Addressing the Global Food Security Challenge – Discovery and Assessment of Sustainable Sources of Ingredients for Aquaculture Feed

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Abstract

Addressing the Global Food Security Challenge – Discovery and Assessment of Sustainable Sources of Ingredients for Aquaculture Feed. By Kieran James Magee.

The world faces the grand challenge of supplying enough food to achieve food security for its rapidly growing population, predicted to reach 9 billion by 2050. Animal meat is an important part of the human diet, despite the global livestock population containing almost 24 billion animals, it is estimated that total food production will need to increase by 70 % to supply the 2050 population. Fish is a highly nutritious food item associated with several health benefits. Global consumption of fish, which is increasing, now constitutes 17 % of animal protein intake. Fish supplied through capture is limited by wild stocks; in 2015 aquaculture was responsible for 53.1 % of fish and seafood produced globally. The aquaculture industry is reliant on fishmeal and fish oil as ingredients for aquafeeds, materials produced from wild stocks or industry waste trimmings; these are finite and costly ingredients. There is great desire to identify cheaper more sustainable ingredients. In order for alternative ingredients to be viable for fish feed inclusion they must be palatable and of sufficient nutritional quality. The aim of this study was to identify alternative ingredients and assess them, through palatability and performance, for aquafeed inclusion. Several alternative ingredients were identified, Natto (fermented full fat soybean), fermented Rapeseed meal, fermented potato protein concentrate (PPC) (all subjected to heating and fermentation to improve nutritional quality), NH Algae (New Horizons Global Ltd Schizochytrium microalgae NHG-002) , Mealworm meal (*Tenebrio molitor*), Silkworm meal (*Bombyx mori*), and Earthworm meal (*Eisenia fetida*). These were tested for palatability using a modified method of behavioural observation based on the work of Alexander Kasumyan, and by analyses of the satiety hormone Cholecystokinin (CCK), released in response to feed. They were then tested in nutritionally balanced feeds for growth and performance in zebrafish (*Danio rerio*) an as initial model species, then in commercially relevant and available species, while partially or completely removing fishmeal and fish oil. Palatability testing via behavioural observation was applied to three species; alternative ingredients were accepted, with only Natto and PPC showing reduced taste response compared with other materials. CCK analyses proved possible, although further development is required in order to identify any significant differences between the responses measured. Growth and performance trials showed that the NH Algae, Natto and Rapeseed meal materials can be included in species specific diets to partially reduce fish meal. The invertebrate meals when used together successfully removed fishmeal completely in diets of three species tested, achieving equal growth and equal or improved performance. Fish oil was only partially reduced with the inclusion of NH Algae, and by Natto in trout diets, the insect diets provided high amounts of linoleic and α-linolenic acid but failed to supply EPA or DHA. This project introduces novel approaches to assess palatability and shows that invertebrate meals have the greatest potential for complete removal of fishmeal, however, fish oil is still required until a suitable source of EPA and DHA can be identified.

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Animal welfare

All the work carried out during the PhD which involved the use of fish was done so under the Establishment Licence for the University (X70548BEB), which by its very nature means all the standards expected for the species in question were fully provided for under the Animals (Scientific Procedures) Act 1986 (ASPA). The work was thoroughly reviewed, ethical approval was granted (ref number AWC0082) via the University's' AWERB (Animal Welfare and Ethical Review Body), and the work was deemed below threshold for regulation. Throughout the trials welfare was monitored independently by the facility named animal and care welfare officer (NACWO).

Contents

Abbreviations

ANF	Anti-nutritional factor
AP	Acidification potential
BBM	Brush border membrane
BSE	Bovine spongiform encephalopathy
CCK	Cholecystokinin
CMC	Carboxymethyl cellulose
CNS	Central nervous system
DHA	Docosahexaenoic acid
EAA	Essential amino acid
EFA	Essential fatty acid
EP	Eutrophication potential
EPA	Eicosapentaenoic acid
FCR	Feed Conversion Ratio
FFINN	Fish Food Innovation
FIFO	Fish-In / Fish-Out ratio
FMR	Fishmeal ratio
GPCR	G-protein-coupled receptor
GWP	Global warming potential
H&E	Haematoxylin and Eosin
HBSS	Hanks balanced salt solution
HSI	Hepatic-somatic index
HUFA	Highly unsaturated fatty acids
IFFO	International Fishmeal and Fish oil Organisation
ISI	Intestinal- somatic index
LCA	Life Cycle Assessment
MSG	Monosodium L-glutamate
MUFA	Mono-unsaturated fatty acid
NBF	Neutrally buffered formalin
NFE	Nitrogen free extract

NH Algae	New Horizons Global Ltd Schizochytrium microalgae (NHG-002)
PAP	Processed animal protein
PER	Protein efficiency ratio
PPC	Potato protein concentrate
PUFA	Polyunsaturated fatty acid
RAS	Recirculation aquaculture system
RSM	Rapeseed meal
SDS-PAGE	Sodium dodecyl polyacrylamide gel electrophoresis
SGR	Specific growth rate
SSI	Splenic- somatic index
TIA	Trypsin inhibitor activity
UAA	Utilised Agricultural Area

Chapter 1

1.0 Introduction: sustainability of aquaculture

1.1 Food security

The world faces the grand challenge of producing sufficient food for a rapidly growing population estimated to reach 9 billion by 2050 (Sanyal, 2011). "Food security exists when all people, at all times, have physical, social and economic access to sufficient safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life" (FAO et al., 2012). It is generally accepted that food security comprises four main issues: availability (supply), access, utilisation (the body's ability to metabolise food nutrients) and stability (continuous provision) (Lawrence and McMichael, 2014). Food security is a complex challenge; this study will tackle the subject by investigating the sustainable production of animal protein, fish, for human consumption. Production of fish through aquaculture is more sustainable than production of conventional terrestrial species, fish are also more efficient at converting feed into protein (Tolkamp et al., 2010, Naylor et al., 2009). Aquaculture does, however, currently rely heavily on finite feed ingredients derived from fish, sourced predominantly through capture fisheries. The aim of this study is to produce a more sustainable fish feed for use in the production of farmed fish. This will be achieved through completion of the following objectives:

