine tissue distribution of specific isoforms and the establishment of a reference range of enolase in healthy salmon production site and change during natural outbreaks of fish disease. This will involve an expansion of study number (n) and the opportunity to validate this marker using samples from fish suffering from diseases other than PD which cause muscle myopathy.

Chapter 5

Serum Precipitate Reaction

5.1 Introduction

5.1.1 General

Pathological damage to tissues during disease is a major problem for the production of salmon by aquaculture with downgrading of fillet quality and reduced cumulative reduction in biomass due to chronic reduced health having a significant effect on the profitability and sustainability of the industry. Presently the most well established and reliable way of assessing pathological disease in fish is by histopathology. However, histopathology, compared to other means of assessment, is time consuming, costly, and takes a high level of expertise. Despite this, it remains the gold standard for diagnosis due to the limited number of reliable and sensitive means of non-destructive health monitoring available. In light of this there is a growing interest in identifying biomolecules which may detect pathological damage or indeed infection using non-destructive techniques, such as sampling of blood derived serum.

5.1.2 The Discovery of a Serum Precipitation Reaction

In the search for a means of detecting infection and disease in salmon we investigated the suitability of the acute phase protein ceruloplasmin (Cp) as a marker using Atlantic salmon serum samples from a pancreas disease (PD) trial. Our hypothesis was that by using the method described by Ceron et al. (2007), where a colorimetric assay based on the oxidation of paraphenylenediamine (PPD) substrate in a sodium acetate buffer is used to determine Cp activity in salmon serum, we could monitor the acute phase response to infection by salmonid alphavirus (SAV) subtype 3 (SAV3) in the experimental model described in Chapter 2. Using this methodology it was found that assumed Cp concentrations in salmon sera were several fold greater than in the serum of species such as man, dog and pig and yet salmon Cp was not identified in the salmon serum proteome (Chapter 3). This observation led to the hypothesis that either Cp gram for gram possesses a higher oxidase activity in salmon than any other thus far studied organism or there were other causes for the apparently elevated Cp level. However, further investigation revealed that upon removal of

the active substrate PPD the simple mixing of sera from salmon with PD and sodium acetate buffer caused a change in turbidity due to the appearance of an opaque precipitate, leading to change in absorbance at 550 nm and an apparent high activity of Cp. Furthermore this turbidity was associated with the disease state of the individual from which the sample was taken. This was an interesting observation especially since the buffer is commonly used as a base buffer for enzymatic/ protein assays to which functional substrates are added. Moreover, similar methodologies have also been used in other finfish species. For example, Haluzova et al. (2010) demonstrated ceruloplasmin activities rising after carp were treated with the pesticide Sparatakus, again with no precipitation of proteins being reported despite pathological damage to a number of tissues. A similar buffer system has also been used with an acetate buffer and sodium azide solution mix (plus PPD substrate) being used by Yada et al. (2004), again with no precipitate formation being noted in Rainbow trout.

The test developed from these initial observations and described in this chapter has the potential to provide results at three levels. The first of these is a qualitative test with simple mixing of serum and sodium acetate trihydrate buffer and observing with the naked eye if the mixture becomes turbid or remains clear. This has shown to be very effective in the laboratory with no visible turbidity occurring when using serum samples from clinically healthy fish. The second level of the test is the quantitative monitoring of precipitation formation over a period of time using a spectrophotometer, where differential precipitation has been shown as an indicator not only of pathological damage to a given tissue but also to attain information on the extent/ severity of the damage. Thirdly precipitated proteins from these tests can be reconstituted in water and separated using electrophoresis to give a band profile relating to the tissues that has been damaged. We can find no evidence in the literature of any similar test in any species. Therefore not only does this test have huge potential in non-destructively diagnosing clinical disease and which tissues are damaged it also represents an interesting novel phenomenon in biological terms.

5.1.3 Aims of Study

- To develop and validate the observed precipitation phenomenon
- To determine correlation between pathological damage and precipitate formation
- To determine whether the test can detect reduction in pathology severity in fish fed different diets
- To investigate which proteins are being precipitated during PD
- To investigate the precipitation potential of other pathologies through use of tissue lysates

N.B. A patent application is in preparation based on the findings of this chapter.

5.2 Materials and Methods

5.2.1 Samples

5.2.1.1 PD Cohabitation Disease Model

Sera used in this chapter, unless otherwise stated, were obtained from the PD trial as described in section 2.2.1. Only samples derived from fish fed fed diets A and B were investigated (n = 54 each time point).

5.2.1.2 Samples from Aquaculture sites and Tissue Lysates

Samples were collected from clinically healthy salmon (n = 23), with no history of PD or any other viral infection, of an average weight of 3kg from an aquaculture production site in Ardnish (UK). Fish were killed by lethal overdose of anaesthetic (MS-222, Pharmaq) and blood taken in non-heparinised vacutainers, spun at 10,000xg for 15 minutes and serum collected and stored at -80°C. A pool of these samples, referred to as 'POOL A' herein, was also used for inter-assay CV calculation upon development of a precipitation assay.

A number of tissue samples from these fish were also collected, with samples of; whole skeletal muscle, liver, spleen, gill, eye, brain, heart, pyloric caeca, kidney and head kidney all being sampled stored immediately on dry ice and subsequently at -80oC until processing. Lysates from these tissues were prepared as in section 4.2.3.

5.2.2 Development and Optimization of Precipitation Assay

In order to develop a rapid and quantifiable assay for the determination of precipitate formation in a given sample, an assay was developed for use on a spectrophotometer using sodium acetate trihydrate buffer and also for use on a micro-titre plate reader. The final optimized conditions for both systems are given and thereafter modifications used in the optimisation process, using the micro-titre plate system are described.

The precipitate assay was performed on a spectrophotometer (Jencons 7315) as follows. An aliquot of 180µl 2% (w/v) bovine serum albumin (BSA) was added to 60µl of sample before and then mixed in a cuvette with 1 ml of 0.6M sodium acetate trihydrate buffer at pH 5.6 was placed in the cuvette. The BSA was shown not to have any effect on the reaction kinetics or precipitation reaction but reduced the sedimentation of precipitate allowing for more reliable OD to be monitored. Monitoring of the OD was at 340nm with readings taken every second for 60 min. This methodology was used primarily in early development of the assay while determining optimum conditions, such as temperature.

For use on a microtitre plate 15µl of sample (in duplicate) was dispensed to a 96 well plate and placed inside the plate reader (Fluorostar Optima BMG Labtech) at 37°C. Sodium acetate trihydrate buffer (0.6 M at a pH of 5.6) was heated to 37°C before addition of 245µl to each well. Absorbance (Ab) was measured at 340nm over a 60 minute duration with readings being taken every 30 seconds. The change in optical density (Δ_{340}) was calculated by subtracting the first reading from the final. When investigating tissue lysate precipitation, conditions

remained the same except 230µl of buffer was added to 30µl of sample in each well.

5.2.3 Statistical analysis

To understand varying precipitation potential of samples and how this might relate to the severity of tissue damage individual Δ_{340} were compared to individual histopathological score of each tissue (2.3.2) via a general linear model procedure in SAS version 9.3 (SAS Institute, Cary, N. Carolina) for regression analysis. Through this analysis it was possible to establish any correlation between precipitate formation and tissue pathologies.

In addition, the Pearson coefficient test was also carried out comparing ALT, AST, and CK scores to investigate the statistical likelihood that these enzymes may be part of precipitate. This test gives the correlation coefficient (r) of two variables with -1 indicating absolute no correlation and 1 indicating total correlation. Interpretation of correlation coefficient was carried out as in Dancey and Reidy (2004) where strength of correlation is interpreted as in Table 5-1.

Table 5-1: Value of Pearson correlation coefficient against corresponding strength of correlation.

Value of the Correlation Coefficient	Strength of Correlation
1	Perfect
0.7 - 0.9	Strong
0.4 - 0.6	Moderate
0.1 - 0.3	Weak
0	No Correlation
-1	Negative Correlation

5.2.4 Electrophoresis and Protein Spot Identification

An observable turbidity is produced upon mixing of 0.6M sodium acetate trihydrate pH 5.6 buffer (SAT) and serum samples. In addition, this turbidity is visibly different depending on the disease status of the individual. To investigate the composition of the proteins forming the precipitate, separate W0pc and W4pc pools were created for examination with equal volumes from each time point. Samples of the serum pools (300µl) were added to 0.6M sodium acetate trihydrate buffer (1ml) and allowed to incubate for one hour in a water bath at 37°C. The solution was then centrifuged at 10,000xg for 10 minutes and supernatant collected. Precipitate was washed by re-suspending in 1ml of SAT buffer and mixed prior to spinning at 10,000xg. This was repeated twice and each time supernatants removed. After the final wash the precipitate was re-suspended in 200µl of distilled water and vortexed until proteins had gone back into solution.

The nature of the protein in the precipitate was examined by two dimensional electrophoresis (2-DE) as in section 3.2.1. In total 36 protein spots from the resulting 2D-E gel were excised and subjected to trypsin digestion before protein identification via electrospray ionisation (ESI) mass spectrometry on an Amazon ion trap MS/MS (Bruker Daltonics) as in section 3.2.3.

One dimensional electrophoresis was also carried out using reconstituted precipitate formed after the mixing of sodium acetate trihydrate buffer and tissue lysates. This methodology is described in section 4.2.4.

5.3 Results

5.3.1 Development and Optimisation of Precipitate Assay

Assay optimization found that the kinetics, overall formation and monitoring of precipitate formation was dependant on a number of factors which were individually optimised. These were pH of buffer (Figure 5.1A), wavelength (Figure 5.1B and 5.1C), temperature (Figure 5.1D), and the molarity of buffer (Figure 5.1E). During optimisation the positive samples used were either pooled serum from W4pc of the infection or were muscle lysates prepared as in Section 4.2.3.

A range of buffers with the desired pH were prepared by titrating sodium acetate (0.2M) with acetic acid (0.2M) and di-sodium hydrogen phosphate (0.2M) with sodium dihydrogen phosphate-2-hydrate to achieve a range from pH 3.7 to pH8.0. Results (Figure 5.1A) indicated that a sodium acetate buffer, pH 5.6 was optimal for maximizing Δ_{340}. The optimal wavelength for detecting changing turbidity by precipitation was examined by assessing a range of fixed wavelength detection filters to establish the optimal wavelength for observing precipitate formation by ELISA plate reader. The 340 nm filter yielded the greatest OD reading (Figure 5.1B). This wavelength also gave higher absorbance using a spectrophotometer when compared to 550nm (Figure 5.1 C), confirming that a wavelength of 340nm was optimal. Figure 5.1D shows the effect of temperature on the spectrophotometer assay. Increasing the temperature from 20^{o}C to 37^{o}C caused a steady increase in the absorbance at 340nm for the precipitation reaction. At 45°C there was a rapid increase followed by a fall in absorbance due to a sedimentation effect with precipitate settling out of the solution. The effect of buffer molarity on precipitation carried out using a plate reader with 60µl muscle lysate being added to 200µl buffer. Figure 5.1E illustrates the wide range of molarity where precipitate is formed, although a significant drop in Δ_{340} being seen when sodium acetate buffers of <0.2 M were used. From these results the optimal assay conditions for the serum precipitate reaction (SPR) were determined: 0.6M sodium acetate trihydrate buffer at pH 5.6, and reaction temperature at 37°C. Other factors such as buffer to sample ratio were also investigated and the optimum was found to be 15µl sample into 245µl buffer for analysis on the ELISA plate reader and 1ml of buffer being added to 240µl of

sample mix (60µl sample and 180µl 2% BSA) in the spectrophotometer with changing absorbance being monitored over a 60 minute period (Δ_{340}).

