IMPACT OF FISHMEAL REPLACEMENT WITH POULTRY MEAL ON RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) NUTRITION, PHYSIOLOGY AND PERFORMANCE

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M.Sc Aquaculture

Submitted in fulfillment of

the requirements for the degree of

Doctor of Philosophy

University of Tasmania

June 2013

DECLARATION

This thesis contains no material which has been accepted for a degree or diploma by any tertiary institution. To the best of my knowledge the thesis does not contain any material written or published by another person, except where due reference is made.

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ETHICAL COUNDUCT

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the ruling of the Safety, Ethics and Institutional Biosafety Committees of the University.

Kamil Latif

ABSTRACT

The future sustainability of aquaculture will depend in part on the reduction of fishmeal (FM) used in fish feeds and the development of economically alternative protein sources. Poultry meal (PM) is one example of an alternative protein source. The aim of this study was to understand the effects of FM replacement with PM on nutrition, physiology and performance of rainbow trout. Four independent experiments using isoenergetic and isonitrogenous feeds (22 MJ GE kg^{-1} DM, 400 g kg^{-1} DM of crude protein) were carried out. An initial study investigated the effect of replacing FM protein with increasing amounts of PM protein in which 0, 20, 30 and 40% FM protein was replaced with PM protein. Fish were grown in freshwater at 15°C for 84 days, during which time they were fed twice daily at 2% bw d^{-1}.The results indicated that PM protein can be used to replace up to 40% of the FM protein without impaired fish growth, apparent digestibility of crude protein, insulin like growth factor I (IGF I) and cortisol. A further study was carried out to determine the effects of replacement on apparent digestibility where twelve isoenergetic and isonitrogenous feeds were formulated in which 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95 and 100% FM protein was replaced with PM protein. Fish were grown in freshwater at 15°C for 27 days, during which time they were fed twice daily until satiation. Faeces were stripped from fish on day 3, 9 and day 27. Effects of poultry meal inclusion level on apparent digestibility showed the significant negative linear relationships between PM inclusion and apparent digestibility measured as AD crude protein, AD total lipid and AD gross energy. There were no significant differences in AD crude protein or AD total lipid between the three adaptation times. However, AD gross energy increased over time. A further experiment evaluated the effects of temperature on FM with increasing PM at two different temperatures (15^{o}C and 20^{o}C). Twelve isoenergetic and isonitrogenous feeds were formulated in which 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95 and

100% FM protein was replaced with PM protein. Fish were fed twice daily at 2% bw d^{-1} for a period of 112 days. Results indicate the high potential of PM as an alternative protein source at optimal and high temperatures. In addition, result on histology of the distal intestine showed that increase in the number of goblet cells (GC) and decrease in the width of supranuclear vacuoles (SNV) in fish fed diets containing greater than 90% of PM protein at both temperatures. The last experiment was designed to test the effects of supplementary essential amino acids (L-lysine and L-histidine) on rainbow trout. Fish was fed either a set daily ration of 1.7% bw·d^{-1} or fed to satiation. The results indicated that supplementary essential amino acids (EAA), L-lysine and L-histidine in PM diet did not improve growth of rainbow trout.

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ACKNOWLEDGEMENTS

I would like to thanks the following people who support, help and guide me during my study period in University of Tasmania.

I am heartily thankful to my supervisors, Prof. Chris Carter, Dr. Ryan Wilkinson and Dr. Robin Katersky whose encouragement, supervision and support from the preliminary to the concluding level enabled me to develop and understanding of my study.

I also would like to thanks to Prof. Natalie Moltschaniwskyj for statistical advice and Dr. Louis Ward for her advice on histology. To the following people; Dr. N.P. Sanga, R. Alhazzaa, N. Amin, S. Fatima and Dr. M.B. Codabaccus from the UTAS fish nutrition team, Dr. M. Adams and D. Plankto from the UTAS aquaculture centre, many thanks for your helped and support during my study.

Finally, thanks to my family who gave me the moral support I required throughout my study. Thank you.

LIST OF ABBREVIATIONS

The following abbreviations are used in this thesis:

AD, apparent digestibility

ANCOVA, analysis of covariance

ANOVA, analysis of variance

BP, break-Point

CMC, carboxymethyl cellulose

CP, crude protein

DM, dry matter

EAA, essential amino acids

FER, feed efficiency ratio

FI, feed intake

FL, final Length

FMG, fishmeal with L-glycine

FMLH, fishmeal with L-lysine and L-histidine

FW, final weight

FM, fishmeal

GC, goblet cells

GE, gross energy

HI, hepatosomatic Index

IGF I, insulin like growth factor I

IW, initial weight

K, condition factor

LP, lamina propria

MF, mucosal fold

MI, maximum inclusion

NEAA, non-essential amino acid

PEV, productive Energy Value

PM, poultry meal

PMG, poultry meal with L-glycine

PMLH, poultry meal with L-lysine and L-histidine

PPV, productive Protein Value

SE, standard error

SGR, specific growth rate

SM, sub-epithelial mucosa

SNV, supranuclear vacuoles

SPSS, statistical analysis software program

TL, total lipid

WG, weight gain

WW, wet weight

CHAPTER 1

General Introduction

GENERAL INTRODUCTION

1.1. **Introduction**

Global capture fisheries and aquaculture provided around 142.3 million tonnes of fish in 2008 to which, aquaculture contributed 52.5 million tonnes. Global capture fisheries have been relatively stable at 89 to 92 million tonnes between 2004 and 2008 whereas aquaculture production increased from 42 to 55 million tonnes for the same period (FAO, 2010). Meanwhile, the demand for fish is increasing with the rising world population (FAO, 2010). Thus, aquaculture has potential to fill the gap between stable fisheries production and increasing demand (Naylor et al., 2000). Since feeding intensively farmed fish depends on commercial and nutritionally complete feeds, the study of fish nutrition is crucial to the sustainability of this industry (Guillaume et al., 2001). Generally, aquafeeds account for 40% to 60% of the production cost for most intensively cultured aquatic species, clearly one way to increase the production profitability is to reduce the cost of aquafeed (Cheng et al., 2003; Yigit et al., 2006; Alami-Durante et al., 2010). The identification of alternative feed ingredients and the relationship to nutritional requirement of specific species is essential in order to improve production and to reduce aquafeed cost (Shamshak and Anderson, 2010).

Fishmeal (FM) is traditionally the major protein source in aquafeed as well as the most expensive readily available protein source (Naylor et al., 2001; El-Shafai et al., 2004). However, in recent years, the use of fish to produce FM has decreased compared to the mid-1990s due to a greater proportion of fish used for direct human consumption (FAO, 2010). Besides the food security issue due to an increase in world population, other factors may influence the availability of fish for FM production (FAO, 2010). The main source of FM is from forage fish such as

anchoveta and other small pelagics (FAO, 2010). Unfortunately, forage fish catch is not stable because it is influenced by the environmental factors such as global warming which leads to el nino and this indirectly affects the production of FM (Broad et al., 2002; Bakun and Broad, 2003). All of these factors contribute to limiting the supply of FM and thusmay compromise the growth and sustainability of the aquaculture industry. The rise in the price of FM is a very good indicator of the demand and supply market dynamics. Tacon and Metian (2008) reported that FM doubled in price, from US $694 to US $1379 per tonne between July 2005 and July 2006. Reducing the dependence on FM for aquafeed will thus address the issue of limited supply of FM and concomitantly will reduce the price of aquafeed (Tacon and Metian, 2008). Therefore, FM replacement is an important part of making intensive aquaculture sustainable (Carter, 2007).

In general, FM is a good protein source in terms of high protein content, excellent level of essential amino acids, high nutrient digestibility; general lack of antinutrients and wide availability (Gatlin et al., 2007; Miles and Jacob, 2011). Alternative protein sources are mainly from animal by-products, plant meals, and other sources including single cell meals (Perera et al., 1995; de Francesco et al., 2004; El-Haroun et al., 2009). Therefore, the potential of new alternatives to FM from animal, plant or biotechnology sources in aquafeeds needs to be investigated; numerous studies on many different aquaculture species have been conducted (Gatlin, et al., 2007; Sales, 2010).

Skretting supplied FM and PM in batches for each experiment this was because the study lasted for 4 years and it was not possible to store FM and PM for this duration. Hence, the need new batch of the meals for every experiment. According to Macan et al., (2006), FM can be stored for at least 3 months at ambient temperature. The main research tools to determine fish

performance are digestibility, nutrients availability and their effect on fish growth performance. Other research tools have been investigated and include measurement of growth-related hormones, health, gene expression or use of proteomic screening (Codabaccus et al., 2011). The principle aim of this study is to evaluate the use of poultry meal (PM) as an alternative protein source for rainbow trout, *Oncorhynchus mykiss*, as a representative for the salmonid aquaculture industry. Previous studies focused on applying parameters from standard nutrition, growth and digestibility analysis to compare the efficiency of replacing FM in aquafeeds. In the current research, different approaches are used and integrated into more standard nutrition experiments in order to understand the effect of different levels of FM replacement with animal-derived alternatives on fish growth, growth-related hormones, digestibility and alimentary tract histology. This introductory chapter presents a detailed review of the effects of replacing FM with PM in farmed fish at different levels of replacement and within varied environmental conditions as a sustainable practice in aquaculture.

1.2. The Importance of Poultry Meal in Aquafeeds

1.2.1 Poultry meal

PM has been extensively studied as an alternative protein sources in animal feeds including aquafeed (Fowler, 1991; Steffens, 1994). Allan et al. (2000) reported PM has a high and relatively consistent protein content of around 60%. A study conducted by Dong et al. (1993) reported the range of PM protein as between 55-74%. The different protein contents in PM may be caused by the manufacturing practice in the production of PM (Bureau and Cho, 1999). Processing involves both physical and chemical methods using a variety of equipment and processes (Meeker and Hamilton, 2006). Differences in PM quality can be influenced by

differences in the processing method including temperature and time of cooking (Meeker and Hamilton, 2006).

The cooking lasted for 40 to 90 minutes and afterwards, the melted fat was separated and the remaining materials were ground and stored (Meeker and Hamilton, 2006).There are two types of rendering namely, wet and dry rendering. The method of wet rendering involves loading raw material into a vertical digester. If the raw material is dry, water is added to moisten it after which steam is injected into the material through perforated plates in a digester (Ockerman and Hansen, 1988). In dry rendering, the raw animal by-products are added to a horizontal steam-jacketed cylinder equipped with an agitator. If the raw material is ground, the vents are closed and at the same time the pressure is increased in the cooker to disintegrate the bones and other large particles of the raw materials (Meeker and Hamilton, 2006).

PM has been tested as a FM replacement in aquafeeds for rainbow trout and results support its use as a promising protein source (Alexis et al., 1985; Steffens, 1994). A study conducted by Fowler (1991) showed that 200g kg^{-1} PM can replace FM in the diet of Chinook salmon (*Oncorhynchus tshawytscha*). Alexis et al., (1985) reported that PM can partially replace FM at 25% of diet without any adverse effects on rainbow trout. Meanwhile, Steffens (1994) indicated that if PM was used at up to 50% in aquafeed, it will also increase fish carcass lipid content. In rainbow trout, the maximum replacement of FM with PM is approximately 50% and ranges from 25 to 50 % of diet (Steffen, 1994; Alexis et al., 1985).

1.2.2 Protein

Critical assessment of alternative protein sources as replacement for FM protein has been conducted by many researchers (Francis et al., 2001; Gatlin, et al., 2007). There are only a limited number of alternative protein sources which have been successfully introduced to replace FM, and one of them is a PM (Sales, 2010). Generally, the protein contents of poultry by-product meal ranges from 55 to 74 % (Dong et al., 1993; El-Haroun, et al., 2009). Poultry by-product meal is a good source of dietary protein for salmonids (Sugiura et al., 1998). This is illustrated by a study conducted on rainbow trout which the refined poultry by-product meal successfully replaced all the FM (Cheng and Hardy 2002). This is in agreement with a study conducted by Sealey et al. (2011) in which the total replacement of FM with poultry by-product meal did not affect the growth of rainbow trout. According to El-Haroun et al. (2009) PM can efficiently replace FM because of its good nutritive value and source of protein for salmonids. This is because PM has good levels of amino acids, vitamins, minerals and fatty acids (Aird and Spragg, 2005; Meeker and Hamilton, 2006). The nutritional value of commercial poultry by-product meal depends primarily on the quantity and quality of protein. Unfortunately, the quality of poultry by-product meal varies from batch to batch (Dong, et al., 1993). Therefore, the effective maximum percentage of replacement depends on the quality of the alternative protein sources (Gomes et al., 1995; Øverland et al., 2009).

The quality of alternative protein in diets depends on its amino acids composition (Drew et al., 2007). Some essential amino acids may be limiting. According to Hauler and Carter (2001), lysine is the first-limiting amino acid in many alternative protein sources used in aquafeeds. When compared to rainbow trout requirements the first limiting essential amino acids

in PM are likely to be lysine or histidine depending on the specific PM (Steffens, 1994; Carter et al., 2002). The requirements for amino acids in most cultured fish species are either known or can now be satisfactorily estimated; therefore it is possible to formulate an "ideal" feed. However, particular attention should be given to balancing amino acids in feed properly since in typical dose-response experiments, there is often an underestimation of essential amino acids (Hauler and Carter, 2001).

FM has the amino acid composition closest to an ideal protein source for rainbow trout (Kaushik and Seiliez, 2010). The availability of essential amino acids in FM is generally greater compared to other protein sources (Kaushik and Seiliez, 2010). To produce high quality feed, the level of amino acids must meet the requirements of the cultured species (Murai, 1992; Gaylord et al., 2010). The deficiency of protein and essential amino acids results in decreased growth efficiency, reduction in weight, decreasing of the immune system and can also increase mortality (Cowey, 1994; Anderson et al., 1995; Li et al., 2007).

A study conducted by Steffen (1994) showed that the first limiting amino acid in PM was lysine, followed by histidine and methionine when compared to rainbow trout requirement. Another study conducted by Carter et al., (2002) indicated that the first limiting amino acid is histidine when compared to the requirement of Atlantic salmon. In contrast, Dong et al., (1993) estimated the first limiting amino acid is methionine and the second limiting amino acid is phenylalanine when compared to the requirement of Chinook salmon. These data show variation in the first limiting amino acid for salmonids fed PM and this is probably due to differences in

method used to estimate the limited AA, type of PM, batches of PM, and fish species (Dong, et al., 1993; Hajen et al., 1993).

However, because the outbreak of bovine spongiform encephalopathy (BSE), the use of animal proteins in fish feed was banned by the European Union to prevent the transmission agent from entering the food chain (Dersjant-Li, 2002; Fumière et al., 2009). Australia is free of BSE and all other transmissible spongiform encephalopathy such as Scrapie and Chronic Wasting Disease (Spragg and Aird, 2003). In Australia, the use of feedstuffs containing animal products is restricted to ruminants only (Spragg and Aird, 2003). Therefore, fish can be fed with animals meals including poultry meal.

1.2.3 Feed intake

Feed acceptance is an important factor in an aquafeed and it is dependent upon the chemical, nutritional and physical characteristics which are influenced by feed ingredients (Jobling et al., 2001). Alternative proteins such as plant protein are known to have negative effects on feed intake of fish (Lovell, 1989; Francis, et al., 2001; Gatlin, et al., 2007). The importance of plant proteins in fish feed has been highlighted by many researchers (Carter and Hauler, 2000; Farhangi and Carter, 2007; Glencross et al., 2008) although they are limited in essential amino acids and it also has antinutritional factors (NRC, 1993). A study conducted by Morales et al.(1994) showed that higher feed intake of lupin fed to rainbow trout resulted in lower total diet digestibility and energy availability compared to other plant protein and FM diets. A study conducted by Sajjadi and Carter (2004) demonstrated that when phytic acid, an antinutritional factor, was added to a feed containing no plant meal protein, digestibility was reduced without any effect on feed intake and growth. In order to reduce reliance on FM in

aquafeed, numerous studies on aquafeed have been undertaken using various sources of plant proteins such as lupin meals known to be high in protein (Farhangi and Carter, 2007; Glencross et al., 2008). A study conducted by Carter and Hauler (2000) indicated that the use of plant meals had a high potential to replace FM for Atlantic salmon. In another study, Barrows et al., (2007) indicated that plant proteins are good sources of proteins and their effects on rainbow trout growth are close that fed FM based diet. For other alternative proteins, such as animal protein, a reduction in feed intake is common.

A study conducted by Poppi et al. (2011) showed reduction of feed intake in the fish fed diets containing different levels of feather meal and this was probably explained by the deficiency of several essential amino acids in the diets (Hauler and Carter, 2001). The same result was observed when the FM was totally replaced with a combination of feather meal and poultry by-product meal (Steffens, 1994). Furthermore, a new feed or ingredients can influence feed intake in fish due to the requirement of an adaptation period when they are fed with a new diet (Carter and Davies, 2004).

1.2.4 Digestibility of aquafeed components

A good knowledge on the apparent digestibility of nutrients is very important in fish nutrition (Sales, 2010) because this will provide information on the availability of energy and nutrients to formulate feeds so as to meet the nutrient requirements for farmed fish (Bureau et al., 1999; Davies et al., 2009). However, measuring digestibility on fish is very complicated compared with terrestrial animals (Sales, 2010). There are many factors that influence apparent digestibility such as the faecal collection method, markers and level of nutrients (Percival et al.,

2001; Glencross et al., 2007; Gaylord et al., 2008). There are a few methods that have been used to collect faecal matter and these methods have a significant influence on the value of apparent digestibility (Storebakken et al., 1998; Percival et al., 2001). A study conducted by Storebakken et al., (1998) showed that sieving methods overestimated apparent digestibility of nitrogen in Atlantic salmon compared with stripping and dissection methods. Other factors which may influence the results are the type of inert marker used. Different markers have been used for digestibility experiments in aquaculture and include chromic oxide, hydrocarbon markers such as cholestane and rare earth metal oxides such as ytterbium oxide, yttrium oxide and other rare earth metal oxides (Austreng, 1978; Austreng et al., 2000; Carter et al., 2003). Austreng, et al. (2000) showed differences in apparent digestibility for nitrogen between chromic oxide and a few types of oxides; lanthanum oxide, ytterbium oxide, yttrium oxide when hydrochloric acid and nitric acid used to dissolved the samples. Another study conducted by Carter et al., (2003) showed the results of digestibility can be influenced by type of markers and indicated the digestibility results for cholestane marker tended to be higher than yttrium. From previous studies conducted by others researchers, it can be concluded that stripping is a suitable method to determine the apparent digestibility of nutrients.

Processing method of protein sources involves both physical and chemical processes and these processes can influence the quality of protein sources (Meeker and Hamilton, 2006). This showed that the differences on digestibility are not only influenced by the different types of protein source, but also by different batch of same protein source or different practices in production of the protein source. For example different batches of poultry by-product meal have different digestibility and may range between 64 and 85% for rainbow trout (Dong, et al., 1993;

Hajen, et al., 1993). A study conducted by Bureau et al., (1999) showed an improvement in the apparent digestibility of protein of two PM by-products with values of 87 and 91% when fed to rainbow trout. All differences in PM quality can be influenced by processing method such as the temperature and length of time of cooking process (Meeker and Hamilton, 2006).

A study conducted by Storebakken and Austreng, (1987) showed when fish fed at a different rations, it does not effects the nitrogen digestibility. In contrast, when FM was partially replaced by bacterial meal, no significant difference was found in feed intake, but the lower digestibility of nitrogen was detected in this experiment (Storebakken, et al., 1998). A study conducted by Sajjadi and Carter (2008) indicated that differences in feeding rate influenced the digestibility of dry matter and crude protein, but had no effect on energy digestibility.

1.3 Protein metabolism and influences on protein retention

The effects of nutritional variables on protein metabolism in fish can be evaluated at a number of levels including the measurement of the activity of specific enzymes (such as trypsin and chymotrypsin), nitrogenous excretion, amino acid flux, protein synthesis, gene expression and proteomics (Carter and Houlihan., 2001; Guillaume et al., 2001; Martin et al., 2003). However, the efficiency of retaining nitrogen (crude protein) represents the most straightforward measurement to interpret in relation to understanding the impact of FM replacement on the use of dietary protein.

The amino acid balance of dietary protein is crucial in fish nutrition and therefore the amounts and balance of protein sources used in fish feed influences protein retention in fish

(Gaylord and Barrows, 2009). A study conducted by Storebakken et al., (1991) showed that in rainbow trout growing from 320 to 462g, about 29% of digestible protein fed was retained. In addition, a study conducted by Glencross et al.(2008) showed the value of protein retention in FM diet when fed to rainbow trout was 39%. Different results were observed when a poultry by-product blend was used as a main protein source and fed to rainbow trout where the level of protein retention was recorded 27% (Sealey, et al., 2011). In the same study, low protein retention was found when FM was replaced with chicken concentrate meal with the value 35% of protein retention. Improvement of protein retention is important in aquafeed as an indicator to the protein quality and fish can utilize feed efficiently and this will reduce the waste material impacts to the environment (Halver and Hardy, 2003).

1.4 Temperature effects on fish

Temperature is recognized as the most important abiotic factor affecting physiology of ectothermic animals and will significantly affect fish growth (Brett and Groves, 1979). Feed intake by fish is increased with increasing temperature until it reaches a peak which is generally a few degrees beyond the optimal temperature of fish and decrease thereafter (Brett and Groves, 1979). There is a reduction of feed intake beyond the optimal temperature range due to the limitations of the respiratory and circulatory system to deliver the oxygen to the tissues (Cocking, 1959).Thus different temperatures will induce changes in feed intake in fish.

Metabolism increases exponentially as temperature increases, within the thermal tolerance range, (Jobling, 1994). Growth can be considered as the balance between feed (nutrient) intake and expenditure, mainly via metabolism (Jobling, 1994). Fish therefore reach

maximum growth at a temperature below the maximum they can survive, this is the optimal temperature (Jobling, 1994). Metabolism includes the energy demand for many processes in fish at a given temperature (Clarke, 2003).

Current global warming models predict a temperature increase of 1.4 to 6.4^{o}C in this century (IPCC, 2001). The change in environmental temperature poses threats to wild and farmed fish (Farrell, 1997; Brander, 2007). Elevated water temperatures are currently occurring in Tasmanian waters, with temperatures in the summer months reaching 19 to 20^{o}C (Miller et al., 2006; Ng et al., 2010). These high temperatures may affect nutrient utilization and digestibility of fish (Miller, et al., 2006; Ng, et al., 2010). This is probably due to a number of different nutritional effects (Katersky and Carter, 2005; Choa et al., 2010). Gastric evacuation rate will be higher at higher water temperatures (Fauconneau et al., 1983). The effect of temperatures on apparent digestibility coefficient of fish is not certain. A study on rainbow trout conducted by Yamamoto et al. (2007) indicated that crude protein digestibility at 18^{o}C was higher at 11^{o}C. Bureau et al. (2008) reported that water temperature significantly influenced apparent digestibility coefficient of protein and energy, but not apparent digestibility coefficient of lipid when rainbow trout were culture at 7.5 and 15^{o}C. Interestingly, an elevated temperature up to 20^{o}C did not show any differences in lipid digestibility in rainbow trout (Ng, et al., 2010). The increasing temperature up to 20^{o}C will affect the protein requirement in fish (Hecht and McEwan, 1992) and at the same time, it also affects the percentage gain of protein and protein conversion efficiency in fish (Selong et al., 2001).

1.5 Nutritional influences on endocrine system

Growth is a complex process and involving many types of hormones in the fish body (Wilkinson, et al., 2006; Benedito-Palos et al., 2007). In order to gain a better understanding of fish nutrition, it is becoming increasingly common for authors to measure various hormones involved in key endocrine systems related to growth which are greatly influenced by nutrition (Pérez-Sánchez and Le Bail, 1999; Gabillard et al., 2006a; Wilkinson et al., 2006). One hormonal axis that has received much attention in recent times is the growth hormone / insulin-like growth factor (GH/IGF) axis. Growth hormone is produced in the alpha-cells of proximal pars distalis of the pituitary gland and has a major role in the control of metabolism and growth in fish (Jobling, 1994).

The various roles of growth hormone in fish are diverse and encompass important functions such as the stimulation of appetite and subsequent growth of salmonids (Devlin et al., 1999). In addition, variations in growth hormone level also influence many important functions in the fish body such as, energy metabolism, reproduction, feeding, osmoregulation and immune function (Chang and Wong, 2009). Many of the roles of GH are believed to be mediated through the production of insulin-like growth factor-I (IGF-I). The production of GH is regulated by hypothalamic hormones; and after being produced, growth hormone binds to its receptors in the target organs mainly in the liver and stimulates synthesis and release of IGF-I (Moriyama et al., 2000). Growth hormone directly influences liver IGF-I gene expression in fish (Wong et al., 2006) with liver considered the major source of circulating IGF-I (Shamblott et al., 1995). Several studies conducted in salmonids show a strong correlation between growth and level of circulating IGF-I (Shimizu et al., 2000; Pierce et al., 2001).

Although the liver is considered to be the major source of circulating IGF-I, this mitogenic hormone is also produced in the pancreatic islets, gastroentero-endocrine cells, cells of the renal proximal tubules, interrenal cells of the head kidney, gill chondrocytes and chloride cells, granulossa cells in the ovary, spermatocytes and sertoli cells in testis, and neurons in the retina and brain of the fish (Shamblott et al., 1995; Le Gac et al., 1996; Reinecke et al., 1997; Greene and Chen, 1999) and is thought to have both systemic and local biological activity which is related with the nutritional status of fish (Duan, 1998; Chauvigné et al., 2003; Gabillard et al., 2003). IGF-I is also directly involved in the regulation of protein, lipid, carbohydrate and mineral metabolism in the cells and growth of fish (Moriyama, et al., 2000).

There a strong evidence linking food restriction with increased circulating levels of GH in fish (Storebakken, et al., 1991; Duan and Plisetskaya, 1993). Increase in plasma GH leads to a decrease in hepatic binding sites for GH (Gray et al., 1992) and as a result, reduces the level of IGF-I mRNA expression in the liver (Duan and Plisetskaya, 1993). This therefore reduces circulating IGF-I levels and leads to retarded somatic growth in salmon (Duan and Plisetskaya, 1993). Refeeding of fish has been shown to reduce circulating GH levels, increase hepatic IGF-I mRNA expression, increase circulating IGF-I levels, and increase growth (Duan and Plisetskaya, 1993). More subtle changes in food intake can also affect the GH/IGF axis. More specifically, it has been reported that the circulating GH increases and IGF-I decreases with a decrease in ration size (Gray et al., 1992; Dyer et al., 2004).

There are a few studies on the effects of replacement of FM with alternative protein sources on the GH/IGF-I system. A study conducted by Gómez-Requeni et al. (2005) showed that replacement of FM with plant protein increased GH but no difference was found in the IGF-I level when compared with fish fed a diet with FM in rainbow trout. In another study conducted by Gómez-Requeni et al. (2004) using plant protein to replace FM in sea bream (*Sparus aurata*) diet, GH level increased and IGF-I level decreased, leading to a reduction in fish growth. Major effects of alternative protein sources on the GH/IGF axis are believed to be due to limitations in essential amino acids. A study conducted by Gómez-Requeni et al.,(2003) showed that changes in dietary essential amino and non essential acids affected the liver GH and growth in sea bream (*Sparus aurata*). This is in agreement with a study conducted by Gómez-Requeni, et al., (2004) where the GH liver axis was affected by dietary essential amino and non essential amino acids in the diets and this was shown in sea bream (*Sparus aurata*) fed with diet consist plant protein with different levels of dietary essential amino acids and non essential amino acids and the results showed a high level of GH and low level of IGF-I in fish.

Temperature has a direct effect on GH secretion directly or indirectly through somatostatin of pancreatic or hypothalamic origin (Gabillard et al., 2005) . Elevated temperature has been shown to reduce hepatic GH-binding and increase growth hormone receptor (GHR) mRNA level (Gabillard et al., 2006b). Since feed intake was influenced by temperature (Brett and Groves, 1979), the differences in feed intake will affect nutritional status and this will influence the GH/IGF-I levels (Gabillard et al., 2005). This is in agreement with a study conducted by Larsen et al. (2001) in which a lower temperature, under the optimal range for coho salmon, reduced feed intake and in turn reduced the level of circulating IGF-I. In contrast,

when rainbow trout were fed with the same ration, temperature no longer affected the level of GH receptor transcript (Gabillard, et al., 2006b).

Another area of interest is the neuroendocrine stress response. Cortisol is the principal corticosteroid in teleosts and it is released from the interrenal tissue, analogous to the adrenal cortex and distributed in the head kidney region (Aluru and Vijayan, 2009; Sandhu and Vijayan, 2011). Cortisol increases when fish are stressed and this is accepted to have a wide range of deleterious effects such as reduction of disease resistance, impairment of fish growth, reductions in fish flesh quality, adverse effects on the reproductive system and increased mortality rates (Carragher et al., 1989; Pickering and Pottinger, 1989; Gregory and Wood, 1999).

Cortisol affects the GH/GF axis since it is involved in regulation of the hypothalamus-pituitary gland-interrenal gland axis which directly affects metabolic processes in the fish (Leatherland et al., 2010; Schreck, 2010). According to Pickering et al. (1991), the elevation of GH level in fish is correlated with an elevation of plasma cortisol. This showed that cortisol has a significant effect on protein synthesis, plasma amino acid level and tissue enzyme and transporter activity (Ballantyne, 2001). In addition, elevation of plasma cortisol had significant negative effects on fish appetite, condition factor and food conversion efficiency (Gregory and Wood, 1999). These will lead to the reduction in fish growth and perhaps can cause high mortality in fish. Increasing in cortisol levels also caused decreasing levels of IGF-I (Peterson and Small, 2005). A study conducted by Vielma et al., (2003) showed an increase in circulating cortisol level in European whitefish (*Coregonus lavaretus*) fed diets containing decreasing levels of protein.

Attempting to understand all physiological and endocrine effects of replacing FM can be a difficult process due to the number of complex systems involved. However, studying the GH/IGF axis can provide important insights into the nutritional effects on fish. In addition, determining dietary effects on cortisol production provides important information on fish growth performance.

1.6 Rainbow trout

Rainbow trout is native to the west coast of North America, from Mexico to Alaska and among the most popular sport fish worldwide as well as for aquaculture production (Willoughby, 1999). Of the 52.5 million tonnes of fish production in 2008, rainbow trout contributed 0.58 million tonnes (FAO, 2010). Based on the percentage of diadromous fish production, rainbow trout contributed 17.4% of the total production of diadromous fish (FAO, 2010). Rainbow trout is anadromous species, but they also can survive in freshwater for their entire life (Willoughby, 1999). This fish is popular as an aquaculture species due to its disease resistance, ease of domestication and fast growth (Willoughby, 1999). There is considerable research on rainbow trout nutrition (Jobling et al., 2001; Farhangi and Carter, 2007; Ng, et al., 2010).