- Understand the nutritional requirements of the target fish species.
- Determine the nutritional profiles of plant materials, along with insect and worm meals and assess suitability as feed ingredients.
- Understand the current view of palatability in fish; methods of determining food preferences will be investigated and palatability of alternative feed ingredients and diets for each species will be determined.
- Understand the interaction between food and the intestinal tract, test satiety response of certain fish species to alternative ingredients.

- Nutritionally balanced diets will be formulated and manufactured. Diets will be tested, in addition to palatability, for growth, efficiency and effects on health.

1.1.1 Protein consumption – animal protein in the human diet

Meat has been an important part of human diet for at least 1.5 million years (Domínguez-Rodrigo et al., 2012). The livestock sector is the largest land use system on Earth, occupying 30 % of the ice-free surface while consuming one-third of the global cropland as a feed source and one-third of the planets freshwater supply (Herrero et al., 2013). The estimated global standing livestock population consists of 1.43 billion cattle, 1.87 billion sheep and goats, 0.98 billion pigs and 19.60 billion chickens (Robinson et al., 2014). In the United Kingdom (UK) the total agricultural area is 18.4 million hectares (Department for Environment et al., 2014). Of which 94 % is utilised already. Therefore, in 2013 the Utilised Agricultural Area (UAA) was 17.3 million hectares, 71 % of the total land area. UAA consists of arable crops, horticultural crops, uncropped arable land, common rough grazing, temporary and permanent grassland and land used to raise outdoor pigs. Woodland and other non-agricultural land is not included. Of the UAA, only 36.6 % was considered croppable land. Alongside crops, a total livestock count of 210.2 million head was also maintained on the UAA, broken down into cattle and calves (9.8 million), sheep and lambs (32.9 million), pigs (4.9 million), and poultry (162.6 million),(Department for Environment et al., 2014). Despite the current livestock numbers it has been estimated that a substantial increase in food production of 70 % will be required to meet the demand of the predicted greater population in 2050, demanding an extra one billion tons of cereal and 200 million tons of meat (FAO, 2009). Producing such a vast amount of extra food is no easy task, as described in the UK for example 94 % of the UAA is already in use, therefore space utilisation (land use) and agriculture methods must be optimised and intensified in order to produce such an increased volume of food. The agricultural production index (PIN) revealed that 68 % less land is being used in 2012 than in 1961 to yield the same amount of produce (Roser,

2015), however, there are still reasons which necessitate the need to find more efficient methods of food production:

- 75 % of human used freshwater, a finite resource, is consumed by agriculture (Wallace, 2000).
- Total available arable land is limited geologically.
- Increased efforts to conserve nature.

Fish tissue is a highly nutritious source of biologically high-value protein which also provides essential micronutrients; vitamins: A and B_3 (nicotinamide), B_6 (pyridoxine), B_{12} (cobalamin), E (d-tocopherol) and D. Minerals include calcium, iodine, zinc, iron and selenium. Essential omega-3 polyunsaturated fatty acids (PUFA's - docosahexaenoic acid - DHA and eicosapentaenoic acid - EPA) are present in high quantities in certain oily species such as European anchovy (*Engraulis encrasicolus)*, Atlantic mackerel (*Scomber scombrus*), Rainbow trout (*Oncorhynchus mykiss*) among others (FAO, 2012, Sidhu, 2003). Consumption of fish, especially oily fish, rich in omega-3's, has been linked to many health benefits in humans such as reducing coronary heart disease mortality (FAO, 2014). In contrast to the benefits gained from eating fish, there are health concerns surrounding the consumption of fish due to environmentally persistent contaminants. The health benefits and risks associated with eating fish are described by the Scientific Advisory Committee on Nutrition and the Committee on Toxicity in 'Advice on fish consumption: benefits & risks' (Nutrition and Toxicity, 2004). They conclude that the health benefits negate the risks of toxicity within recommended guidelines. Fortunately such contaminants are not an issue in farmed fish.

Global fish consumption has been rising for many years, from 9.9 kg per capita in the 1960s, to more than 19 kg per capita in 2012, which constitutes 17 % of animal protein intake (FAO, 2014). Fish consumption is dependent on a diverse array of variable factors: availability and cost of fish and alternative foods, income, taste preference, health and knowledge. Due to the complex interactions of said factors

fish consumption can vary drastically between countries from less than 1 kg per capita per year to more than 100 kg. Commonly countries in coastal or riverine areas have greater levels of consumption (FAO, 2014). In 2013 fish and seafood supply and consumption within the UK alone was 1.32 million tonnes, while world total has steadily increased to reach 133 million tonnes (FAOSTAT, 2015), Figure 1.1. FAOSTAT (2015), where consumption data was collected, provides data for total fish and seafood, this consists of figures for freshwater fish, demersal fish, pelagic fish, marine fish and others, and it also includes cephalopods, crustaceans, molluscs and others. For the purpose of consistency the term 'fish and sea food' will refer to the same food categories. The term 'Finfish' will refer to only fish; freshwater, demersal, pelagic, marine and others.