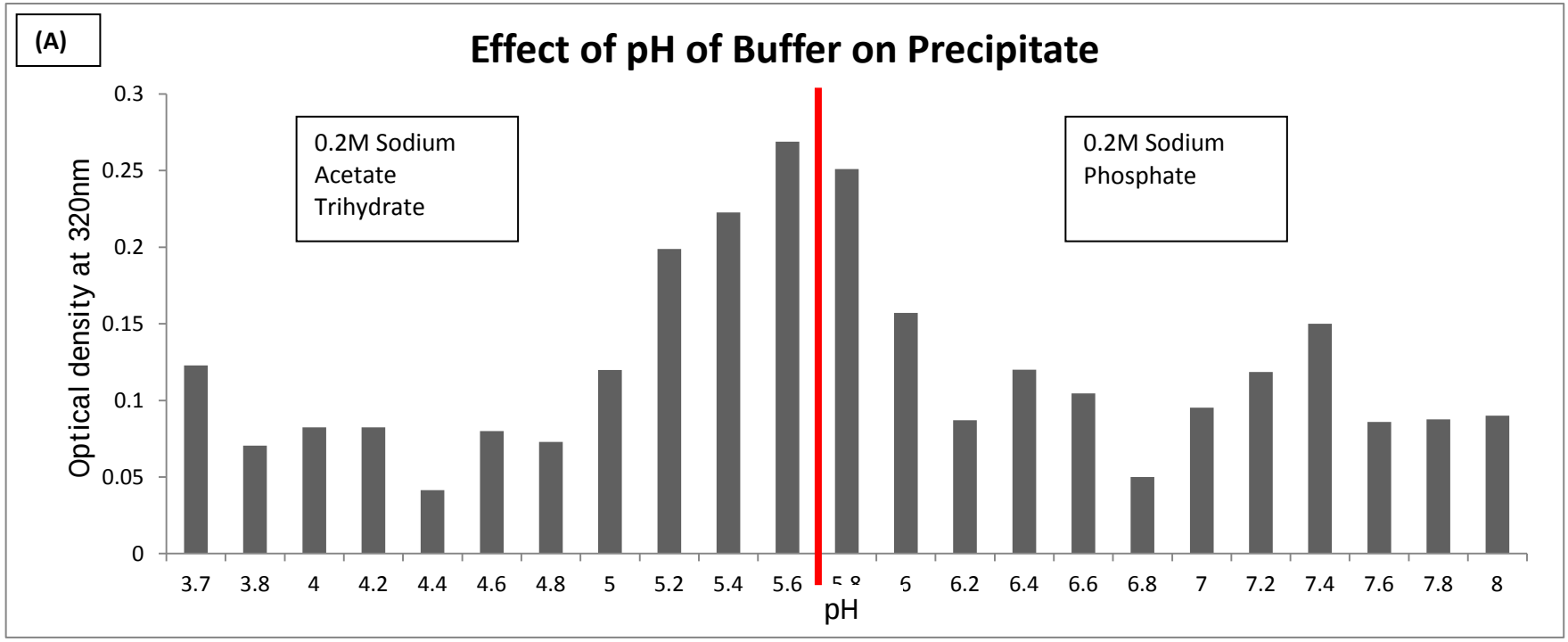

Figure 5.1: Optimization of precipitate assay.(A) Illustrates the effect of pH of buffer (pH 3.7 to 5.6 sodium acetate trihydrate buffer while pH range 5.8 to 8 is a sodium phosphate buffer) of delta OD of muscle lysate over a one hour period.

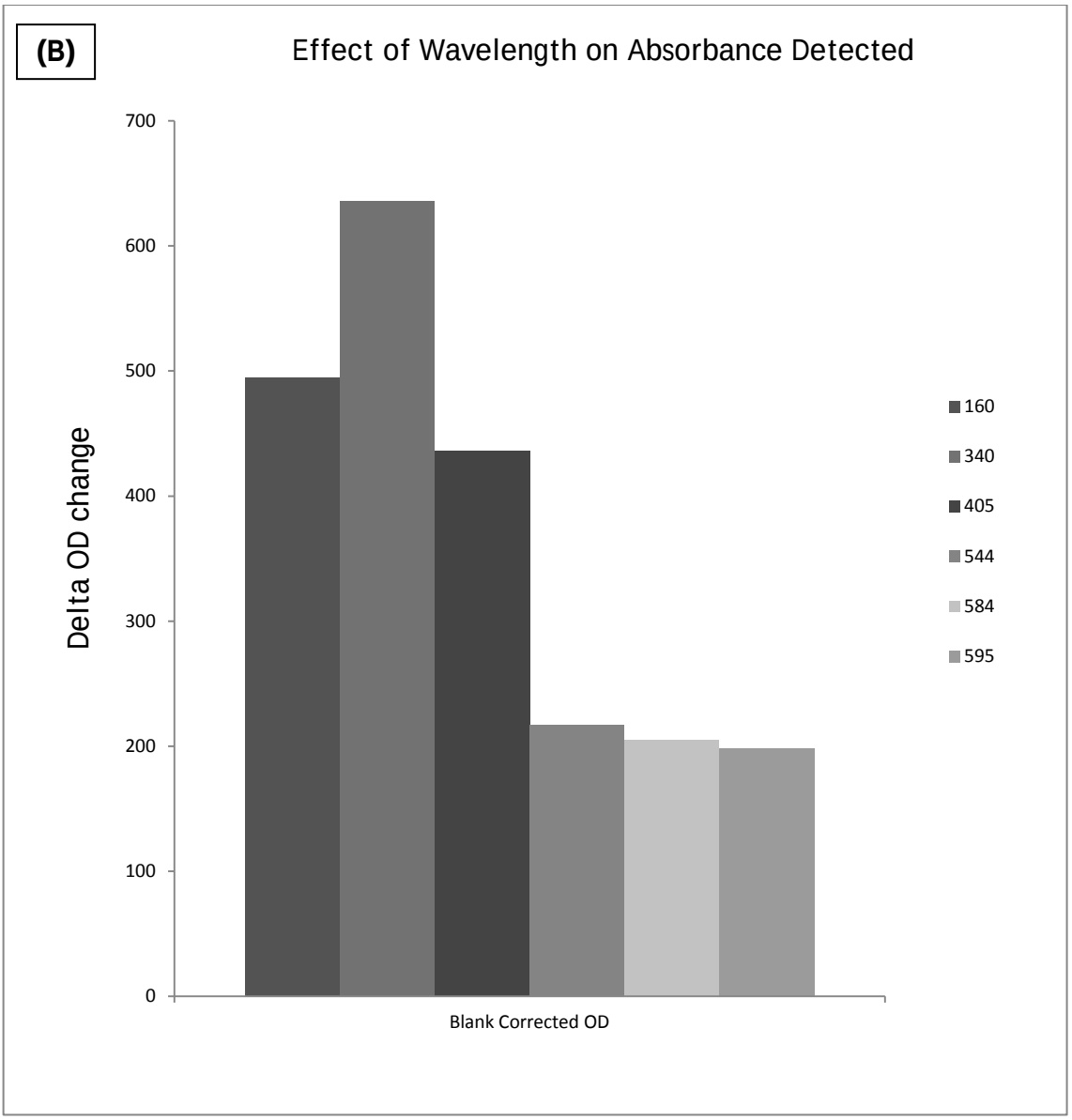

Figure 5.1: Optimization of precipitate assay. (B) Blank corrected optical density of resulting precipitation reaction from the mixing of muscle lysate and buffer when using a range of different wavelengths

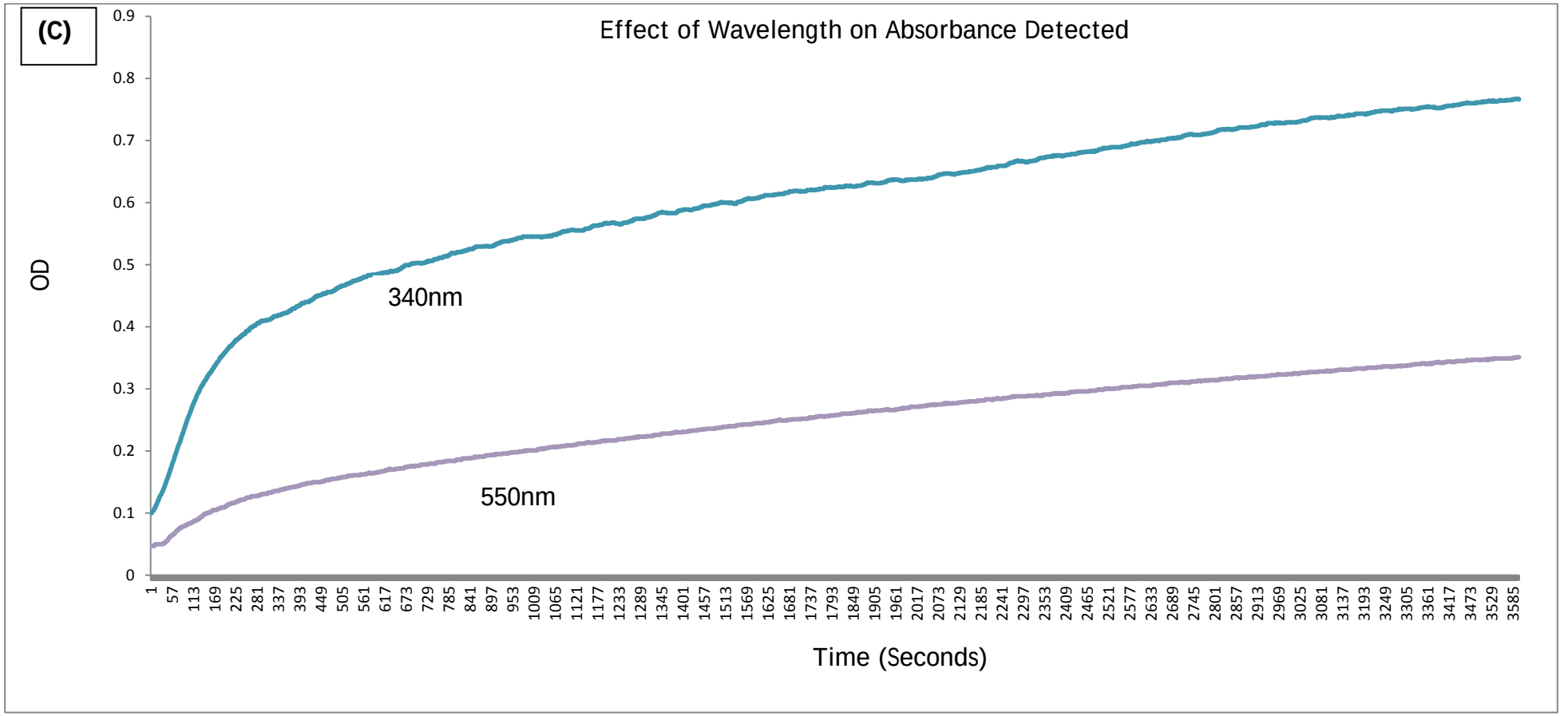

Figure 5.1: Optimization of precipitate assay. (C) The kinetics of muscle lysate precipitation reaction when monitored at either 340nm or 550nm.