Rainbow trout is a carnivorous fish and the main protein source in rainbow trout feed is FM. Due to the increasing in price of FM, it is predicted that the inclusion level will decrease in the long term (Tacon and Metian, 2008). Reducing FM levels in aquafeed has been widely reported (Farhangi and Carter, 2001; Glencross et al., 2004b; Farhangi and Carter, 2007).

However, total replacement of FM with alternative protein sources affects rainbow trout growth (Farhangi and Carter, 2001; Glencross et al., 2004a).

1.7 Aims of the present study

The overall aim of this study was to understand the patterns of hormones, digestibility and the appearance morphology of the distal intestine of rainbow trout at different levels of substitution of FM with PM (Chapters 2 and 3), low and high temperatures (Chapter 4) and the use of indispensible amino acids (Chapter 5).

To address this aim, the following strategies were examined:

1) To test the hypothesis that at an undefined inclusion level PM will restrict growth due to nutrient limitation (compared with using FM) (Chapter 2).
2) To test the hypothesis that rainbow trout requires an adaptation time when it is introduced to a feed with PM (Chapter 3).
3) To test the hypothesis that differences in the inclusion level of PM in rainbow trout are influenced by temperature and will eventually affect fish growth (Chapter 4).
4) To test the hypothesis that the growth of rainbow trout will not be significantly different upon supplementing PM with essential amino acids compared to that of fish meal (Chapter 5).

The approaches in chapter 2 were to determined the effectiveness of partial replacement of FM with PM. Initially, this experiment was carried out to investigate the minimum replacement of FM and effect on growth, physiology and protein digestibility in rainbow trout

Chapter 3 was designed to investigated whether the total replacement of FM with PM affects the nutrients digestibility in rainbow trout. The apparent digestibility of protein, lipid and energy were analyzed in order to assessing the effects of replacement.

Chapter 4 is a continuation from chapter 3. The total replacement of FM with PM was assessed at two different temperatures, $15.4 \pm 0.01^{o}C$ and $20.2 \pm 0.01^{o}C$. This was to examined whether sub-optimal temperatures influence protein replacement.

Chapter 5 was designed to assesses the potential of total replacement of FM with PM which in this experiment, PM was supplemented with indispensible amino acids, L-lysine and L-Histidine. In general, PM was supplemented with complete EAA. This diet was compared with a FM diet, which was also supplied with the same amino acids. The effects of diets supplemented with indispensible amino acids were evaluated to in order to determine the quality of these diets.

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CHAPTER 2

Effects of replacing fishmeal with increasing levels of poultry meal on growth performance, plasma insulin-like growth factor-I and plasma cortisol in rainbow trout (*Oncorhynchus mykiss* Walbaum)

2.1 ABSTRACT

The effects of replacing fishmeal with poultry meal on rainbow trout (*Oncorhynchus mykiss*) was evaluated using four isoenergetic and isonitrogenous feeds (22 MJ GE kg^{-1} DM, 394 g kg^{-1} DM of crude protein) in which 0, 20, 30 and 40% fishmeal was replaced. Fish with mean weight 190.0 ± 39.6 g were fed 2% of body weight twice a day for a period of 84 days. Poultry meal inclusion did not have a significant effect on daily feed intake (1.5 ±0.02 % day^{-1}), final weight (561.4 ± 13.9 g), weight gain (370.8 ± 12.6 g), specific growth rate (1.3 ± 0.03 %·day^{-1}), feed efficiency ratio (0.8 ± 0.02), or condition factor (1.7 ± 0.01). In addition, poultry meal inclusion did not have a significant effect on whole body chemical composition (dry matter [309.7 ±0.4 g kg^{-1} WW], crude protein [123.5 ± 0.12 g kg^{-1} WW], total lipid [164.9 ±0.23 g kg^{-1} WW], ash [19.0 ±0.05 g kg^{-1} WW], or gross energy [8.2 ±0.16 MJ kg^{-1} WW]). There was no significant difference between apparent digestibility of crude protein. Furthermore, plasma concentrations of insulin-like growth factor–I and cortisol were not affected by poultry meal replacement. This study showed that poultry meal can replace up to 40% of fishmeal without effecting growth performance, protein digestibility or selected plasma hormone concentrations.

Keywords: Rainbow trout, Cortisol, IGF-I, Poultry meal, Fishmeal

2.2 INTRODUCTION

Fishmeal (FM) is an excellent source of protein. It has a good level of essential amino acids (Gatlin et al., 2007; Miles and Jacob, 2011). The increase in FM and fish oil demand will potentially increase the price of aquaculture feed (Tacon and Metian, 2008). Therefore, alternative sources of protein such as spray-dried blood meal (BM), feather meal (FEM), meat and bone meal (MBM) and poultry by-product meal (PM) which are less expensive, and of good quality, are needed to replace or partially substitute FM (El-Haroun et al., 2009). PM is the most likely alternative animal derived protein source to replace the FM. PM are processing wastes from the poultry industry and provide relatively cheap but high quality protein sources for a range of fish species (Steffens, 1994). PM is an alternative protein source which has been used widely in fish feed and has potential for use in rainbow trout diets at high levels of incorporation (Steffens, 1994; Cheng and Hardy, 2002; El-Haroun et al., 2009), however the maximum level of inclusion of PM in fish feed depends on both protein quantity and quality. The previous studies showed in rainbow trout, the maximum replacement of FM with PM is approximately 50% and ranges across 25 to 50 % (Steffen, 1994; Alexis et al., 1985). However, the maximum replacement may vary depending on the quality of PM. This is because the quality of PM varies based on the processing method (Ockerman and Hansen, 1988; Meeker and Hamilton, 2006). The processing method involves temperature, time and in some cases, pressure. If there is overheating , the nutritional value and digestibility of the finished product decreases (Meeker and Hamilton, 2006).

Although many studies have investigated the effect of FM replacement on growth performance, relatively few have investigated underlying endocrine changes. Measuring key

hormones may provide important information on how alternative protein sources influence growth physiology (Duan and Plisetskaya, 1993; Pérez-Sánchez and Le Bail, 1999; Gómez-Requeni et al., 2004). For example, plasma insulin like growth factor-I (IGF-I) concentrations increase with increasing growth rates in a number of aquaculture species and provide a measure of growth performance (Dyer et al., 2004a). Plasma IGF-I is positively correlated with fish growth and some research has indicated that IGF-I is nutritionally regulated (Ketelslegers et al., 1995). When fish are not feeding, plasma IGF-I level will decrease due to an increase in liver growth hormone (GH) resistance and at the same time the level of GH will increase (Pierce et al., 2005). Similarly when feed intake decreased, IGF-I mRNA expression decreased, resulting in lower plasma IGF-I levels and fish growth (Duan and Plisetskaya, 1993). Atlantic salmon (*Salmo salar* L.) which were fed a diet consisting of 46% protein, of which 33% came from replacement plant protein and 13% from marine protein, resulted in decreased feed intake, a reduction in essential amino acids intake which therefore resulted in lower pituitary GH and muscle IGF-I mRNA levels (Hevrøy et al., 2008).

Stress is another critical factor that affects growth but which is rarely considered when investigating novel dietary components (Gregory and Wood, 1999; Carter and Davies, 2004; Bernier, 2006). The corticotropin-releasing factor (CRF) system is the main centre in coordinating the neuroendocrine, autonomic and behavioural responses to the stress (Bernier, 2006). Therefore, the measurement of plasma cortisol (the primary corticosteroid in the teleost stress response) is typically performed as an indicator of the primary physiological stress response of fish (Ellis et al., 2007). Some feed ingredients have been shown to directly affect cortisol level in fish. For example, dietary sunflower oil increased plasma cortisol in Atlantic

salmon (Jutfelt et al., 2007). No studies have investigated the effect of FM replacement with PM on cortisol levels in rainbow trout. Consequently cortisol was measured in the current experiment to determine the relationship between cortisol level and effects of replacement on fish performance. The aim of this study was to determine whether partial FM replacement with high-quality PM affects the plasma IGF-I, cortisol levels and apparent digestibility in rainbow trout.

2.3. MATERIALS AND METHODS

2.3.1 Experiment Diet

Diets were formulated to be isonitrogenous and isoenergetic to contain 394 $g \cdot kg^{-1}$ DM of crude protein and 22 $MJ.kg^{-1}$ DM gross energy (Table 2.1). Three experimental diets contained graded levels of PM (20% [PM20], 30% [PM30] and 40% [PM40]) to replace the FM protein. An additional control diet contained FM as the only protein source [PM00]. Ingredients were added with 10% w/w water and were mixed using a Hobart mixer, cold pelleted with a laboratory pelletizer (California Pellet Mill Co., San Francisco, CA, USA) and oven dried for 24 h at 30^{o}C. Diets were kept refrigerated at 4^{o}C. FM, PM and fish oil were supplied by Skretting (Cambridge, Tasmania, Australia). Vitamins, minerals, carboxymethyl cellulose, bentonite, calcium phosphate, α-cellulose and yttrium oxide as a marker were supplied by Sigma-Aldrich Pty. Ltd (Sydney, Australia). Vitamin C was supplied as Stay-C from Roche Pharmaceuticals (Roche Vitamins Australia Ltd., Sydney, Australia) and choline chloride was supplied by ICN Biomedicals, INC. (Aurora, Ohio, USA).

2.3.2 Growth Experiment

The experiment was conducted according to Ethics Approval A0009762 the Aquaculture Centre, University of Tasmania (Launceston, Tasmania, Australia). Four identical recirculating systems each containing three, 1000 L fibreglass tanks and each system was fitted with a submerged biofilter and UV steriliser. A total of 250 rainbow trout (*Oncorhynchus mykiss*) with a mean initial weight 190.0 ± 39.6 g were obtained from Mountain Stream Fishery, (Tasmania, Australia). Fish were stocked in three 1000 L tanks for a 3 week acclimation period and held in freshwater at 15^{o}C.

At the start of the experiment each tank was stocked with 20 fish. An additional 10 fish were killed with an overdose of anaesthetic with a concentration 60 mgL^{-1} (AQUI-S New Zealand LTD, Lower Hutt, NZ) and immediately frozen for whole body chemical composition. During the experiment, fish were held under constant conditions (15°C, 12:12 light:dark photoperiod, freshwater (0 ‰ salinity)). Fish were not fed for three days prior to the start of the experiment. Tanks were randomly divided between the four dietary treatments (n = 3). Fish in all treatments were hand-fed 2% body $weight.day^{-1}$ at 0900 and 1600h for 84 days. Sampling was carried out every three weeks. Every three weeks, the feed ration was recalculated based on the fish weight . Feed intake can vary between replicate tanks on a daily basis, to account for a fixed ration that was in excess of normal intake. Fixed ration was selected and actual feed intake measured each day.

All fish were anaesthetized before sampling. Sampling order of tanks was conducted randomly on each sample occasion. Fish were quickly captured using a net and were transferred immediately to 300L tank containing 30 mg L^{-1} anaesthetic. Fish fork length (cm) and weight (g) were measured and blood samples were collected from the caudal vein into heparinised tubes from all fish in each tank where the average sampling time for each fish was 3 minutes. Fish were recovered in a tank of aerated freshwater following sampling. Weight and length were measured on day 1, 21, 42, 63 and 84; blood samples were collected on day 1, 42 and 84. At the end of the experiment, five fish from each tank were killed and the tissues were analyzed for whole-body chemical composition.

The remaining fish were maintained for an additional three weeks in order to measure protein digestibility. Fish faeces were collected using stripping techniques (Austreng, 1978; Percival et al., 2001). Fish were anaesthetized (30 mg L^{-1} AQUI-S) and the faeces were collected using gentle abdominal pressure from the ventral fins to the anus to ensure the faeces were not contaminated by urine or mucous (Austreng, 1978). Faecal samples were stored at -20^{o}C prior to analysis.

Apparent digestibility of nutrients was calculated as described by Maynard and Loosli (1969) using the following formula:

$$ADC_{diet} = 1 - (Marker_{diet} * Nurient_{faeces}/Marker_{faeces} * Nutrient_{diet}) \quad (1)$$

The diets included yttrium oxide (1 g kg^{-1}) as a digestibility marker. During the experimental period, one tank of fish from the 30% replacement treatment was lost and therefore all data from this replicate was excluded from the analysis.

2.3.3 IGF-I radio-immunoassay

Plasma IGF-I levels were determined using an IGF-I Radio-immunoassay (RIA) as previously described (Dyer et al., 2004b; Wilkinson et al., 2006). Plasma samples were first extracted to separate IGF-I/IGFBP complexes using acid-ethanol. Extraction involved mixing 40µL of plasma with 160µL acid-ethanol (12.5% 2 N HCl, 87.5% ethanol) and incubating at room temperature for 30 minutes. The acid-ethanol solution was neutralized using 80µL 0.855M Tris and samples were centrifuged at 10,000g for 10 minutes at 2-8^{o}C. The supernatant was collected and assayed for IGF-I as previously described using iodinated recombinant tuna IGF-I

(RIA label, recombinant salmon IGF-I assay standards and anti-barramundi IGF-I primary antibody (GroPep Ltd., Adelaide, Australia)). The antibody-bound hormone complexes were separated from free tracer by adding the 10µL rabbit gamma globulin (1:20) and 50µL sheep anti-rabbit gamma globulin (1:200). After mixing and incubating for 30 minutes at 2-8^{o}C, one mL of cold 5% (v/v) polyethylene glycol was added and the precipitate was centrifuged at 4000g for 30 minutes at temperature 2-8^{o}C. The supernatant was removed and the level of bound radioactivity was determined using a gamma counter. The minimum detectable limit of the assay was 0.15 ng mL^{-1}.

2.3.4 Cortisol radio-immunoassay

Cortisol concentration levels were determined in trout plasma samples using a tritiated RIA method (Pankhurst and Carragher, 1992) following ethyl acetate extraction. The minimum detectable limit of the assay was 6 ng mL^{-1}. Any values which were below the detection limit were reported as 6ng mL^{-1}. Tablecurve 2D v5.01 was used to determine cortisol value.

2.3.5 Chemical analysis

Fish carcasses were autoclaved (Williams et al., 1995) and dried using a freeze drier to a constant weight. Chemical analyse of fish carcass, diets and faeces were performed using standard methods. Dry matter was determined by drying the sample at 135^{o}C for 2 h (AOAC, 1995), ash content was determined by incineration in a muffle furnace at 600^{o}C for 2 h (AOAC, 1995), total lipid was determined according to Bligh and Dyer (1959), total nitrogen was determined by Kjeldhal (crude protein content was estimated as Nitrogen ((N) X 6.25) and gross

energy was determined by an auto bomb calorimeter. Yttrium were determined according to Ward et al., (2005).

2.3.6 Statistical analysis

Data are presented as mean ± standard error. All data were analysed using SPSS Statistical Analysis Software Program (version 15.0 for Windows). One-way analysis of variance (ANOVA) was performed to evaluate all data and Tukey's multiple range tests carried out to compare means among treatments. Levene's test was used to test the homogeneity of variance. Results with probability values less than 0.05 were considered as significant.

2.3.7 Calculations

The following equations were used to calculate weight gain (WG), specific growth rate (SGR), feed intake (FI), feed efficiency ratio (FER) and condition factor (K).

Weight gain (WG) (g) = Final weight – Initial weight (1)

Specific growth rate (SGR) (%·d^{-1}) = [(ln final weight (g) – ln initial weight (g))·

Time $(Days)^{-1}$] * 100 (2)

Feed intake (FI) (%·d^{-1}) = [(feed intake (g)·mean mid weight $(g)^{-1}$)·no $days^{-1}$] * 100 (3)

Feed efficiency ratio (FER) (g g^{-1}) = (wet weight gain (g)·total food consumption $(g)^{-1}$) (4)

Condition factor (K) = (whole wet weight (g))·(fork length $(cm)^3)^{-1}$ * 100 (5)

Table 2.1. Ingredient and chemical composition of experimental diets (mean ± standard error).

	Diets			
	PM00	PM20	PM30	PM40
Ingredient composition ($g \cdot kg^{-1}$)				
Fishmeal	564.0	450.7	394.4	338.1
Poultry meal	00.0	121.9	182.5	243.1
Pre-gelatinized starch	155.0	150.1	148.0	146.3
Fish oil	149.0	142.3	138.8	135.1
Carboxyl methyl cellulose (CMC)	10	10	10	10
Alpha cellulose	68.1	71.1	72.4	73.5
Bentonite	33.2	33.2	33.2	33.2
Choline chloride	0.7	0.7	0.7	0.7
Vitamin C	3.0	3.0	3.0	3.0
Vitamin Premix[a]	3.0	3.0	3.0	3.0
Mineral Premix[b]	3.0	3.0	3.0	3.0
Yttrium oxide	1.00	1.00	1.00	1.00
Calcium phosphate	10.0	10.0	10.0	10.0
Chemical composition ($g \cdot kg^{-1}$ DM)				
Dry matter	917.7 ±4.47	920.1 ±6.74	929.2 ±4.32	921.4 ±4.88
Crude protein	392.7 ±5.49	391.6 ±4.20	391.8 ±1.23	398.6 ±2.18
Total lipid	241.8 ±2.62	240.7 ±1.41	239.1 ±2.05	247.1 ±2.82
Ash	149.4 ±0.80	148.0 ±1.47	145.3 ±0.38	149.6 ±2.26
Gross energy ($MJ\ kg^{-1}$ DM)	22.4 ±1.55	22.6 ±1.55	22.0 ±2.67	22.6 ±0.90

Means with similar or no superscripts were not significantly different ($p>0.05$) between diets.

[a] Vitamin premix ($g\ kg^{-1}$): Vitamin A (4.50), Vitamin D3 (5.40), Rovimix E50 (90.00), Menadone sodium bisulphate (1.80), Riboflavin (3.60), Calcium D-pantothenate (19.57), Nicontinic acid (9.00), Vitamin B12 (0.01), D-biotin (0.14), Folic acid (0.90), Thiamin HCL (1.01), Pyridoxine HCL (3.29), myo-Inositol (270.00), α-cellulose (490.79)

[b] Mineral premix ($g\ kg^{-1}$):$CuSO_4.5H_2O$ (23.58), $FeSO_4.7H_2O$ (363.10), $MnSO_4.H_2O$ (61.51), Na_2SeO_4 (0.66), $ZnSO_4.7H_2O$ (131.94), KI (1.44), $CoSO_4.7H_2O$ (9.54), α-cellulose (408.23)

2.4. RESULTS

The mean initial weight for all treatments was 190.6 ± 4.1g. After 12 weeks, there were no significant differences (F=1.032; df=3,7, P=0.435) in growth performance among all treatments and the mean final weight was 561.4 ± 13.9g. Assessment of fish weight gain (g) showed no significant differences between treatments (F=0.363; df=3,7; P=0.782) and the overall mean was 370.8 ±12.6g. Specific growth rates (% d^{-1}) were not significantly different between treatments (F=0.070; df=3,7, P=0.974) with a mean value of 1.29 ±0.03 %·d^{-1}. Similarly feed efficiency ratio (F=0.224; df=3,7, P=0.877) with overall mean of 0.78 ± 0.02, daily feed intake rates (%·d^{-1}) (F=0.580; df=3,7, P=0.647) with overall mean of 1.52 ± 0.02 %·d^{-1}, and condition factor (F=3.271; df=3,7, P=0.089) with overall mean of 1.74 ± 0.01 again showed no significant differences between dietary treatments (Table 2.2).

The chemical composition of fish showed no significant differences in terms of dry matter (F=0.017; df=3,7, P=0.997), crude protein (F=0.237; df=3,7, P=0.868), crude fat (F=0.096; df=3,7, P=0.960), ash (F=0.748; df=3,7, P=0.557) and gross energy (F=0.169; df=3,7, P=0.914). The range of dry matter was 307.8 ± 1.79 to 311.1 ± 2.32 g kg^{-1} WW, crude protein was 120.7 ± 3.15 to 125.0 ± 1.70 g kg^{-1} WW, total lipid was 162.3 ± 1.67 to 168.4 ± 14.33 g kg^{-1} WW, ash was 18.4 ± 0.52 to 19.6 ± 0.60 g kg^{-1} WW and gross energy was 8.0 ± 7.70 to 8.5 ± 1.00 MJ kg^{-1} DM (Table 2.3).There was no significant difference in apparent digestibility of protein (F=4.093; df=3,7, P=0.057). The range of apparent digestibility of protein was 82.5 % to 86.2 % for all treatments (Table 2.4).

After 42 days plasma IGF-I levels were not significantly different between fish fed the four experimental diets (F=1.154; df=3,7, P=0.392). The range in plasma IGF-I concentration was 39.3 ± 6.17 ng mL^{-1} to 51.9 ± 6.34 ng mL^{-1}. Similarly after 84 days no significant differences in plasma IGF-I level between the diets (F=1.893; df=3,7, P=0.219) were observed with a range of 48.8 ± 3.29 ng mL^{-1} to 55.6 ± 1.57 ng mL^{-1} (Table 2.5).

No significant differences in plasma cortisol concentrations (F=0.257; df=3,7, P=0.854) were found between dietary treatments after 42 days. The range in plasma cortsiol concentrations was 3.4 ± 1.26 ng mL^{-1} to 5.6 ± 2.37 ng mL^{-1}. After 84 days the range of cortisol was 7.8 ± 3.66 ng mL^{-1} to 14.1 ± 1.18 ng mL^{-1} which was not significantly different between the dietary treatments (F=1.209; df=3,7, P=0.375) (Table 2.5).

Table 2.2 Growth response and feed utilization (mean ± standard error) of rainbow trout (*Oncorhynchus mykiss*) fed four different experimental diets.

	Diets				*One Way Anova*		
	PM00	PM20	PM30	PM40	*F*	*df*	*P*
Initial weight (g)	198.9 ±6.91	198.3 ±2.38	190.2 ±9.88	175.0 ±6.07	3.530	3,7	*0.077*
Final weight (g)	575.1 ±24.03	586.1 ±28.71	559.5 ±15.71	524.4 ±31.15	1.032	3,7	*0.435*
Weight gain (g)	376.2 ±23.94	387.9 ±28.21	369.3 ±5.83	349.4 ±33.53	0.363	3,7	*0.782*
SGR (% day^{-1})	1.3 ±0.06	1.3 ±0.06	1.3 ±0.03	1.3 ±0.09	0.070	3,7	*0.974*
FI (% day^{-1})	1.6 ±0.02	1.5 ±0.06	1.5 ±0.03	1.5 ±0.04	0.580	3,7	*0.647*
FER (g g^{-1})	0.8 ±0.03	0.8 ±0.07	0.8 ±0.00	0.8 ±0.06	0.224	3,7	*0.877*
K	1.8 ±0.01	1.8 ±0.02	1.7 ±0.02	1.7 ±0.03	3.271	3,7	*0.089*

Means with no superscripts were not significantly different ($p>0.05$) between dietary treatments.

PM (PM replacement in feeds of 0% (PM00%), 20% (PM20), 30% (PM30), PM40% (PM40%)

SGR (% day^{-1}) = Specific growth rate
FI (% day^{-1}) = Feed intake (FI)
FER (g g^{-1}) = Feed efficiency ratio
K = Condition factor

Table 2.3. Whole body chemical composition (mean ± standard error) of rainbow trout (*Oncorhynchus mykiss*) fed four different experimental diets (g kg^{-1} of wet weight).

	Diets				*One Way Anova*		
	PM00	PM20	PM30	PM40	*F*	*df*	*P*
Dry matter (g kg^{-1} WW)	311.0 ±3.84	311.1 ±2.32	307.8 ±1.79	308.9 ±2.54	0.017	3,7	*0.997*
Crude Protein (g kg^{-1} WW)	125.0 ±1.70	123.6 ±5.74	120.7 ±3.15	124.8 ±2.25	0.237	3,7	*0.868*
Total Lipid (g kg^{-1} WW)	162.3 ±1.67	168.4 ±14.33	166.6 ±3.08	162.4 ±11.10	0.096	3,7	*0.960*
Ash (g kg^{-1} WW)	18.9 ±0.45	19.2 ±0.58	18.4 ±0.52	19.6 ±0.60	0.748	3,7	*0. 557*
Gross energy (MJ kg^{-1} WW)	8.5 ±1.00	8.1 ±8.12	8.0 ±7.70	8.1 ±1.06	0.169	3,7	*0.914*

Means with similar or no superscripts were not significantly different (p>0.05) between diets.

PM (PM replacement in feeds of 0% (PM00%), 20% (PM20), 30% (PM30), PM40% (PM40%)

Table 2.4: Apparent digestibility of protein (mean ± standard error) of rainbow trout (*Oncorhynchus mykiss*) fed four different experimental diets

	Diets				*One Way Anova*		
	PM00	PM20	PM30	PM40	*F*	*df*	*P*
AD (%)							
Protein	86.2 ±0.73	82.8 ±1.07	82.5 ±0.82	82.5 ±0.86	4.093	3,7	0.057

Means with no superscripts were not significantly different (p>0.05) between diets.

PM (PM replacement in feeds of 0% (PM00%), 20% (PM20), 30% (PM30), PM40% (PM40%)

Table 2.5. IGF-I and cortisol (mean ± standard error) of rainbow trout (*Oncorhynchus mykiss*) fed four different experimental diets (ng mL^{-1}).

Hormones	Day	Diets				*One Way Anova*		
		PM00	PM20	PM30	PM40	*F*	*df*	*P*
IGF I ($ng\ ml^{-1}$)	42	51.9 ± 6.34	43.0 ± 1.89	45.1 ± 2.96	39.3 ± 6.17	1.154	3,7	*0.392*
	84	50.2 ± 1.57	55.6 ± 1.57	52.1 ± 0.52	48.8 ± 3.29	1.893	3,7	*0.219*
Cortisol ($ng\ ml^{-1}$)	42	7.4 ± 1.41	7.0 ± 1.01	7.0 ± 0.98	6.0 ± 0.00	0.375	3,7	*0.774*
	84	14.1 ± 1.18	13.6 ± 1.95	12.1 ± 3.90	7.8 ± 3.66	1.209	3,7	*0.375*

Means with no superscripts were not significantly different ($p>0.05$) between diets.

PM (PM replacement in feeds of 0% (PM00%), 20% (PM20), 30% (PM30), PM40% (PM40%)

2.5. DISCUSSION

This present trial we investigated replacement of FM protein with PM protein up to a level of 40% with results indicating that substitution did not affect the final weight, SGR, WG, DIR, FER and K of rainbow trout fed the test diets and as compared to the control diet. Some studies have reported that partial replacement of FM with alternative protein sources such as bacterial protein meal and plant protein did not affect the feed intake of the rainbow trout (Gomes et al., 1995; Aas et al., 2006). The same result was observed in the present study where feed intake was not affected, even at 40%, however, at higher inclusion levels it is possible that feed intake may start to be affected. A study conducted by Teskeredžić et al., (1995) indicated that growth rate, feed efficiency and protein and energy utilization of rainbow trout were affected when dried whole herring meal was totally replaced by rapeseed protein concentrate but did not significantly affect feed intake.

A study by Gomes et al., (1995) showed that the total replacement of FM with vegetable meal reduced the feed intake of fish. A study conducted by Carter and Davies (2004) showed that fish required certain time before being able to adapt to a new protein source. This is also related to palatability of fish, an aspect that affects feed intake and feed utilization (Glencross et al., 2007). In the present study, fish were fed at set ration of 2% of body weight. Fish were fed based on the fixed ration which was calculated every three weeks. The fish were growing and this caused a decrease in actual feed intake compared to set ration of 2%. The feed intake reduces as the fish increases in weight. An investigation of higher PM inclusion levels is therefore justified for future research.

Digestibility has a major influence on fish growth and differences among feed ingredient categories can be large (Storebakken et al., 1998). In the present study, the apparent digestibility (AD) of protein was evaluated. The AD protein is a very important parameter to determine the quality of PM compared with AD energy and AD lipid. This is due to the nutritional value of the commercial poultry by-product meal in fish feed depends on the quality and quantity of protein in the product (Dong et al., 1993). There was no difference in AD protein as would be predicted given the similarity in performance across the diets; the overall mean digestibility of around 84% was similar to generally found for good quality PM (Dong, et al.,1993) A study conducted by Bureau et al., (1999) showed there were no differences in AD protein when PM was included at 30%. The effect of PM replacement on AD protein would probably be different if the replacement level was higher than 40%.

Despite the large amount of published research on PM, effects of PM replacement on the hormones such as cortisol and IGF-I have not previously been investigated in rainbow trout. Insulin-like growth factors (IGFs) play a major role in fish development and metabolism (Company et al., 2001) and their measurement can be used as an indicator for the assessment of fish growth and performance (Shimizu et al., 2000). IGF-I has been related with fish growth and a study by Dyer et al., (2004b) showed that IGF-I is positively correlated with growth rates of Atlantic salmon. Additionally, the level of IGF-I was decreased in Chinook salmon (*Oncorhynchus tshawytscha*) during fasting (Pierce et al., 2005). Similar results were found where food deprivation resulted in lower IGF-I levels in juvenile coho salmon (*Oncorhynchus kisutch*) with affects on fish growth (Duan and Plisetskaya, 1993). In addition a recent study has shown that different levels of protein in the diets resulting in differences in circulating IGF-I levels in fish (Dyer et al., 2004a). The results of this study clearly showed that the use of PM up

to 40% did not affect circulating levels of IGF-I. Not surprisingly, there were no effects on fish growth observed among the treatments. It has previously been shown that it is possible to use IGF-I level in fish blood as tool to asses fish growth performance, however higher inclusion levels of PM should be tested to determine the potential effects on the IGF-I axis.

Cortisol, is the predominant corticosteroid in fish and is used as a stress indicator (Ellis et al., 2007). Previously, cortisol levels have been shown to increase when food deprivation occurred (Blom et al., 2000). In this trial, plasma cortisol levels were not affected by PM replacement. Although blood samples were only taken on day 42 and 84, and there can be no speculation on potential cortisol levels at other times throughout the experiment (i.e. soon after the transition to the PM diets) this result indicates that the diets with PM replacement were as good as the diet with FM as a sole protein source. Furthermore, plasma cortisol levels can provide insight into fish growth performance, where fish will grow faster when the level of cortisol is low compared with fish with higher levels of cortisol (Øverli et al., 2006). For example increasing plasma cortisol level will result in reduction of fish appetite and growth rates (Gregory and Wood, 1999). At the same time, fish with high cortisol levels will produce more feed waste and reduce overall feed efficiency (Øverli et al., 2006). Plasma cortisol not only will reduce the growth performance of fish, but also can also lead to adverse effects on feeding behaviour, physical condition and survival of the individual fish (Gregory and Wood, 1999). Cortisol level can also be affected by social hierarchy. It appeared that fish with low social status in a tank will have higher levels of plasma cortisol compared with dominant fish (Sloman *et al.*, 2001).