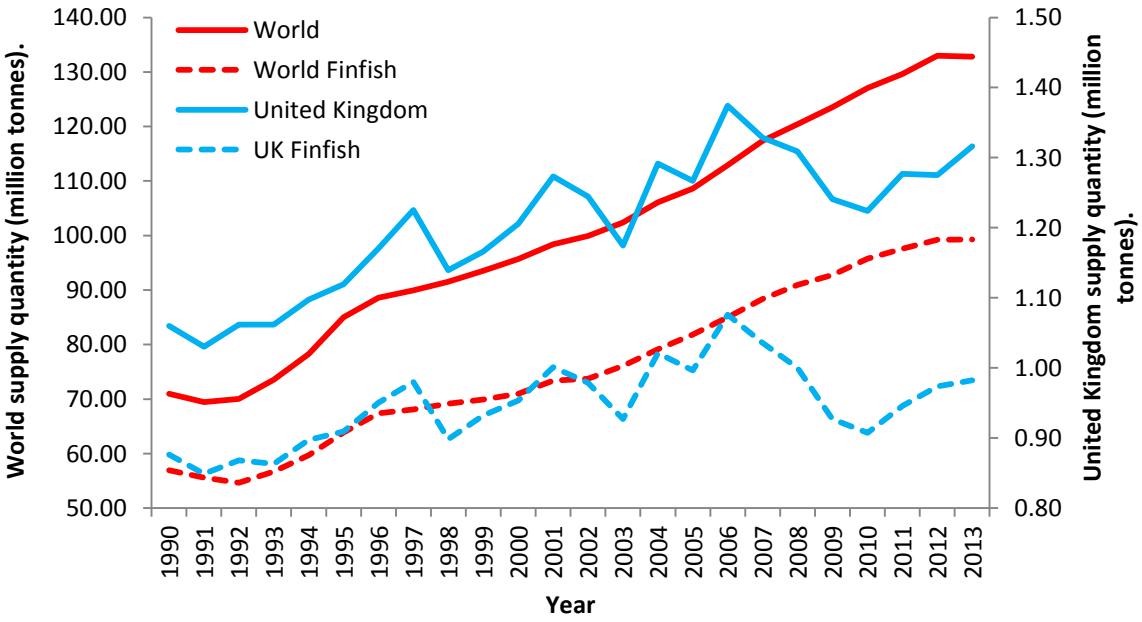


Figure 1.1. United Kingdom and World total quantity (million tonnes) of fish and sea food supplied annually for consumption between 1990 - 2013, data collected from FAOSTAT (2017).

Data sourced from FAOSTAT (2017) shows average annual increases in world consumption of traditional terrestrial animal protein sources between 1990 - 2013: bovine meat (0.81 ± 1.26 % per annum), pig meat (2.18 ± 1.49 % per annum) and

poultry meat (4.28 ± 1.4 % per annum), in conjunction, consumption of fish and other seafood products has increased at a rate of 2.78 ± 2.13 % per annum while consumption of finfish alone increased by 2.47 ± 2.2 % per annum. In 2013 world total fish and seafood consumption (132.83 million tonnes) greatly exceeded consumption of other terrestrial animal protein sources. In the UK fish and seafood is less preferred over traditional sources of animal protein, although consumption of fish and seafood is increasing slowly, see Figure 1.2 for UK and World consumption (million tonnes) of each animal food type. During the past 25 years, China has experienced a dramatic increase in pig meat production and consumption along with fish and sea food, the great amounts of each critically influences global statistics. China, in 2013, consumed 37.0 % of the world total annual fish and sea food supply, which includes 30.1 % of world finfish supply. They also consumed 48.8 % of the world total annual pig meat supply (FAOSTAT, 2017). Although world statistics are affected by the figures for China, this data is clearly an indicator of the vital global importance of a sustainable fish and seafood supply to partly maintain the growing annual animal protein requirement across the planet.