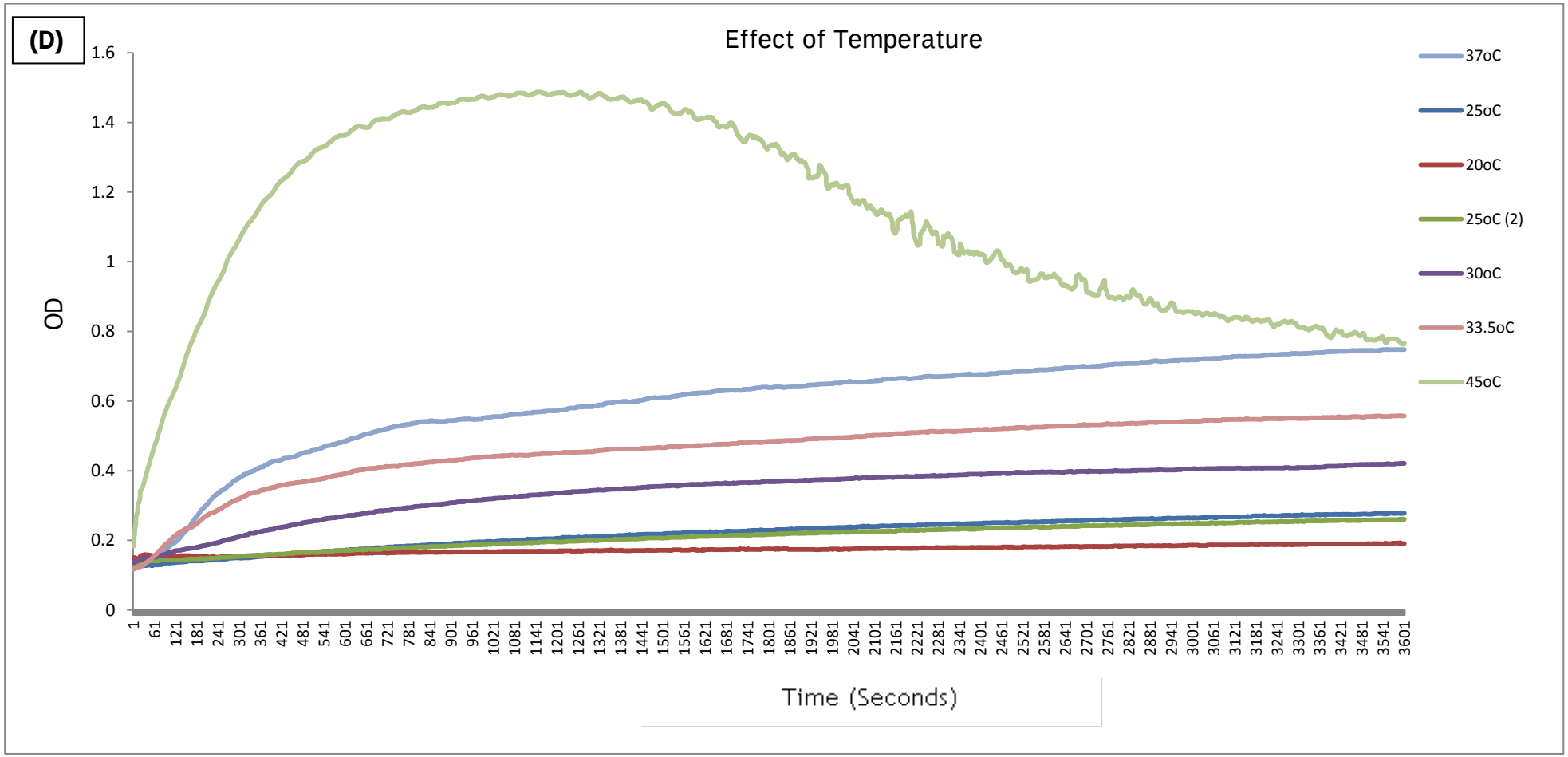

Figure 5.1: Optimization of precipitate assay. (D) Temperature effect on the formation of precipitate, monitored at 340nm using muscle lysate with OD reading taken every second.

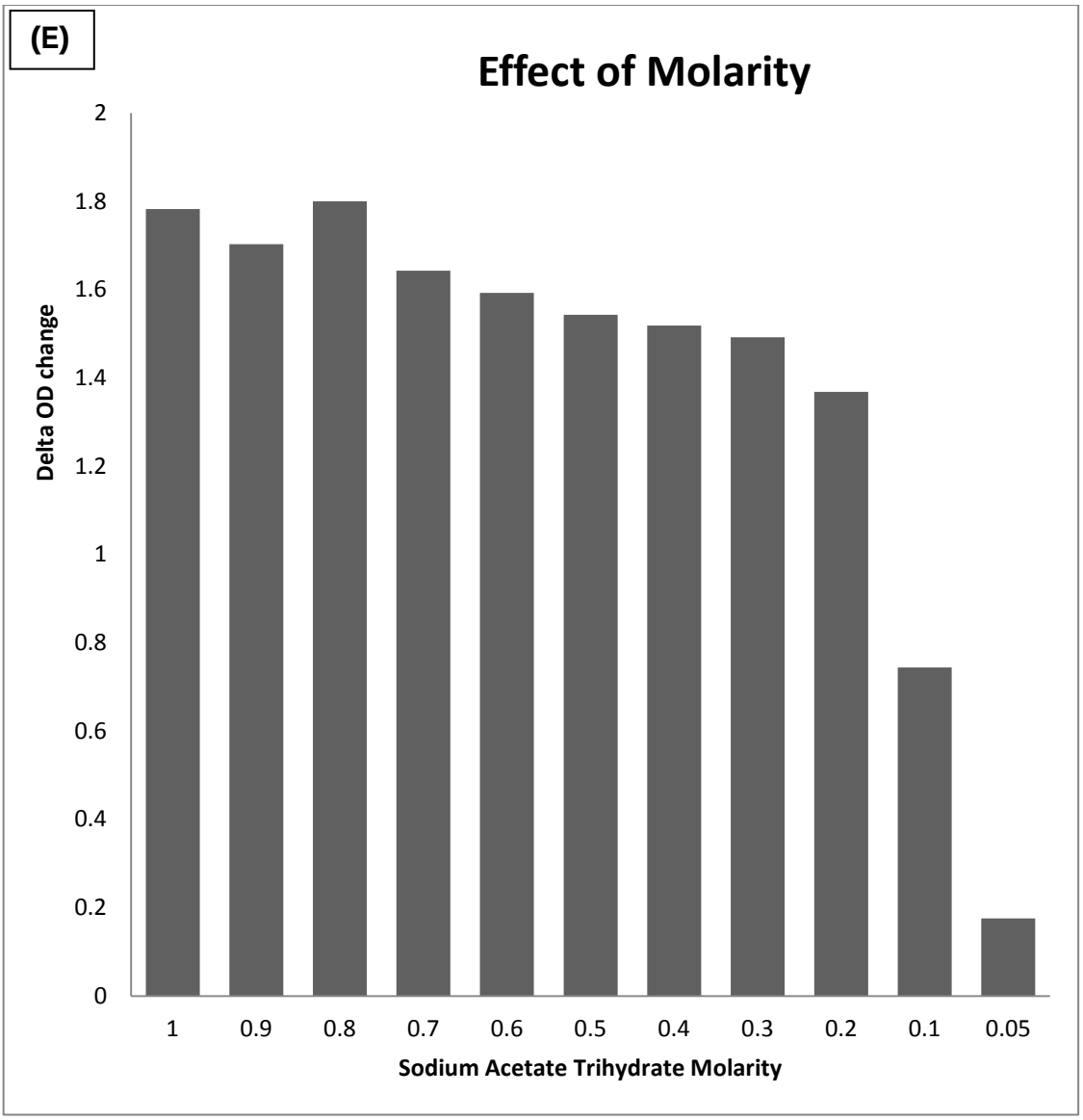

Figure 5.1: Optimization of precipitate assay. (E) The effect of molarity on the delta OD (over a one hour time period) when using W6pc sera.

5.3.2 Quantification of the Serum Precipitation Reaction during Pancreas Disease

The developed SPR assay was carried out on individual samples from the PD trial to investigate the changes in precipitation over time of the trial and the relationship with pathological damage of PD. A pool of sera from clinically healthy fish 'POOL A' and a pool of week 4 post challenge (W4pc) fish were used to calculate interassay %CV, which were 10.6% and 9.7% respectively (n = 25 plates), average intra assay %CV was 7.2% (n= 25 plates.

Figure 5.2 shows SPR measured by the mean Δ_{340} of serum from fish according to week post challenge (Wpc). There is a clear increase in precipitation of sera samples post W3pc, peaking at W6pc and returning to near homeostasis levels by W12pc. In addition, the mean result of pool A is shown with Δ_{340} comparable with healthy fish from the trial (W0pc).