In the current study, replacement of FM with PM did not result in differences in plasma cortisol or IGF-I. This result is not surprising given the comparable growth of fish in this study. Overall, fish fed with diets containing PM to an inclusion of 40% grew as well as fish fed with FM with no detectable effect on plasma cortisol or IGF-I levels. It is clear that PM is a good protein source and can be used to replace up to 40% FM protein. Consequently, further study should be carried out to determine the maximum replacement of FM by PM. This would also reveal whether higher inclusion impacts on the IGF axis.

2.6. ACKNOWLEDGEMENTS

We would like to thank to Skretting Australia for ingredients, to Prof. Natalie Moltschaniwskyj for statistical advice, Dr Ashley Townsend CSL-UTAS for yttrium analysis and to the technical staff at the Aquaculture Centre, University of Tasmania for their assistance. Financial support was provided by Grant from Universiti Putra Malaysia (UPM), Malaysia.

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CHAPTER 3

Effects of poultry meal inclusion and adaptation time on apparent digestibility in rainbow trout (*Oncorhynchus mykiss* Walbaum)

3.1 ABSTRACT

This experiment aimed to investigate the effect of poultry meal inclusion and three adaptation times on apparent digestibility (AD) of crude protein, total lipid and gross energy in rainbow trout (*Oncorhynchus mykiss*). Twelve isoenergetic and isonitrogenous (22 MJ GE kg^{-1} DM, 400 g kg^{-1} DM of crude protein) experimental diets were formulated to contain graded levels of poultry meal protein(0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95 and 100%) to replace the fishmeal protein. A total of 360 rainbow trout with mean initial weight of 128.0 ± 0.78 g were randomly stocked into twelve 300 L tanks at 30 fish per tank. The tanks were supplied with freshwater at 15^{o}C under ambient photoperiod. The fish were hand-fed to satiation twice a day for 27 days and faeces were stripped from 10 fish per tank after 3, 9 and 27 days. There were significant negative linear relationships between PM inclusion and apparent digestibility measured as AD crude protein, AD total lipid and AD gross energy. There were no significant differences in AD crude protein or AD total lipid between the three adaptation times. However, AD gross energy increased over time. This was most probably explained by a change in carbohydrate digestibility and more research is needed to determine how fish adapt to this dietary component. Poultry meal contains little carbohydrate and the results from the present experiment suggest that rainbow trout do not require an adaptation period longer than 3 days for poultry meal when first included in a new feed.

3.2 INTRODUCTION

Finding good quality and economically sustainable alternative protein sources to replace fishmeal presents important challenges for aquafeed research (Watanabe, 2002; Hardy, 2010). To understand the effects of alternative protein sources on fish, information on ingredient digestibility is important because it has a major impact on nutrient intake (Austreng 1978; NRC 1993). Therefore, it is important to know the apparent digestibility (AD) of protein, lipid and energy in order to formulate high quality aquafeeds (Drew et al., 2007; Sales, 2010). Numerous studies have identified the potential of various protein sources of animal origin as replacements for fishmeal (FM) in aquafeeds (Sugiura et al., 1998; Lesiow et al., 2009). Animal protein sources, including meat meal, poultry feather meal, meat and bone meal, blood meal and poultry by-product meal all have high digestible protein content (NRC, 1993; Bureau et al., 2000; Carter et al., 2002). Poultry by-product meal is an example of a good quality alternative for FM that is currently used in aquafeeds (Erturk and Sevgili, 2003; Yanik et al., 2003). One important issue is that quality differs between poultry by-product meals (Dong et al., 1993), with this quality reflected in differences in digestibility (Cheng and Hardy, 2002).

AD can be measured either as a complete feed digestibility value (Austreng, 1978) or it can be calculated for an ingredient (Cho et al., 1982). Cho et al. (1982) proposed a method that combined 30% of the ingredient with a reference diet and marker to make a test feed. This approach has been adopted in many studies although the correct formula to calculate ingredient digestibility is required (Sugiura et al., 1998). This approach focuses on comparing AD values across a range of single ingredients; the reference diet and the ingredient are maintained at a similar proportion (3:7). It should be noted that if inclusion level is investigated using a ratio

between ingredient and reference feed then two variables are being tested simultaneously (inclusion level of the ingredient and the reference diet) and interpretation becomes relatively complex. An alternative approach is to measure digestibility in complete and nutritionally balanced diets, which allows a more practical assessment of factors such as inclusion level to be assessed, albeit indirectly. In this case, differences between diets would be explained by the variation among the digestible nutrients of the alternative ingredients and a regression analysis would reveal a systematic trend in these differences (Bureau, et al., 2000).

In addition, although an alternative feed ingredient may have different palatability compared to FM based diets fish can adapt to alternative ingredients if given enough time (Refstie et al., 1997; Carter and Davies, 2004). Therefore the length of the experiment may influence feed intake in fish (Refstie, et al., 1997; Wybourne and Carter, 1998). Feed intake is determined by the palatability of the pellets which is defined as the combination of both attractiveness and ingestion of the diets (Glencross et al., 2007). Thus, increasing the experimental period will allow fish to adapt to new ingredients and this will allow fish to reach maximum feed intake and to maximise the availability of nutrients and energy from the feed (Carter and Davies, 2004; Glencross et al., 2007). Austreng (1978) suggested fish should be fed for 7 days before sampling and this is to allow the marker to be present throughout the fish intestine. In the present experiment, sampling was performed on day 3, 9 and 27. This is to determine the effects on AD nutrients if sampling is performed before and after the supposed adaptation time.

In the present study, the effect of different inclusion levels of PM in the diet on rainbow trout (*Oncorhynchus mykiss* Walbaum) was evaluated. The AD of crude protein, total lipid and gross energy was measured after 3, 9 and 27 days in order to determine the effects of adaptation time. This aimed to demonstrate whether rainbow trout adapt to different levels of PM and the time required for this adaptation to occur.

3.3. MATERIALS AND METHODS

3.3.1. Experimental Diet

Twelve isoenergetic and isonitrogenous (22 MJ GE kg^{-1} DM, 400 g kg^{-1} DM of crude protein) experimental diets were formulated to replace FM protein using graded levels of poultry meal (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95 and 100%, Table 3.1) . Ingredients were mixed using a Hobart mixer and cold pelleted into 3 mm pellets with a laboratory pelletizer (California Pellet Mill Co., San Francisco, CA, USA) and oven dried for 24 h at 30^{o}C. Diets were kept in cold storage at 4^{o}C. FM, poultry meal and fish oil were supplied by Skretting (Cambridge, Tasmania, Australia). Vitamins, minerals, carboxymethyl cellulose, bentonite, calcium phosphate, α-cellulose and ytterbium oxide were supplied by Sigma-Aldrich Pty. Ltd (Sydney, Australia). Vitamin C was supplied as Stay-C by Roche Vitamins Australia Ltd. (Sydney, Australia) choline chloride was supplied by ICN Biomedicals, INC. (Aurora, Ohio, USA).

3.3.2. Digestibility Experiment

The experiment was conducted according to Ethics Approval A0009762 at the University of Tasmania (Launceston, Tasmania, Australia) in a freshwater recirculation system. The system contained twelve 300 L fibreglass tanks and was fitted with a submerged biofilter and UV sterilizer (Bransden et al., 2001). In the present study, each tank represents a treatment. There were no replications for each treatment. A total of 360 rainbow trout (supplied by the University of Tasmania) with mean initial weight 128.0 ± 0.78 g were randomly stocked into the twelve (30 fish·$tank^{-1}$) and maintained for a 1 week acclimation period. Throughout the acclimation period, fish were fed with 3 mm Spectra sinking pellet (Skretting, Cambridge, Tasmania) twice daily to satiation. Water quality was monitored daily to ensure it was within the limits for rainbow trout

(Willoughby, 1999) and maintained with a 10% daily water replacement. Throughout acclimation and during the experiment, water temperature was kept at 15^{o}C under ambient photoperiod.

The fish were not fed for three days prior to the beginning of the experiment. Tanks were randomly allocated to the twelve dietary treatments. The fish in all treatments were hand-fed to satiation at 0900 and 1600h for 27 days. The fish faeces were collected from 10 fish per tank on days 3, 9 and 27 by stripping (Austreng, 1978; Percival et al., 2001). During faecal collection, all fish were anaesthetized (AQUI-S, 30 mg·L^{-1}) and the tanks were sampled in random order on each sample occasion. The 10 randomly selected fish were stripped only once and then removed from the experiment. Faecal samples for 10 fish were pooled in each sampling. Faecal samples were stored at -20^{o}C prior to analysis. The diets included ytterbium oxide (1 g kg^{-1}) as a digestibility marker.AD of nutrients was calculated as described by Maynard and Loosli (1969) using the following formula:

$$AD_{diet} = 1 - (Marker_{diet} * Nurient_{faeces}/Marker_{faeces} * Nutrient_{diet})$$

Where, Marker is the % of ytterbium oxide in the diets and faeces; and Nutrient is the % of protein, lipid and energy in the diets and faeces. The value were ranged between 0 to 1, and to convert to percentage, the equation was multiplied by 100.

3.3.3. Chemical analysis

The chemical analysis of fish faeces was performed using standard methods. Total lipid was determined by Bligh and Dyer (1959), total nitrogen was determined by Kjeldhal (crude

protein content was estimated as Nitrogen ((N) X 6.25) and gross energy was determined by an auto bomb calorimeter. Ytterbium was determined according to Ward et al., (2005).

3.3.4. Statistical analysis

The data were analysed using linear regression analysis; $y = ax + b$, where y is the AD of crude protein, total lipid or gross energy (%) and x is the PM inclusion (%) (Shearer, 1994). Linear regression analysis is part of statistics that studies the relationship between two variables and develops linear probabilistic model (Johnson and Bhattacharyya, 2006). Analysis of covariance (ANCOVA) was used to determine the differences between the regression lines from the 3 sampling days, with PM inclusion as the covariate. The analysis was performed with SPSS Statistical Analysis Software Program (version 17.0 for Windows).

Table 3.1. Ingredient and chemical composition of experimental diets (mean ±standard error).

	Diets											
	PM00	PM10	PM20	PM30	PM40	PM50	PM60	PM70	PM80	PM90	PM95	PM100
Ingredient composition (g kg^{-1})												
Fishmeal	564.0	507.0	450.7	394.4	338.1	285.2	225.4	169.1	112.6	56.3	28.1	00.0
Poultry meal	00.0	61.0	121.9	182.5	243.1	300.0	364.3	424.9	485.6	546.2	576.5	606.7
Pre-gelatinized starch	155.0	153.0	150.1	148.0	146.3	144.1	141.8	139.0	137.0	134.9	133.6	132.3
Fish oil	149.0	145.6	142.3	138.8	135.1	131.7	128.3	125.1	121.6	118.1	116.5	114.9
Carboxyl methyl cellulose (CMC)	10	10	10	10	10	10	10	10	10	10	10	10
Alpha cellulose	68.1	69.5	71.1	72.4	73.5	75.1	76.3	78.0	79.3	80.6	81.4	82.2
Bentonite	33.2	33.2	33.2	33.2	33.2	33.2	33.2	33.2	33.2	33.2	33.2	33.2
Choline chloride	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Vitamin C	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Vitamin Premix[a]	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Mineral Premix[b]	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Ytterbium oxide	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Calcium phosphate	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Chemical composition (g kg^{-1} DM)	PM00	PM10	PM20	PM30	PM40	PM50	PM60	PM70	PM80	PM90	PM95	PM100
Dry matter	911	905	909	909	912	910	908	911	907	907	907.8	906
	±1.0	±0.1	±0.0	±0.4	±2.3	±0.8	±0.0	±2.6	±4.5	±3.6	±0.1	±0.0
Crude protein	403	396	397	396	397	396	401	404	408	401	400	399
	±4.3	±1.0	±0.8	±0.5	±03.8	±2.6	±1.7	±2.9	±0.5	±4.1	±0.2	±0.4
Total lipid	230	228	229	226	220	225	218	220	222	219	215	226
	±0.9	±2.8	±3.1	±0.5	±3.6	±3.6	±7.1	±1.7	±1.0	±3.7	±0.2	±6.4
Ash	115	118	118	117	117	113	116	118	112	112	107	106
	±3.9	±0.0	±0.1	±0.3	±0.5	±0.7	±2.5	±1.7	±5.0	±3.7	±02.0	±0.0
Gross energy (MJ kg^{-1})	22.3	22.3	22.4	22.3	22.3	22.4	22.1	22.2	22.3	22.2	22.3	22.5
	±0.13	±0.07	±0.07	±0.02	±0.05	±0.09	±0.06	±0.02	±0.06	±0.28	±0.09	±0.03

Means with similar or no superscripts were not significantly different ($p>0.05$) between diets.

[a] Vitamin premix ($g \cdot kg^{-1}$): Vitamin A (4.50), Vitamin D3 (5.40), Rovimix E50 (90.00), Menadione sodium bisulphate (1.80), Riboflavin (3.60), Calcium D-pantothenate (19.57), Nicontinic acid (9.00), Vitamin B12 (0.01), D-biotin (0.14), Folic acid (0.90), Thiamin HCL (1.01), Pyridoxine HCL (3.29), myo-Inositol (270.00), α-cellulose (490.79)

[b] Mineral premix ($g\ kg^{-1}$):$CuSO_4.5H_2O$ (23.58), $FeSO_4.7H_2O$ (363.10), $MnSO_4.H_2O$ (61.51), Na_2SeO_4 (0.66), $ZnSO_4.7H_2O$ (131.94), KI (1.44), $CoSO_4.7H_2O$ (9.54), α-cellulose (408.23)

Poultry meal (PM) accounted for between zero (PM00) and 100 (PM100) percent of the dietary protein

3.4. RESULTS

This analysis was designed to determine the effects of digestibility on different days. The graphs were plotted to show the patterns of digestibility on different days of sampling. There was a significant negative linear relationship between PM inclusion and AD crude protein at all three adaptation times (Figure 3.1). The AD crude protein after 3, 9, and 27 days can be determined from the equations: AD_{3days} (%)= -0.070 PM_{Inc}(%) + 84.629 (r^2=0.578; p=0.000; n=12); AD_{9days} (%) = -0.065 PM_{Inc}(%) + 83.597 (r^2=0.364; p=0.004; n=12); AD_{27days} (%) = -0.067 PM_{Inc}(%) + 88.246 (r^2=0.541; p=0.000; n=12), respectively. AD of crude protein decreased at a rate of 0.070, 0.065 and 0.067% per 1% increase in PM inclusion after 3, 9 and 27 days, respectively. AD crude protein at 0% inclusion level of PM was 7.04, 6.46 and 6.67% higher than at 100% replacement of FM after 3, 9 and 27, respectively (Figure 3.1). The intercept and PM inclusion were significantly affected by the day of sampling however, the day of sampling and interaction between day and PM inclusion were not affected (Table 3.2A).

There was a significant negative linear relationship between PM inclusion and AD total lipid (Figure 3.2). The AD total lipid after 3, 9 and 27 days can be calculated from the following equations: AD_{3days} (%) = -0.099PM_{Inc}(%) + 84.888 (r^2=0.616; p=0.000; n=12), AD_{9days} (%) = -0.146 PM_{Inc}(%) + 84.308 (r^2=0.455; p=0.001; n=12) and AD_{27days} (%) = -0.066 PM_{Inc}(%) + 90.567 (r^2=0.456; p=0.001; n=12), respectively. AD of total lipid decreased at rates of 0.10, 0.15 and 0.07 % per 1% increase in PM inclusion after 3, 9 and 27 days, respectively. AD total lipid at 0% inclusion level of PM was 9.40, 14.64 and 6.58% higher than at 100% replacement of FM after 3, 9 and 27, respectively (Figure 3.2). Furthermore, the intercepts and PM inclusion were

highly influenced by the day of sampling, however, the day of sampling and interaction between day and PM inclusion were not influenced by the day of sampling (Table 3.2B).

There was a significant negative linear relationship between PM inclusion and AD gross energy (Figure 3.3). The AD gross energy after 3, 9 and 27 days can be determined from the following equations: $AD_{3\ days}$ (%) = -0.065 PM_{Inc}(%) + 86.922 (r^2=0.626; p=0.000; n=12), AD_{9days} (%) = -0.084 PM_{Inc}(%) + 86.229 (r^2=0.507; p=0.000; n=12) and AD_{27days} (%) = -0.053 PM_{Inc}(%) + 90.919 (r^2=0.569; p=0.000; n=12), respectively. AD gross energy decreased at a rate of 0.07, 0.08 and 0.05% per 1% increase in PM inclusion after 3, 9 and 27 days, respectively. AD gross energy at 0% inclusion level of PM was 6.54, 8.41 and 5.31% higher than at 100% replacement of FM after 3, 9 and 27, respectively (Figure 3.3). In addition, AD gross energy showed significant differences between intercepts, day of sampling and PM inclusion, but no differences on interaction between day and PM inclusion (Table 3.2C).

Table 3.2. Results of ANCOVA that were performed to compare the lines between poultry meal (PM) inclusion and apparent digestibility (AD) of (A) protein, (B) total lipid and (C) gross energy in rainbow trout (*Oncorhynchus mykiss*), respectively.

A.

ANCOVA - Protein	DF	SS	MS	*F*	*P*
Intercept	1	71176.34	71176.34	11305.45	0.00
Day	2	38.70	19.35	3.07	0.06
PM Inclusion	1	174.25	174.25	27.68	0.00
Day and PM Inclusion	2	0.22	0.11	0.02	0.98
Error	30	188.87	6.30		

B.

ANCOVA – Total Lipid	DF	SS	MS	*F*	*P*
Intercept	1	73013.95	73013.95	4683.67	0.00
Day	2	77.66	38.83	2.49	0.10
PM Inclusion	1	401.73	401.73	25.77	0.00
Day and PM Inclusion	2	43.07	21.54	1.38	0.27
Error	30	467.67	15.59		

C.

ANCOVA-Gross Energy	DF	SS	MS	*F*	*P*
Intercept	1	75455.88	75455.88	15204.42	0.00
Day	2	41.61	20.80	4.19	0.03
PM Inclusion	1	175.96	175.96	35.46	0.00
Day and PM Inclusion	2	6.26	3.13	0.63	0.54
Error	30	148.88	4.96		

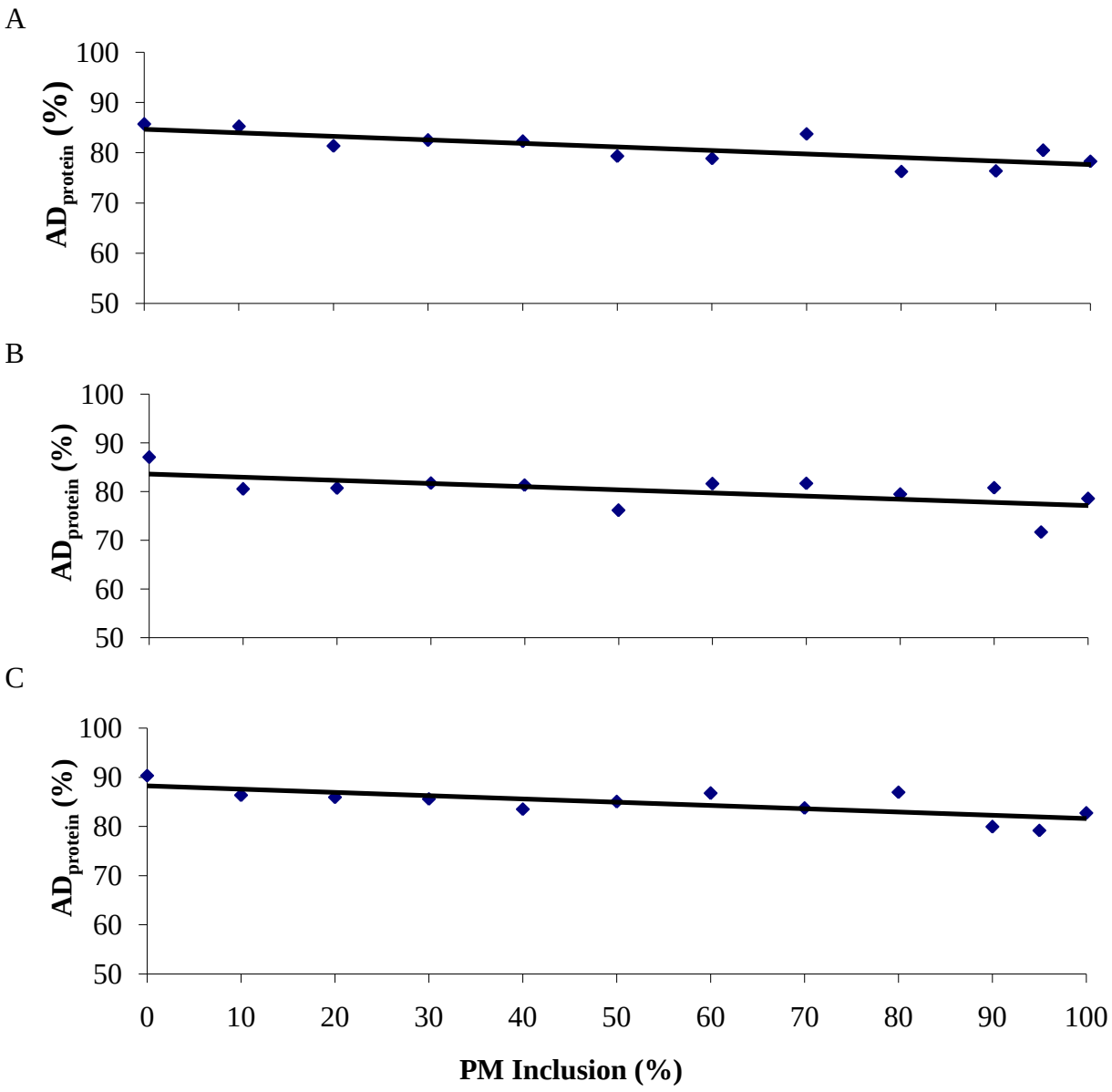

Figure 3.1.The relationship between poultry meal (PM) inclusion and apparent digestibility (AD) of protein in rainbow trout (*Oncorhynchus mykiss*) after 3 (A), 9 (B) and 27 days (C). The relationship between PM inclusion (%) and AD (%) was described by AD_{3days} (%)= -0.070 PM_{Inc}(%) + 84.629 (r^2=0.578; p=0.000; n=12); AD_{9days} (%) = -0.065 PM_{Inc}(%) + 83.597 (r^2=0.364; p=0.004; n=12); AD_{27days} (%) = -0.067 PM_{Inc}(%) + 88.246 (r^2=0.541; p=0.000; n=12), respectively.

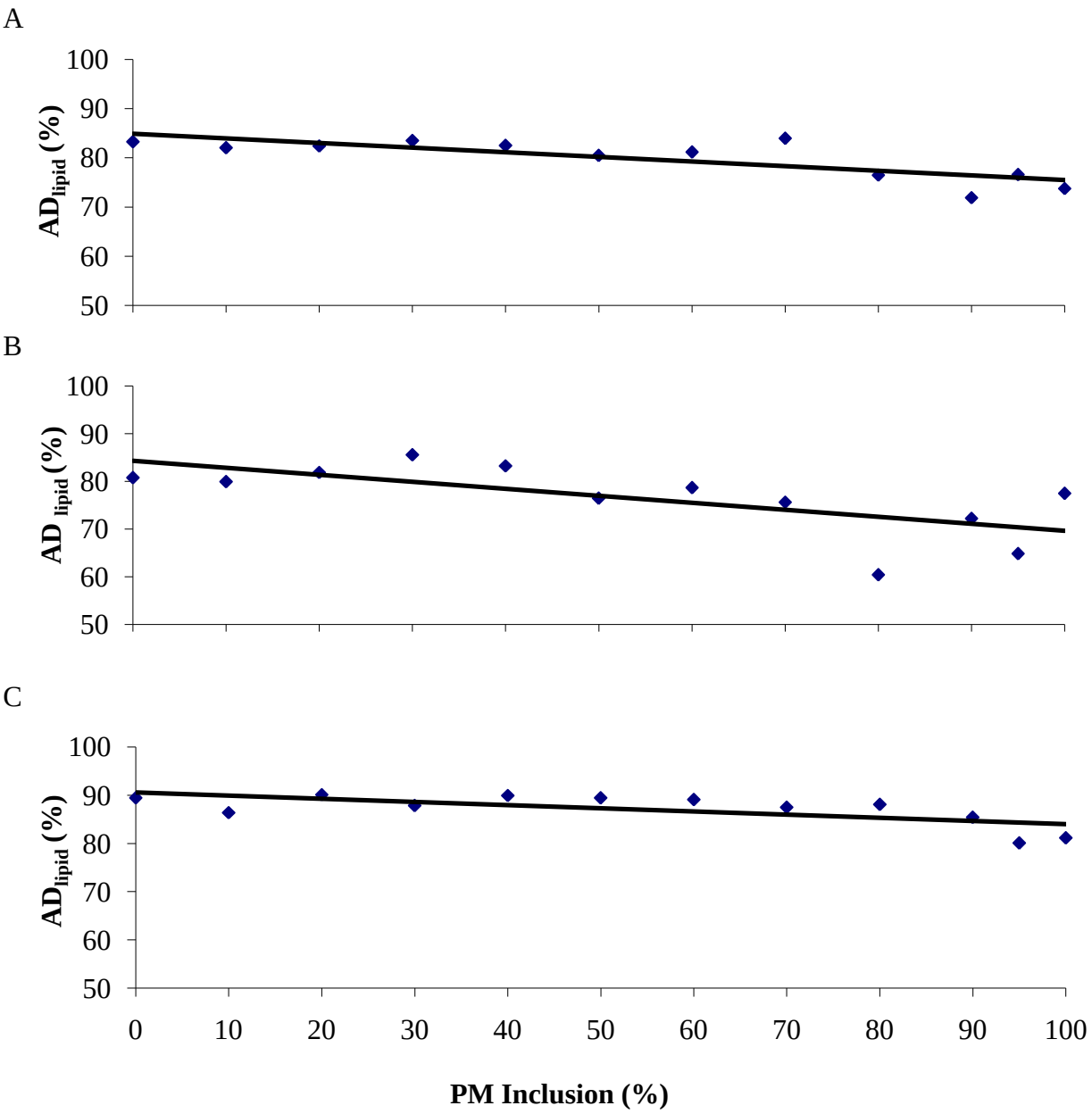

Figure 3.2.The relationship between poultry meal (PM) inclusion and apparent digestibility (AD) of total lipid in rainbow trout (*Oncorhynchus mykiss*) after 3 (A), 9 (B) and 27 days (C). The relationship between PM inclusion (%) and AD (%) was described by AD_{3days} (%) = -0.099PM_{Inc}(%) + 84.888 (r^2=0.616; p=0.000; n=12), AD_{9days} (%) = -0.146 PM_{Inc}(%) + 84.308 (r^2=0.455; p=0.001; n=12) and AD_{27days} (%) = -0.066 PM_{Inc}(%) + 90.567 (r^2=0.456; p=0.001; n=12), respectively.

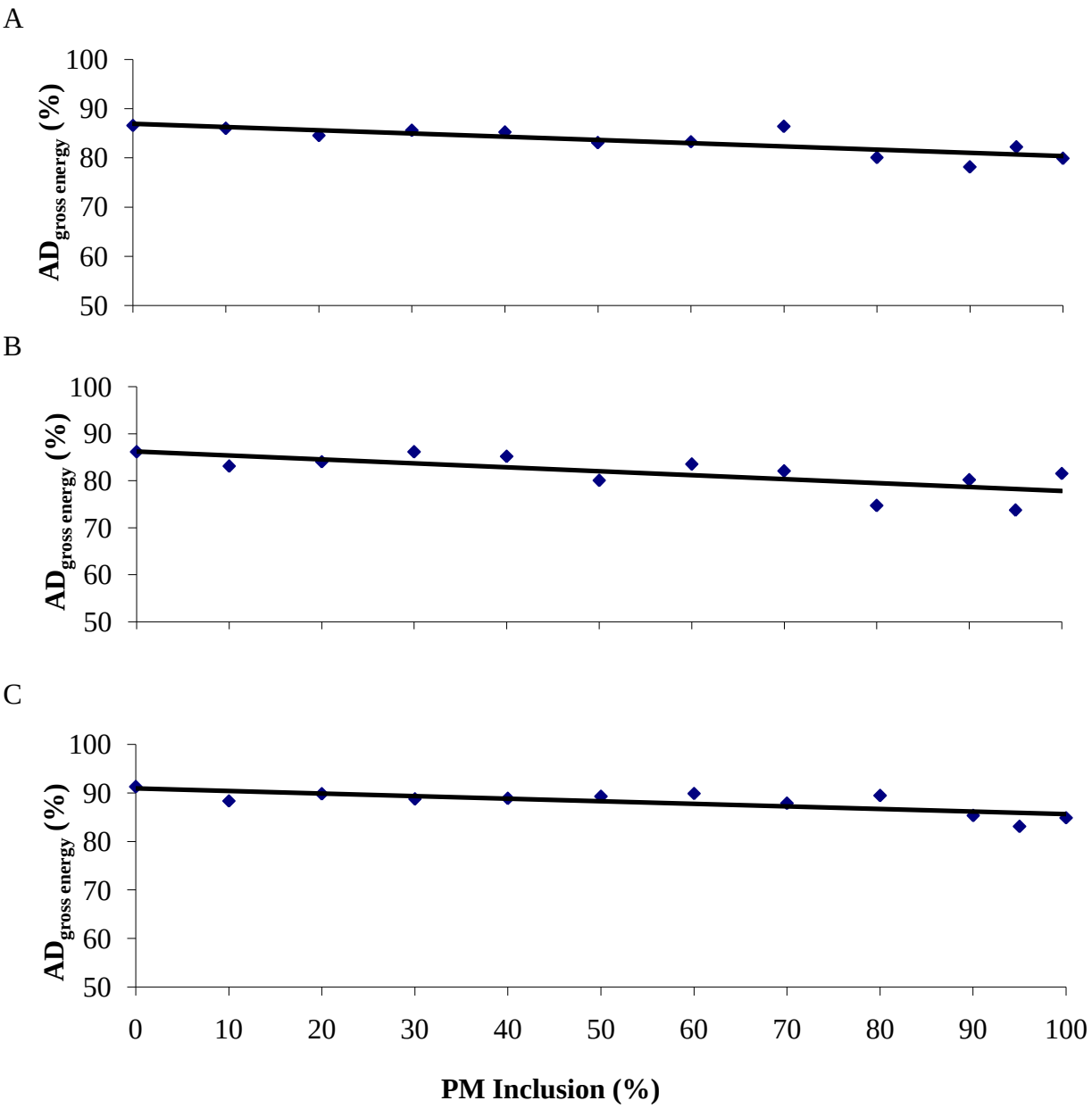

Figure 3.3. The relationship between poultry meal (PM) inclusion and apparent digestibility (AD) of gross energy in rainbow trout (*Oncorhynchus mykiss*) after 3 days (A), 9 days (B) and 27 days (C). The relationship between PM inclusion (%) and AD (%) was described by $AD_{3\ days}$ (%) = -0.065 PM_{Inc}(%) + 86.922 (r^2=0.626; p=0.000; n=12), AD_{9days} (%) = -0.084 PM_{Inc}(%) + 86.229 (r^2=0.507; p=0.000; n=12) and AD_{27days} (%) = -0.053 PM_{Inc}(%) + 90.919 (r^2=0.569; p=0.000; n=12), respectively.