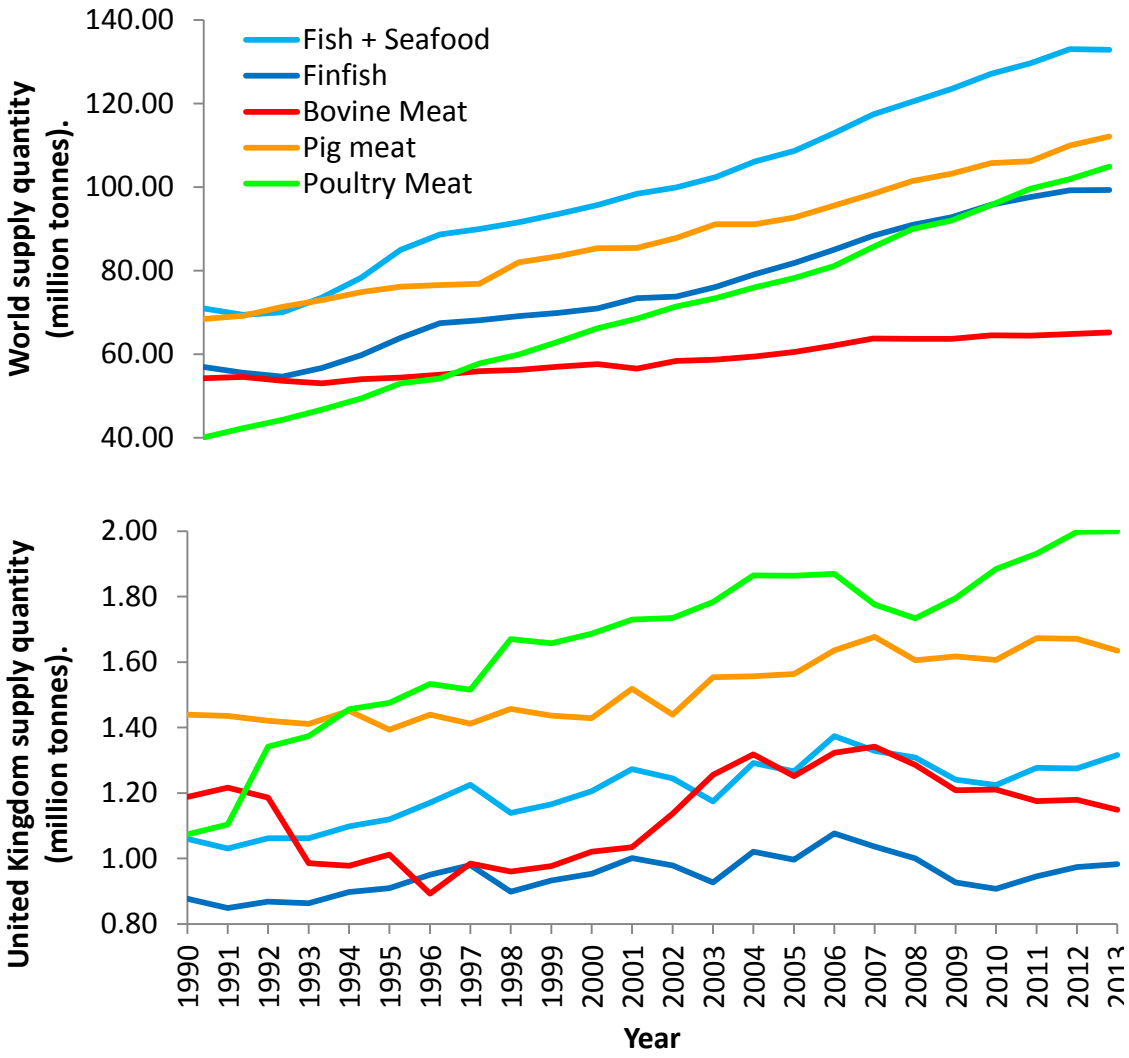


Figure 1.2. Annual United Kingdom (bottom) and world (top) consumption quantity (million tonnes) of traditional terrestrial animal protein food sources compared with fish and sea food including finfish since 1990. Data collected from FAOSTAT (2017).

1.1.2 Animal protein conversion:

Feed conversion ratio (FCR), is a mathematical relationship between the amount of feed input and the weight gain output, providing a measure of how efficiently an animal converts feed into a desired product. For animal protein production the desired product is meat.

A lower FCR expresses higher efficiency at converting the feed into product. The average FCR for beef cattle is 7.42 ± 0.37, when calf rearing costs are included this increases to 10.5 ± 0.31. FCR for pigs is 2.54 ± 0.04, when piglet rearing is

considered it rises to 2.94 ± 0.04. For poultry (broiler chickens) FCR is 1.69 ± 0.03 (Tolkamp et al., 2010). In comparison to terrestrial livestock fish have low FCRs. For farm raised Atlantic salmon *(Salmo salar)* for example, FCR is typically 1.2 (Austreng, 1994), and some studies achieved a lower FCR of 1.04 (Mørkøre and Rørvik, 2001), 0.88 (Einen and Roem, 1997), and 0.76 (Hevrøy et al., 2004). Rainbow trout (*Oncorhynchus mykiss*) express an FCR as low as 0.9 ± 0.1 (Kheyrabadi et al., 2014). FCR for omnivorous or herbivorous fish is usually higher than that of carnivorous species; studies on common carp (*Cyprinus carpio*) have found optimum FCRs ranging from 4.76 (Kiaalvandi et al., 2011), to 1.46 (Cremer et al., 2002), and 1.43 ± 0.03 (Przybyl and Mazurkiewicz, 2004). Fish can exhibit very low FCRs, below 1.0 in some species, especially when juvenile fish develop rapidly during the growth phase. As an FCR of 1.0 indicates that 1 kg of feed is required to obtain 1 kg of fish, an FCR below 1.0 seems mistaken. However, a lower FCR might be expected as FCR is calculated using dry feed weight as is (feed is usually approximately 90 % dry matter and 10 % moisture) and wet fish weight (culture species usually contain 25 % dry matter and 75 % moisture) (Boyd et al., 2007).

Fish also achieve lower FCR values than terrestrial livestock species as the feed used is more concentrated compared with terrestrial species: beef cattle finisher diets contain between 12.5 – 14.4 % crude protein (CP) (DM) (Galyean, 1996), pig finisher diets contain 14% CP (DM), chicken broiler finisher diets contain 19% CP (DM) while salmonid fish smolt diets contain between 40-46% CP (DM) (Miller, 2002).

1.2 Production:

1.2.1 Efficiency of producing fish protein

Efficient livestock production will be fundamental to the production of sufficient volumes of animal protein to satisfy future demand. Livestock production is assessed in terms of environmental impact during the life cycle of an animal or the final meat product. Life cycle assessment (LCA) is the internationally recognised and standardised method of evaluating environmental impact (ISO, 2006). LCA from an

attributional perspective, which focuses on current environmental impacts based on past averages, is considered here as this provides a comparable snapshot of the efficiency of current animal protein food production processes. LCA is commonly conducted from cradle to farm gate boundaries; assessments, however, should ideally extend beyond the farm gate to the final product and include waste disposal (cradle to grave). Environmental impact is calculated from evaluation of all inputs, outputs and any other impact factors. Product inputs consist of resource use both for species housing, growing the animal and for growing food crops; this includes land, water, fossil fuels (energy use). Outputs are formally considered as emissions or pollutants (Guinée et al., 2004).