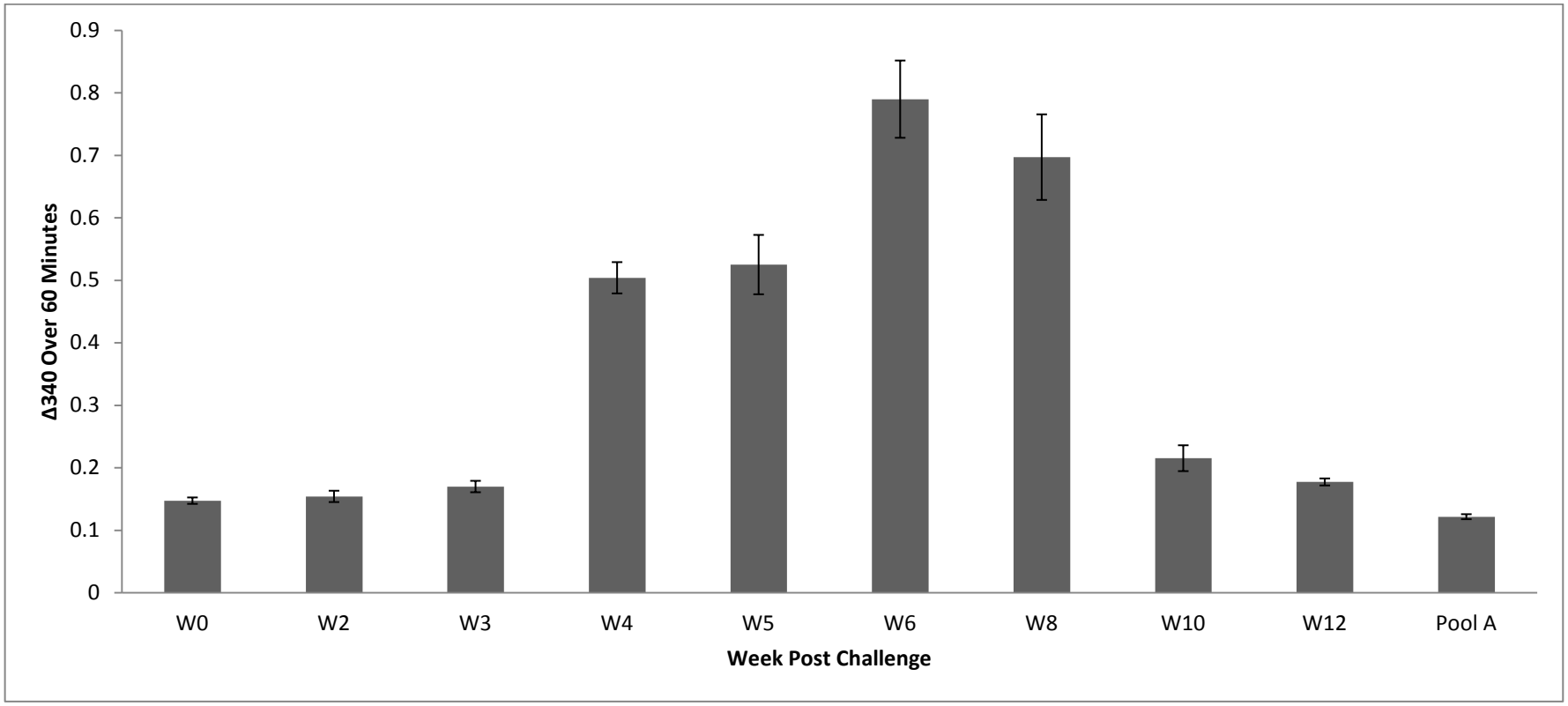

Figure 5.2: Mean Δ_{340} of samples by week post challenge. Serum precipitation reaction (mean ± SEM) measured by change in absorbance over 60 minutes at 340nm (Δ_{340}) according to week post challenge (Wpc) using microtiter plate assay. In addition pool A quantified precipitation formation is shown. SEM bars on Pool A result indicates SE of inter assay replicates (n = 33 plates).

Given the profile of precipitate it was thought that this may be influenced by general pathology of PD. Therefore, statistical analysis of individual fish Δ_{340} of samples was carried out against corresponding histopathological lesion scores. Precipitate reaction correlated positively with heart ($p = 0.001$), pancreas ($p = <0.001$), red muscle ($p = <0.001$), and white muscle ($p = <0.001$) histopathology scores, thus indicating that pathological damage to all tissues had a significant effect on the precipitate potential of serum. Pearson correlation coefficient scores of precipitate reaction with serum activities of AST, ALT and CK were 0.62, 0.86, and 0.92 respectively. These moderate to strong associations further strengthened the hypothesis precipitation potential was dependent on the existence/ severity of pathological damage to tissues in fish with leakage of these enzymes (ALT, AST, and CK) into the humoral system causing a differential serum precipitate potential.

5.3.3 Dietary Difference

Given that pathological damage apparently causes the release of proteins which appear to be components of the precipitate described, quantified precipitation (Δ_{340}) results were separated by diet to establish if this novel SPR test could also reflect the reduction in severity of pathology to tissues and thus lower enzyme activities (at certain time points post challenge). Figure 5.3 shows the separation of results by diet through sampling time course from W0pc to W12pc. This analysis showed that from W4pc to W8pc that mean Δ_{340} was lower in fish fed diet B than those fed diet A indicating a lowering of severity of pathological damage.

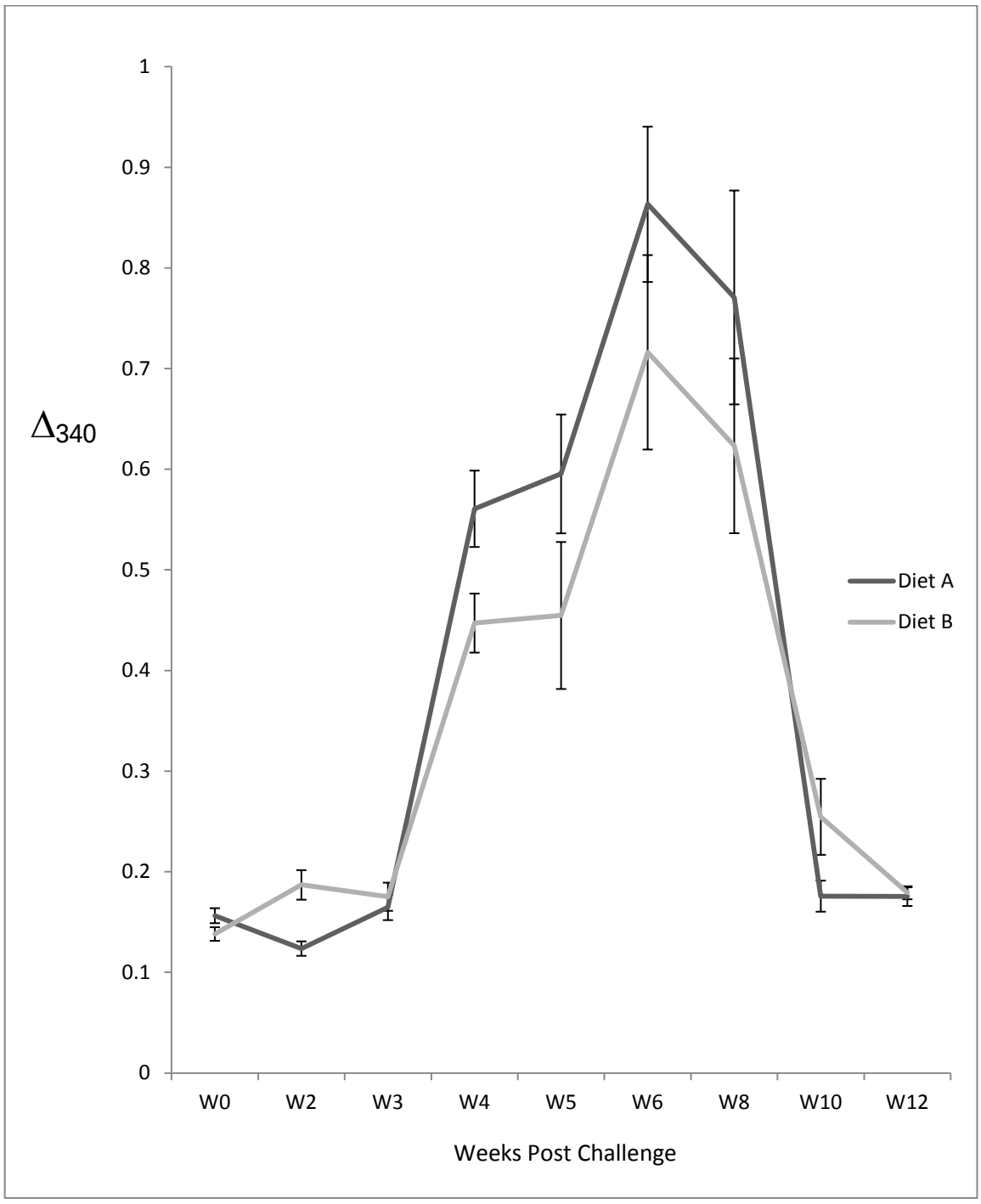

Figure 5.3: Dietary difference in mean Δ_{340} of samples by week post challenge. Line graph of mean delta change, of each diet separately, in absorbance over 60 minutes at 340nm (Δ_{340}) according to week post challenge (Wpc). Error bars indicate standard error (SE) of the mean at each time point. Diet A is indicated in black and diet B with grey line.

5.3.4 Composition of Precipitate

The composition of the precipitate formed by the SPR was examined by 2-DE and subsequent spot excision for identification of the proteins involved via electrospray ionisation (ESI) mass spectrometry. This precipitation was found to be dependent on the health status of fish. Samples from healthy fish produced very little visible change in turbidity thus forming a very small pellet, in comparison to the precipitate formed with serum from diseased fish, post centrifugation. Therefore, to examine the changes that occur in serum proteins that cause this SPR in salmon with PD, the precipitates formed from pools of W4pc and W0pc were compared (Figure 5.4A and 5.4B respectively). The change in precipitate composition was clearly visible and 36 protein spots were removed for identification by mass spectrometry (Table 5-2).

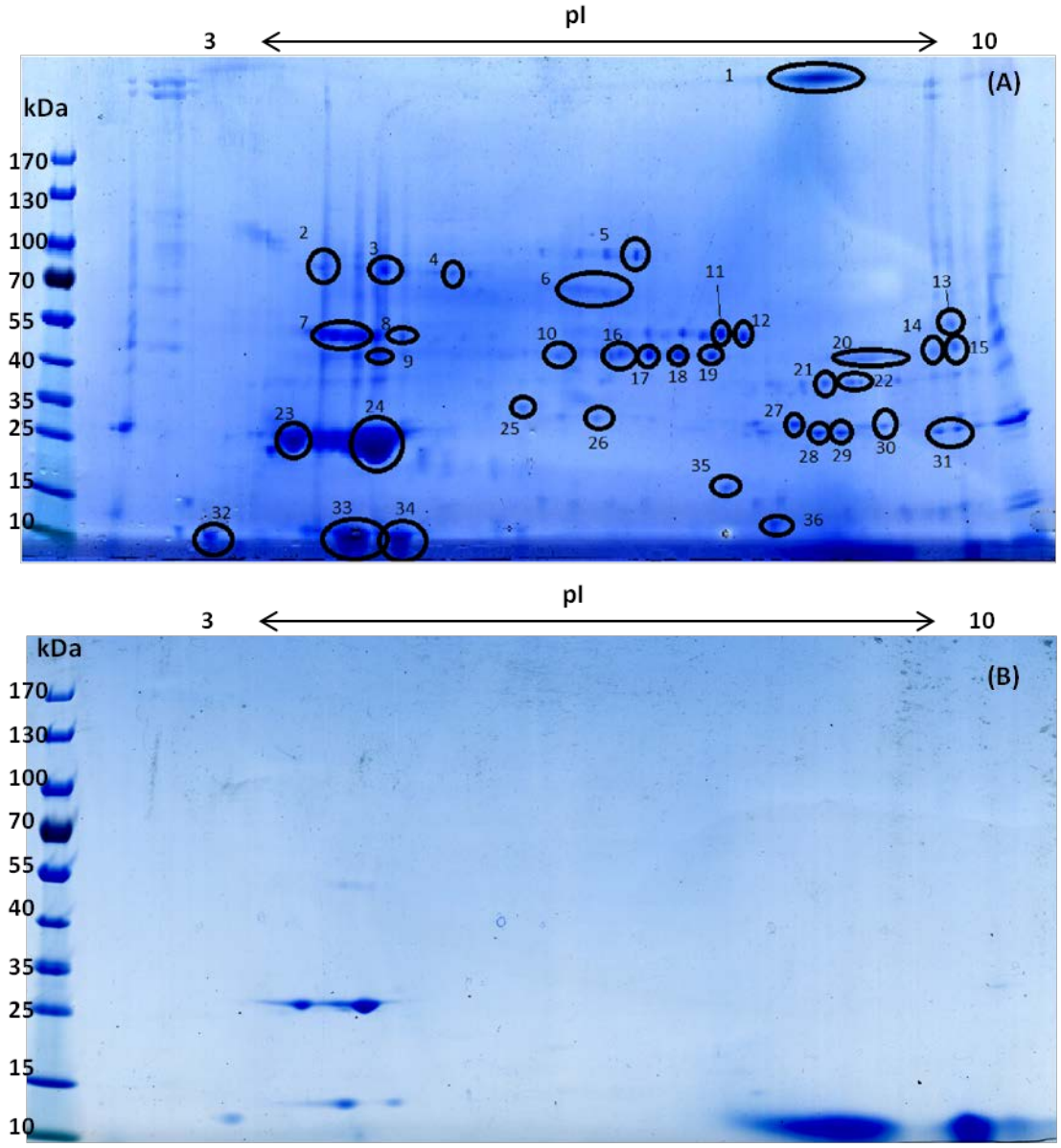

Figure 5.4: 2-DE separation of precipitate W0pc vs W4pc.The 2-DE separation of protein content of precipitate; proteins were separated over a pI range 3-10 and then by mass. Spots which were excised for identification are circled and numbered. (A) W4pc serum (B) W0 serum