3.5. DISCUSSION

The present study showed that AD for protein, lipid and gross energy decreased as poultry meal (PM) inclusion increased. The relationships were similar for all three nutrients and the diet with 100% FM had the highest AD for protein, lipid and gross energy compared to the 100% PM diet after each of the 3 adaptation periods. Numerous studies have shown that various alternative protein sources can partially replace FM in aquafeeds (Glencross et al., 2004; Farhangi and Carter, 2007; Brinker and Reiter, 2011). The complete replacement of FM with rendered animal protein fed to rainbow trout resulted in significant reductions in AD of protein, lipid and gross energy (Bureau et al., 1999) which concurred with our findings in rainbow trout. This is most probably explained by the replacement ingredient having a lower digestibility than FM. FM is a good protein source in terms of high nutrient digestibility (Gatlin et al., 2007; Miles and Jacob, 2011). However, it should be noted that some processing techniques can negatively affect the AD of nutrients when PM is included in diets (Dong et al., 1993; Bureau et al., 1999). This manufacturing practice of PM involves both physical and chemical transformations using a variety of equipment and processes where the temperature and length of time of the cooking process are critical in determining the quality of the final product with cooking generally accomplished with steam at 115 to 145^{o}C for 40 to 90 minutes depends on the types of system and materials (Meeker and Hamilton, 2006).

In the present study, AD protein gradually decreased with increasing level of PM inclusion in the diet. In general, PM has a lower level of protein compared to FM and the gradual replacement of FM with PM will increase the amount of PM in the diets. In the present study, all diets were balanced in protein content. This indicated that the only difference was the ratio between FM and

PM since the levels of protein were same in all diets. This showed that the source of protein influenced AD protein in rainbow trout. Dong, et al. (1993) reported the protein levels in PM obtained from 6 different batches ranged from 55.5 to 73.8%. A study conducted by Bureau, et al. (1999) indicated the protein level in PM was 64.7 and 69.2% which are within the ranged reported by Dong, et al.(1993). The low percentage of AD protein in the fish fed diet with PM is also related to the essential amino acid (EAA) composition as previously described by Cheng and Hardy(2002). According to Cowey (1994) the most important factor in determining the efficiency of protein utilization of a protein is the EAA profile. Cheng and Hardy (2002) reported significant differences in the digestibility of essential amino acids except for tryptophan and phenylalanine between FM and PM when fed to rainbow trout. However, in the present study, there was no analysis on digestibility of EAA. However, there are some reports on the effects of EAA digestibility on fish growth (Cheng and Hardy., 2002; Chowdhury et al., 2012). A study conducted by Chowdhury et al., (2012) indicated the differences in digestibility of arginine when rainbow trout were fed with a feed consisting of 30% of Indian mustard protein concentrate. A study conducted by Cheng et al., (2003) found differences in lysine and protein digestibility in the same diet, when L-lysine was supplied in the diet of rainbow trout that consists of herring and soybean meals as protein sources.

As reported in other studies AD total lipid was higher in fish fed with a diet with 100% FM protein compared with PM (Bureau et al., 1999). In the study conducted by Bureau et al.,(1999), AD lipid was 78% for a poultry by-product meal diet compared to 88% for the FM diet when fed to rainbow trout. Bureau, et al.(1999) also reported the percentages lipid in two PM batches were 11.4 and 13.6%. In the present study, the lipid composition in all diets was

balanced; this meant the presence of fat in the PM reduced the amount of fish oil added to the diets as PM protein proportion increased. Poultry fat is extremely low in omega-3 fatty acids compared with fish oil and a report from NRC (1993) showed the percentage of omega-3 in poultry fat is only 1% compared with fish oil which is 31.2%. Liu et al. (2004) reported that poultry fat had a 28.66% saturated FA. Thus, fat from PM used in the present study also has low percentage of unsaturated fatty acids (FA) (NRC, 1993). Therefore the gradual replacement of FM by PM will result in an increase of saturated FA and decrease in unsaturated fatty acids. Thus, in the present study, the decrease in total lipid digestibility is related with the composition of FA in the diets due to a low digestibility of saturated FA (Austreng et al., 1980). In addition, Glencross (2009) reported that a lack of some of essential fatty acids will reduce the ability of fish to digest and absorb lipids. There are clear associations between effects on essential fatty acids (EFA) and digestibility of total lipid. Therefore, when formulating diets containing alternative protein ingredients, thought should also be given to the FA profile of that raw material as it may influence the digestibility of total lipid when fed to the fish.

A similar pattern of results was found in AD gross energy with decreasing values observed as PM inclusion is increased. Hajen et al., (1993) reported a lower AD gross energy (72.4%) when poultry by-product meal was fed to chinook salmon (*Oncorhynchus tshawytscha*) and this value was greatly different from the AD gross energy in a herring meal diet (92.6%) Another study conducted by Bureau, et al. (1999) demonstrated a low AD gross energy (77%) when poultry by-product meal was fed to rainbow trout. In the present study, the changes in AD protein and total lipid affected the AD energy because the energy is derived from protein and lipid. Another factor may influence AD energy is AD of carbohydrate. In the present study, the

main sources of cabohydrate are from carboxylmethylcellulose (CMC) and pre-gelatinized starch. CMC is indigestible and changes with diet but will contribute to gross energy of the diets and starch is partly digested but digestibility can vary with inclusion level. All these factors may contribute to the AD energy.

There were no significant differences in AD crude protein or AD total lipid between the three adaptation times. This showed that after 3 days no further adaptation time is required for rainbow trout to process poultry meal. This is most probably due to a favorable palatability and feed intake throughout the experimental period. Decreased feed intake associated with poor palatability will in turn affect the nutrient digestibility in fish (Glencross et al., 2007). In the present study, fish were fed until satiation and feed intake was not recorded. Therefore this discussion about feed intake can only be based on observation during the experimental period. Although feed intake was not quantified, fish readily accepted the feed containing high inclusion of PM. Although the effect of feed intake is investigated further in the next experiment (Chapter 5) future work should be carried out to specifically determine the relationship between feed intake and adaptation to the new diet. The positive results on AD of protein and lipid may also have been influenced by the favorable quality of poultry meal used in the diets (Cheng and Hardy, 2002). Interestingly AD gross energy increased over time. This was likely to have resulted from changes in carbohydrate digestibility from pre-gelatinized starch since poultry meal contains little carbohydrate. Based on the diet formulation in the present study, the level of pre-gelatinized starch decreased with increasing inclusion level of PM. A study conducted by Kim and Kaushik (1992) indicated an increase in dietary carbohydrate will decrease the AD of

energy. This is in agreement with a study conducted by Storebakken et al. (1998) where the AD of gross energy was affected by increasing levels of dietary carbohydrate in the diets .

In conclusion, nutrient AD decreases linearly with increasing PM inclusion. Furthermore, between 3 and 29 days there was no evidence for improved digestibility and hence adaptation to the new ingredient. We showed that nutrient AD in rainbow trout fed either PM, FM or gradual mixture of both protein sources will not change significantly with time. It is still unknown how different levels of FM replacement with PM can affect the histology of digestive system in rainbow trout which may assist in understanding the limitations of dietary protein replacement in aquafeeds.

3.6. ACKNOWLEDGEMENTS

We would like to thank to Skretting Australia for ingredients, to Dr Ashley Townsend CSL for analysis of ytterbium and to the technical staff at the Aquaculture Centre, University of Tasmania for their assistance. Financial support was provided by Grant from Universiti Putra Malaysia (UPM), Malaysia.

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CHAPTER 4

Effects of fishmeal replacement with poultry meal on rainbow trout (*Oncorhynchus mykiss* Walbaum) growth performance at optimal and high temperatures

4.1 ABSTRACT

This experiment aimed to investigate the effect of fishmeal (FM) replacement with poultry meal (PM) in diets for rainbow trout (*Oncorhynchus mykiss*Walbaum) cultured at optimal and elevated temperatures. Twelve isoenergetic and isonitrogenous (22 MJ GE kg^{-1} DM, 400 $g \cdot kg^{-1}$ DM crude protein) diets were formulated to contain graded levels of PM protein; PM00, PM10, PM20, PM30, PM40, PM50, PM60, PM70, PM80, PM90, PM95 and PM100% to replace the FM protein. A total of 528 rainbow trout (28.77 ± 0.10 g) were randomly allocated to 24 experimental tanks (22 fish per tank). Twelve tanks were held at a temperature of 15.4 ± 0.01^{o}C while the other twelve tanks were held at 20.2 ± 0.01^{o}C. Fish in all treatments were fed twice a day with 2% of body weight for 112 days. This set ration was above the actual amount eaten by fish. Intermittently, fish were fed based on the fixed ration which was calculated every three weeks. Feed intake decreased as the fish grew. The growth and growth efficiency data were analysed using a polynomial regression analysis or a break point analysis and all results indicated that PM can be used to replaced FM at high level without any adverse effects on fish. The histology of the distal intestine showed an increase in the number of goblet cells (GC) and a decrease in the width of supranuclear vacuoles (SNV) in the fish fed diets containing greater than 90% of poultry meal protein. Overall, these results indicate that the maximum inclusion was slightly influenced by temperature. The range of performance indicates poultry meal could be included at up to 89 % and 83% at 15 and 20^{o}C.Thus, poultry meal has an excellent potential as an alternative protein source in feeds for rainbow trout grown at both optimal and elevated temperatures.

4.2 INTRODUCTION

In recent years, water temperature has become a growing concern in aquaculture, especially in a context of global warming (Alborali, 2006). In Tasmania, summer water temperatures in the main salmonid growing areas can increase to 22^{o}C (Battaglene et al., 2008). CSIRO and the Bureau of Meteorology (BoM) (2007) reported that by 2030, the sea surface temperature will increase 0.6-0.9^{o}C in the southern Tasman Sea and off the north-west shelf of Western Australia and 0.3 to 0.6^{o}C elsewhere. These water temperatures have many implications for the aquaculture of salmonids, likely impacts include effects on biological processes such as reproduction, growth, metabolism and disease resistance (Wedemeyer, 1996, Barnes et al., 2011; Pankhurst et al., 2011).

Most of fish are ectothermic and do not have the ability to control their body temperature, consequently water temperature has important influences on fish physiology (Brett and Groves, 1979). Each species of fish has a range of temperature over which it survives, the thermal tolerance range, and a narrower range where growth occurs (Brett and Groves, 1979; Katersky and Carter, 2007). Fish growth increases within the thermal tolerance range and then decreases after the optimal temperature or prior to the upper thermal limit (Jobling, 1994). The thermal tolerance for rainbow trout (*Oncorhynchus mykiss)* is between approximately 0.0 and 29.8^{o}C with an optimal temperature range between 12 and 18°C (Currie et al., 1998). The optimum temperature for rainbow trout is 15^{o}C (Coghlan and Ringler, 2005; McMahon et al., 2008). A study conducted by Ng et al., (2010 and unpublished) indicated that 20^{o}C was a sub-optimum elevated water temperature for rainbow trout because growth efficiency was reduced compared to at 15^{o}C. The optimal temperature range is the point where the growth of fish is maximized

(Jobling, 1994). Beyond the optimal temperature range is considered as a high temperature and at this stage, feed intake will keep increasing as more food is required to support increased metabolic demand (Jobling, 1994). However, after a certain point feed intake will decrease due to the limitations of the respiratory and circulatory system to deliver oxygen to the tissues (Cocking, 1959). In addition, when held under high temperatures, post-prandial metabolic increase contributes more stress to fish further resulting in a reduction in the ability of fish to feed (Katersky and Carter, 2007). A study conducted by Bear et al., (2005) indicated the decreasing in relative growth rate of rainbow trout culture at 20^{o}C compared to rainbow trout culture below 20^{o}C. Therefore, an increase in water temperature will contribute to changes in feed intake, affect physiology and directly impact the growth of fish (Koskela et al., 1997; Guderley, 2004).

Temperature also has indirect effects on growth through changes in the endocrine system. The neuroendocrine stress response is one important factor which is very sensitive to changes in temperature (Pickering and Pottinger, 1987). Exposure to temperature above the optimal range has been found to affect the level of cortisol in fish (Sundh et al., 2010). When an environmental temperature is elevated above the normal range fish will exhibit abnormal behavior and physiological indices of stress such as plasma cortisol level are elevated (Ellis, 1981; Wendelaar Bonga, 1997; Barton, 2002). Elevation of cortisol is a characteristic response of fish to stress (Barton, 2002). Cortisol is the principal corticosteroid in teleosts and it is released from the interrenal tissue, analogous to the adrenal cortex and distributed in the head kidney region (Aluru and Vijayan, 2009; Sandhu and Vijayan, 2011). Cortisol is involved in many biological actions in fishes (Barton, 2002). The hypothalamic portion of the brain stimulates the release of

adrenocorticotropic hormone (ACTH) which circulates to the anterior kidney; where it stimulates the interregnal cells to produce cortisol and other corticosteroid hormones when fish are exposed to stressful situations (Wedemeyer, 1996). Cortisol production will lead to the changes in features (or secondary responses) related to metabolism, hydromineral balance, cardiovascular, respiratory and immune function (Barton, 2002). Tertiary responses to stress include reduced fish growth, reduced feed intake, impaired resistance to infectious diseases, changes in behavior, adverse effects on the reproductive system, reductions in flesh quality and can lead to high mortality in fish (Wedemeyer, 1996; Pickering, 1998; Barton, 2002).

Insulin-like growth hormone-I (IGF-I) is a mitogenic hormone produced mainly in the liver which is directly involved in the regulation of protein, lipid, carbohydrate and mineral metabolism and can be influenced by stress and increasing cortisol levels in fish (Moriyama et al., 2000). This was demonstrated in a study conducted by Peterson and Small (2005) using exogenous cortisol where fish were fed with feed mixed with exogenous cortisol with final results indicating a decreased level of IGF-I in channel catfish (*Ictalurus punctatus*). A reduction of IGF-I level was also detected in tilapia (*Oreochromis mossambicus*) when cortisol was injected intraperitoneally (Kajimura et al., 2003). This indicates that cortisol is involved in the regulation of IGF-I (Davis and Peterson, 2006) and environmental or husbandry related factors which result in the stimulation of a stress response are also likely to influence plasma and tissue levels of IGF-I.

Another study conducted by Moriyama et al. (1997) indicated that fish growth is positively correlated with plasma IGF-I levels. At the same time, the level of IGF-I is also

closely related to water temperature (Gabillard et al., 2003). A study conducted by Beckman et al. (1998) on chinook salmon (*Oncorhynchus tshawytscha*) showed that fish raised in warm water had higher growth and plasma IGF-I level compared to fish raised in cold water. Similarly, research conducted by Larsen et al. (2001) showed that the plasma level of IGF-I declined with a decreased in water temperature in coho salmon (*Oncorhynchus kisutch*).

It is therefore clear that environmental conditions (i.e. water temperature) can influence both the stress response and other endocrine systems (i.e. IGF) important for growth. At the same time, other studies have shown that IGF-I can be influenced by nutrition where replacing FM with alternative protein sources which are not naturally present in the diet of fish may influence IGF-I secretion. Gómez-Requeni, et al. (2003) reported low levels of IGF-I when plant protein supplemented with non essential amino acids was added in the diet of gilthead sea bream (*Sparus aurata*). A reduced level of IGF–I in plasma was also observed in rainbow trout fed with diets containing plant protein also resulting in a reduction of growth (Gómez-Requeni et al., 2005). In addition to IGF-I, cortisol may also be affected by the replacement of fishmeal (FM). Elevated levels of cortisol level in the European whitefish (*Coregonus lavaretus*) were associated with a reduction in FM content in the diet (Vielma et al., 2003). In addition, cortisol level in fish is also related to feed intake by fish and the feed intake is greatly influenced by dietary ingredients (Martins et al., 2006).

One of the previously identified problems when using alternative protein sources (i.e plant proteins) has been the development of enteritis. Krogdahl et al., (2003) reported that increasing levels of soybean meal in fish feed can reduce the enzymatic activity in the distal

intestine which leads to enteritis. The symptoms, which include changing in mucosal folds, supranuclear vacuoles, goblet cells, eosinophilic granulocytes, lamina propria and sub-epithelial mucosa, have been shown to occur in the fish intestine when soybean meal is used to feed Atlantic salmon (*Salmo salar*) for 20 days. Few studies that have examined the effects of FM replacement on the morphology of fish intestine (van den Ingh et al., 1991; Glencross et al., 2004; Heikkinen et al., 2006). All of the changes in fish intestine which have been characterized as enteritis were observed in the distal intestine when rainbow trout and Atlantic salmon were fed with a diet with soybean meal and FM as protein sources (van den Ingh, et al., 1991; Urán et al., 2008). Temperature can also influence the onset of enteritis with lower temperature delay the problem (Urán, et al., 2008). As yet there have been no reported studies investigating the effects of PM on intestine morphology of rainbow trout.

Previous studies have shown that replacement of FM with poultry meal (PM) up to 40% does not affect growth performance of rainbow trout. However nutrient digestibility in diets with high inclusion levels of PM is lower (Chapter 2). This study examines the effects of FM replacement with PM on growth performance of rainbow trout at optimal and elevated thermal conditions (15 and 20^{o}C). The data were analysed using a polynomial regression analysis (Zeitoun et al., 1976) and a break point analysis (Robbins et al., 1979) to predict the maximum dietary inclusion based on a variety of growth performance parameters. In addition, plasma cortisol, IGF-I levels and intestine histology were performed to evaluate the effects of replacement and at two temperatures.

4.3. MATERIALS AND METHODS

4.3.1 Experimental Diets

A total of 12 diets (PM00, PM10, PM20, PM30, PM40, PM50, PM60, PM70, PM80, PM90, PM95 and PM100) were formulated to be isonitrogenous and isoenergetic to contain 400 $g \cdot kg^{-1}$ DM crude protein and 22 MJ GE$\cdot kg^{-1}$ DM (Table 4.1). The diets contained graded levels of PM protein (0-100%) to replace the FM protein in the diet. Water (10% w/w) was added to the ingredients and they were mixed using a Hobart mixer (Hobart Corp, Troy, Ohio, USA). The feed mixes were cold pelleted with a laboratory pelletizer (California Pellet Mill Co., San Francisco, CA, USA) and oven dried for 24 hours at 30^{o}C. The diets were kept in cold storage at 4^{o}C throughout the experimental period. FM, PM and fish oil were supplied by Skretting (Cambridge, Tasmania, Australia). Vitamins, minerals, carboxymethyl cellulose, bentonite, calcium phosphate, α-cellulose and ytterbium oxide were supplied by Sigma-Aldrich Pty (Sydney, Australia). Vitamin C was supplied as Stay-C by Roche Vitamins Australia Ltd. (Sydney, Australia). Choline chloride was supplied by ICN Biomedicals, INC. (Aurora, Ohio, USA).

4.3.2 Growth Experiment

The experiment was conducted according to Ethics Approval A0009762 the Aquaculture Centre, University of Tasmania (Launceston, Tasmania, Australia) in two identical freshwater recirculation systems. Each system contained 12 x 300 L fiberglass tanks and was fitted with a submerged biofilter and UV steriliser. A total of 528 rainbow trout (*Oncorhynchus mykiss*) with a mean initial weight of 28.77 ± 0.10 g were obtained from Mountain Stream Fishery (Tasmania, Australia). Fish were randomly allocated to the 24 tanks (22 fish per tank). Twelve tanks were

held at a temperature of 15.4 ± 0.01^{o}C while the other twelve tanks were held at 20.2 ± 0.01^{o}C. Prior to the start of the experiment, all 24 tanks were kept at 15^{o}C. Temperature for the 20^{o}C system was gradually increased at a rate of 1^{o}C per day until the desired experimental temperature was achieved. A study conducted by Barnes et al., (2011) indicated that the increasing in water temperature will decreasing the dissolved oxygen in the water. Water quality was monitored daily to ensure it was within the limits for rainbow trout (Willoughby, 1999). Every day 10% of the water in the tanks was replaced. The dissolve oxygen was maintained at 5mg L^{-1} and above.

During the experiment, fish were held under natural photoperiod and in freshwater (0 ‰ salinity) and were not fed for three days prior to the start of the experiment. Tanks within each system were randomly divided between the 12 dietary treatments (n = 1). Fish in all treatments were fed a total of 2% body weight per day at 0900 hours and 1600 hours for 112 days. Fish bulk weight was measured every two weeks for all treatments in order to adjust the feed ration. This sampling was conducted at initial and last days of the experiment in order to prevent fish from stress and at the same time to determine the maximum effects of treatment without tampering with fish condition during the experimental period. A study conducted by Percival et al. (2001) reported that when the Atlantic salmon were stripped twice at the same faecal collection period, the lower apparent digestibility coefficients for organic matter and crude protein were detected. This was supported by a study conducted by Stone et al. (2008) which reported that the repeated faecal collection increased the cortisol level in rainbow trout.

Sampling of fish was conducted on day 1 and day 112 and tanks were sampled in random order. All fish were anaesthetized before sampling and fish were quickly netted into a 300 L tank containing 30 $mg.L^{-1}$ AQUI-S anesthetic (AQUI-S New Zealand LTD). At the initial sampling, fork length (cm) and wet weight (g) were measured for all fish. Blood samples were collected from the caudal vein into heparinised tubes from one fish per tank. An additional fish from each tank was killed by overdose of AQUI-S anesthetic (60 $mg.L^{-1}$) and sampled for whole-body chemical composition (see below). The remaining fish were recovered in a tank containing aerated freshwater before being returned to experimental tanks. At the final sampling, fish fork length (cm) and wet weight (g) were measured for all fish and blood samples were then collected from the caudal vein into heparinised tubes from 10 fish from each tank. The average sampling time for each fish was 3 minutes. Five fish from each tank were killed for whole-body chemical composition analysis and an additional 10 fish were killed for histological analysis.

4.3.3 IGF-I radio-immunoassay

Plasma IGF-I levels were determined using an IGF-I radio-immunoassay (RIA) as previously described (Dyer et al., 2004; Wilkinson et al., 2006). Plasma samples were first extracted to separate IGF-I/IGFBP complexes using acid-ethanol. Extraction involved mixing 40 µL of plasma with 160 µL acid-ethanol (12.5% 2 N HCl, 87.5% ethanol) and incubating at room temperature for 30 minutes. The acid-ethanol solution was neutralized using 80 µL 0.855 M Tris and samples were centrifuged at 10,000 g for 10 minutes at 2-8^{o}C. The supernatant was collected and assayed for IGF-I using iodinated recombinant tuna IGF-I RIA label, recombinant salmon IGF-I assay standards and anti-barramundi IGF-I primary antibody (GroPep Ltd., Adelaide, Australia). The antibody-bound hormone complexes were separated from free tracer

by adding the 10 µL rabbit gamma globulin (1:20) and 50 µL sheep anti-rabbit gamma globulin (1:200). After mixing and incubating for 30 minutes at 2-8^{o}C, one mL of cold 5% (v/v) polyethylene glycol was added and the precipitate was centrifuged at 4000 g for 30 minutes at temperature 2-8^{o}C. The supernatant was removed and the level of bound radioactivity was determined using a gamma counter. The minimum detectable limit of the assay was 0.15 ng.mL^{-1}.

4.3.4 Cortisol

Cortisol levels were determined using a commercially available enzyme-linked immune-sorbent assay (ELISA) Kit for Cortisol (Enzyme Immunoassay, reference # KS18EW, RADIM, Pomezia, Italy). Ten µL of plasma from each fish was used in the assay. In the ELISA sample cortisol competes with the cortisol conjugated with horseradish peroxidase (HRPO) for binding to anti-cortisol antiserum coated to the microplate walls. After the incubation, all unbound material was removed by aspiration and washing. Following incubation with a chromogen solution enzyme activity bound to the solid phase was inversely proportional to the cortisol concentration in standards and samples. Colorimetric reading was carried out using a spectrophotometer at 450 and 405 nm. The assay sensitivity was 5 ng.mL^{-1}.

4.3.5 Histology of intestines

Fish distal intestines and half portion of the liver were sampled for histological analysis. All dissected samples were taken immediately and rinsed in saline water (9 g.L^{-1}NaCl). Tissues were fixed in 10% neutral buffered formalin and were embedded in paraffin, cut (5 µm thick) and stained with haematoxylin-eosin. All distal intestine samples were observed under a light

microscope (Olympus BH-2) to observe the intestinal epithelium and measure the height of mucosal fold (Urán, et al., 2008). A semi-quantitative scoring system was used to evaluate the intestine from all treatments. Five separate parameters (Table 4.2) were quantified independently. Each parameter was scored on a scale from 1 to 5. After a preliminary assessment, fish fed diets PM10 to PM80 for 15^{o}C and diet PM10 to PM70 for 20^{o}C did not show any signs of differences from the PM0 control diet therefore were excluded from further analysis. Representative images from fish fed diet PM00 for 15^{o}C and 20^{o}C were taken as a 'normal' control to compare effects of diets. Images for fish fed diets PM90 and PM100 were taken from both temperatures to represent these particular diets. No images were taken from fish fed diet PM95 as the dietary effects were similar to fish fed diets PM90 and PM100. All images were taken using a Leica DC 300F digital camera connected to a light microscope.

All liver samples were observed under a light microscope (Olympus BH-2) to observe the hepatocytes. After the preliminary assessment, no differences were found between samples originating from the different dietary treatments. Representative images were therefore taken from fish fed diets PM00 and PM100 at both temperatures to represent the characteristics of these diets. All images were taken using a Leica DC 300F digital camera connected to a light microscope.

4.3.6 Chemical analysis

Fish carcasses were autoclaved (Williams et al., 1995) and dried to a constant weight using a freeze drier. The chemical analysis of fish carcass, diets and faeces were performed using standard methodology. Dry matter (DM) was determined by drying sample at 135^{o}C for 2 hours

(AOAC, 1995), ash content was determined by incineration in a muffle furnace at 600^{o}C for 2 hours (AOAC, 1995), total lipid was determined according to the methods of Bligh and Dyer (1959), total nitrogen was determined by Kjeldhal (crude protein content was estimated as Nitrogen ((N) X 6.25) and gross energy was determined by an auto bomb calorimeter.

4.3.7 Calculations

The following equations were used to calculate weight gain (WG), specific growth rate (SGR), feed intake (FI), feed efficiency ratio (FER), condition factor (K), productive protein value (PPV), productive energy value (PEV), hepatosomatic index (HI) and mesenteric fat index (MFI).

Weight gain (WG) (g) = Final weight – Initial weight (1)

Specific growth rate (SGR) (%·d^{-1}) = [(ln final weight (g) – ln initial weight (g))·

Time (Days)$^{-1}$] * 100 (2)

Feed intake (FI) (%·d^{-1}) = [(feed intake (g)·mean mid weight (g)$^{-1}$)·no days^{-1}] * 100

(3)

Feed efficiency ratio (FER) (g g^{-1}) = (wet weight gain (g)·total food consumption (g)$^{-1}$) (4)

Condition factor (K) = (whole wet weight (g))·(fork length (cm)^{3})$^{-1}$ * 100 (5)

Protein productive value (PPV) (%)=

(fish protein gain (g Crude Protein)·total protein (g Crude Protein)$^{-1}$) * 100 (6)

Productive energy value (PEV) (%) = (fish energy gain (g MJ)·total energy (g MJ)$^{-1}$)

* 100 (7)

Hepatosomatic index (HI) (%W) = (liver weight (g)·wet weight (g)$^{-1}$)*100 (8)

Mesenteric fat index (MFI) (%W) = (mesenteric fat weight (g)·wet weight (g)$^{-1}$) * 100 (9)

4.3.8 Statistical analysis

All data are presented as means ± standard error. The data were analysed using polynomial regression analysis ($Y = a + bX + cX^2$) or break point analysis. Results with probability values less than 0.05 were considered as significant. Polynomial analysis was used to determine the maximum PM inclusion level above and below which there were decreases in growth performance parameters. Polynomial analysis was analysed using SPSS Statistical Analysis Software Program (version 17.0 for Windows). Break point analysis (Robbins et al., 1979) determined the maximum PM inclusion above which there was a decrease in performance. The break point regression was performed using excel (Microsoft Office Excel 2007) based on the piecewise regression by Ryan et al. (2007). Break point analysis was conducted on FW, FL, FI, K, PPV, PEV, HI, MFI, IGF-I, cortisol, WG, SGR and FER. Maximum inclusion levels for polynomial and break point analysis were presented when the results showed significant differences. The data from parameters with no significant differences in both models were pooled together and were analysed using independent t test to determine the differences between two temperatures. The result for 80% inclusion level of PM at 20^{o}C was not presented except for initial weight (Table 4.3), IGF-I and cortisol (Table 4.4). This is due to high mortality in this treatment as a result of a power failure.

Table 4.1. Ingredient and chemical composition of experimental diets (mean ± standard error).

	Diets											
	PM00	PM10	PM20	PM30	PM40	PM50	PM60	PM70	PM80	PM90	PM95	PM100
Ingredient composition ($g \cdot kg^{-1}$)												
Fishmeal	564.0	507.0	450.7	394.4	338.1	285.2	225.4	169.1	112.6	56.3	28.1	00.0
Poultry meal	00.0	61.0	121.9	182.5	243.1	300.0	364.3	424.9	485.6	546.2	576.5	606.7
Pre-gelatinized starch	155.0	153.0	150.1	148.0	146.3	144.1	141.8	139.0	137.0	134.9	133.6	132.3
Fish oil	149.0	145.6	142.3	138.8	135.1	131.7	128.3	125.1	121.6	118.1	116.5	114.9
Carboxyl methyl cellulose (CMC)	10	10	10	10	10	10	10	10	10	10	10	10
Cellulose	68.1	69.5	71.1	72.4	73.5	75.1	76.3	78.0	79.3	80.6	81.4	82.2
Bentonite	33.2	33.2	33.2	33.2	33.2	33.2	33.2	33.2	33.2	33.2	33.2	33.2
Choline chloride	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Vitamin C	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Vitamin Premix[a]	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Mineral Premix[b]	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Ytterbium oxide	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Calcium phosphate	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Chemical composition ($g \cdot kg^{-1}$ DM)												
Dry matter	908	907	914	912	911	910	912	909	912	913	910	907
	±4.6	±4.1	±2.5	±1.3	±1.5	±1.2	±0.2	±2.6	±2.2	±5.1	±0.5	±0.9
Crude protein	399	398	401	400	396	397	400	402	398	396	395	406
	±1.9	±2.7	±2.0	±3.1	±4.5	±1.9	±8.1	±5.1	±8.6	±11.6	±11.6	±1.1
Total lipid	228	224	230	225	228	230	226	220	221	230	227	224
	±3.1	±4.3	±0.6	±0.6	±5.0	±9.7	±11.2	±17.3	±1.7	±8.1	±11.3	±7.4
Ash	119	117	116	116	112	113	119	108	111	106	109	111
	±1.1	±1.1	±0.2	±0.7	±1.2	±2.1	±5.6	±1.0	±6.1	±4.0	±5.3	±0.4
Gross energy	22.3	22.2	22.1	22.2	22.5	22.4	22.7	22.8	22.4	23.0	22.4	22.5
($MJ \cdot kg^{-1}$ DM)	±0.36	±0.32	±0.59	±0.27	±0.33	±0.79	±0.04	±0.05	±0.13	±0.55	±0.63	±0.01

Means with no superscripts were not significantly different ($p>0.05$) between diets.