Livestock production and environmental impact studies which conformed to specific criteria have been reviewed (Vries and Boer, 2010); providing a comprehensive comparison between them and the livestock production methods discussed within each. For comparison of livestock products to be possible, standard functional units are required for each measure; which provide the basis on which alternative products or processes can be compared (Rebitzer et al., 2004, Ayer and Tyedmers, 2008) such as those described below. Livestock is produced primarily to meet the nutritional requirements of humans (Schau and Fet, 2008), predominantly protein, therefore common units for the target product include: kg of product (wet meat in this case) and kg of protein. Units for resource use; land use is expressed as meters squared per year (m^2/kg of product) and fossil energy used is expressed as mega joules of primary energy (MJ/kg of product), (Vries and Boer, 2010). Water use is expressed as total water footprint (L/kg of product); this consists of drinking water, service water, feed mixing water and water footprint of feed crops. The units of emission varies with type of emission, terrestrial livestock production releases carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O). Each gaseous emission persists in the atmosphere for varied time periods and contributes differently towards global warming. They are therefore converted into carbon dioxide-equivalents (CO_2-e) for comparison; calculated as the amount of CO_2 in kilograms required to be released to equal the effects of one kilogram of CH_4 (x 28-

36) or N_2O (x 265-298) (EPA, 2017). CO_2-e is used in the calculation of global warming potential (GWP) which is expressed as kg CO_2-e/kg of product (Vries and Boer, 2010). Acidification potential (AP) of water, fresh or marine, which is the process of decreasing pH resulting from absorption of atmospheric CO_2 (Society, 2005), is expressed as sulphur dioxide equivalents per kilogram of product (SO_2-e/kg of product). Eutrophication potential (EP), of soil or water, is expressed as phosphate equivalents per kilogram of product (PO_4^{3-}-e/kg of product) (Vries and Boer, 2010).

Environmental impact varies considerably for each meat product depending on the production methods used. For the terrestrial livestock protein products discussed so far, bovine meat exhibits the highest overall environmental impact with the highest resource use and GWP, followed by pig meat and then poultry meat. Based upon the limited data presented (Table 1.1), poultry meat is the most efficient livestock product of the three.

Table 1.1. Life Cycle Assessment (LCA) impact factors; resource use (land, water and energy) and environmental impact (global warming potential (GWP), acidification potential (AP) and eutrophication potential (EP)) for production of three terrestrial livestock meat products: bovine, pig and poultry.

Animal protein source	Resource use			Output		
	Land (m^2/kg of product)[1]	Fossil energy (MJ/kg of product)[1]	Water foot print (L/kg of product)[2]	AP (kg SO_2-e/kg of product)[1]	EP (kg PO_4^{3-}-e/kg of product)[1]	GWP (CO_2-e kg/kg of product)[1]
Bovine meat	27–49	34–52	15400	0.008–0.055	0.009–0.025	14–32
Pig meat	8.9–12.1	18–45	6000	0.004–0.062	0.008–0.019	3.9–10
Poultry meat	8.1–9.9	15–29	4300	0.005–0.022	0.006–0.011	3.7–6.9

[1] data from Vries and Boer (2010).
[2] data from Mekonnen and Hoekstra (2010).

Fish and seafood is captured from the wild, referred to as capture fisheries, or farmed, referred to as aquaculture. The term 'aquaculture' encompasses all species of plants and animals in all forms of aquatic environments through all production stages: breeding, rearing and harvesting. Aquaculture species consist of predominantly finfish by number of species cultured, quantity produced and value (FAO, 2014). It also includes crustaceans, molluscs and other invertebrates, algae and aquatic plants, turtles, frogs and all other aquatic species destined for the food market. Comparison between terrestrial livestock products and fish is difficult due to the nature of fish supply or production. LCAs have been conducted for fish products supplied through capture fisheries and aquaculture, mainly targeting finfish and conducted from an attributional LCA perspective (Samuel-Fitwi et al., 2013b). Reviews of the literature for LCA of capture fisheries, aquaculture and fish feeds (Parker, 2012, Henriksson et al., 2012) found comparisons between studies and fish products are hindered by the variety of functional units used. The differences in length of product life cycle investigated and the methodologies used to carry out LCA on fish products, are diverse and many of the LCA studies published on aquaculture focused on one method of production. This leaves large gaps in the data and prevents thorough aquaculture comparisons of production methods. LCA criteria of capture fisheries and aquaculture are difficult to compare like-for-like because some of the variables are not comparable or simply not available. For example, the impact of wild stock removal is also extremely difficult to quantify. The most commonly used functional units for LCA of aquaculture fish products are acidification potential, eutrophication potential, energy use and global warming potential (Henriksson et al., 2012), along with land use and water dependence. Table 1.2 displays results from papers which use at least four of the six functional units described above, which facilitates a basic comparison of efficiency of producing fish through capture fisheries and various aquaculture methods. Table 1.2 uses the same format as Table 1.1 to enable an insight into the efficiency of producing animal protein in the form of fish compared to other animal meats; beef, pig and poultry.

Table 1.2. Life Cycle Assessment (LCA) impacts factors, Resource use (land use, water dependence (total water input) and energy) and environmental impact (global warming potential (GWP), acidification potential (AP) and eutrophication potential (EP)), for production of Fish (per kg of product: live weight, frozen fillet or market ready product) through capture fisheries and various aquaculture methods.