Table5-2: Spot mass spectrometry identities. Spots excised, from W4pc precipitate gel, and their corresponding identities found via mass spectrometry, score, peptide matches and percent (%) sequence coverage.

Spot #	ID	Score	Matches	% Seq. Coverage
1	No sig. Matches	-	-	-
2	Complement component C9 (Q4QZ25)	185	13	13
3	Complement Component C9 (Q4QZ25)	408	32	19
4	Complement Component C9 (Q4QZ25)	91	4	5
5	Serotransferrin-1 (P80426)	797	58	33
6	Enolase 3-1 (B5DGQ6)	112	7	14
7	Enolase 3-2 (B5DGQ7)	261	24	26
	Enolase 3-1 (B5DGQ6)	213	20	22
	Cathespin M (Q70SU8)	175	63	40
8	Cathespin M (Q70SU8)	150	29	30
9	Fructose-bisphosphate aldolase (B5X0T0)	309	20	25
	Creatine Kinase-2 (B5DGP0)	290	35	40
	Creatine Kinase-3 (B5DGP2)	257	34	36
10	Pyruvate Kinase (C0H8V3)	109	4	6
11	Enolase 3-2 (B5DGQ7)	767	79	41
	Enolase 3-1 (B5DGQ6)	635	71	39
12	Enolase 3-1 (B5DGQ6)	583	51	41
13	Enolase 3-2 (B5DGQ7)	596	50	29
	Enolase 3-1 (B5DGQ6)	547	41	27
14	Aldolase a, fructose-bisphosphate 1 (B5DGM9)	251	16	26
15	Creatine Kinase-2 (B5DGP0)	400	57	42
	Creatine Kinase-3 (B5DGP2)	340	53	42
	Creatine Kinase (B5DGN9)	263	45	31
16	Creatine Kinase-2 (B5DGP0)	462	63	44
	Creatine Kinase-3 (B5DGP2)	427	60	40
	Creatine Kinase (B5DGN9)	285	44	35

Spot #	ID	Score	Matches	% Seq. Coverage
17	Creatine Kinase-2 (B5DGP0)	501	68	45
	Creatine Kinase-3 (B5DGP2)	452	64	45
	Creatine Kinase (B5DGN9)	356	56	40
18	Creatine Kinase-2 (B5DGP0)	426	67	39
	Creatine Kinase-3 (B5DGP2)	365	60	35
	Creatine Kinase (B5DGN9)	191	36	27
19	Creatine Kinase-2 (B5DGP0)	491	77	39
	Creatine Kinase-3 (B5DGP2)	415	64	35
	Creatine Kinase (B5DGN9)	181	44	27
20	Aldolase a, fructose-bisphosphate 1 (B5DGM9)	727	60	48
21	Glyceraldehyde 3-phosphate dehydrogenase (O42259)	210	29	36
22	Glyceraldehyde 3-phosphate dehydrogenase (O42259)	251	31	48
23	Apolipoprotein A-I (P27007)	773	129	55
	Apolipoprotein A-I-2 (O57524)	264	55	23
24	Apolipoprotein A-I (P27007)	1970	241	76
	Apolipoprotein A-I-2 (O57524)	350	66	30
25	Apolipoprotein A-I (P27007)	571	92	56
	Serotransferrin-2 (P80429)	182	9	10
26	Apolipoprotein A-I (P27007)	216	37	56
27	Creatine Kinase-2 (B5DGP0)	279	57	30
	Creatine Kinase (B5DGN9)	149	45	17
28	Triosephosphate isomerise B (NP_001133174.1)	652	60	40
	Triosephosphate isomerase (B5DGL3)	365	18	8
29	Triosephosphate isomerise B (NP_001133174.1)	471	33	40
	Triosephosphate isomerase (B5DGL3)	295	14	8
30	Fructose-bisphosphate 1 (B5DGM9)	349	30	30

Spot #	ID	Score	Matches	% Seq. Coverage
30	fructose-bisphosphate 2 (B5X0T0)	332	26	28
31	Triosephosphate isomerase (Q70I40)	197	9	27
32	Type-4 ice-structuring protein LS-12	664	33	54
33	Apolipoprotein A-I (P27007)	149	24	52
	Apolipoprotein A-I-2 (O57524)	131	12	23
34	Apolipoprotein A-I (P27007)	214	52	39
	Apolipoprotein A-I-2 (O57524)	154	16	46
35	Creatine Kinase-3 (B5DGP0)	112	13	19
36	Hemoglobin subunit beta (Q91473)	385	39	79
	Alpha-globin 1 (A9YVA1)	90	5	18

The 2-DE gel profile of precipitate formed from diseased (W4pc) serum is much more complex and diverse than that of the W0pc serum. The main difference in the proteome associated with this differential precipitation is an increase in proteins (spots 6-22, 27-31, 35) that are described as intra cellular enzymes which are increased in the serum due to pathological damage during PD. For instance, a number of intracellular enzymes were identified three isoforms of creatine kinase, enolase, aldolase, glyceraldehyde 3-phosphate dehydrogenase, and pyruvate kinase. Moreover, proteomic analysis found that these proteins increased in serum abundance (section 3.3.4). However, there also appears to be an increase in apolipoprotein A1 (spot 23-25, 33, 34) complement C9 (spots 2-4) serotransferrin (spot 5, 25) cathepsin (spot 8) and haemoglobin (36). The small precipitate formed with W0pc (healthy fish) sera comprises mainly apolipoprotein and haemoglobin (Figure 5.4B).

5.3.5 Tissue Lysate Precipitation

The correlation of SPR and the health status of the individual coupled with the over representation of intracellular enzymes in the precipitate whose presence can only be explained by pathological damage during PD led to the investigation of precipitation of tissue lysates, to assess the potential of the SPR test as a diagnostic tool of diseases of differing pathology. Figure 5.5 illustrates the Δ_{340} of different tissue lysates over a 60 minute period when 30µl of lysate was added to230µl 0.6M sodium acetate trihydrate (pH 5.6) to per well. Average replicate CV was 5.3% (n=3). The graph illustrates that whilst all tissue lysates studied possess precipitate potential there is a difference in Δ_{340} between them. However, whilst pyloric cacae appears to form little precipitate it did not behave in the same manner as other tissue lysates and as soon as lysate was added to buffer there was an instant precipitation making it hard to quantify over a period of time.

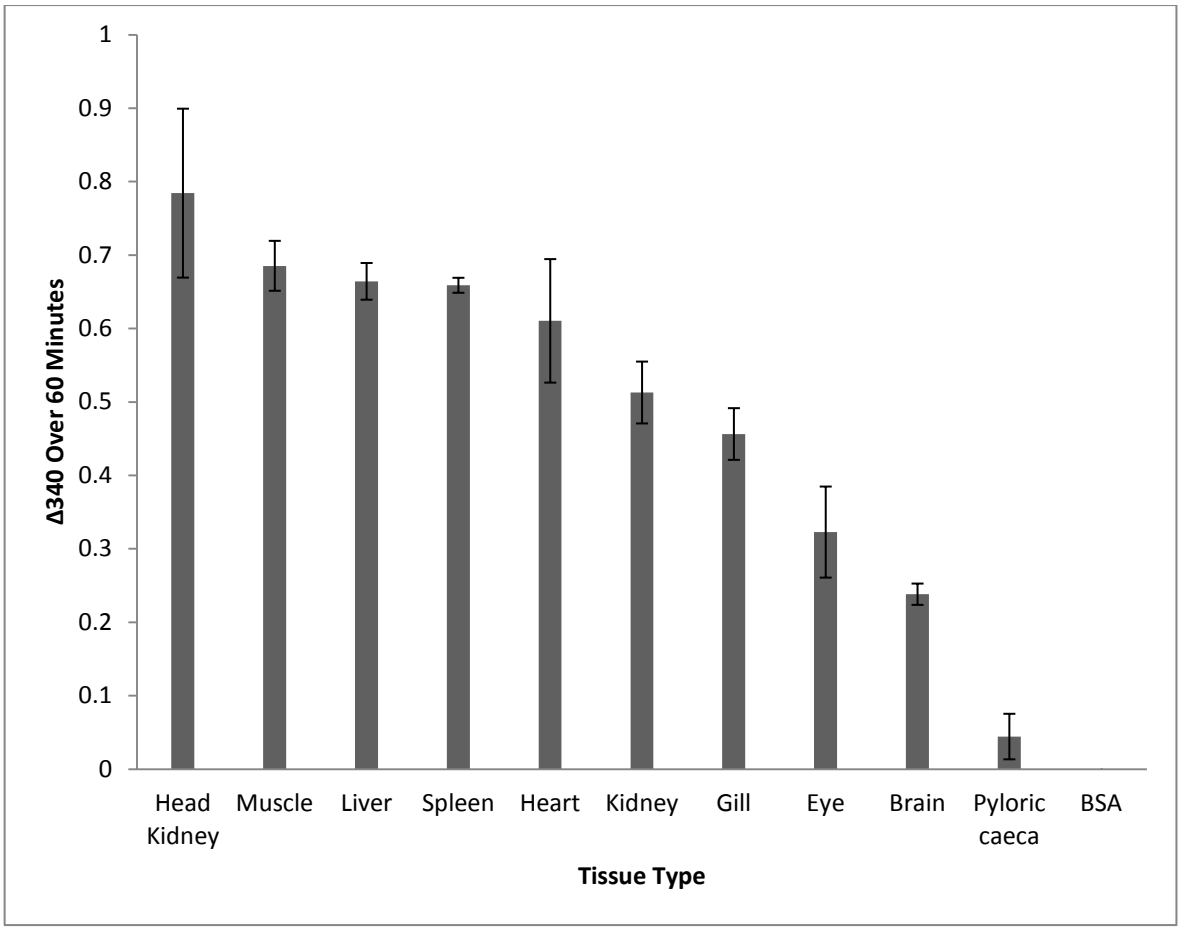

Figure 5.5: Tissue lysate precipitation Δ_{340}. The Δ_{340} over 60 minutes caused by precipitation of protein when using tissue lysates. Bovine serum albumin (2% BSA) was used as a blank. Error bars indicate standard error of mean (SEM) (n = 8).