[a] Vitamin premix (g kg^{-1}): Vitamin A (4.50), Vitamin D3 (5.40), Rovimix E50 (90.00), Menadone sodium bisulphate (1.80), Riboflavin (3.60), Calcium D-pantothenate (19.57), Nicotinic acid (9.00), Vitamin B12 (0.01), D-biotin (0.14), Folic acid (0.90), Thiamin HCL (1.01), Pyridoxine HCL (3.29), myo-Inositol (270.00), α-cellulose (490.79)

[b] Mineral premix (g kg^{-1}):$CuSO_4.5H_2O$ (23.58), $FeSO_4.7H_2O$ (363.10), $MnSO_4.H_2O$ (61.51), Na_2SeO_4 (0.66), $ZnSO_4.7H_2O$ (131.94), KI (1.44), $CoSO_4.7H_2O$ (9.54), α-cellulose (408.23)

Poultry meal (PM) accounted for between zero (PM00) and 100 (PM100) percent of the dietary protein

4.4. RESULTS

PM inclusion level resulted in a significant negative polynomial in final weight (FW) ($p=0.001$ at 15^oC; $p=0.015$ at 20^oC), final length (FL) ($p=0.003$ at 15^oC; $p=0.007$ at 20^oC), feed intake (FI) ($p=0.001$ at 15^oC; $p=0.015$ at 20^oC), weight gain (WG) ($p=0.000$ at 15^oC; $p=0.006$ at 20^oC) and specific growth rate (SGR) ($p=0.000$ at 15^oC; $p=0.005$ at 20^oC) when analysed using polynomial. The significant break point results were recorded at both temperatures for final weight (FW) ($p=0.000$ at 15^oC; $p=0.004$ at 20^oC), final length (FL) ($p=0.001$ at 15^oC; $p=0.000$ at 20^oC), feed intake (FI) ($p=0.000$ at 15^oC; $p=0.000$ at 20^oC), weight gain (WG) ($p=0.001$ at 15^oC; $p=0.004$ at 20^oC) and specific growth rate (SGR) ($p=0.000$ at 15^oC; $p=0.002$ at 20^oC).

Maximum FW occurred at a PM inclusion levels of 36.7% and 38.6% at 15^oC and 20^oC, respectively as determined by polynomial regression (Table 4.3). For FW the, maximum PM inclusion percentage, as determined by break-point analysis, was 83.0% and 78.0% at 15^oC and 20^oC, respectively (Table 4.3). In addition, maximum FL occurred at PM inclusion levels of 34.1% and 37.1% at 15^oC and 20^oC, respectively as determined by polynomial regression (Table 4.3). Conversely, the break point analysis indicated maximum PM inclusion levels of 85.0% and 80.0% for 15^oC and 20^oC, respectively (Table 4.3).

The maximum response of FI was determined at 31.4% and 37.5% of inclusion level of PM at 15^oC and 20^oC, respectively as determined by polynomial regression (Table 4.3). However, maximum PM inclusion percentage as determined by break point analysis was 85.0% and 80.0% at 15^oC and 20^oC, respectively (Table 4.3). Maximum WG was determined at inclusion level of 36.9% and 37.9% at 15^oC and 20^oC, respectively as analysed by polynomial

regression (Fig 4.1) whereas maximum inclusion levels in break point analysis were 83.0% and 78.0% at 15^{o}C and 20^{o}C, respectively (Fig 4.2 A and B). Moreover, the maximum SGR was found at inclusion levels of 60.7% and 73.1% at 15^{o}C and 20^{o}C, respectively when analysed using polynomial regression (Fig 4.4 A and B). On the other hand, the maximum inclusion level was determined to be 83.0% and 79.0% at 15^{o}C and 20^{o}C, respectively (Fig 4.4 A and B) when determined by break point analysis. There was no significant negative polynomial relationship between PM inclusion level and K at either temperature (Table 4.3). Therefore, the maximum inclusion level could not be determined for K (Table 4.3).

There was a significant negative polynomial relationship between PM inclusion level with PPV (Table 4.4) when analysed using polynomial regression and break point analysis. The maximum PPV was found to be 62.0% inclusion levels when analysed by polynomial and 89.0% inclusion levels when analysed using break point analysis (Table 4.4). The maximum FER was found at a PM inclusion levels 34.6% and 35.3% at 15^{o}C and 20^{o}C, respectively. However, the maximum PM inclusion percentage as determined by break-point analysis was 86.0% and 81.0% at 15^{o}C and 20^{o}C, respectively (Fig 4.6 A and B). In addition, MFI also shows a significant negative polynomial relationship at 15^{o}C in polynomial analysis (Table 4.4). The maximum MFI was detected at a PM inclusion levels 48.3% (Table 4.4) when analysed using polynomial regression. There was no significant negative polynomial relationship between PM inclusion level with, PEV and HI when analysed using polynomial or break point at both temperatures (Table 4.4).

There was a significant negative polynomial relationship between PM inclusion level and IGF-I at 15^{o}C. The maximum IGF-I occurred at inclusion levels 44.9% and 62.0% when analysed by polynomial regression and break point, respectively (Table 4.4). No significant relationship was determined at 20^{o}C in both analyses (Table 4.4). In addition, there was no significant negative polynomial relationship was found between cortisol level and PM inclusion level for polynomial and break point analysis at both temperatures (Table 4.4).

The results for chemical composition of rainbow trout showed that dry matter, total lipid and gross energy content at the two temperatures were unaffected by the PM inclusion level (Table 4.5). However, protein content at 20^{o}C was significantly affected by the PM inclusion level whereas, no significant negative polynomial relationship was found for protein content at 15^{o}C (Table 4.5). Similarly ash content was significantly affected at 20^{o}C but not at 15°C (Table 4.5).

Representative images of the morphological appearances of the distal intestine of fish reared at two different temperatures and fed with either FM or PM diets are shown in figure 4.7. The diet with 100% FM source (PM0) is considered to represent the normal morphology at 15^{o}C and 20^{o}C. The size of sub-epithelial mucosa (SM), lamina propria (LP) and mucosal folds (MF) were not different between treatments. Differences were found when the FM was replaced up to 90% with PM in both temperatures. Changes were related to the irregular shape and size of supranuclear vacuoles (SNV) and the increased number of goblet cells (GC) among the enterocytes (Table 4.6). Width of villi in the SNV was significantly reduced with increasing PM inclusion. Increases in the number of GC were found in 100% inclusion level of PM at 15^{o}C and

when the inclusion level reached 95 and 100% at 20^{o}C. No pathological changes were observed in the liver of all treatments (Fig 4.8). The hepatocytes were similar with vacuoles in all treatments (Fig 4.8). The hepatocytes were not considered to be atrophied (Fig 4.8).

Table 4.2 Histological scoring system for morphological changes by soybeans in the distal intestine of Atlantic salmon (*Salmo salar* L.) (Urán, et al., 2008) adapted in the current study to investigate the effects of PM inclusion level on the morphology of distal intestine.

Score	Parameter	Score	
	Mucosal folds (MF)		**Supranuclear vacuoles (SNV)**
1	Basal length	1	Basal SNV size
2	Some shrinkage and bloating	2	Some size reduction
3	Diffused shrinkage and onset of tissue disruption	3	Diffused size reduction
		4	Onset of extinction
4	Diffused tissue disruption	5	No SNV
5	Total tissue disruption		
	Goblet cells (GC)		**Sub-epithelial mucosa (SM)**
1	Scattered cells	1	Normal SM
2	Increased number and sparsely distributed	2	Increased size SM
		3	Medium size SM
3	Diffused number widely spread	4	Large SM
4	Densely grouped cells	5	Largest SM
5	Highly abundant and tightly-packed cells		
	Lamina propria (LP)		
1	Normal size LP		
2	Increased size of LP		
3	Medium size LP		
4	Large LP		
5	Largest LP		

Table 4.3: Initial weight, final weight, final length, feed intake and condition factor of rainbow trout (*Oncorhynchus mykiss*) fed with 12 different experimental diets.

	Temp (°C)	Diets												*Polynomial Regression*				*Break Point*		
		PM 00	PM 10	PM 20	PM 30	PM 40	PM 50	PM 60	PM 70	PM 80	PM 90	PM 95	PM 100	*F*	*df*	*P*	*MI (%)*	R^2	*P*	*BP (%)*
IW	15	28.9	28.9	29.1	29.6	29.6	29.4	29.0	29.1	28.5	29.5	29.4	28.9	-	-	-	-	-	-	-
(g)	20	28.1	28.2	28.4	28.5	28.4	28.5	28.3	28.4	28.3	28.8	28.4	28.3	-	-	-	-	-	-	-
FW	15	166.5	157.2	173.9	176.1	174.2	179.7	166.4	156.8	162.1	146.8	122.2	114.8	35.2	2, 11	0.001	36.7	0.88	0.000	83.0
(g)	20	133.2	147.3	139.9	129.7	150.7	139.9	142.4	150.3		126.3	111.4	101.7	10.6	2, 10	0.015	38.6	0.86	0.004	78.0
FL	15	22.7	22.0	23.2	22.8	23.0	23.2	22.6	21.8	22.9	21.6	20.4	19.6	15.3	2, 11	0.003	34.1	0.87	0.001	85.0
(cm)	20	21.0	21.5	21.3	20.7	21.6	21.2	21.9	21.4		20.3	19.4	18.4	15.5	2, 10	0.007	37.1	0.92	0.000	80.0
FI	15	0.8	0.9	0.8	0.8	0.9	0.8	0.9	0.9	0.9	0.9	1.0	1.0	22.1	2, 11	0.001	31.4	0.95	0.000	88.0
(%·d^{-1})	20	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9		0.9	1.0	1.1	8.7	2, 10	0.015	37.5	0.90	0.000	83.0
K	15	1.4	1.5	1.4	1.5	1.4	1.4	1.4	1.5	1.3	1.5	1.4	1.5	0.2	2, 11	0.837	-	0.25	10.079	-
	20	1.4	1.5	1.4	1.5	1.5	1.5	1.4	1.5		1.5	1.5	1.6	4.3	2, 10	0.058	-	0.61	0.473	-

MI and BP (Maximum Inclusion) value only presented when the $p < 0.05$

MI (%) = Inclusion which gives the maximum response of measured parameters
BP (%) = Break-Point
IW (g) = Initial Weight
FW (g) = Final Weight
FL (cm) = Final Length
FI(%·day^{-1}) = Feed Intake
K = Condition Factor
Poultry meal (PM) accounted for between zero (PM00) and 100 (PM100) percent of the dietary protein

Table 4.4: Productive protein value, productive energy value, hepatosomatic index, mesenteric fat index, insulin-like growth factor I and cortisol of rainbow trout (*Oncorhynchus mykiss*) fed with 12 different experimental diets.

	Temp (°C)	Diets												*Polynomial Regression*				*Break Point*		
		PM 00	PM 10	PM 20	PM 30	PM 40	PM 50	PM 60	PM 70	PM 80	PM 90	PM 95	PM 100	*F*	*df*	*P*	*MI (%)*	R^2	*P*	*BP (%)*
PPV	15	25.1	25.6	25.5	25.5	24.9	26.2	27.8	22.7	26.3	24.8	22.7	16.3	4.8	2, 11	0.039	62.0	0.80	0.008	89.0
(%)	20	27.0	21.1	22.1	20.7	22.3	22.4	22.5	23.8		26.1	21.8	19.5	0.3	2, 10	0.780	-	0.28	9.214	-
PEV	15	32.1	31.1	37.8	27.9	35.1	28.3	31.3	33.9	27.9	28.4	21.9	25.7	4.3	2, 11	0.048	-	0.53	0.728	-
(%)	20	32.8	26.0	26.9	31.5	27.1	26.7	24.6	27.8		30.1	23.6	26.4	1.2	2, 10	0.352	-	0.24	12.803	-
HI	15	1.5	1.6	1.5	1.6	1.5	1.5	1.5	1.5	1.5	1.7	1.5	1.8	3.0	2, 11	0.105	-	0.49	1.171	-
(%)	20	1.3	1.4	1.3	1.3	1.3	1.4	1.3	1.2		1.5	1.3	1.6	1.6	2, 10	0.262	-	0.43	2.972	-
MFI	15	3.8	4.6	5.1	5.4	5.4	4.2	5.4	6.3	4.6	4.7	4.1	3.9	4.6	2, 11	0.041	48.3	0.56	0.525	-
(%)	20	4.1	4.2	4.5	5.1	5.1	5.1	4.8	5.3		4.5	5.5	4.2	4.1	2, 10	0.063	-	0.44	2.591	-
IGF-I	15	34.2	27.4	31.2	29.6	24.8	27.8	20.8	25.7	29.2	40.7	40.0	35.8	8.8	2, 11	0.011	44.9	0.77	0.028	62.0
($ng \cdot mL^{-1}$)	20	37.9	24.9	36.8	38.8	50.4	26.3	43.0	43.7	48.3	48.3	35.9	33.5	0.9	2, 11	0.429	-	0.33	4.996	-
Cortisol	15	18.2	43.8	48.1	50.1	45.6	31.4	56.2	27.5	41.3	19.0	39.3	17.9	3.25	2, 11	0.096	-	0.44	12.803	-
($ng \cdot mL^{-1}$)	20	60.2	54.1	46.6	53.2	48.6	43.7	50.8	51.7	40.1	74.4	50.6	21.9	0.36	2, 11	0.717	-	0.49	0.063	-

MI and BP (Maximum Inclusion) values presented when $p < 0.05$

MI (%) = Inclusion which gives the maximum response of measured parameters
BP (%) = Break-Point
PPV (%) = Productive Protein Value
PEV (%) = Productive Energy Value
HI (%) = Hepatosomatic Index
MFI (%) = Mesenteric Fat Index
IGF-I ($ng \cdot mL^{-1}$) = Insulin-like Growth Factor I
Cortisol ($ng \cdot mL^{-1}$)
Poultry meal (PM) accounted for between zero (PM00) and 100 (PM100) percent of the dietary protein

Table 4.5: Chemical composition (mean) of rainbow trout (*Oncorhynchus mykiss*) fed with 12 different experimental diets culture at two different temperatures, 15°C and 20°C (g kg^{-1} of wet weight)

	Tem (°C)	Diets												*Polynomial Regression*		
		PM00	PM10	PM20	PM30	PM40	PM50	PM60	PM70	PM80	PM90	PM95	PM100	*F*	*df*	*P*
Dry matter (g kg^{-1} WW)	15	223.2	227.6	272.0	257.0	244.8	224.4	255.2	239.2	250.9	227.7	225.4	239.5	1.2	2, 11	0.349
	20	253.7	200.0	220.0	241.0	209.1	216.4	210.1	216.5	-	252.0	220.5	247.4	2.0	2, 10	0.210
Crude Protein (g kg^{-1} WW)	15	101.7	107.6	104.1	103.4	101.5	104.6	115.7	99.9	109.1	109.5	111.1	92.3	0.3	2, 11	0.720
	20	117.1	91.4	100.9	97.3	95.4	98.7	98.2	100.7	-	116.9	110.1	109.0	5.0	2, 10	0.039
Total Lipid (g kg^{-1} WW)	15	98.7	103.0	128.5	98.5	120.9	96.8	111.1	123.8	98.1	89.0	93.2	101.7	1.6	2, 11	0.258
	20	109.9	85.3	90.0	124.8	93.6	91.2	84.4	90.6	-	111.6	89.5	115.5	0.6	2, 10	0.595
Ash (g kg^{-1} WW)	15	16.6	13.8	19.1	13.4	15.3	15.6	18.8	14.8	14.9	16.7	17.6	20.7	1.5	2, 11	0.271
	20	19.3	15.4	15.7	16.5	14.8	16.0	17.4	14.7	-	20.0	18.0	19.5	6.0	2, 10	0.025
Gross energy (MJ·kg^{-1} WW)	15	6.1	6.2	7.2	5.4	7.0	6.0	6.7	7.2	5.7	6.1	5.3	6.3	0.8	2, 11	0.488
	20	6.7	5.2	5.5	6.6	5.5	6.0	5.3	5.7	-	6.5	5.8	6.5	1.1	2, 10	0.388

Poultry meal (PM) accounted for between zero (PM00) and 100 (PM100) percent of the dietary protein

Table 4.6: Histological evaluations of distal intestine of rainbow trout (*Oncorhynchus mykiss*) reared in two different temperatures and fed with either FM or PM diets (mean).

Parameters	Temp (^{o}C)	*Diets*			
		PM00	PM90	PM95	PM100
SNV	15	1.0	1.5	2.2	3.0
	20	1.0	1.4	2.8	3.0
GC	15	1.0	1.0	1.0	2.0
	20	1.0	1.0	1.8	2.0

SNV = Supranuclear vacuoles
GC = Goblet cells
Poultry meal (PM) accounted for between zero (PM00) and 100 (PM100) percent of the dietary protein

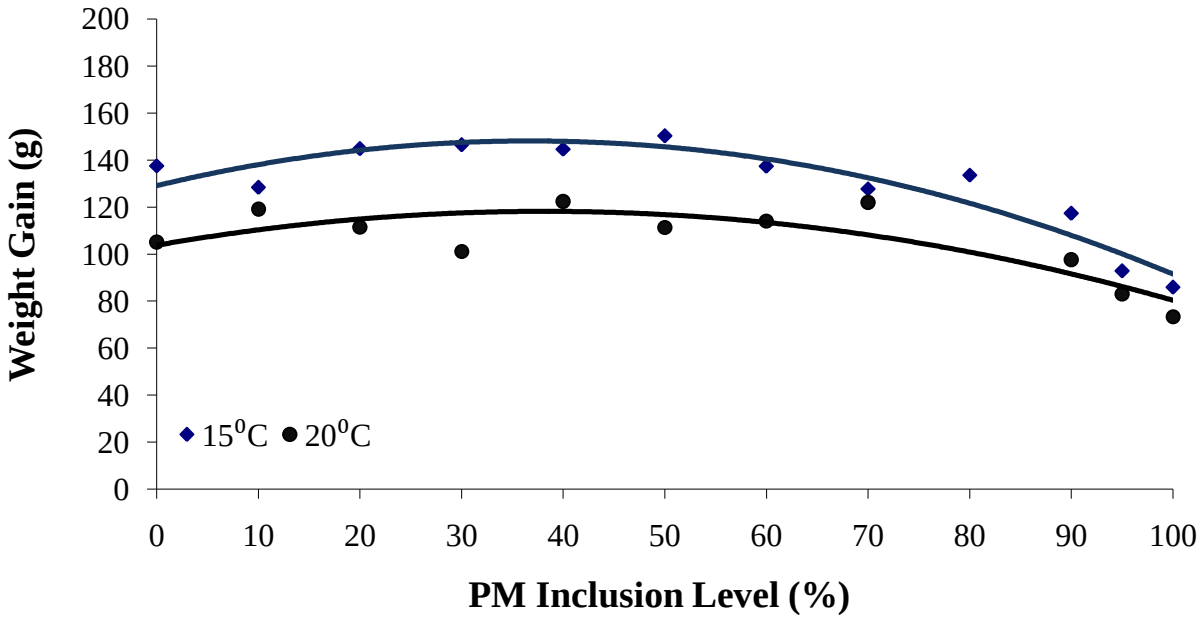
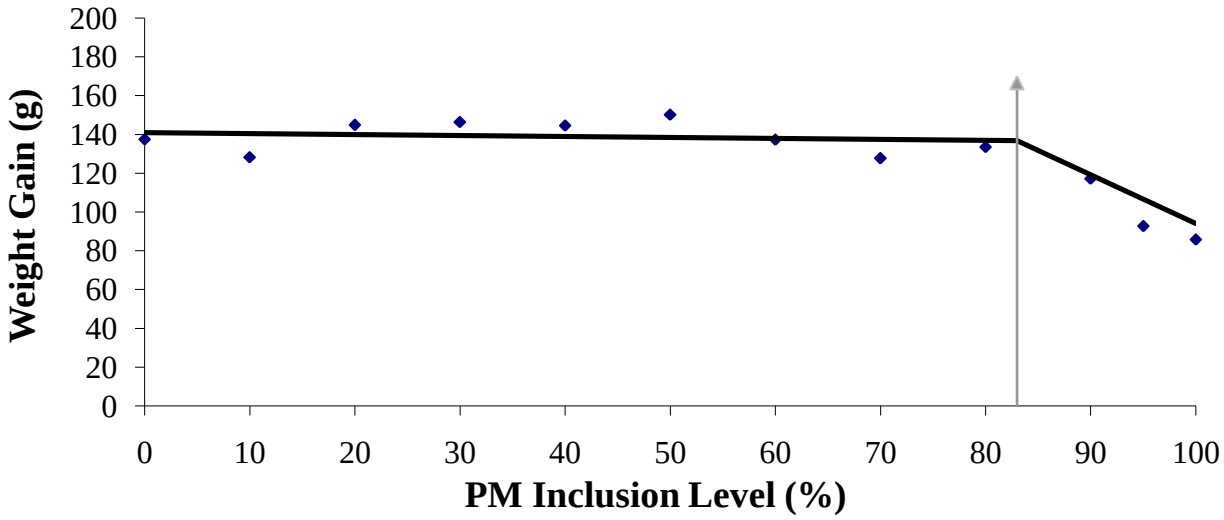


Figure 4.1.Weight gain of rainbow trout (*Oncorhynchus mykiss*) after 112 days culture at 15°C and 20°C. The relationship between PM inclusion level and weight (g) was expressed by the equation $y = -0.0141x^2 + 1.04x + 129.09$ ($r^2 = 0.884$, n = 12, PM inclusion level = 36.9%, p = 0.000) and $y = -0.0099x^2 + 0.75x + 103.70$ ($r^2 = 0.725$, n = 11, PM inclusion level = 37.9%, p = 0.006), respectively.

A.



B.

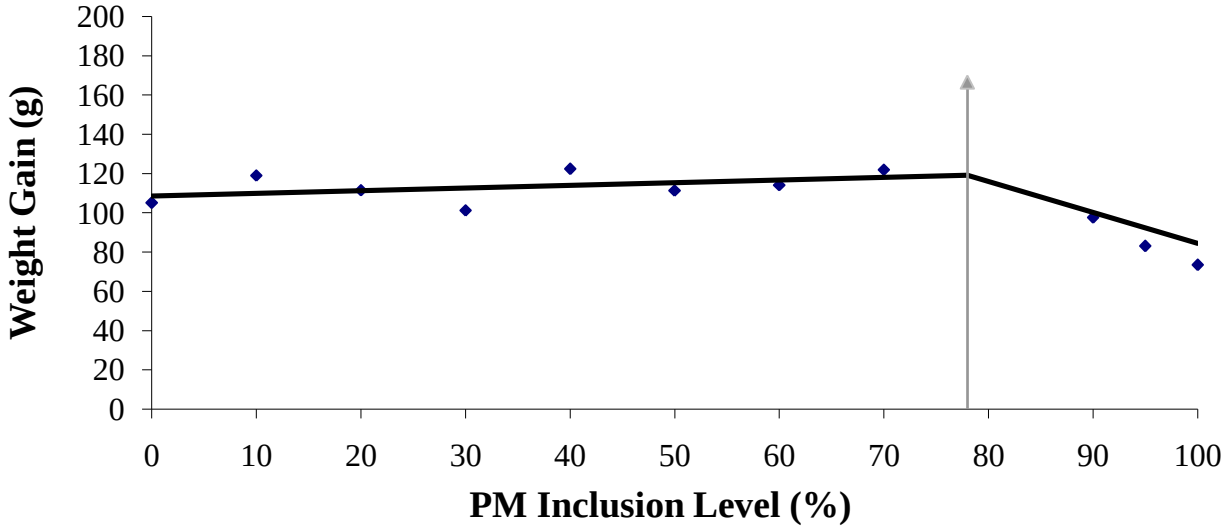


Figure 4.2. Broken-line analysis of weight gain of rainbow trout (*Oncorhynchus mykiss*) after 112 days cultured. (A) 15°C (PM inclusion level = 83.0%, r^2 = 0.88, p = 0.001) and (B) 20°C (PM inclusion level = 78.0%, r^2 = 0.86%, p = 0.004).

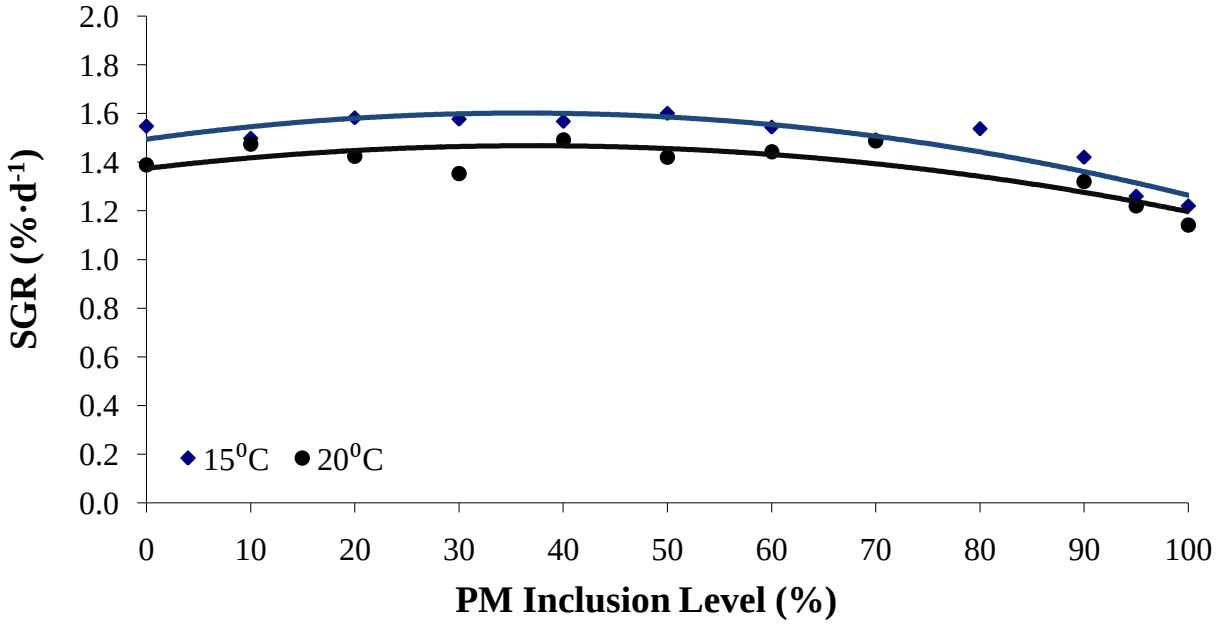
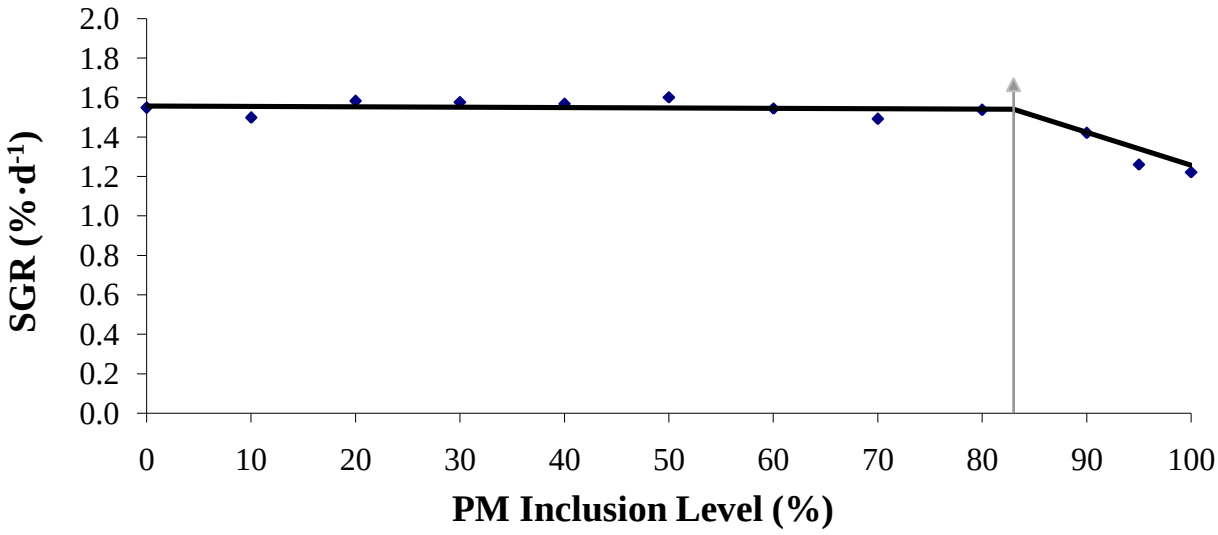


Figure 4.3. Specific growth rate of rainbow trout (*Oncorhynchus mykiss*) after 112 days culture at 15°C and 20°C. The relationship between PM inclusion level and SGR (%/day) was expressed by the equation $y = -0.0000824x^2 + 0.01x + 1.49$ ($r^2 = 0.858$, n = 12, PM inclusion level = 60.7%, p =0.000) and $y = -0.0000684x^2 + 0.01x + 1.37$ ($r^2 = 0.743$, n = 11, PM inclusion level = 73.1%, p = 0.005), respectively.

A.



B.