Production Methods	Resource use			Output		
	Land (m^2/kg of product)	Fossil energy (MJ/kg of product)	Water dependence (L/kg of product)	AP (kg SO_2-e/kg of product)	EP (kg PO_4^{3-}-e/kg of product)	GWP (CO_2-e kg/kg of product)
Pelagic fish species						
Capture fisheries [1]	N/A	37	N/A	0.017	0.004	2.14
Atlantic salmon (*Salmo salar*)						
Conventional marine net-pen [2]	*No Data*	26.9	*No Data*	0.018	0.035	2.073
Marine floating bag [2]	*No Data*	32.8	*No Data*	0.015	0.031	1.9
Saltwater flow-through [2]	*No Data*	97.9	*No Data*	0.016	0.029	2.77
Arctic char (*Salvelinus alpinus*)						
Freshwater recirculation [2]	*No Data*	353	*No Data*	0.255	0.02	28.2
Sea bass (*Dicentrarchus labrax*)						
Marine cages [3]	*No Data*	54.656	48782.2	0.025	0.108	3.601
Turbot (*Scophtalmus maximus*)						
Marine recirculation [3]	*No Data*	290.986	4.8	0.048	0.076	6.017
Rainbow trout (*Oncorhynchus mykiss*)						
Freshwater flow-through [3]	*No Data*	78.229	52.6	0.019	0.065	2.753
Extensive freshwater flow through [4]	1.279	*No Data*	473040	0.011	0.06	2.239
Intensive freshwater flow through [4]	1.008	9.194	4380	0.011	0.06	3.561
Freshwater recirculation [4]	1.474	70.639	10	0.041	0.004	13.622
Brook trout (*Salvelinus fontinalis*), **Brown trout** (*Salmo trutta fario*), **Rainbow Trout** (*Oncorhynchus mykiss*) and **Arctic char** (*Salvelinus alpinus*) combined production.						
Flow through [5]	2.737	34.869	98804	0.013	0.029	2.015
Hypothetic recirculation [5]	2.097	57.659	6634	0.011	0.018	1.602
Striped catfish (*Pangasianodon hypophthalmus*)						
Intensive freshwater pond culture [6]	*No Data*	13.2	6125	0.048	0.065	8.93
Common carp (*Cyprinus carpio*)						
High stocking density in freshwater lake cage [7]	1.624	29.68	899	0.014	0.1	1.747
Low stocking density in freshwater lake cage [7]	1.876	33.61	1144	0.016	0.15	2.065
Tilapia (*Oreochromis niloticus*)						
High stocking density in freshwater lake cage [7]	1.138	20.785	629	0.009	0.07	1.253
Low stocking density in freshwater lake cage [7]	1.312	23.501	800	0.011	0.105	1.444

Table 1.2 continued, source material.

[1] (E. A. M. Schau, 2012). LCA end point of study is the retail store; the product is frozen fish fillet.

[2] (Ayer & Tyedmers, 2008). LCA end point of study is farm gate; the product is live weight of fish.[3] (Aubin, Papatryphon, Werf, & Chatzifotis, 2009). LCA end of study is farm gate / shore; the product is live weight of fish. Hatchery of fish was excluded from analyses due to lack of available data.

[4] (B. Samuel-Fitwi, Nagel, Meyer, Schroeder, & Schulz, 2013). LCA end point of the study is farm gate; the product is live weight of fish. Figures given for energy use exclude transport of product and materials such as feed. This study was however, conducted from a consequential view point.

[5] (d'Orbcastel, Blancheton, & Aubin, 2009). LCA end point is farm gate; the product is weight of fish. For this LCA study Land use and water use fail to account for feed growing and production, only land occupation by the infrastructure and water used in the systems in included. Data for the hypothetic recirculation system, based on a two year pilot system, was presented for a Feed Conversion Ratio (FCR) of 0.8 and 1.1; data presented here is for an FCR 0.8 to represent the most efficient system.

[6] (Bosma, Anh, & Potting, 2011). LCA end point of study is farm gate; the product is weight of fresh fish ready for delivery. This study excludes fish hatching and nursing in its assessment.

[7] (Mungkung et al., 2013). LCA end point of study is market; the product is fresh fish delivered. The aquaculture system investigated is a twin net system containing *C. carpio* as the primary product in the top nets with *O. niloticus* produced as a by-product underneath in secondary nets.

The data compiled in Table 1.2 highlights the overall differences in environmental impacts between the fish production methods from research so far. As fish production intensifies and stocking densities increase, improvements are seen in land use, water dependence (although data is somewhat lacking), and EP per kg of product. In recirculation systems the advanced filtration and reuse of water significantly reduces system water input. Total feed input increases as a consequence of increasing stocking density (intensity), subsequently water and land use for growing feed ingredients increases, however, land use for farm infrastructure is reduced compared to other extensive aquaculture methods. In contrast AP increases as intensity of production increases, GWP also increases; a direct consequence of the increased energy consumption by the filtration equipment and continuous pumping of water which amplifies related emissions. Therefore, determining which aquaculture method is considered most environmentally sustainable depends highly on which environmental impact factor is given precedence as most important.

Despite the difficulty comparing water use in fish production with terrestrial livestock production, from the functional units used here it can be seen that generally fish production requires less land and results in less emissions which contribute to GWP. Therefore, it appears fish represent a more sustainable source of animal protein. However, the current literature on LCA of aquaculture and fish products must be interpreted with caution due to such high variance between studies.

FCR can significantly affect the cost and environmental efficiency of fish production using aquaculture methods, a lower FCR ultimately requires less feed per kg of product, subsequently land and water use for growing feed is reduced, as is waste output. Feed production and use is the major contributor to most environmental impact factors across the current production methods (Bosma et al., 2011, Mungkung et al., 2013, Aubin et al., 2009, Ayer and Tyedmers, 2008), energy use only contributing more in recirculation aquaculture systems (RAS's) (Samuel-Fitwi et al., 2013a).