This differential precipitate led to the investigation of proteins which precipitate from lysates from a number of tissues. After reconstitution with water of the precipitate pellet, 1-DE was carried out and band profile compared. Figure 5.6 shows these band profiles (samples 1-10) demonstrating the different nature of protein content of precipitate and also the unique profiles of each tissue type's precipitate. In addition, W4pc precipitate (sample 11) is shown as reference and demonstrates that its band profile is not influenced by one tissue alone, again in agreement with statistical analysis. Moreover, it appears to share a combination of the band profiles present in heart and skeletal muscle precipitate with the possibility of pancreas but given this is not a distinct organ this is hard to ascertain.

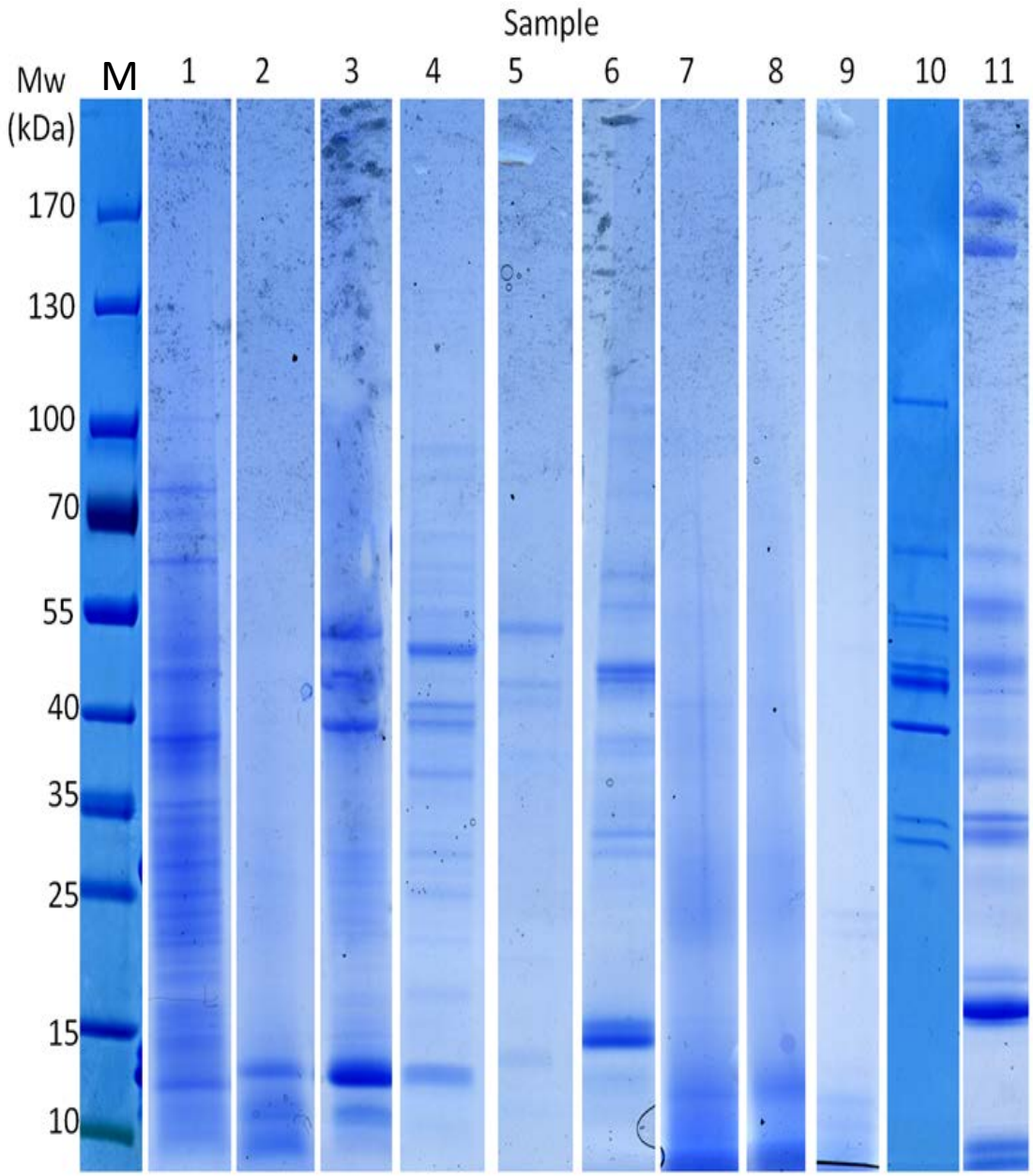

Figure 5.6: 1-DE separation of tissue lysate precipitation. Protein precipitate (after reconstitution in water) of tissue lysate and W4pc pool precipitate 1-DE band profile. Corresponding tissue/ sample are as follows:M = Molecular weight (Mw) ladder, 1 = Liver, 2 = Spleen, 3 = Gill, 4 = Eye, 5 = Brain, 6 = Heart, 7 = Kidney, 8 = Head kidney, 9 = Pyloric caeca, 10 = Whole skeletal muscle, 11 = W4pc

5.4 Discussion

This study has demonstrated that a precipitation reaction occurs when serum from Atlantic salmon is mixed with either sodium acetate trihydrate or sodium phosphate buffers in the pH range of 3.7-8. The reaction is much more pronounced when the salmon are infected with SAV, the causative agent of PD. This interaction is interesting biologically as it is possible that it may be a salmon specific phenomenon, as despite the many applications of similar buffers in a number of assays for Cp in studies of many species, such an effect (precipitation) has not been reported.

In its simplest form, observable turbidity upon addition of sera from PD positive salmon to the buffer, this pathophysiologal reaction has promise as a quick onsite inexpensive point of care qualitative test. Although precipitate formation does occur using healthy serum (as in Figure 5.4B), it is not visible to the naked eye thus making a clear distinction between sera from healthy and diseased salmon simple. Furthermore, a quantifiable test was also developed which has a potential role in diagnostic laboratories devoted to aquaculture.

Through development and optimization it was found that this phenomenon is affected by a number of parameters such as temperature and the molarity pH of the buffer used (Figure 5.1). For instance, the rate of precipitation formation is much faster at higher temperatures and also more is formed over a given time when using a buffer of higher molarity, with an optimal concentration of 0.7 M. In addition, the precipitate leading to the quantifiable turbidity has an optimal absorbance at a wavelength of 340 nm. Using this optimised method the precipitation potential of individual samples from the PD cohabitation trial described was assessed with Δ_{340} measured from 0 to 60 minutes.

Protein precipitation is a well described phenomenon where proteins become insoluble through a change in environment thus altering surface or charge characteristics. This is achieved through a number of techniques including

altering temperature and pH, adding metal ions, organic solvents and salts, all of which enable for the separation of desirable proteins (Asenjo, 1990). One of the most widely used methodologies for precipitating proteins is ammonium sulphate precipitation, where proteins are separated through the gradual increase of ammonium sulphate percentage. For instance, the protocol is commonly used to fractionate proteins in order to make samples less complex to then carry out purification of specific targets (Sarikaputi et al., 1991) or for proteomic investigation (Saha et al., 2012). However, sodium acetate trihydrate has never been described to be used in such a way. Moreover, it is currently used as a base buffer for investigation of protein activity on substrates with no such phenomenon being described (Ceron et al., 2007).

Analysis of individual samples from the pancreas disease trial described in section 2.2.1 showed that there was a significant difference in SPR through the trial with an initial rise in precipitation potential of sera at W4pc, before its peak at W6pc and returning to homeostasis near levels at W12pc (Figure 5.2). Using a pool of clinically healthy sera collected from fish at aquaculture sites it was possible to validate the Δ_{340} results of W0 fish as not an artefact of the trial and facilitate the establishment of a healthy range of precipitate formation. The increase in precipitate and subsequent fall was shown to be influenced by all tissues which are affected during PD, with histopathological lesion scores from pancreas, heart, red and white muscle all significantly correlating with Δ_{340} of individual serum. Moreover, ALT, AST and CK, which were previously shown to be indicators of general pathology, appear from Pearson coefficient analysis to have a strong correlation with the phenomenon. Therefore, it was hypothesised that pathological damage by the virus caused enzymes to leak into the sera (in agreement with ontology and previous work) forming components of a precipitate due to a lower stability in the presence of the buffer than the normal serum proteins such as albumin and immunoglobulins.

Diet had an effect on the SPR. When these results were split according to diet (Figure 5.3) it was found that fish fed diet B (functional feed Biomar plus 3) had

lower mean Δ_{340} in W4pc to W8pc compared to those who were fed diet A control. This reduction was in the weeks post challenge where the most severe pathological damage was seen in tissue pathology and serum enzyme activities (of ALT, AST and CK) were at their greatest. The precipitate assay is sensitive enough to reflect differential severity of pathology, as evidenced through histopathology and enzyme activity, on application of a functional feed. Furthermore, this mean reduction in SPR was much more pronounced and consistent than the semi-quantitative histopathology scoring systems and enzyme activity assays as previously discussed (2.3.4). Having linked precipitate formation to pathology it was necessary to investigate the composition of precipitate in more detail by gel based proteomic techniques.

Figure 5.4 shows the precipitate composition of SPR of diseased (W4pc) and healthy (W0pc) sera after it was mixed with sodium acetate buffer and after extensive washing and reconstitution in water, by 2-DE. A clear difference in complexity of precipitate was observed and 36 spots were excised for ion trap MS/MS identification. These excised spots are labelled in Figure 5.4A with their corresponding identities in Table 5-2. The vast majority of spots in identified that were present in W4pc precipitate but absent in W0pc were intracellular enzymes which are not present in high abundance during homeostasis. Indeed a number of them had previously been found (section 3.4.2) to be raised in serum abundance during PD due to pathological damage. For example, creatine kinase (CK), enolase, aldolase, and glyceraldehyde glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were found in precipitate of W4pc serum likely due to their increasing humoral abundance due to pathological damage to tissues as described in chapter 3. However, precipitation is not exclusive to proteins which are present in serum due to damage to tissues. For instance, complement component 9 (C9) was found to be present in precipitate of W4pc sera but the protein spot was absent at W0pc though found in proteomic investigation of sera (section 3.4.3) rising in abundance as part of the humoral response to PD. Therefore, there appears to be potential for certain humoral proteins to precipitate and could mean diseases which do not cause extensive pathologies such as in PD or sub-clinical cases could also be diagnosed by this approach,

although it may be that the increase in released glycolytic enzymes causes the complement C9 to co-precipitate. Despite this, certainly during PD pathological damage to the tissues is the main driving force of the SPR. In addition, the finding of multiple isoforms of enolase (ENO1, ENO2 and ENO3) and creatine kinase (CK1, CK2, and CK3) further validates GLM statistical analysis which indicates that the precipitate phenomenon is influenced by multiple tissue pathologies. While this makes the assay not specific to a given tissue as in the case of enolase 3 ELISA described in chapter 4, it has added power that precipitated proteins can be run using electrophoresis which through further development may give band profiles specific to a given disease and or pathology. It would be important to continue this research by proteomic analysis of the protein precipitated from all the tissue lysates and the precipitate generated with serum from other diseases of salmon and other finfish.