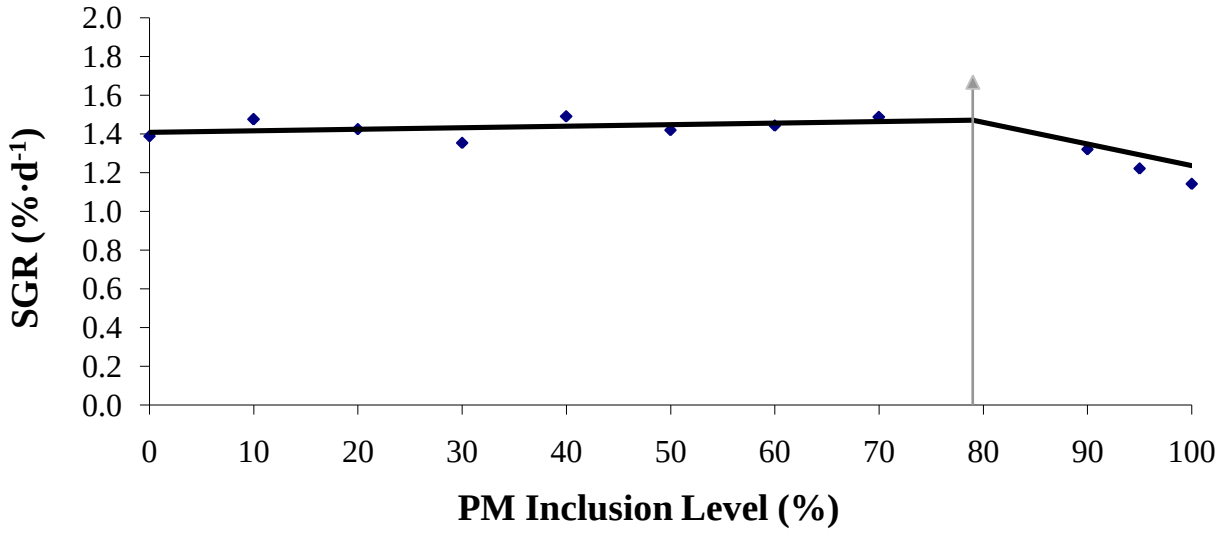


Figure 4.4. Broken-line analysis of specific growth rate of rainbow trout (*Oncorhynchus mykiss*) after 112 days cultured. (A) 15°C (PM inclusion level = 83.0%, r^2 = 0.92, p = 0.000) and (B) 20°C (PM inclusion level = 79.0%, r^2 = 0.88, p = 0.002).

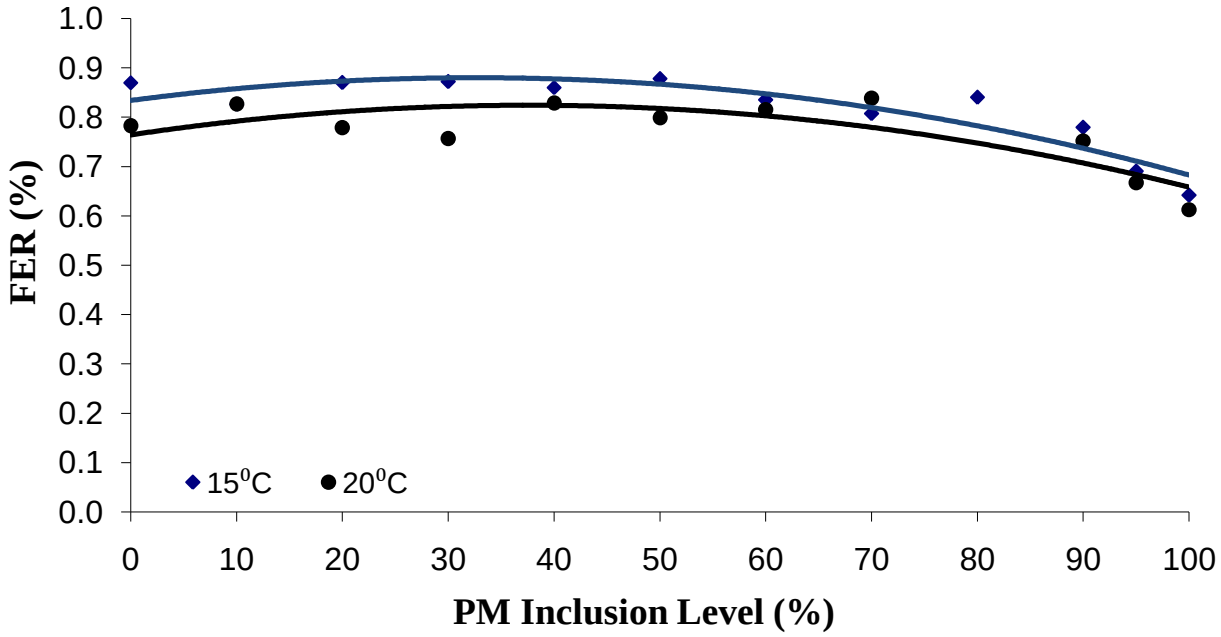
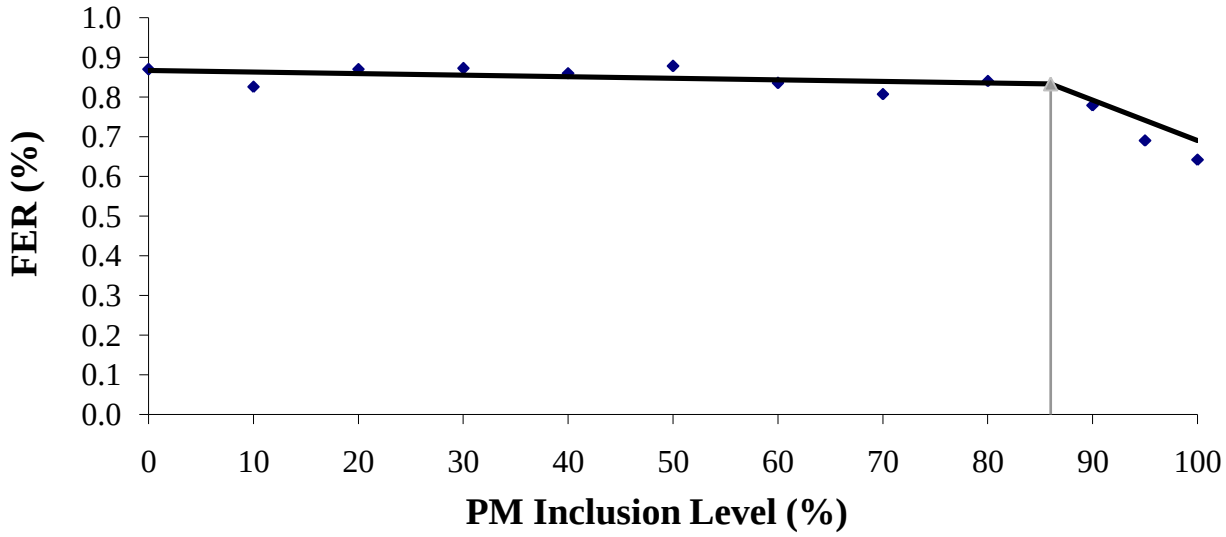


Figure 4.5 Feed efficiency ratio of rainbow trout (*Oncorhynchus mykiss*) after 112 days culture at 15°C and 20°C. The relationship between PM inclusion level and FER was expressed by the equation $y = -0.0000434x^2 + 0.003x + 0.83$ ($r^2 = 0.837$, n = 12, PM inclusion level = 34.6%, p = 0.000) and $y = -0.0000425x^2 + 0.003x + 0.76$ ($r^2 = 0.697$, n = 11, PM inclusion level = 35.3%, p = 0.007), respectively.

A.



B.

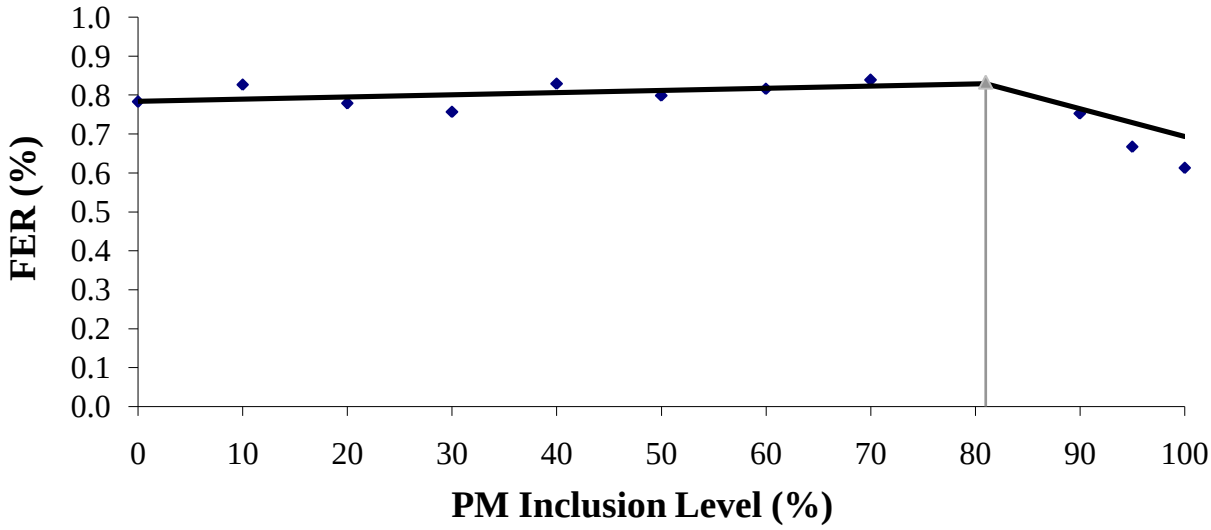


Figure 4.6. Broken-line analysis of feed efficiency ratio of rainbow trout (*Oncorhynchus mykiss*) after 112 days cultured. (A) 15°C (PM inclusion level = 86.0%, r^2 = 0.93, p = 0.000) and (B) 20°C (PM inclusion level = 81.0%, r^2 = 0.89, p = 0.001).

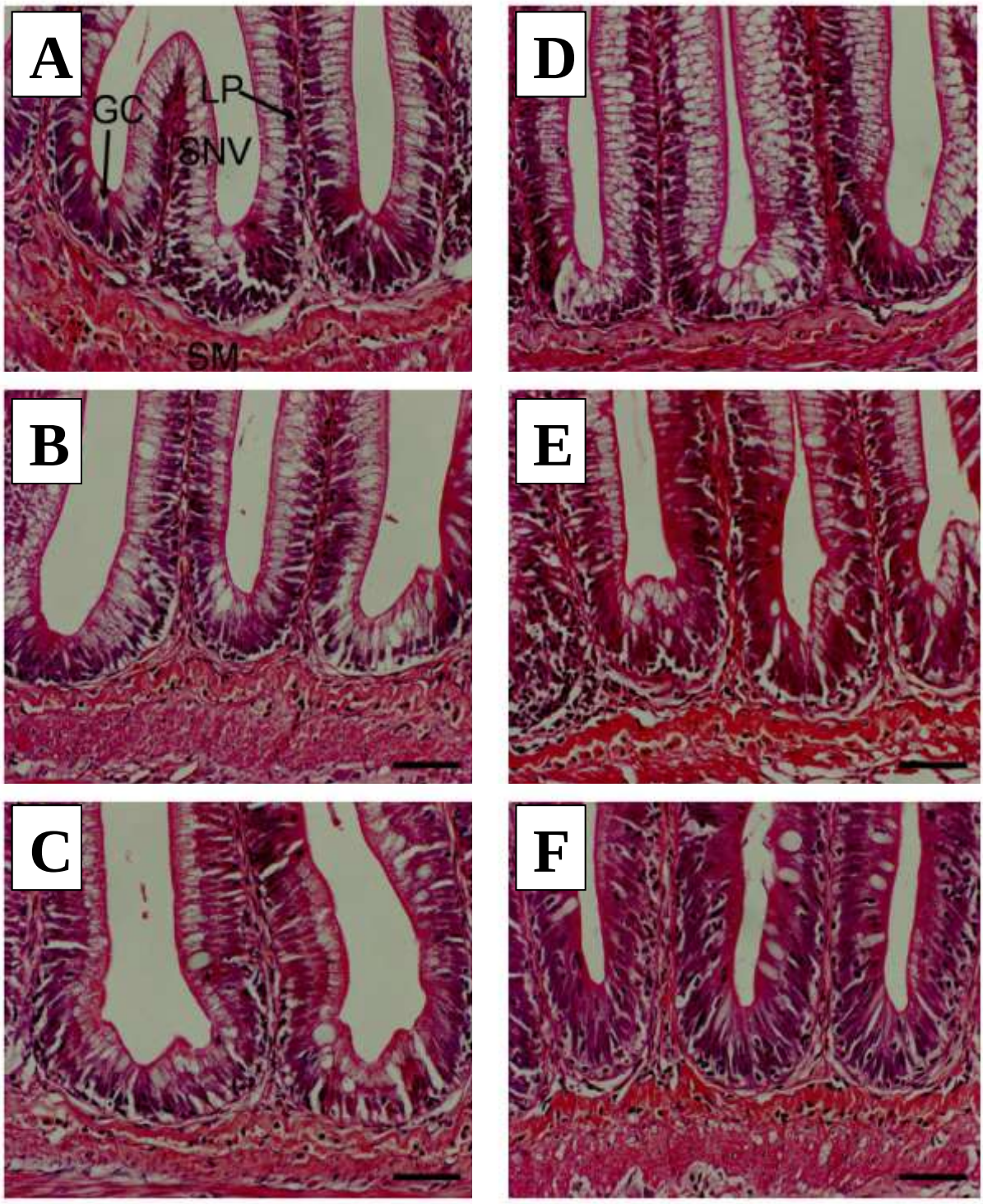
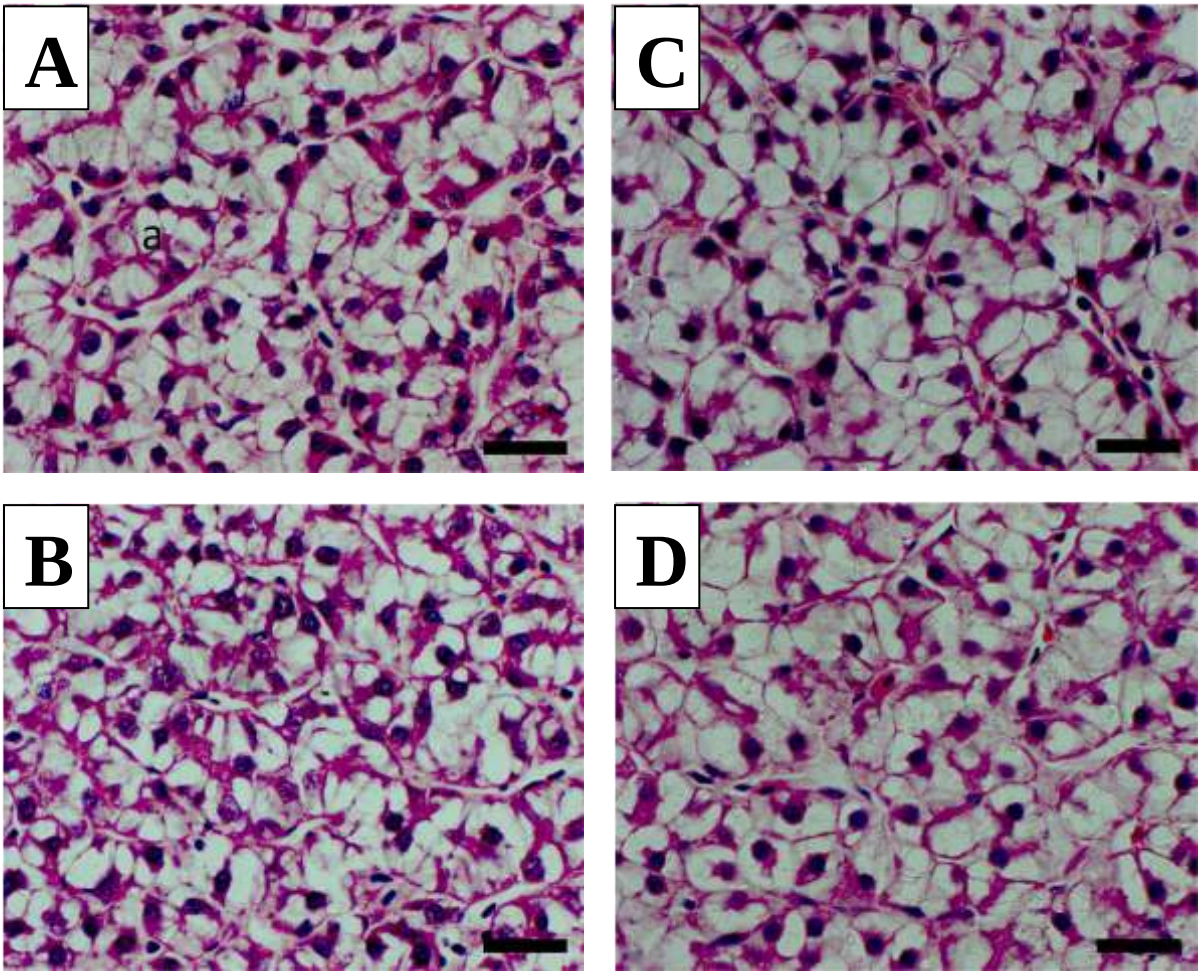


Figure 4.7: Morphological appearances of the distal intestine of rainbow trout (*Oncorhynchus mykiss*) reared at two different temperatures and fed with either FM or PM diets. A) PM00 (100% FM, 15^{o}C) **B**) PM90 (90% PM, 15^{o}C) and **C**) PM100 (100% PM, 15^{o}C) **D**) PM00 (100% FM, 20^{o}C) **E**) PM90 (90% PM, 20^{o}C) and **F**) PM100 (100% PM, 20^{o}C). Scale bar 50 µm.



a = Hepatocytes

Figure 4.8: Morphological appearances of the liver of rainbow trout (*Oncorhynchus mykiss*) reared at two different temperatures and fed with either FM or PM diets. A) PM00 (100% FM, 15^{o}C) **B**) PM100 (100% PM, 15^{o}C) and **C**) PM00 (100% FM, 20^{o}C) **D**) PM100 (100% PM, 20^{o}C). Scale bar 50 µm.

4.5. DISCUSSION

This experiment was designed to examine the effects of FM protein replacement with PM protein in rainbow trout cultured at an optimal and high temperature. An underestimation of maximum FM replacement can occur in nutrition studies and to overcome this problem, correct statistical methods are needed (Shearer, 2000). Approaches that are based on analysis across a broad range of dietary inclusion are required (Shearer, 2000) and in the present study two different statistical analyses were performed; a polynomial analysis (Zeitoun et al., 1976) and a breakpoint analysis (Robbins et al., 1979). Both models were used to predict the maximum dietary inclusion of PM, however the models use the data differently and the interpretation and practical use of the predicted maximum inclusion is different as will be discussed

4.5.1 Growth

The polynomial analysis was performed in order to determine the maximum PM inclusion level above and below which there was a decrease in the growth performance parameter. This, in effect, predicts the optimum dietary inclusion although both precision and the practical value depend on how much of a peak there is in the data (Zeitoun et al., 1976). As the model only determines the maximum response of the fish the disadvantage with this method is that the calculated maximum replacement percentage is often quite low and therefore the aim of reducing the FM at a high percentage in the diet may not achievable. Increasing the percentage of replacement beyond the calculated maximum point may slightly decrease the fish growth. This indicates that the level of FM replacement can be increased after the maximum point until it reaches a certain point at which negative effects are observed. One approach to overcome this limitation is to use confidence limits to define the range in the independent variable over which

the dependent variable is considered not different to the maximum response (Katersky and Carter 2007). Alternatively the highest value in the independent variable (PM inclusion) can be estimated using break point analysis.

Break point analysis was used to determine the maximum inclusion level of PM above which there was a significant negative effect on the measured growth parameter. The horizontal line represents nutrient sufficiency and the descending line represents a decrease in response when the PM inclusion is increased. As noted above, the two models predict maximum inclusion but the values require interpretation in relation to the distribution of the data and how the maximum value is to be used. This indicated more parameters need to be considered before the maximum replacement can be decided for rainbow trout when the research involved the replacement of FM. In the present experiment, only responses measured in terms of FW, FL, FI, WG, FCR and SGR were found to be a significantly different with FM replacement at both temperatures. All of these parameters were representative for growth of fish which are important parameters when evaluating the effects of nutrition in fish. The problem with this model is that the replacement does not represent the maximum effect on the measured parameter.

Robbins, et al. (1979) also reported that break point analysis method frequently underestimates the requirement and this was supported by Baker (1986). The only advantage in the break point analysis is the percentage of replacement is high compared to the polynomial regression. In the present study, all data were analysed and elaborated using both models in order to compare the differences between maximum replacement and effects or maximum replacement without adverse effects on fish.

Another factor to consider is the duration of the experimental period. For example there is still a chance that negative effects of the maximum replacement of FM on rainbow trout will become apparent if the rearing period is extended to more than 16 weeks. The observations during the experimental periods showed that fish fed a diet where FM protein was gradually replaced by PM protein showed a decrease in growth, and although no significant relationship may become more prominent as the culture duration is extended. If the culture period is more than 16 weeks, the inclusion level of PM may need to be reduced to a lower level. Dissolved oxygen (DO) was maintained at above 5 mg L^{-1}. Barnes et al. (2011) explained that increasing water temperature decreases dissolved oxygen in water and demonstrated the critical oxygen threshold for salmon at 18 and 22^{o}C was 3.4 and 4.6 mgL^{-1}, respectively.

Feed intake is clearly an important determinant of fish growth and influenced by many nutritional factors (Jobling et al., 2001; Toften and Jobling, 1997). The polynomial regression analysis revealed that the inclusion level of PM for maximum FI was higher at 20^{o}C compared to 15^{o}C, although in break point analysis, the maximum inclusion levels of PM was higher at 15^{o}C compared to 20^{o}C. Various studies have determined the relationship between FM replacement and feed intake at various temperatures (Gomes et al., 1993; Oliva-Teles et al., 1994; de Francesco et al., 2004). A study conducted by Oliva-Teles et al., (1994) indicated that FI in rainbow trout fed with plant proteins was significantly lower compared with fish fed FM diet at 13.9^{o}C. Interestingly, another study conducted by de Francesco, et al. (2004) showed no differences in FI when rainbow trout were fed a diet consisting of plant protein as a sole protein compared to the fish fed FM diet cultured at 17^{o}C. In addition, Gomes, et al. (1993) reported no

significant differences in voluntary intake when rainbow trout were fed with an isoprotein diet; FM partially replaced by plant protein compared to FM diet cultured at 17^{o}C temperature. The differences between polynomial regression and break points models most probably relate with the nutritive value in the feed because each inclusion level will consist of a different composition of amino acids and fatty acids in the diets. Jobling et al.(2001) reported that feed acceptance is dependent upon chemical, nutritional and physical characteristics which can be influenced by feed ingredients.

The FI results are consistent with the growth efficiency results. The FER in break point analysis showed the fish utilise feed more efficiently at high inclusion levels of PM at 15^{o}C compared to 20^{o}C, however, in polynomial analysis, temperature did not appear to affect inclusion levels of PM. The reduction in growth efficiency is probably reflective of the amino acids composition in the diets. This results is supported by Bransden et al. (2001) where the PPV value decreased for a poultry feather meal diet fed to Atlantic salmon due to low quality of protein source. The same result was acquired when FM, replaced by combined PM by-product and feather meal lead to a decrease in PPV value (Steffens, 1994). A study conducted by Bureau et al., (2000) showed that when rainbow trout were fed with 20% feather meal, FER decreased which was attributed to the low level of lysine and other amino acids. The same study showed that high inclusion level of meat and bone meal decreased the digestible nutrients and led to a low FER value. These results are in agreement with a study by El-Haroun et al., (2009) who found that rainbow trout fed diets containing rendered animal protein (meat and bone meal with feather meal or meat and bone meal with poultry by-product meal) had a lower FER than that of the fish fed the control diet.

In addition, growth; WG and SGR are also affected by the replacement level of FM in fish diets (Gomes et al., 1995; Sealey et al., 2009). In the present study, decreased growth was detected when the inclusion level of PM exceed a certain point in both analyses. This is most probably related to the composition of essential amino acids in the diets.Gomes, et al. (1995) reported where a favorable SGR was found in fish fed a diet with 66% of vegetable protein supplemented with L-lysine and L-methionine. A study conducted by Kim (1997) shows the presence of high and low essential amino acid levels affected growth of rainbow trout. Generally, the levels of chemical compositions of diets were balanced, but not the levels of ingredients of the diets. For example, the fatty acids composition of the lipid fraction is not balanced since the PM also contributed to the source of oil. This may contribute to the lower growth rate of trout when FM replaced by 100% PM in the present study. This is in agreement with study conducted by Liu et al., (2004) where different oil source affected fish growth in different ways. In addition, the effect also related to an increase in metabolic rate in fish reared at high temperatures (Goddard, 1996). Metabolism increases exponentially as temperature increases within the thermal tolerance range (Jobling, 1994). In fish, differences in the metabolic rate indicate differences in thermal optima of fish (Atkins and Benfey, 2008). Metabolism includes the energy demand for many processes in fish body at given temperature (Clarke, 2003). The increasing temperature leads to increasing metabolism, which at the same time will cause an increase in fish appetite (Linton et al., 1998; Dabrowski and Guderley, 2002).

A study conducted by De Francesco et al. (2004) showed the K was positively correlated with the MFI content. Unfortunately, in the present study, the pattern was different from that

study where no interaction between K and PM, while in MFI, the interaction was found at optimum temperature. The PEV values were also not affected by the replacement of PM in both temperatures. In the present study, fish in all treatments were fed with isoenergetic diets although the energy sources were derived from different sources (FM or PM). A study conducted by Sveier et al.(1999) showed the PEV was not affected by the dietary protein concentration and qualities (different in particle size).

In the present study, body composition showed no significant interaction with FM protein replacement with PM protein at 15^{o}C, however, at 20^{o}C, a significant interaction was detected in crude protein and ash as determined by the polynomial analysis. This showed that the inclusion levels of PM influenced body composition of fish. This indicated that fish reared in high temperature sensitive with the types of protein sources. There also a correlation between protein and ash content. A study conducted by Gary (1983) showed the increase of protein in fish carcass will decrease the ash content.

4.5.2 Hormones

Circulating plasma levels of IGF- I are a useful tool to predict fish growth (Dyer et al., 2004). Hevrøy et al. (2007) demonstrated that nutritional factors influence regulation of IGF-I in fish with dietary lysine levels regulating the signal of the growth hormone (GH)-IGF system. Pierce, et al. (2005) reported that the differences in IGF-I levels in fish body are influenced by fish activity and temperature. The observed decrease in IGF-I after the maximum inclusion in the current study is most probably due to the low quality of the PM as unbalanced dietary amino acid profiles lead to a reduction in IGF-I (Gómez-Requeni, et al., 2003). Interestingly, the

significant relationship were only observed at 15^{o}C in both models, and not at 20^{o}C. In addition, elevated water temperature has a direct effect on growth hormone secretion through somatostatin of pancreatic or hypothalamic origin and leads to a decrease in plasma IGF-I (Gabillard, et al., 2005). This finding is very important in relation to FM substitution by PM in aquafeeds in the event of future predicted rises in water temperature as a result of global warming.

Plasma cortisol is a hormone which can affects fish appetite, growth rate, condition factor and food conversion efficiency (Gregory and Wood, 1999). In the present study, there was no significant interaction between level of cortisol and level of PM inclusion on rainbow trout for both polynomial and break point models. This shows that replacing FM with PM protein did not affect plasma cortisol levels in rainbow trout. This is in agreement with a study conducted by Leatherland et al. (1987) when rainbow trout were fed a diet consisting of canola meal and soybean meal, no significant difference in cortisol level was found. This indicated the level of cortisol is not influenced by the level of replacement.

In addition, there was no sign that cortisol was influenced by temperature in this experiment. This is in agreement with a study conducted by Wagner et al.(1997) where an elevated temperature of 22^{o}C did not affect the level of cortisol in rainbow trout. In the present study, the introduction of new diets did not affect cortisol levels in rainbow trout. This is most probably due to cortisol sampling at the end of the experiment allowing sufficient time for fish to adapt to the new diets. A study conducted by Leatherland et al (1987) showed levels of cortisol were significantly different in rainbow trout fed soybean and canola meal based diets

supplemented with different levels of thyroid hormone for 12 weeks. This showed that the introduction to a new diet in rainbow trout will affect the cortisol levels in fish.

4.5.3 Organ and specific effects

The liver is an important part of the fish body for biological activities and changes in the diet have been shown to alter liver morphology. Alteration of liver histology was observed when FM was replaced by alternative proteins; especially plant protein (Glencross, et al., 2004). In the current study there was no evidence to suggest that PM inclusion can affect liver histology. No pathological changes were observed in the liver of all treatments and the vacuolation in the liver was probably due to lipid droplets. A study conducted by Caballero et al. (2002) showed an increase of lipid droplets within the hepatocytes in the liver when fish oil was replaced by vegetable oil, but this result did not affect fish growth. In the present study, the diets were formulated to be isolipidic, but the sources of the lipids were derived either directly from fish oil or fat from PM. These factors probably affected the vacuolation in the liver. Moreover, high HI was associated with the high lipid content in the liver (Purchase and Brown, 2001; Figueiredo-Silva et al., 2005). In the present study, HI was not affected by temperature and this is in agreement with study conducted by Sato et al. (1983) where is no differences in HI were found when rainbow trout were cultured at 15^{o}C and 20^{o}C.

The intestine is the most important part of the fish for the absorption of nutrients. Nutrient absorption depends on the distal intestine and differences in feed quality can influence the macromorphology of intestine and therefore absorption efficiency (Urán, et al., 2008). In the present study, no significant differences in liver morphology were observed in fish fed diets with

1% to 80% inclusion level of PM for 15^{o}C and 1 to 70% inclusion level of PM at 20^{o}C when compared to PM0 control diets. However, at higher substitution, changes to the macromorphology of the intestine were observed. The main difference was an increase in the number of goblet cells (GC) and decrease in the width of supranuclear vacuoles (SNV) with reductions in size of the absorptive vacuoles. GC secrete mucus to facilitate the passage of food and to protect the mucosa by neutralising the acidity (Khojasteh et al., 2009).

The increase of goblet cell numbers changes the properties of the mucus layer and may affect the absorption of nutrients in fish, which can change the process of intracellular digestion (Ostaszewska et al., 2005). Therefore, the increase number of goblet cells in the current study may decrease the nutrient absorption (Ostaszewska, et al., 2005). SNVs are an important for the absorption of macromolecules (Rombout et al., 1985). Therefore a change in the regular alignment of SNV will affect nutrient absorption. This has been observed previously in salmonid species after feeding with soybean meal based diets (Baeverfjord and Krogdahl, 1996; Refstie et al., 2000; Urán, et al., 2008). In the present study, the effects of FM replacement were clear where the growth of fish with high PM was decreased compared to fish fed with FM diet. The observed changes in intestines macromorphology might go some way to help explain this reduced performance at high PM inclusions however; overall, the observed intestine changes were relatively minor as other measured parameters such as LP, SM and MF were not affected. For example, a study conducted by Urán, et al. (2008) showed that the changes in macromorphology of salmon intestine was observed in as little as 7 days when fish were fed with a diet consisting of soybean meal. In the present study, sampling of intestine was conducted after 16 weeks and the effects of timeline on the macrmorpholgy of intestine cannot be observed

however at that point only SNV and GC were affected in 16 weeks of experiment. To determine the effects of PM on morphological of intestine, a longer trial should be conducted in the future.