1.2.2 Fish production – capture and aquaculture

Traditionally fish were derived from capture fisheries, which refers to all methods of harvesting of naturally occurring living resources in all aquatic environments (Greenfacts, 2015). Capture fishery includes inland capture, freshwater species and marine capture. Total capture fishery production increased annually from 1950; however, since approximately 1990, production remained unchanged over consecutive years with peak production in 1996 (95.16 million tonnes), see Figure 1.3. Capture of finfish follows the same trend during the same time period. Capture fisheries are under great pressure, yet they have reached a limit in quantity of finfish landed due to the status of wild fish stocks; insufficient numbers remain to sustain a growth in harvest. Each year the health of fish stocks is subject to stock assessment. Once a stock assessment is complete the result is used to set the fishing quota for that species for that year. Fishing quotas are used to aid in the prevention of overfishing and depleting fish stocks beyond a sustainable or

recoverable level. However, in 2013, 68.6 percent of the assessed fish stocks were considered to be fished within biologically sustainable levels. The remaining 31.4 percent of stocks were considered to be overfished (FAO, 2016).

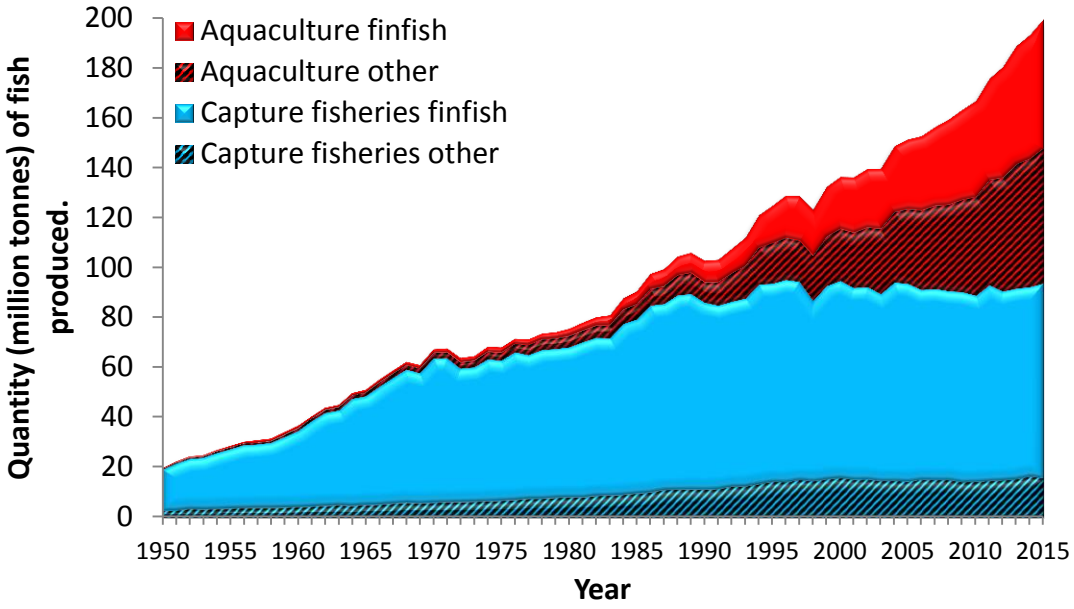


Figure 1.3. Total quantity (million tonnes) of fish and sea food (divided into finfish and other) produced annually by capture fisheries and aquaculture between 1950 - 2015. Data collected from FishstatJ (FAO, 2017a).

The capture of wild fish remained roughly static since approximately 1990, the continued rise in demand has been balanced largely by increased aquaculture production, which has grown rapidly in recent years; 53.1 % of fish and seafood produced globally in 2015 were produced by aquaculture up from just 16.4 % in 1990. The proportion of finfish produced annually via aquaculture has also increased significantly from 10.4 % in 1990 to 40.0 % in 2015 (FAO, 2017a) (Figure 1.3).

Fish have been cultivated as a food source since as early as 5000 BC in China. This was initially dominated by carp species. Cultivation of other species such as Tilapia

was widespread by 1500 BC, for example in Egypt (AANS, 2015). Early fish farming might have started with the utilisation of fish trapped in lakes as a result of flood waters subsiding from nearby rivers. This progressed into fish being captured, transferred to lakes then raised on insects and by-products of the silk industry, namely nymphs and silkworm faeces (Durgappa, 2006). Modern aquaculture methods can generally be categorised into either open systems e.g. floating cages situated in lakes or at sea either in coastal regions or open water for larger species such as tuna, semi-closed systems such as raceways or pond culture farms, which have the nearby river diverted through to maintain water quality, and closed systems i.e. recirculation aquaculture systems (RAS's), usually situated inland. The same methods can be applied to freshwater, brackish and marine culture.

1.2.3 Feed in aquaculture (fishmeal, fish oil) and sustainability

Farmed fish can be either fed or unfed; those which are unfed are raised using pond culture with low stocking densities, the fish feed on natural feed sources such as algae, zoo plankton and insects. Ponds may be supplemented with fertilizer to enhance the population densities of feed sources, however, the fish are not directly fed a feed source. Species that can be produced using such methods are herbivorous or omnivorous, including carp and certain catfish species. Those which are fed receive daily feed rations with manufactured aquaculture pellets formulated for the target species; feed is used when fish are produced in high stocking densities. As could be predicted, when fish of the same species receive daily rations of nutritionally balanced and formulated feeds, aimed at maximizing growth, compared to fish that partake in natural feeding, they grow much faster. This has led, in recent years, to a decrease in the share of non-fed species from 33.5 % in 2010 to 30.8 % in 2014, consequently the share of fed aquaculture species has risen in parallel (FAO, 2014, FAO, 2016).

In terms of providing fish with the nutrition they need, there are broadly two types of aquaculture diet, a complete diet is formulated to meet the complete nutritional

requirements for the target species, whereas a supplementary feed is not designed to meet the full nutritional needs of the animal, but is fed in addition to another, usually natural, feed source. Protein source is the primary and most expensive component of formulated aquafeeds (Wilson, 2002).