Clearly the potential of this precipitation test to be used as a diagnostic tool for diseases of differing pathologies to PD depends on the ability of proteins found in other tissues to precipitate. Therefore, initial investigation focused on testing this ability using tissue lysates from a number of tissues using the precipitation assay where Δ_{340} was taken over a 60 minute period after mixing of lysate and sodium acetate buffer (Figure 5.5). This showed that all lysates had precipitation potential but extent of OD change was tissue dependent, although it is important to note that pyloric caeca behaved very differently from other tissue types by precipitating very rapidly (within seconds) and sedimenting making it difficult to monitor using the same methodology. Differing optimal conditions may be needed when using this tissue. However, this difference indicated that different tissues possess differing concentrations of precipitatable protein or even different proteins which can be precipitated. If pathological damage to tissues leads to release of different protein from individual tissues it could prompt modifications of the diagnostic test as electrophoresis profiles and ultimately immunoassay could be used to diagnose damage to specific tissues. Moreover, this may identify specific markers which could be used downstream. Therefore, an initial investigation in which 1-DE was carried out on precipitated proteins from these lysates, was performed to assess this potential use. Figure

5.6 shows the one dimensional separation of precipitated proteins derived from liver, spleen, gill, brain, heart, kidney, head kidney, pyloric caeca, and whole skeletal muscle. While it appears many bands are shared between tissues there are clear observable differences. For example, there is a prominent band at 15KDa present in heart (sample 6) that is absent in skeletal muscle (sample 10) precipitate. In addition to this, W4pc precipitate band profile is also shown and appears to share a band profile similar to both muscle and heart lysates again supporting previous analysis that pathological damage to all tissues has an effect on precipitation. Unfortunately given that the pancreas is not a distinct organ in salmon it was not possible to sample this, however, W4pc precipitate has some very low molecular weight (<10KDa) bands not present in either heart or skeletal muscle but are observed when using pyloric caeca (where the pancreas is found discreetly) which may be potential target markers of this pathology. In addition the bands present in W4pc precipitate challenge at around 170KDa do not appear in the separation of any tissues studied and thus may represent, from molecular weight, precipitatble components of the immune system such as C9. Future investigation of these band profiles by mass-spectroscopy is warranted but it seems from initial investigation there is potential that the SPR is representative of whichever tissues are damaged thus other pathologies out with PD could be diagnosed by investigation of the proteins present in the precipitate. In light of this future work should focus on the further development of this test and its validation through investigation of different diseases.

This discovery of the SPR with serum from salmon with PD holds promise in diagnosing pathogenesis and fish health assessment. It has been shown that a quantifiable change in SPR occurs when examining sera from PD affected fish compared with clinically healthy fish. In addition, this test may be used as an economical and rapid test for assessing the efficacy of protective/ functional feeds, disease trial models and vaccines in PD research. In addition, future work aims to assess its usefulness as an indicator of many flesh quality issues that are currently a problem in salmon aquaculture.

Chapter 6

General Discussion and Future Directions

6.1 General Discussion

Infectious diseases pose a great threat to the long term sustainability of Atlantic salmon, *Salmo salar*, aquaculture (Kibenge et al., 2012; Yousaf et al., 2013). Therefore, limiting the impact of any outbreak which may occur is paramount to the industry and is achieved by diagnosis of a disease problem and subsequent implementation of disease management strategies. However, despite recent improvements in diagnostic methods (Adams and Thompson, 2014) and the development of novel ways to reduce disease severity such as functional feeds (Tacchi et al., 2012) the tools available possess a number of limitations and there is industrial demand for non-destructive means of health assessment. Moreover, while there is growing evidence for the usefulness in altering diet composition during disease, there are very few studies which show the effects in terms of disease severity. Therefore, the aim of this project was to assess the potential of non-destructive markers of pathological damage, using a pancreas disease (PD) model, which could be used as part of a proactive health monitoring programme.

Initial work focussed on establishing that the disease trial was successful in causing PD in the experimental population with a number of routine diagnostic methods carried out. Detection of SAV3 infection was performed, as described in Graham et al. (2011), by attempting to isolate SAV and detection of virus neutralizing antibodies from the sera of fish at 0, 4, and 5 Wpc. To confirm that fish were SAV free at the start of the experiment the viraemia of the serum was investigated. Reverse transcriptase polymerase chain reaction (RT-PCR) was also carried out with heart tissue from sacrificed fish from W2pc to W5pc to investigate the horizontal spread of infection. These tests confirmed that fish were naive pre-trial and that the virus spread throughout the population as anticipated. In addition to this, histopathological assessment of pancreas, heart, red and white skeletal muscle attained results similar to previous studies with the sequential pathology characteristic of PD being shown (McLoughlin et al., 2006; Christie et al., 2007). The pancreas was the first tissue to develop lesions at week 2 and was also the slowest to recover, with a minority of samples showing that the fish had still not fully recovered by week 12. Conversely, the

heart pathology demonstrated an extremely quick recovery, with a peak in lesion severity in fish sampled in week 4 and then a rapid recovery. Moreover, dietary analysis found that fish fed functional feed (diet B: Plus 3 Biomar product) had lower pathological lesion scores at specific time points during the trial compared to those fed a control diet. Histopathology scores at each time point for all assessed tissues were compared between diets finding fish fed diet B had significantly lower lesion scores at specific time points during the trial, often when pathological damage was at its peak in heart, pancreas and red muscle at W4pc ($p = 0.073$; $p = 0.0206$; $p = 0.0006$ respectively) and W6pc ($p = 0.0288$; $p = 0.014$; $p = 0.049$). There was also a significant reduction in lesion severity of the white muscle at W6pc ($p = 0.0331$). However, given that histopathology is destructive and the scoring system used is semi- quantitative the serum activities of three analytes were assayed. Creatine kinase (CK) serum activities have previously been investigated by Ferguson et al. (1986) and Rodger at al. (1991) showing that activities increase significantly during the disease. This was also found to be the case in this study increasing to a peak at W6pc before falling to homeostasis levels by W12pc. Similar profiles were also observed through analysis of two novel targets of aspartate transaminase (AST) and alanine transaminase (ALT) which had not previously been investigated in salmon disease. In addition, statistical analysis found that fish fed diet B possessed significantly lower activities at W4pc and W6pc for both CK ($p = 0.011$; $p = 0.0076$ respectively) and ALT ($p = 0.0013$; $p = 0.0017$ respectively. Lower AST activities were observed in fish fed diet B at W6pc ($p = <0.001$). As anticipated serum activities were found to be influenced by pathological damage to heart, red muscle, and white muscle thus not being tissue specific. Moreover, despite the apparent usefulness in non-destructively assessing general fish health these assays have a number of limitations which may impact on their application in the field. First, the range of concentrations in healthy sera, due to a high and variable activity, is large as described in the literature (Ferguson et al., 1986; Roger et al., 1991; Yousaff & Powell 2012) thus making it hard to identify subtle changes in concentrations on which to base health management decisions at a given time point. This is especially a problem when a fish farm site has an outbreak of HSMI where changes may be quite small and fall within this range. Indeed, it would appear that values (U/L) of CK are extremely high when fish are

affected with PD compared to HSMI. In addition, publications only report total CK concentration, which is ubiquitous, thus not indicating a specific pathology. These two issues are much more of a problem when the nature of sampling at an aquaculture site is considered where there is a low probability that a fish will be sampled multiple times over a given period. Therefore, meaning that individual variability cannot be easily accounted for as in other livestock species. Therefore, it would be useful in terms of diagnostics to have other non-destructive targets that may add to information attained from serum analysis and may be more sensitive, accurate, and reliable in diagnosing pathogenesis. Therefore, there is a high industrial demand for non-destructive markers of disease which can indicate specific tissue pathologies for diagnosing clinical disease and also infection of infectious agents.

In the search for potential targets a gel based proteomic study was carried out comparing the two dimensional electrophoresis (2-DE) profile of protein composition of pooled serum (according to week) from the nine sampling points from W0pc to W12pc. In total 72 spots were found to differ significantly over the course of the disease trial which were excised, subjected to trypsin digestion and proteins identified via ion trap mass spectrometry. Bioinformatic analysis of spot profile intensities by Hierarchical Cluster Analysis made it possible to understand the effect of disease progression on the serum proteome in general terms with a clear separation between those recorded from 0 – 4 wpc and those from 5 – 10 wpc. However, the clustering of W12pc with those W0pc to W4pc indicated by this time there was a return to near homeostasis. Further analysis of spot profiles were against histopathological scores of damage to specific tissues in order to establish if a given spots abundance change was statistically linked to pathology or not. In this regression generalised linear model each spot was regressed on the mean value of each tissue's histopathological score at each sampling time point in a separate model. Therefore, the probability that a protein spot increased or decreased in intensity in association with tissue damage as determined by histopathology was determined, with those which did not correlate significantly being classed as components of the humoral immune system. This analysis has a number of advantages in simply basing categorization

on ontology mainly being that it allows for the identification of potential tissue specific isoforms of the same protein. However, in the main, ontology and this analysis were in agreement. For example, complements C3 & C9, complement factor B, and the complement inhibitor C1 which were found to be altered in abundance irrespective of tissue pathologies have been previously characterised as part of the fish innate immune system. In addition, other well-known humoral components of the immune system were found to alter in abundance, such as hemopexin and transferrin. However, the main focus of the project was to establish potential markers of pathological damage and thus these were studied in more detail.

Potential markers identified in the study were mainly for red and white muscle, with few spots being correlated to pancreas and heart pathology. This apparent lack of biomarkers for these tissues was most likely because the abundances of other potential specific protein biomarkers for heart and pancreas were not raised sufficiently in the serum to be detected by the proteomic methodology used in this study. Alternatively it may be due to differential size of the tissues. However, there was a significant correlation between one spot (656) containing serotransferrin and heart pathology. This was interesting given that the protein is not generally thought of being present in the tissue, indeed most spots containing the protein correlated with pathological damage to any tissue. However, the protein has been synthesised in the heart of rats (Aldred et al., 1987) and possesses transferrin receptors (Upton et al., 2003) as iron is pivotal to its function. Many protein spots were found to correlate with histopathological lesion scores of more than one tissue. For example, the spot abundance of creatine kinase (CK), as previously indicated by enzyme activity, was found to be significantly linked to pathological damage to the pancreas as well as red and white skeletal muscle. Indeed many proteins did not appear to be influenced by a single pathology rather correlating with two or more tissue's histopathology scores which could identify tissue specific isoforms or be an artefact of the sensitivity of proteomic approach or histopathological scoring system's semi- quantitative nature. However, the most promising candidates were those which correlated largely with white muscle pathology, namely

enolase and aldolase A. Thus these were chosen for validation by other approaches. In addition, glyceraldehyde-3-phosphatedehydrogenase (GAPDH) was chosen for further investigation to test whether the finding that spots containing the protein did not correlate with white muscle pathology was an artefact of the proteomic methodology used.