The present study showed the differences in the macromorphology were found at both temperatures. These results in agreement with previous research, where it was found the temperature can influence intestine structure and function thereby affecting the absorption of nutrients (Buddington et al., 1997; Ruyter et al., 2006). This is probably due to the high selected temperatures in the present study. A study conducted by Urán, et al., (2008) considered 12^{o}C to be a high temperature and 5^{o}C to be a low temperature. Any temperature related differences may have been lost as the current study was conducted at 15^{o}C and 20^{o}C.

In conclusion, this study has demonstrated that growth efficiency, circulating hormone levels and the macromorphology of distal intestine were affected by the level of FM replacement with PM. Interestingly; the effects of increasing levels of FM replacement on growth efficiency parameters and hormones were different between optimal and high temperatures. There were no analysis on apparent digestibility on fatty acids in this study. A study conducted by Ng et al., (2010) indicated that elevated water temperature of 20^{o}C did not significantly affect the AD of monounsaturated fatty acids and saturated fatty acids in rainbow trout. In addition, the present study provided the information regarding the prediction of the maximum inclusion levels for PM and maximum response of rainbow trout to the diets.

4.6 ACKNOWLEDGEMENTS

We would like to thank to Skretting Australia for ingredients, to Dr. Louise Ward for advice on histology and to the technical staff at the Aquaculture Centre, University of Tasmania for their assistance.

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CHAPTER 5

Evaluation of feed intake and growth of rainbow trout (*Oncorhynchus mykiss* Walbaum) fed poultry meal supplemented with L-lysine and L-histidine

5.1 ABSTRACT

This study aimed to examine the effects of total replacement of fishmeal protein (FM) with poultry meal protein (PM) supplemented with two essential amino acids on the growth performance of rainbow trout (*Oncorhynchus mykiss*) when fed either a set ration or to satiation. A diet in which PM supplied all the protein and was supplemented with essential amino acids (EAA) L-lysine and L-histidine (PMLH) was compared to PM with a non-essential amino acid (NEAA) L-glycine (PMG) and to FM diets with L-lysine and L-histidine (FMLH) or with L-glycine (FMG). All diets were formulated to be isonitrogenous (404 ± 2.2 crude protein $g \cdot kg^{-1}$ wet weight), isoenergetic (20.4 ±0.09 $MJ \cdot kg^{-1}$ DM of gross energy) and to all contain the same amounts of crystalline amino acids, fish oil and poultry fat. Rainbow trout (1.50 ±0.01 g) were held under constant conditions (15°C), and fed at either a set ration of 1.7% of body weight per day or to satiation. The results indicated significant interaction between diet and feeding regime for feed intake (FI), final weight (FW), weight gain (WG), specific growth rate (SGR), feed efficiency ratio (FER), productive energy value (PEV) and protein productive value (PPV). However, the interaction between diet and feeding regime was not significant for condition factor (K) and survival. At satiation, supplementary essential amino acids (EAA), L-lysine and L-histidine, improved the performance of FM but decreased performance of PM. In addition, L-lysine and L-histidine did not improve the growth performance of fish fed FM and PM in fixed rations. There were significant differences in the histology of the distal intestine in terms of supranuclear vacuoles (SNV) and goblet cells (GC) for PMG and PMHL diets. These differences may partially explain decreased nutrient utilisation and reduced growth efficiency results. The SNV had reduced villi width and the number of GC increased for both the PM diets. This study

indicates that the total replacement of FM with PM, even when supplemented with limiting essential amino acids L-lysine and L-histidine, is not possible for small rainbow trout.

5.2 INTRODUCTION

Fishmeal (FM) is a major protein source in aquafeeds and has the amino acid composition closest to an ideal protein source for rainbow trout (*Oncorhynchus mykiss*) (Kaushik and Seiliez, 2010). To formulate a high quality aquafeed essential amino acids must meet or slightly exceed the requirements of the cultured species (Murai, 1992; Gaylord et al., 2010). A limitation in the intake of essential amino acids will have several effects including changes in feed intake, decreased growth and decreased growth efficiency (Guillaume et al., 2001). Unlike FM, alternative protein sources such as poultry meal (PM) limit intake of some essential amino acids and the first limiting essential amino acids in PM are likely to be lysine or histidine depending on the specific PM (Steffens, 1994; Carter et al., 2002). Therefore, when formulating diets with PM, it is important to either include proteins rich in potentially limiting EAA or supplement the diet with crystalline amino acids. There have been many studies that have used this approach and crystalline amino acids have been added as supplementary ingredients in aquafeeds to test FM replacement with alternative proteins (Carter and Hauler, 2000; Cheng et al., 2003; Nang Thu et al., 2007).

The effects of crystalline EAA have been studied extensively as supplements when testing alternative protein sources in rainbow trout (Davies and Morris, 1997; Luo et al., 2006; Gaylord and Barrows, 2009). A study conducted by Poppi et al. (2011) on rainbow trout fed feather meal supplemented with a mixture of EAA showed comparable weight gain. In addition, a favorable response of rainbow trout fed crystalline EAA was found by Rodehutscord et al. (1995a). However, crystalline amino acids were not as effective when compared to protein

bound amino acids in some studies on salmonids (Rodehutscord et al., 1995b; Zarate et al., 1999; Watanabe et al., 2001).

The replacement of FM with alternative protein sources may also affect the macromorphology of the fish intestine (Burrells et al., 1999; Urán et al., 2008; Chapter 4). Furthermore, it has been shown that enteritis is a common pathological change related to soybean meal inclusion in salmonid diets (Baeverfjord and Krogdahl, 1996) and with plant proteins in general (Penn et al., 2011). According to a study conducted by Penn, et al. (2011), feeding the pea protein concentrate resulted in decreased fish weight and inflammation in the distal intestine similar to that described for soy enteritis. Chapter 4 revealed changes to the macromorphology of the distal intestine of rainbow trout and a similar analysis was included as part of the present chapter. However, these effects were only observed when FM was replaced at very high levels of PM at up to 90%.

This study aimed to examine the effects of total replacement of FM with PM supplemented with two essential amino acids on the growth performance of rainbow trout when fed either a set ration or to satiation. A diet in which PM supplied all the protein was supplemented with crystalline essential amino acids (EAA) L-lysine and L-histidine (PMLH) and compared to three diets: PM with a non-essential amino acid (NEAA) L-glycine (PMG); FM diets with L-lysine and L-histidine (FMLH); FM with L-glycine (FMG). The study was designed to test the importance of supplementary essential amino acids and take into account differences in feed intake due to diet.

5.3 MATERIALS AND METHODS

5.3.1 Experimental Diets

The diets were formulated to be isonitrogenous and isoenergetic to contain 404 ± 2.2 g CP·kg^{-1} wet weight (WW) of crude protein (CP) and 204 ±0.9 MJ·g^{-1} DM of gross energy (Table 5.1). A diet in which PM supplied all the protein and was supplemented with L-lysine and L-histidine (PMLH) was compared to diets which contained FM, L-lysine and L-histidine (FMLH); FM and L-glycine (FMG); PM with L-glycine (PMG). Up to 10% water was added to the ingredients and mixed using a Hobart mixer (Hobart Corp, Troy, Ohio, USA). The mixtures were cold pelleted with a laboratory pelletizer (California Pellet Mill Co., San Francisco, CA, USA) and oven dried for 24 hours at 30^{o}C. The diets were kept in cold storage at 4^{o}C throughout the experimental period. FM, PM, fish oil, poultry fat, L-lysine and vitamin C in the form of Stay-C were supplied by Skretting (Cambridge, Tasmania, Australia). Vitamins, minerals,carboxymethyl cellulose, bentonite, calcium phosphate, α-cellulose, L-histidine, L-glycine and ytterbium oxide were supplied by Sigma-Aldrich Pty (Sydney, Australia). Choline chloride was supplied by ICN Biomedicals, INC. (Aurora, Ohio, USA).

5.3.2 Growth Experiment

The experiment was conducted according to Ethics Approval A0009762 at the University of Tasmania (Launceston, Tasmania, Australia) in two identical freshwater recirculation systems. Each system contained 12 x 300L fibreglass tanks and was fitted with a submerged biofilter and UV sterilizer (Codabaccus et al., 2011). A total of 1728 rainbow trout (*Oncorhynchus mykiss*) with mean initial weight of 1.50 ±0.01 g were obtained from Springfield hatchery (Tasmania, Australia). The fish were randomly allocated to each of the 24 tanks with each and were stocked

with a total 72 fish each. An additional 48 fish were killed with an overdose of anaesthetic at a concentration 60 $mg.L^{-1}$ (AQUI-S New Zealand LTD, Lower Hutt, NZ) and were immediately frozen for whole body chemical composition and were pooled and analysed as an initial whole body chemical composition. During the experiment, the fish were held under constant conditions with a water temperature of 15°C, natural photoperiod and in freshwater (0 ‰ salinity). The fish were not fed for three days prior to the start of the experiment.

Tanks from both systems were randomly divided between 8 treatments (n = 3), 4 diets and 2 rations. Each diet was fed either a set daily ration of 1.7% of body weight per day (split evenly between two feeds) or fed to satiation. Both groups were fed at 0900 hours and 1600 hours for 56 days. To determine the set daily ration, all treatments were fed until satiation from day 1 to day 4. This was to measure the minimum feed consumption for all treatments. After that, 4 treatments continued to be fed until satiation and the other 4 treatments were fed 1.7% of body weight, the minimum average feed consumption was recorded for diet PMLH over the initial 4 days of feeding. In order to balance the excess feed consumption in rationed treatments which were initially fed until satiation in the first 4 days, the amount of feed was recalculated to balance the diets over a further 9 days. The bulk tank fish weight was measured every 2 weeks in order to adjust the feed ration for each tank. On days 1 and 56, all fish were anaesthetized (30 mgL^{-1} AQUI-S anaesthetic, AQUI-S New Zealand LTD. Fork length (cm) and weight (g) were then measured for all fish and samples were taken from 20 fish from each tank for whole-body chemical composition (see below).

5.3.3 Histology

Fish distal intestine was sampled for histological analysis. All dissected samples were taken immediately and rinsed in saline (9 $g.L^{-1}$NaCl). Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, cut (5 µm thick) and stained with haematoxylin-eosin. All intestinal epithelium samples were observed under a light microscope (Olympus BH-2) and the height of mucosal folds measured (Urán, et al., 2008). A semi-quantitative scoring system was used to evaluate the intestine using five parameters (Table 5.2). Each parameter was scored on a scale from 1 to 5. Images were taken to represent each diet. All images were taken using a Leica DC 300F digital camera connected to a light microscope.

5.3.4 Chemical composition

The total 20 fish per tank were analysed for whole-body chemical composition. Fish carcasses were autoclaved (Williams et al., 1995) and dried to a constant weight using a freeze drier. The chemical analysis of fish carcass, diets and faeces were performed using standard methodology. Dry matter (DM) was determined by drying sample at 135^{o}C for 2 hours (AOAC, 1995), ash content was determined by incineration in a muffle furnace at 600^{o}C for 2 hours (AOAC, 1995), total lipid was determined according to the methods of Bligh and Dyer (1959), total nitrogen was determined by Kjeldhal (crude protein content was estimated as Nitrogen ((N) X 6.25) and gross energy was determined by an auto bomb calorimeter.

5.3.5 Calculations

The following equations were used to calculate weight gain (WG), specific growth rate (SGR), feed intake (FI), feed efficiency ratio (FER), condition factor (K), productive protein value (PPV) and productive energy value (PEV).

Weight gain (WG) (g) = Final weight – Initial weight (1)

Specific growth rate (SGR) (%·d^{-1}) = [(ln final weight (g) – ln initial weight (g))·

Time (Days)$^{-1}$] * 100 (2)

Feed intake (FI) (mg g^{-1} day^{-1}) = ((Feed consumption*1000)·((Final weight + Initial Weight)·2^{-1})

^{1})·Day^{-1} (3)

Feed efficiency ratio (FER) (g g^{-1}) = (wet weight gain (g)·total food consumption (g)$^{-1}$) (4)

Condition factor (K) = (whole wet weight (g))·(fork length (cm)^{3})$^{-1}$ * 100 (5)

Protein productive value (PPV) (%) = (fish protein gain (g Crude Protein)·total protein (g Crude

Protein)$^{-1}$) * 100 (6)

Productive energy value (PEV) (%) = (fish energy gain (g MJ)·total energy (g MJ)$^{-1}$)

* 100 (7)

5.3.6 Statistical analysis

Data are presented as mean ± standard error. All data were analysed using SPSS Statistical Analysis Software Program (version 17.0 for Windows). Two-way analysis of variance (ANOVA) was performed to evaluate all data based on diet and feeding regime. Where there was a significant interaction between diets and feeding regime one-way ANOVA was used followed by a Tukey's multiple range test to compare means among treatments. Levene's test

was used to test the homogeneity of variance. Results with probability values less than 0.05 were considered as significant. The histological scoring results and tests of equality of score medians among the groups were analysed by Kruskal-Wallis One-way ANOVA. A multiple comparisons test with mean ranks (Student-Newman-Keuls, $\alpha = 0.05$) was used to determine the differences of post hoc in order to compare mean ranks for all diets (Knudsen et al., 2008).

Table 5.1. Ingredients and chemical composition of experimental diets (mean ±standard error) fed either to satiation or at a set ration with a fishmeal or poultry meal based diets supplemented either with L-glycine or L-lysine and L-histidine

Ingredient composition (g kg^{-1})	Diets			
	FMG	FMLH	PMG	PMLH
Fishmeal	598.0	598.0	0.00	0.00
Poultry meal	0.00	0.00	600.0	600.0
Pre-gelatinized starch	155.0	155.0	155.0	155.0
Fish oil	60.0	60.0	117.0	117.0
Poultry fat	91.0	91.0	0.00	0.00
Carboxyl methyl cellulose (CMC)	10.0	10.0	10.0	10.0
Alpha cellulose	30.0	30.0	30.0	30.0
Bentonite	7.3	3.7	39.3	35.7
Choline chloride	0.7	0.7	0.7	0.7
Vitamin C	3.0	3.0	3.0	3.0
Vitamin Premix[a]	3.0	3.0	3.0	3.0
Mineral Premix[b]	3.0	3.0	3.0	3.0
Ytterbium oxide	1.0	1.0	1.0	1.0
Calcium phosphate	10.0	10.0	10.0	10.0
L-Histidine	0.0	16.0	0.0	16.0
L-Lysine	0.0	15.6	0.0	15.6
L-Glycine	28.0	0.0	28.0	0.0
Chemical composition (g kg^{-1} DM)				
Dry matter	887.5 ±10.3	890.3 ±13.2	907.4 ±11.2	901.3 ±11.4
Crude protein	401.5 ±3.3	406.3 ±10.2	406.5 ±0.5	405.6 ±2.0
Total lipid	225.4 ±7.6	217.1 ±4.4	215.5 ±2.7	219.3 ±3.2
Ash	122.2 ±0.6	117.4 ±0.05	115.7 ±3.2	119.6 ±5.4
Gross energy (MJ kg^{-1})	20.3 ±0.30	20.3 ±0.20	20.6 ±0.19	20.5 ±0.09

Means with no superscripts were not significantly different ($p>0.05$) between diets.

[a] Vitamin premix (g kg^{-1}): Vitamin A (4.50), Vitamin D3 (5.40), Rovimix E50 (90.00), Menadione sodium bisulphate (1.80), Riboflavin (3.60), Calcium D-pantothenate (19.57), Nicotinic acid (9.00), Vitamin B12 (0.01), D-biotin (0.14), Folic acid (0.90), Thiamin HCL (1.01), Pyridoxine HCL (3.29), myo-Inositol (270.00), α-cellulose (490.79)

[b] Mineral premix (g kg^{-1}):$CuSO_4.5H_2O$ (23.58), $FeSO_4.7H_2O$ (363.10), $MnSO_4.H_2O$ (61.51), Na_2SeO_4 (0.66), $ZnSO_4.7H_2O$ (131.94), KI (1.44), $CoSO_4.7H_2O$ (9.54), α-cellulose (408.23)

FMG = Fishmeal with L-glycine
FMLH = Fishmeal with L-lysine and L-histidine
PMG = Poultry meal with L-glycine
PMLH = Poultry meal with L-lysine and L-histidine

5.4. RESULTS

There was a significant interaction between diet and feeding regime for final weight (FW) (F=1003.78; df=3,16; p=0.00), weight gain (WG) (F=1050.58; df=3,16; p=0.00), specific growth rate (SGR) (F=256.80; df=3,16; p=0.000) and feed intake (FI) (F=86.03; df=3,16; p=0.00) (Table 5.3). The comparison of individual means showed the highest FW and WG were for fish fed FMLH diet to satiation. In contrast, the lowest FW and WG were for fish fed PMLH diet to satiation and for fish fed PMG and PMLH fed to a set ration. In addition, fish fed FMG and FMLH diets to satiation had the highest SGR. Conversely, the lowest SGR was for fish fed PMLH diet to satiation and for fish fed PMG and PMLH fed to set ration. At satiation, the highest FI were for fish fed FMG and FMLH diets and the lowest FI was for fish fed PMLH diet (Table 5.3).

There were significant dietary and feeding regime differences in condition factor (K) (F=2.45; df=3,16; p=0.10) (Table 5.3) with fish fed to satiation having higher K values and fish fed FMLH had the higher K value in satiation. In addition, no significant differences was found in survival (F=1.99; df=3,16; p=0.16) (Table 5.3).

The interaction between diet and feeding regime was significant for dry matter (DM) (F=18.81; df=3,16; p=0.000); crude protein (CP) (F=8.35; df=3,16; p=0.001); total lipid (TL) (F=31.05; df=3,16; p=0.000); and gross energy (GE) (F=32.45; df=3,16; p=0.000) (Table 5.4). DM, CP, TL and GE were higher in fish fed FMG and FMLH to satiation. However, there was no significant interaction between diet and feeding regime for ash (F=0.12; df=3,16; p=0.947) (Table 5.4).

There was a significant interaction between diet and feeding regime for productive energy value (PEV) (F=5.54; df=3,16; p=0.008) and feed efficiency ratio (FER) (F=16.00; df=3,16; p=0.000) (Table 5.5). The highest PEV for fish fed FMG and FMLH diets fed to satiation. The lowest PEV was found for fish fed PMLH diet fed to satiation and fish fed PMG and PMLH diets fed to a set ration. Furthermore, the highest FER was for fish fed FMLH to satiation, FMG and FMLH fed at a set ration. In contrast, the lowest FER was for fish fed PMLH to satiation and PMG fed to set ration. There were significant dietary and feeding regime differences in protein productive value (PPV) (F=1.71; df=3,16; p=0.205) (Table 5.5) with fish fed to set ration having higher PPV values and fish fed FMG and FMLH diets showed the highest PPV in both feeding regimes.

Changes in the histology of the distal intestine were associated with the different treatments and were related to the irregular shape and size of supranuclear vacuoles (SNV) and the increased number of goblet cells (GC) among the enterocytes (Fig. 5.1). The SNV had reduced villi width and the number of GC increased for both the PM diets. There were no differences in the size of sub-epithelial mucosa (SM), lamina propria (LP) and mucosal folds (MF) among treatments (Table 5.6). The LP, MF and SM showed the normal form for all treatments.

Table 5.2 Histological scoring system for morphological changes by soybeans in the distal intestine of Atlantic salmon (*Salmo salar* L.) (Urán, et al., 2008) adapted in the current study to investigate the effects of total replacement of FM with PM supplemented with amino acids on the morphology of distal intestine of rainbow trout (*Oncorhynchus mykiss*) fed at a set ration

Score	Parameter	Score	Parameter
	Mucosal folds (MF)		**Supranuclear vacuoles (SNV)**
1	Basal length	1	Basal SNV size
2	Some shrinkage and bloating	2	Some size reduction
3	Diffused shrinkage and onset of tissue disruption	3	Diffused size reduction
		4	Onset of extinction
4	Diffused tissue disruption	5	No SNV
5	Total tissue disruption		
	Goblet cells (GC)		**Sub-epithelial mucosa (SM)**
1	Scattered cells	1	Normal SM
2	Increased number and sparsely distributed	2	Increased size SM
		3	Medium size SM
3	Diffused number widely spread	4	Large SM
4	Densely grouped cells	5	Largest SM
5	Highly abundant and tightly-packed cells		
	Lamina propria (LP)		
1	Normal size LP		
2	Increased size of LP		
3	Medium size LP		
4	Large LP		
5	Largest LP		

Table 5.3: Growth response and feed utilization (mean ±standard error) of rainbow trout (*Oncorhynchus mykiss*) fed either to satiation or at a set ration with a fishmeal or poultry meal based diets supplemented either with L-glycine or L-lysine and L-histidine

	Treatments								*Two Way ANOVA*	
	Satiation				Ration					
	FMG	FMLH	PMG	PMLH	FMG	FMLH	PMG	PMLH	*Factor effects*	*P*
IW (g)	1.5 ±0.02	1.5 ±0.01	1.5 ±0.01	1.5 ±0.01	1.5 ±0.02	1.5 ±0.02	1.5 ±0.01	1.5 ±0.02	Feeding regime Diet Interaction	*0.198* *0.474* *0.825*
FW (g)	14.2 ±0.16^{b}	15.5 ±0.17^{a}	3.5 ±0.31^{c}	2.2 ±0.02^{d}	3.4 ±0.01^{c}	3.4 ±0.05^{c}	2.5 ±0.02^{d}	2.2 ±0.07^{d}	Feeding regime Diet Interaction	*0.000* *0.000* *0.000*
WG (g)	12.7 ±0.16^{b}	14.0 ±0.17^{a}	2.0 ±0.31^{c}	0.7 ±0.02^{d}	1.9 ±0.03^{c}	1.9 ±0.05^{c}	1.0 ±0.02^{d}	0.7 ±0.05^{d}	Feeding regime Diet Interaction	*0.000* *0.000* *0.000*
SGR (% day^{-1})	4.3 ±0.03^{a}	4.4 ±0.02^{a}	1.6 ±0.17^{b}	0.8 ±0.02^{c}	1.59 ±0.03^{b}	1.59 ±0.03^{b}	0.7 ±0.04^{c}	1.0 ±0.02^{c}	Feeding regime Diet Interaction	*0.000* *0.000* *0.000*
FI (mg g^{-1} day^{-1})	31.8 ±0.76^{a}	30.3 ±1.01^{a}	23.0 ±1.06^{b}	16.0 ±0.20^{c}	13.5 ±0.09^{c}	13.3 ±0.15^{c}	14.5 ±0.06^{c}	14.4 ±0.06^{c}	Feeding regime Diet Interaction	*0.000* *0.000* *0.000*
K	1.3 ±0.04*^{xy}	1.4 ±0.04*^{x}	1.3 ±0.06*^{xy}	1.2 ±0.02*^{y}	1.2 ±0.01^{x}	1.3 ±0.02^{x}	1.1 ±0.04^{x}	1.2 ±0.03^{x}	Feeding regime Diet Interaction	*0.001* *0.005* *0.101*
Survival (%)	97.6 ±1.72	98.6 ±0.83	97.1 ±1.43	99.0 ±0.48	99.5 ±0.48	98.1 ±0.48	99.5 ±0.48	97.6 ±0.48	Feeding regime Diet Interaction	*0.375* *0.991* *0.156*

Means with superscripts (a,b,c,d) represent an interaction effect. Means with similar or no superscripts are not significantly different ($p>0.05$) between diets.

Means with superscripts (x,y) represent differences between diets. Means with similar superscripts not significantly different ($p>0.05$) between diets.

*Represent feeding regime effects

FMG	=	Fishmeal with L-glycine
FMLH	=	Fishmeal with L-lysine and L-histidine
PMG	=	Poultry meal with L-glycine
PMLH	=	Poultry meal with L-lysine and L-histidine
IW (g)	=	Initial weight
FW (g)	=	Final weight
WG (g)	=	Weight gain
SGR (% d^{-1})	=	Specific growth rate
FI (mg g^{-1} day^{-1})	=	Feed intake
K	=	Condition factor

Table 5.4: Chemical composition (mean ±standard error) of rainbow trout (*Oncorhynchus mykiss*) fed either to satiation or at a set ration with a fishmeal or poultry meal based diets supplemented either with L-glycine or L-lysine and L-histidine (g kg^{-1} of wet weight)

	Treatments								*Two Way ANOVA*	
	Satiation				Ration					
	FMG	FMLH	PMG	PMLH	FMG	FMLH	PMG	PMLH	*Factor effects*	*P*
Dry Matter (g kg^{-1} WW)	235.9 ±4.1^{a}	245.8 ±9.1^{a}	158.3 ±10.2^{bc}	128.5 ±7.7^{c}	159.0 ±8.1^{bc}	167.4 ±2.2^{b}	136.9 ±4.7^{bc}	134.4 ±4.1^{bc}	Feeding regime Diet Interaction	*0.000* *0.000* *0.000*
Crude Protein (g kg^{-1} WW)	99.9 ±0.7^{a}	104.5 ±1.7^{a}	80.8 ±3.8^{bc}	73.1 ±1.4^{d}	87.4 ±3.8^{bc}	89.8 ±0.7^{b}	73.8 ±1.4^{bcd}	78.2 ±1.0^{cd}	Feeding regime Diet Interaction	*0.000* *0.000* *0.001*
Total Lipid (g kg^{-1} WW)	121.0 ±5.3^{a}	123.8 ±7.1^{a}	56.8 ±6.5^{b}	33.5 ±2.0^{b}	49.4 ±3.1^{b}	46.2 ±8.1^{b}	39.9 ±1.5^{b}	36.4 ±1.6^{b}	Feeding regime Diet Interaction	*0.000* *0.000* *0.000*
Ash (g kg^{-1} WW)	17.1 ±0.7	17.2 ±0.4	16.0 ±1.1	17.2 ±2.3	17.4 ±1.9	16.9 ±0.3	15.0 ±0.7	16.2 ±1.0	Feeding regime Diet Interaction	*0.574* *0.539* *0.947*
Energy (MJ kg^{-1} WW)	6.82 ±0.12^{a}	7.1 ±0.31^{a}	4.1 ±0.3^{bc}	3.1 ±0.15^{d}	4.0 ±0.22^{bc}	4.3 ±0.03^{b}	3.4 ±0.06^{bcd}	3.3 ±0.10^{cd}	Feeding regime Diet Interaction	*0.000* *0.000* *0.000*

Means with similar or no superscripts were not significantly different (p>0.05) between diets.

FMG = Fishmeal with L-glycine
FMLH = Fishmeal with L-lysine and L-histidine
PMG = Poultry meal with L-glycine
PMLH = Poultry meal with L-lysine and L-histidine

Table 5.5: Productive protein value, productive energy value and feed efficiency ratio (mean ±standard error) of rainbow trout (*Oncorhynchus mykiss*) fed either to satiation or at a set ration with a fishmeal or poultry meal based diets supplemented either with L-glycine or L-lysine and L-histidine

	Treatments								*Two Way ANOVA*	
	Satiation				Ration					
	FMG	FMLH	PMG	PMLH	FMG	FMLH	PMG	PMLH	*Factor effects*	*P*
PPV (%)	28.4 ±1.02*^{x}	31.2 ±1.38*^{x}	16.8 ±1.92^{*y}	12.6 ±0.42^{*y}	33.8 ±2.12^{x}	35.1 ±0.28^{x}	16.8 ±0.70^{y}	15.9 ±0.75^{y}	Feeding regime Diet Interaction	*0.003* *0.000* *0.205*
PEV (%)	39.1 ±1.50^{ab}	43.4 ±3.25^{a}	17.0 ±2.47^{d}	7.6 ±1.02^{e}	29.1 ±2.60^{c}	32.4 ±0.29^{bc}	13.6 ±0.75^{de}	10.1 ±0.89^{de}	Feeding regime Diet Interaction	*0.001* *0.000* *0.008*
FER (%)	1.0 ±0.03^{b}	1.0 ±0.03^{ab}	0.6 ±0.05^{c}	0.5 ±0.01^{d}	1.1 ±0.02^{a}	1.1 ±0.01^{a}	0.5 ±0.03^{d}	0.7 ±0.01^{c}	Feeding regime Diet Interaction	*0.001* *0.000* *0.000*

Means with superscripts (a,b,c,d, e) were represent an interaction effects and with similar or no superscripts not significantly different ($p>0.05$) between diets.

Means with superscripts (x,y) were represent a different between diet effects and with similar superscripts not significantly different ($p>0.05$) between diets.

*Represent feeding regime effects

FMG = Fishmeal with L-glycine
FMLH = Fishmeal with L-lysine and L-histidine
PMG = Poultry meal with L-glycine
PMLH = Poultry meal with L-lysine and L-histidine
PPV (%) = Protein productive value
PEV (%) = Productive energy value
FER (%) = Feed efficiency ratio

Table 5.6: Histological evaluation of distal intestine of rainbow trout (*Oncorhynchus mykiss*) (mean score ± standard error) fed a set ration with either FM or PM diets supplemented with L-glycine or L-histidine and L-lysine

Parameters	Diets			
	FMG	FMHL	PMG	PMHL
SNV	1.0 ±0.00 [b]	1.0 ±0.00 [b]	2.0 ±0.00 [a]	2.1 ±0.00 [a]
GC	1.0 ±0.00 [b]	1.0 ±0.00 [b]	1.5 ±0.00 [a]	1.3 ±0.00 [a]

Means without or with similar superscripts were not significantly different ($p>0.05$) between diets.