The ultimate aim of Atlantic salmon aquaculture is to produce flesh, comprised of skeletal muscle and fat, which is desirable in the market place. Whilst downgrading of fillet can be caused by nutritional factors, toxic myopathies, and muscle breakdown due to excessive exertion (Larsson et al., 2012) one of the most important causes is following infectious diseases which cause skeletal muscle myopathy. Therefore, given white muscle is the main constituent of this product, any reliable biomarker of its pathology would be extremely useful to industry in diagnosing clinical disease and as a marker of flesh quality. Arguably the biggest hurdle in taking potential biomarkers through a production pipeline, and indeed why despite much research into diseases in general very little impact on industry usually occurs, is the nature of validation and finding of suitable makers which fit the requirements of industry. In brief, for a biomarker to be a useful tool in diagnostics it must be accurate and sensitive for disease state, have low variation within a population undergoing the same stimuli and reproducible and consistent over time and circumstance (Moore et al., 2007). Moreover, if normally present in biological fluid or tissue under homeostatic conditions a 'healthy range' of analyte concentration must be established so a problem may be identified swiftly when out with this range. In addition, any diagnostic test target must also be consistent and accurate with robustness to biological and analytical variation. With these criteria in mind it was decided that enolase, aldolase, and GAPDH should be taken forward for initial validation of proteomic results as serum abundance of the enzymes in healthy individuals seemed extremely low/ not present using gel based proteomics. Initial validation was carried out using a number of commercial antibodies against the enzymes by western blotting to ensure cross reactivity and specificity. This investigation found that western blot band profiles, which were semi-quantified using imageJ (http://rsbweb.nih.gov/ij/), correlated significantly with statistical analysis

with general spot profiles from proteomic investigation containing these three enzymes. Moreover, it was shown that a signal for GAPDH was only observed at W5pc and W6pc and thus did not seem to correlate with any pathology. While this did not definitively answer the original question posed the most plausible explanation was that antibody sensitivity may have been low and thus any presence of the enzyme may have been too low for detection pre W5pc and at W8pc onwards. Therefore, it was ruled out for future development. In addition, western blotting using a commercial antibody against aldolase A showed that band intensities and proteomic profiles demonstrated similar profiles through the time course of the PD trial. Despite this, initial attempts to develop an ELISA for this marker were not successful due to a high level of non-specific binding, which to some degree can also be seen on western blots. Despite this, three antibodies were used in initial validation of enolase, two of which were against enolase 1 isoform (ENO1a and ENO1b here in) while the other was against enolase 3 (antibody referred to as ENO3) isoform of the protein as both of these were discovered by proteomics.

Western blots using these three antibodies were initially carried out after one dimensional electrophoresis and subsequent transfer and the profiles of spots corresponded well with band intensities through the course of the trial. Moreover, it was observed that antibodies ENO1a and ENO3 had minimal non-specific binding. Therefore, the use of these antibodies in a more sensitive and quantitative manner was explored through the development of an ELISA. This work demonstrated that while both of these antibodies were suitable for this platform, ENO3 much better represented the histopathological lesion scores of white muscle. This result agreed with ontology as in most studied species enolase 1 isoform is expressed in all tissue types while enolase 3 is tissue specific, and while this is not clearly defined in Atlantic salmon the initial hypothesis was that the latter would be the more appropriate biomrker. Subsequent work therefore focused on validating enolase 3 as muscle specific. To do this a section of 50 fish from the trial with varying tissue (all tissues) histopathological scores were chosen depending on score of different tissues and statistical analysis of their enolase 3 concentrations against these scores carried

out. From this it was found that enolase 3 concentrations only correlated significantly with pathological damage to white muscle. Moreover, immunohistochemical staining of a section of whole skeletal muscle revealed that only white muscle cells stained after treatment with ENO3 antibody again indicating that this tissue type was the only location of the enzyme isoform. While this study has not fully validated enolase 3 serum concentration as a tool for diagnosis of PD it has gone some way in doing so. Future work must attempt to do this, through the use of the assay in higher samples numbers. In addition, the dietary difference leading to alteration in serum enolase 3 concentrations was not established and thus must be investigated. Despite this, the ELISA developed has potential uses as a non-destructive health monitoring tool, not only for PD induced white muscle myopathy but also as a means of assessing flesh quality and sensitivity of histopathological scoring systems such as the one used in this study.

Chapter five describes the discovery and subsequent development of a novel tool for diagnosing pathological damage to Atlantic salmon. The discovery came about through the attempt to use a well-established protocol in mammalian species, for the determination of serum concentrations of ceruloplasmin (Cp) using an assay where the oxidation of a substrate paraphenylenediamine (PPD) is monitored through time. From this study assumed levels of Cp were extremely high even if oxidation activity in salmon was greater gram for gram than in other species. Subsequent observation lead to the finding that if the oxidation substrate was removed completely and diseased sera was mixed with the base buffer commonly used in this assay, sodium acetate trihydrate, it resulted in a visible turbidity. This observed turbidity was found to be due to the precipitation of proteins out of solution and was variable, in terms of degree of cloudiness, being dependent on disease state (initially at a qualitative level by eye). Optimization of the test found that it was sensitive to a number of parameters, such as, temperature, molarity of buffer, pH, and wavelength of light used. This optimization of conditions allowed for the quantification of turbidity leading to the development of an assay where the change of optical density (Δ) was represented by this phenomenon. The developed assay was then

used with all fish fed diet A (control) and B (Plus 3 functional feed) were it was found that from W4pc to W8pc that fish fed a functional feed had lower Δ_{340} than those fed the control diet, indicating as with previous results that this functional feed lowers disease severity. This was strengthened through subsequent analysis which found that this differential turbidity was influenced by pathological damage to all tissues affected by PD, with significant correlations with pancreas, heart, red and white muscle histopathological lesion scores. Moreover, serum enzyme activities of CK, ALT, AST were found to have a strong association with precipitate formation indicating that proteins which are leaked into the serum due to pathological damage to tissues are the main constituents. Subsequently, the composition of precipitate was investigated by gel based proteomics to investigate this further. In total 36 major protein spots were removed after two dimensional electrophoresis separation of precipitate formed after W4pc was mixed with sodium acetate trihydrate. Identification of the proteins by mass spectrometry found that many enzymes already discussed as markers of pathology, including; creatine kinase (CK), enolase, aldolase, and glyceraldehyde glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were found in precipitate with W4pc serum. However, precipitation is not exclusive to proteins which are present in serum due to damage to tissues. For instance, complement component 9 (C9) was found to be present in precipitate of W4pc sera, but this protein spot was absent when W0pc precipitate was separated by 2-DE. Moroever, C9 was also found in chapter 3 by proteomic investigation of sera through the disease trial to rise in abundance as part of the humoral response to PD. Despite this, certainly during PD pathological damage to the tissues is the main driving force of differential precipitation In addition, the finding of multiple isoforms of enolase (ENO1, ENO2 and ENO3) and creatine kinase (CK1, CK2, and CK3) further validates statistical analysis which indicates that precipitate phenomenon is influenced by multiple tissue pathologies. This led to the hypothesis that it may be possible for tissues other than those affected by PD to leak proteins into serum which could be detected by the precipitation test described. However, clearly this is dependent on ability of proteins found in other tissues to precipitate. Therefore, initial investigation focused on testing this ability using tissue lysates from a number of tissues using the precipitation assay where Δ_{340} was taken over a 60 minute period after

mixing of lysate and sodium acetate buffer. All investigated tissue lysates possessed proteins which precipitated out of solution when added to buffer. Although protein precipitation by adjustment of buffer conditions is an established technique and has been used as an indicator of IgG in the serum of neonatal animals when barium sulphate has been used (Pfieffer et al., 1977), the precipitation of serum protein following pathological release of protein from tissue has not been used previously as a diagnostic test. Furthermore, when resulting precipitate was examined by one dimensional electrophoresis there appeared to be tissue specific band profiles which may represent pathology specific markers. Moreover, the separation of W4pc precipitate when compared to tissue lysate bands shared bands with those damaged during PD indicating the potential of this test of diagnosing pathologies of different kinds.

6.2 Future Directions

This project through the use of a pancreas disease cohabitation disease model has identified a number of novel markers of non-destructive markers pathological damage in Atlantic salmon and demonstrated the reduction in disease severity through the administration of a functional feed.

Initial investigation into the potential of non-destructive markers of pathological damage focused on serum biochemistry activities of CK, ALT, and AST. However, there are a number of limiting factors limiting their application in industry as a diagnostic tool. Despite this, CK is becoming increasingly used in general health assessment. Therefore, future work into this test must focus on the apparent high activity compared to other species and try to control for this. Moreover, the individual and disease variability observed in literature and in this study means that it would be highly useful for industry wide establishment of a healthy reference range of CK activity so subtle changes in abundance can be identified as soon as possible.

Gel based proteomics and statistical analysis identified a number of potential markers of pathology and immune responses to SAV infection. Following studies

should focus on the validation of these and try to investigate the tissue specific isoforms of proteins that have been potentially found. In addition, to this more sensitive gel free proteomic methodologies would be useful in finding more targets thus increasing our understanding of the pathology and pathogenesis of PD. Moreover, it would be interesting to attempt to see if the same proteins correlate to pathological damage to the same tissues during different diseases.

Further development of work described in the validation of enolase should also be carried out in diseases of other aetiologies and investigating its use as a marker of reduction of fillet quality due to other myopathies. In addition, while it was found that healthy fish from aquaculture sites had no detectable level of the enzyme in their serum it would be useful for further investigation of fish at different ages to confirm that this is the case.

The discovery of the serum precipitation test holds promise in diagnosing pathological damage in fish in a non-destructive manner. Moreover, this test is able to quantify the severity of damage to tissues which are damaged. This quantification of turbidity caused by precipitation and the ability to reconstitute precipitated proteins in water for examination by electrophoresis appears to be a powerful tool in diagnosing disease and illustrating which tissues seem to be damaged. However, much work is still to be done in order to maximize its potential. Firstly, the identification via mass spectrometry of unique bands that result from electrophoresis separation of tissue lysate precipitate may indicate tissue specific markers of disease. In addition, to this, the test's usefulness in other diseases which differ in pathological damage to PD must be assessed. Clinical samples from the field must also be tested to validate this test in an aquaculture setting.

In general the findings of this study have great potential application in being used in proactive monitoring schemes in industry with fish health being assessed non-destructively. Monitoring in such a way may have economic benefits as early

diagnosis of a disease problem may permit implementation of other disease management strategies such as functional feeds at the most optimal time. Moreover, these tools add to the diagnostic tools available to farm managers to diagnose clinical disease thus informing them on the best action to take during an outbreak.

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Appendices