FMG = Fishmeal with L-glycine
FMLH = Fishmeal with L-lysine and L-histidine
PMG = Poultry meal with L-glycine
PMLH = Poultry meal with L-lysine and L-histidine
SNV = Supranuclear vacuoles
GC = Goblet cells

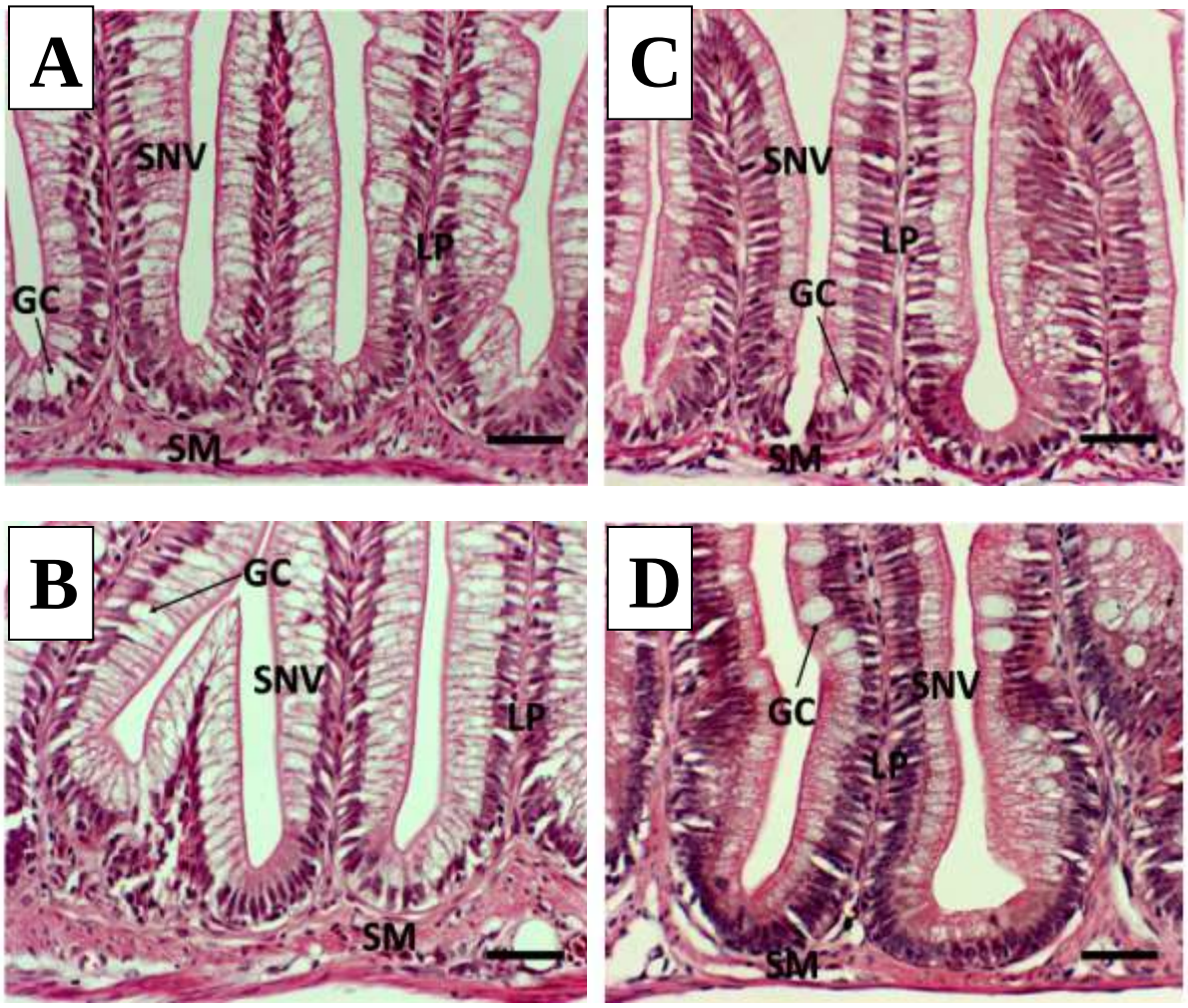

Figure 5.1: Morphological appearances of the distal intestine of rainbow trout (*Oncorhynchus mykiss*) fed a set ration with either FM or PM diets supplemented with L-glycine or L-histidine and L-lysine. A) FMG (100% FM + L-glycine) **B**) FMHL (100% FM, + L-histidine and L-lysine) **C**) PMG (100% PM + L-glycine) **D**) PMHL (100% PM + L-histidine and L-lysine). Scale bar 50 µm.

FMG = Fishmeal with L-glycine
FMLH = Fishmeal with L-lysine and L-histidine
PMG = Poultry meal with L-glycine
PMLH = Poultry meal with L-lysine and L-histidine
SNV = Supranuclear vacuoles
GC = Goblet cells
SM = Sub-epithelial mucosa
LP = Lamina propria

5.5 DISCUSSION

The present study was designed to determine the importance of several factors that may influence the successful use of PM. Several studies have evaluated the effects of using PM as a source of protein in the rainbow trout diet (Erturk and Sevgili, 2003; Sealey et al., 2011; Gaylord et al., 2010) with most of the results indicating that PM cannot be used to replace 100% of the FM. This is because of the low level of some EAA in the PM that result in the poor growth performance of fish. The most important factor in determining the efficiency of protein utilization of a protein is the EAA profile (Cowey, 1994). In the previous chapters, FM replacement was evaluated with different sized fish. The replacement up to 40% FM with PM showed no adverse effects on rainbow trout with a size of 190 g (Chapter 2).

However, the replacement up to 100% of FM with PM affected the digestibility of nutrients in 128 g rainbow trout (Chapter 3). Smaller 29 g rainbow trout successfully grew to the highest final weight of 177 g at 15^{o}C treatments on a 36.7% replacement diet for 112 days (Chapter 4). However, complete FM replacement with PM reduced the fish growth to a 115 g final weight after 112 days (Chapter 4), presumably as a result of an incomplete supply of EAA. These were interesting results which led to the current experiment. In the present chapter, smaller rainbow trout with an initial weight of 1.5 g were used in order to determine the effects of PM diets supplemented with crystalline EAA. Unfortunately, the complete replacement of FM with PM supplemented with L-lysine and L-histidine did not increase rainbow trout growth. A previous study conducted by Sealey et al.(2011) indicated that rainbow trout with initial weight of 0.5 g showed the same final weight gain when fed either a control diet or a diet consisting of a poultry by-product blend as the sole protein. Interestingly, the quality of poultry meal by-

product blend used in that study was defined as ‘high’ because there were no limiting amino acids (Sealey, et al., 2011). Unfortunately, in the present study, the PM was not of high quality and was deficient in lysine and histidine. The information of EAA in PM is based on a study conducted by Carter et al., (2002) and EAA requirements of rainbow trout (NRC, 1993).

The previous chapters evaluated the effects of PM in the diets of rainbow trout using different sizes of rainbow trout where the results showed unfavorable results on fish growth and apparent digestibility of nutrients. The same PM was used in the present study and supplemented with EAA. The results indicated the growth of fish fed with PM diet was lower than fish fed a FM diet even when the diet was supplemented with crystalline L-lysine and L-histidine. This is comparable with study conducted by Steffens (1994) using rainbow trout with initial weight of 16.7 g fed with PM supplemented with EAA (methionine and lysine) in which growth of fish was reduced compared to fish fed a control diet. Another study conducted by Santigosa et al. (2011) using 19 g rainbow trout indicated the replacement of FM with plant protein supplemented with crystalline EAA decreased the total uptake and nutrient absorption of rainbow trout. In addition, Davies and Morris (1997) indicated that when 50g rainbow trout were fed with soya based diets supplemented with crystalline amino acids no improvement in growth rate was observed compared to the fish fed FM as a sole protein. All these results suggest a difference in fish sizes would not influence the effects on alternative protein sources supplemented with EAA.

In the present study, the supplementations of L-lysine and L-histidine in the diets were adjusted to meet the requirements of rainbow trout and glycine was added to the control diets.

The complete replacement of FM with PM supplemented with L-lysine and L-histidine resulted in the same growth performance as fish fed PM supplemented with L-glycine. However, low growth performance was found when compared with fish fed FM diet supplemented with L-lysine and L-histidine or L-glycine. This indicates that the primary problem was caused by PM in the diets. In addition, EAA supplementation showed no improvement in growth performance and feed efficiency of rainbow trout. The total replacement of FM by PM but in which the limiting EAA was supplemented; suggest that factors other than balanced EAA may play a negative role in fish growth.

This indicated that supplementation of crystalline amino acids did not significantly affect the growth of fish fed diets with PM supplemented either with L-lysine and L-histidine or L-glycine fed either satiation or at a set ration. There was, however, no apparent indication that the supplementation of crystalline amino acids stunted the growth of rainbow trout. In the current research lysine and histidine were calculated as the two most limiting EAA in the PM used and were added in crystalline form. In general, lysine is the first limiting essential amino acid (EAA) in many alternative protein sources, including PM, used to replace FM in aquafeed (Hauler and Carter, 2001). The lysine level significantly affects fish growth performance and health (Hauler and Carter, 2001; Hauler et al., 2007; Peng et al., 2009). Lysine is an important substrate for synthesis of carnitine, which is required for the transport of long chain fatty acids from the cytosol into mitochondria for oxidation (Peng, et al., 2009). Carnitine is also important as a growth promoter, providing protection against toxic levels of ammonia and xenobiotics, alleviating stress due to extremes of water temperature and facilitating better acclimation to water temperature changes (Harpaz, 2005).

Additionally, histidine is also an important EAA in fish because it is involved in one-carbon unit metabolism and can affect deoxyribonucleic acid (DNA) and protein synthesis (Peng et al., 2009). Histidine can also provide the energy fuel during starvation and is the major component of the non-carbonate buffering system which can protect fish against changes in pH resulting from hypoxia, burst-swimming and lactacidosis (Peng et al., 2009). In addition, it also acts as a precursor for bioactive chemicals such as histamine, carnosine and anserine (Glover and Wood, 2008). Studies on histidine supplementation in rainbow trout are limited and supplementing histidine in PM-based diets has not been investigated.

The effects of supplementing EAA in the diets has been studied in FM replacement with alternative protein sources in rainbow trout and Atlantic salmon (Cheng, et al., 2003; Nang Thu, et al., 2007). These studies showed that supplementing EAA to alternative protein sources promoted fish growth. However, the alternate protein source investigated was not PM. In another study, where a combination between PM and feather meal were used as alternate protein sources in the diet of rainbow trout, supplementation with EAA (methionine and lysine) did not result in better growth of fish (Steffens, 1994). Similarly, in this present study, the supplementation of EAA (L-lysine and L-histidine) in diets for rainbow trout where PM was used as alternate protein source resulted in reduced growth. This indicates that the crystalline amino acids had a limitation and lack the ability to sustain good growth of rainbow trout.

This was explained by Ambardekar et al. (2009) where the efficiency of using crystalline amino acids in the diet could be affected by the rate at which intact proteins in a mixture of

dietary ingredients are digested. This is because the differences in the time course of absorption of different amino acids could reduce the efficiency of protein synthesis if the dietary purified amino acids are absorbed and catabolized before protein-bound amino acids are released by digestion (Ambardekar, et al., 2009). This was indicated in few studies where the crystalline amino acids in the diet might not be utilized efficiently compared to the protein bound amino acids because of temporal differences in the peak absorption of amino acids from the two sources (Thebault, 1985; Cowey and Walton, 1988). Besides, it has been previously shown that the supplementation of crystalline amino acids was not able to sustain fish growth (NRC, 1993; Cowey, 1994). In the present study, EAAs were added in order to determine the effects of EAAs on small size of fish compared to the others studies which used large size fish as an experimental fish. To avoid the use of crystalline amino acids, it has been proposed to instead use protein-bound amino acids (Hauler and Carter, 2001). A few studies have been conducted using different species of aquatic animals to determine the effects of protein-bound lysine (Berge et al., 1994; Zarate, et al., 1999; Fox et al., 2009). A study conducted by Zarate et al. (1999) on channel catfish (*Ictalurus punctatus*) showed that fish fed a diet containing protein-bound lysine from soybean meal had a greater weight gain compared to fish fed diet supplemented with free lysine. Another study conducted by Berge et al.(1994) indicated that Atlantic cod (*Gadus morhua*) showed the greatest retention of lysine in the muscle tissue when fed with lysine bound into intact protein. In light of above discussion, it would be of interest to further investigate the effect of supplementing EAA in PM as protein-bound.

In the present study, the comparison between satiation feeding and restricted ration showed that fish fed PMLH and PMG had the lowest growth performance. It appeared as though

the reduced growth performance was linked to a reduction in feed intake which may be influenced by several factors. In the present study, the reduction in feed consumption in fish fed PM diet was most probably due to the fish not being able to adapt to a new alternative protein source (Carter and Davies, 2004). Despite PM having a high protein content, it cannot be used as a sole protein in fish diet because of the quality of the PM. The composition, freshness of the raw material and the processing method influence the quality of PM (Dong et al., 1993). In addition, the problem to PM may also relate with the presence of a potential deterrent in the diets. In the present study, the existence of a deterrent is possible since PM is an animal origin protein. The total volatile nitrogen and biogenic amine concentration can influence the quality of protein of animal origin (Jobling et al., 2001). Reduced feed intake has been found when rainbow trout have been fed a diet which contained feather meal (Poppi, et al., 2011) and this was associated with the palatability which directly affected the feed intake and feed utilization (Glencross et al., 2007).

In the present study, when fish were fed to a set ration, growth was lower in the two PM-based diets even though feed intake was the same as the fish fed FM diets. Therefore, besides feed intake, there were clearly other factors influencing fish growth. The differences between fish fed PM-based diets and FM-based diets also showed fish can utilize FM diets better than PM diets. This was also explained in the chapter 3 where the apparent digestibilities of nutrients decreased with increasing inclusion level of PM in the diets. Steffens (1994) reported that low weight gain and SGR were found when FM was completely replaced by a combination of poultry by-product meal and feather meal supplemented with lysine when rainbow trout were fed at a set ration. In this study, Steffens (1994) indicated the decline in fish growth was caused by

the low level of lysine in the diet of rainbow trout. The same results were reported in a study conducted by Teskeredžić et al., (1995) where no differences were found in the feed intake when steam-dried whole herring meal was totally replaced by rapeseed protein concentrate, but this significantly reduced the growth rate, feed efficiency and protein and energy utilization of rainbow trout.

The effects of FM replacement with plant protein on the intestines of salmonids have been reported (Knudsen, et al., 2008; Urán, et al., 2008; Borquez et al., 2011). This is in agreement with a study conducted by Borquez et al.(2011) which indicated a reduction in the number of basophils and abundant numbers of lipid drops were observed in the intestine of rainbow trout fed a diet with FM and lupin meal supplemented with crystalline amino acids, L-lysine and DL-methionine. Interestingly, in a study conducted by Heikkinen et al.(2006) morphological changes in trout intestine were observed when fish were fed with a diet consisting of soybean meal supplemented with crystalline amino acids, L-lysine and DL-methionine, but despite the morphological changes, the growth of fish was not affected. In the present study, histological analysis on distal intestine exhibited morphological changes in the intestine including a decrease in the width of SNV and increased number of GC. Both of GC and SNV had an important function in salmonid which the GC secrete mucus to facilitate the passage of food and to protect the mucosa by neutralising the acidity (Khojasteh et al., 2009) and SNVs are an important for the absorption of macromolecules (Rombout et al., 1985). The increasing number in GC influenced absorption of nutrients due to changes in the mucus layer (Ostaszewska et al., 2005). The same effects on SNV which changes the regular alignment of SNV will also decrease nutrient absorption (Baeverfjord and Krogdahl, 1996; Refstie et al.,

2000; Urán, et al., 2008). This result was same as in chapter 4. This indicated that the supplementation of EAA in the diets not improve the quality of feed and decreased the absorption efficiency in fish. Several authors have reported histological alteration in the intestine when alternative protein sources were used to replace FM in the salmonid diets (Romarheim et al., 2006; Knudsen, et al., 2008; Urán, et al., 2008). Romarheim, et al. (2006) reported that abnormal tissue consistent with the criteria of soybean meal induced enteritis of salmon described by Urán, et al. (2008) was found in the distal intestine of rainbow trout fed with soybean meal and white flakes diets. This happens because of decreased carrier-mediated transport and permeability of the distal epithelium. At the same time, the higher trypsin activity in the distal intestine may be caused by increased secretion from the pancreas or by decreased degradation and reabsorption in the digestive tract (Romarheim, et al., 2006). A study conducted by Knudsen, et al. (2008) indicated that soya saponin concentrate also caused the inflammatory reaction in distal intestine of the Atlantic salmon. This is because soya saponins trigger the onset of enteritis through increased epithelial permeability, which exposes the local immune system to foreign antigens from the gut lumen, leading to an inflammation reaction.

The present study indicates that the complete replacement of FM with PM supplemented with L-lysine and L-histidine did not positively affect the growth of rainbow trout. This showed it is not possible to improve the quality of PM as an alternative protein source for rainbow trout through the use of crystalline L-lysine and L-histidine. In addition, the size of fish may also influence the results. The present study indicated that small fish are not able to utilize diets supplemented with EAA efficiently. There also the possibility of fish to utilize experimental

diets if larger fish. In addition, PM in the diet changed the macromorphology of intestine and the complete replacement of FM with PM is not suitable for fish intestine.

5.6 ACKNOWLEDGEMENTS

We would like to thank to Skretting Australia for ingredients, to Dr. Louise Ward for advice on histology and to the technical staff at the Aquaculture Centre, University of Tasmania for their assistance.

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CHAPTER 6

General Discussion

6.1 Overview

This research, involving a series of four separate experiments, was designed to evaluate poultry meal (PM) as an alternative to fishmeal (FM) in aquafeeds for rainbow trout (*Oncorhynchus mykiss*). Rainbow trout was used because its nutrition is well understood (Willoughby, 1999) and does not require a large proportion of dietary protein to be supplied from FM (Farhangi and Carter, 2001; Sealey et al., 2011; Burr et al., 2012). An initial experiment was performed to determine whether partial protein replacement with PM was possible in rainbow trout diets and to expand previous research in this area by incorporating an investigation of insulin-like growth factor-I (IGF-I) and cortisol to investigate endocrine correlates of growth and stress. The aim of these additional measurements was to further explore the relationships between nutrient availability, and hormone systems influencing growth and growth performance.

The initial study (Chapter 2) indicated that FM protein can be replaced by at least 40% PM protein, the maximum used in the experiment, without affecting fish growth or fish hormones such as cortisol and IGF-I; this stimulated further research investigating the effects of gradual replacement of FM up to 100%. In the second experiment, the graded replacement of FM by PM did not result in significant differences in AD crude protein or AD total lipid following three adaptation times, 3, 9 and 27 days. Adaptation time has rarely been studied and the present study provided an important validation of using the more standard time of 9 days (Glencross et al 2007) for protein and lipid. However, AD gross energy increased over time. This was most probably explained by a change in carbohydrate digestibility from pre-gelatinized starch and carbohydrate in the diets with PM (Storebakken et al., 1998). In the third experiment, an additional environmental factor (water temperature) was investigated in light of future concerns

regarding the culture of salmonids at increasingly high water temperatures resulting from climate change (Miller et al., 2006; Ng et al., 2010). This study indicated that fish reared at an optimal temperature (15^{o}C) displayed better performance at high dietary PM inclusion measured in terms of final weight, final length, feed intake, weight gain, specific growth rate and feed efficiency ratio compared to fish reared at high temperature (20^{o}C). This was explained because the growth efficiencies will decrease if the temperature is too high and metabolic expenditure increases (Katersky and Carter 2005).

Histology of the distal intestine also showed a decreased width of supranuclear vacuoles and increased numbers of goblet cells in fish fed diets containing greater than 90% of PM at both temperatures. This was the first time that intestinal morphology has been investigated and it is important in order to understand the effects of PM on distal intestine of rainbow trout. Finally, the last experiment focused on the quality of PM, particularly that of the essential amino acid (EAA) profile and supplementation of EAA in rainbow trout diets. This study indicated that total replacement of FM with PM, even when supplemented with limiting essential amino acids L-lysine and L-histidine, was not possible for rainbow trout. In addition, PM in the diets affected the macromorphology of the distal intestine with decreased width of supranuclear vacuoles and increased numbers of goblet cells occurring in fish fed PM diets. This thesis indicated that PM can be used to replace FM up to 90% without any significant impact on the fish intestine. In addition, the supplementation of EAA in PM diets will not improve the quality of fish feed for rainbow trout.

6.2 Poultry meal as an alternative protein source in rainbow trout diet

PM has good potential as an alternative protein source in rainbow trout diets and this thesis has further explored the effects of PM on rainbow trout culture. From the present study, PM protein can be used effectively to replace up to 90% of the protein, and result in good growth performance and no change in intestinal morphology of experimental fish.This shows that the partial replacement of FM with PM is possible and this can be applied in rainbow trout culture. This is in agreement with previous research conducted in this species by Steffens, (1994) where 50% replacement of FM with PM can be utilized as well as FM in the diet. In the present study, the replacement of FM protein with PM protein at levels as high as 100% replacement and the result indicated that FM can be replaced with PM if the replacement of FM with PM not exceed 90%. This strategy can reduce the FM consumption by rainbow trout and at the same time can reduce the feed cost without impairing fish growth.

A slightly different approach was attempted here in order to determine the effects of protein source on fish physiology. Two main hormones, cortisol and IGF-I, were investigated which to my knowledge, have never been used to assess the effects of PM in the diet of rainbow trout. Feed intake has been related to hormones and a study conducted by Gregory and Wood, (1999) indicated an increasing level of plasma cortisol reduced the appetite and growth performance of rainbow trout. Circulating cortisol levels may be increased due to many factors such as differences in dietary protein source (Martins et al.,2006) and elevated water temperature affecting the oxygen level in the water (Pickering and Pottinger, 1987). In addition, IGF-I was selected as an assessment tool in this study because this hormone has been used previously as an indicator to evaluate fish growth and performance (Shimizu *et al.*, 2000). This is in agreement

with a previous study conducted by Ketelslegers *et al*. (1995) where plasma IGF-I was positively correlated with the growth of fish. Therefore any growth related impacts of PM inclusion may have been as a result of modification of the IGF axis. It was therefore unknown whether PM inclusion would result in changes to the hypothalamus-pituitary-interrenal axis (HPI), in turn affecting cortisol secretion and growth performance.

The analysis of cortisol and IGF- I levels may play an important role in fish nutrition research given the possibility that these hormones will be affected by different protein sources or other dietary manipulations. In the present study, however, the use of PM as an alternative protein source failed to result in major changes in circulating levels of cortisol or IGF-I, with the only effect noticed when PM was substituted at 100% in fish reared at optimum temperature (Chapter 4). Together with the other traditional measures used in nutrition studies (i.e. fish length, fish weight, weight gain, specific growth rate, feed intake, condition factor, productive protein value, productive energy value, feed efficiency ratio and chemicals composition of fish) this indicates that PM is a promising alternative protein source for rainbow trout.

Digestibility is an important aspect to consider when evaluating protein quality in fish feed (Gauthier et al., 1982). In addition, information on specific nutrient digestibility will provide crucial information in order to formulate efficient fish feeds (Bureau and Cho, 1999; Davies et al., 2009). In the present study (Chapter 3), nutrient digestibility was evaluated after 3, 9 and 27 days in order to determine whether fish need the adaptation time to accept the new diets. Furthermore, 9 days is a common adaptation period used in digestibility studies and this study was designed to determine whether this is an appropriate time or whether shorter studies

would also be acceptable. There were no significant differences in AD crude protein or AD total lipid between the three adaptation times. However, AD gross energy increased over time. This is most probably due to a change in carbohydrate digestibility from pre-gelatinized starch added equally to all the feeds. One potential explanation regarding decreased AD was observed changes in the macromorphology of the distal intestine when replacement of FM protein by PM protein reached 90% and above. Two main effects were observed with an increase in the number of goblet cells (GC) and decrease in the width of supranuclear vacuoles (SNV) with reductions in size of the absorptive vacuoles known to be related to nutrient absorption (Urán, et al., 2008). Although subtle, these changes could explain the reduced AD of nutrients and subsequent reduced growth performance observed at high PM inclusion.

Interestingly, despite the fact that all of the feeds were isonitrogenous and isoenergetic and balanced in all aspects of nutrient, reduced growth performance was observed when PM inclusion exceeded 90% for rainbow trout of average size 29g (Chapter 4). One potential reason for this is because the EAA levels were unbalanced due to relatively low levels of specific EAA (Steffens 1994). The first limiting essential amino acids in PM are likely to be lysine or histidine depending on the specific PM (Steffens, 1994; Carter et al., 2002). Results of Chapter 3 and 4 demonstrated unfavorable effects of high inclusion level of PM on rainbow trout. In the last experiment (Chapter 5), particular attention was focused on the EAA profile of the diets where PM diets were supplemented with crystalline EAA, L-lysine and L-histidine, in order to ensure the level of EAA was balanced in the diet. The results of this study were not clear since huge differences were observed between rainbow trout fed PM diet supplemented with L-lysine and L-histidine compared to those fed the FM diet in terms of growth. Rainbow trout with an average

initial weight 1.50 ±0.01 g were used in this study, whereas in all previous studies larger fish were used. The used of EAA in the present study was to determine either the small rainbow trout able to utilise feed with PM supplemented EAA. A study conducted by Steffens (1994) using bigger rainbow trout with initial weight 16.7g, fed with PM supplemented with EAA (methionine and lysine) showed reduced growth despite the EAA supplementation. This work and previously published studies indicate that crystalline have a limitation in order to support fish growth (NRC, 1993; Cowey, 1994). A possible reason for this is that small rainbow trout were not able to utilize crystalline EAA efficiently. This may in part be due to the temporal differences in the peak absorption of amino acids where peak levels are reached quickly compared to amino acids digested from intact proteins (Thebault, 1985; Cowey and Walton, 1988).

The replacement up to 90% FM protein with PM protein showed the high potential of PM to be used in aquafeed. In addition, complete replacement of FM protein may be a reality in the near future if processing techniques can be improved. The amino acid profile should meet the requirements of the cultured species (Murai, 1992; Drew et al., 2007; Gaylord et al., 2010). EAA deficiency will affect fish growth and feed utilization (Cowey, 1994; Anderson et al., 1995). In order to reduce the percentage of FM, partial replacement is recommended, but not total replacement using only one other protein source. Under commercial conditions several alternative protein sources would be combined to meet the specific EAA requirements. The current research demonstrates that, with the exception of small trout, the maximum inclusion level for PM is very high and theoretically, only small amounts of other protein sources are required. Whether this is achievable depends on EAA composition of these other proteins. Thus,

using the partial replacement method, significant level of FM may be reduced in the production of fish feed.

6.3 Feeding regime and the acceptance of alternative protein sources

Feeding regime is another factor that needs to be considered when feeding fish especially with feed containing alternative protein sources. From observations in the present study, palatability and feed intake problems occurred in the diet with PM as a sole protein supplemented with EAA. This indicates, supplementing EAA did not positively increase growth of fish fed PM diet. PM is an alternative protein source of animal origin and is known to have some adverse effects on fish as highlighted by the current study (Chapter 2, 3, 4 and 5). Therefore, suitable feeding regimes need to be considered before feeding fish with diets consisting of PM as a source of protein. In Chapter 5, a comparison between two different feeding regimes, satiation and restricted was evaluated.

Arguably different fish species have unique nutrient requirements, so different effects may be observed in terms of feed intake when introducing a novel diet. In the present study, rainbow trout was used as a model species due to its significance as a commercial aquaculture species arising from its disease resistance, ease of domestication and fast growth (Willoughby, 1999). The effects of PM in rainbow trout also had been presented in previous studies (Alexis, et al., 1985; Bureau et al., 1999; Cheng and Hardy, 2002).

Interestingly, different results were found for other species of fish. A study conducted by El-Sayed (1998) indicated that poultry by-product meal can be used for Nile tilapia

(*Oreochromis niloticus*) diet without any adverse effects on fish growth. Additionally, Webster et al. (1999) indicated that a combination between soybean meal and poultry by-product meal can be used to totally replace FM the sunshine bass (*Moronechrysops x M. saxatilis*) diet. Another study conducted by Shapawi et al. (2007) using humpback grouper (*Cromileptes altivelis*) also indicated no significant difference between fish fed a FM diet and fish fed pet food grade poultry by-product meal as a sole protein without any supplemented crystalline EAA. In addition, a study conducted by Goda et al. (2007) showed comparable growth performance of African catfish (*Clarias gariepinus*) when FM was totally replaced by poultry by-product meal. This showed that different species of fish will be affected differently when fed a PM diet. In the present study, PM can be used in rainbow trout diet without any additional EAA in the diet without adverse effects; however, the replacement should not exceed 90% of total protein in the diet.

6.4 Temperature and effects on fishmeal replacement

Temperature is a major parameter which can influence fish physiology and fish growth performance (Brett and Groves, 1979). In the present study, rainbow trout was used as an experimental fish because rainbow trout has a wide range of thermal tolerance between 0.0 and 29.8^{o}C (Currie et al., 1998). However, an increase above the optimum temperature (15^{o}C) gave adverse effects on rainbow trout. Elevated water temperature can affect nutrient utilization, digestibility and gastric evacuation rate in fish (Fauconneau et al., 1983; Miller, et al., 2006; Ng, et al., 2010).

Fish growth is a complex process influenced by many parameters such as fish size, nutrients in the feed and water quality. An optimum temperature of 15^{o}C is acceptable for rainbow trout based on the present findings (Chapter 4). This is because fish growth will decrease if the temperature is too high for fish to maintain the growth (Katersky and Carter, 2005). In addition, a reduction on feed intake was recorded when the temperature was above the optimum temperature and this is caused by the limitations of the respiratory and circulatory system to deliver the oxygen to the tissues (Cocking, 1959). Since fish are ectothermic animals increasing temperature beyond the optimum will significantly affect fish growth (Brett and Groves, 1979). Murray et al. (1977) reported that fish require more energy when reared at higher temperature.

In the present study, the difference between optimal and high temperature affected the amount of FM replacement with PM when fed to rainbow trout (Chapter 4). Fish reared at optimum water temperature showed better acceptance of a diet with a high inclusion level of PM compared to fish reared at high water temperature. However, the results on histology of the distal intestine showed increase in the number of goblet cells (GC) and decrease in the width of supranuclear vacuoles (SNV) in fish fed diets containing greater than 90% of PM at both temperatures.

6.5 Outcomes and recommendations

This thesis has further explored the utilization of PM as an alternative protein source in rainbow trout diets. A major focus was high and complete replacement of FM with PM and a determination of how water temperature affected fish growth, efficiency and the morphology of

distal intestine of rainbow trout in the replacement studies. In the present study, the use of PM as a sole protein in rainbow trout diet was a very effective way to reduce FM in aquafeed. Unfortunately, the total replacement of FM reduced fish growth and also had adverse effects on the distal intestine of rainbow trout which changed the macromorphology of the distal intestine and reduced nutrient absorption. From this study, the inclusion up to 90% PM would not require additional supplementation of EAA. Moreover, partial replacement did not affect the digestive system and intestinal health of rainbow trout. At the same time, the diet with partial replacement can also be used at optimum (15^{o}C) and high temperature (20^{o}C) without impaired fish growth.

This thesis has explained and improved the information regarding the use of PM particularly in rainbow trout diet. However, more work on FM replacement with PM and effects on fish growth are still required in order to make it better in the near future. Future experiments should focus on crystalline EAA and effects on different fish sizes and effects of PM on rainbow trout at low water temperature. With this information, the use of FM in aquafeed can be reduced and at the same time can improve the sustainability of FM in the aquafeed industry.

6.6 REFFERENCES

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