Commercially produced complete and compound feeds are traditionally formulated to contain fishmeal and fish oil; inclusion levels are dependent on the desired nutritional profile of the final pellet. Both fishmeal and oil are derived from marine capture fish, fish offal and other fish by-products. These aquaculture by-products now account for one-third, 35 % in 2012, of raw material (FAO, 2014) but are predicted to increase (FAO, 2016). Often only part of the fish, the fillets, is suitable for human consumption, leaving heads, tails, and entrails. The production process involves reduction of whole fish or by-products using heating to coagulate the protein, thereby liberating bound oil and water, pressing (or centrifuging) to separate liquids and solids, separation of oil and water (stickwater) which is converted into fish solubles via evaporation, drying of the solid material (presscake) in which the fish solubles are re-added. Dried material is finally ground to the desired particle size or pelletised and packaged, oil is packaged or further refined then packaged (FAO, 1986a). Global average oil recovery is approximately 5 % and fishmeal recovery is approximately 22.5 % of the fish material used for this process (Shepherd, 2005, Tacon and Metian, 2008). Capture species commonly used for fishmeal and oil production include small oily pelagic species that are too small or bony for direct human consumption such as anchoveta, Chilean jack mackerel, Atlantic herring, chub mackerel, Japanese anchovy, round sardinella, Atlantic mackerel and European anchovy (Naylor et al., 2000). Fishmeal is valued as it contains high quality protein, vitamins and minerals; the amino acid profile also closely matches the amino acid profile required for many aquaculture finfish species. Fishmeal is especially high in lysine and methionine content. Fish oil is valued as a source of long chain HUFAs (highly unsaturated fatty acids) EPA and DHA (FAO, 1986b). Fishmeal and fish oil are mostly destined for agriculture feed markets; fish oil also enters the human food market as a supplement. Diets

formulated for carnivorous species usually contain higher amounts of fishmeal and oil in order to fulfil specific nutritional requirements. Cultured species such as Atlantic salmon (*S. salar*) and Rainbow trout (*O. mykiss*) currently rely on such diets.

Annual production of both fishmeal and fish oil is highly dependent on the annual catch of these pelagic species. El Niño is a climatic phenomenon whereby the thermocline across the Pacific Ocean is interrupted, in turn, causing disruption of the ocean-atmosphere in the Tropical Pacific. It is characterized by unusually warm ocean surface temperatures, preventing the upwelling of nutrient rich cold water, the band of cold water descends from approximately 50m to 150m. The consequences of this event are far-reaching and have global weather effects (NOAA, 2015). During El Niño years the warmer ocean reduces in productivity (NOAA, 2015) and capture fishery production is reduced, the eastern Pacific is particularly effected (Peru and Chile) (Naylor et al., 2009). As Peru and Chile remain the dominant exporters of fishmeal and oil (FAO, 2014, FAO, 2016), 40 % of global production in 2007 (Naylor et al., 2009), the El Niño event plays a pivotal role in the annual availability of both commodities. Due to the health status of fish stocks, total capture volume reached a peak in 1996 and has remained static since (FAO, 2017a). Of the total catch volume, a small percentage is destined for fishmeal and fish oil production, which in 2012 amounted to 10.5 % of the total catch (FAO, 2014). Fishmeal production peaked at 30.1 million tonnes (live weight) in 1994, since then production has fluctuated but overall declined, with production in 2014 being 15.8 million tonnes (FAO, 2016). This shortfall in production has been compensated by production from fish by-products of the aquaculture industry, however, the resulting meal is nutritionally inferior, limiting the inclusion and use of the product. Prices of both fishmeal and fish oil fluctuate (Figure 1.4) with availability, although they have steadily increased since January 2000. Due to increasing prices along with availability of these commodities becoming increasingly limited, inclusion levels have been reduced in recent years (Table 1.3), and the use of alternative ingredients has risen. This decline in dietary inclusion of fish-based ingredients is predicted to continue (Green, 2012). In parallel, effort has been targeted towards

reducing and improving FCR of aquaculture diets, in turn reducing the fish in/fish out (FIFO) ratio (volume of fish required in feed ingredients compared to the volume of fish produced) of modern aquaculture feeds. This figure is affected by the inclusion levels of both fishmeal and oil, Due to low yields of oil compared to fishmeal from source fish material, inclusion of fish oil in a diet has a stronger influence on the FIFO figure than fishmeal. Calculation of FIFO ratios conducted by the International Fishmeal and Fish oil Organization (IFFO – The Marine Ingredient Organization) accounts for any excess fishmeal and oil (calculated per tonne of raw material required to produced dietary levels) being used elsewhere and not wasted. The IFFO formula:

$$\text{FIFO Ratio} = \frac{\text{Amount of fishmeal in the diet} + \text{Amount of fish oil in the diet}}{\text{Yield of fishmeal from wild fish} + \text{Yield of fish oil from wild fish}} \times \text{FCR}$$

(Jackson, 2009).

Feeds formulated for production of carnivorous species such as Atlantic salmon (*S. salar*) have a high FIFO ratio, but FIFO ratios across all aquaculture species are improving. Using the IFFO method, the global FIFO average in 2006 was 0.53:1, with salmon, the highest consumer species of fishmeal and oil, being 1.68:1 (Jackson, 2009). This data shows aquaculture to be a more sustainable industry than previously thought, however, with increasing human populations and increasing financial and environmental pressures on the industry, efforts to find alternative sources of protein with satisfactory bioavailability and sources of lipids with high enough content of HUFAs are therefore ongoing.

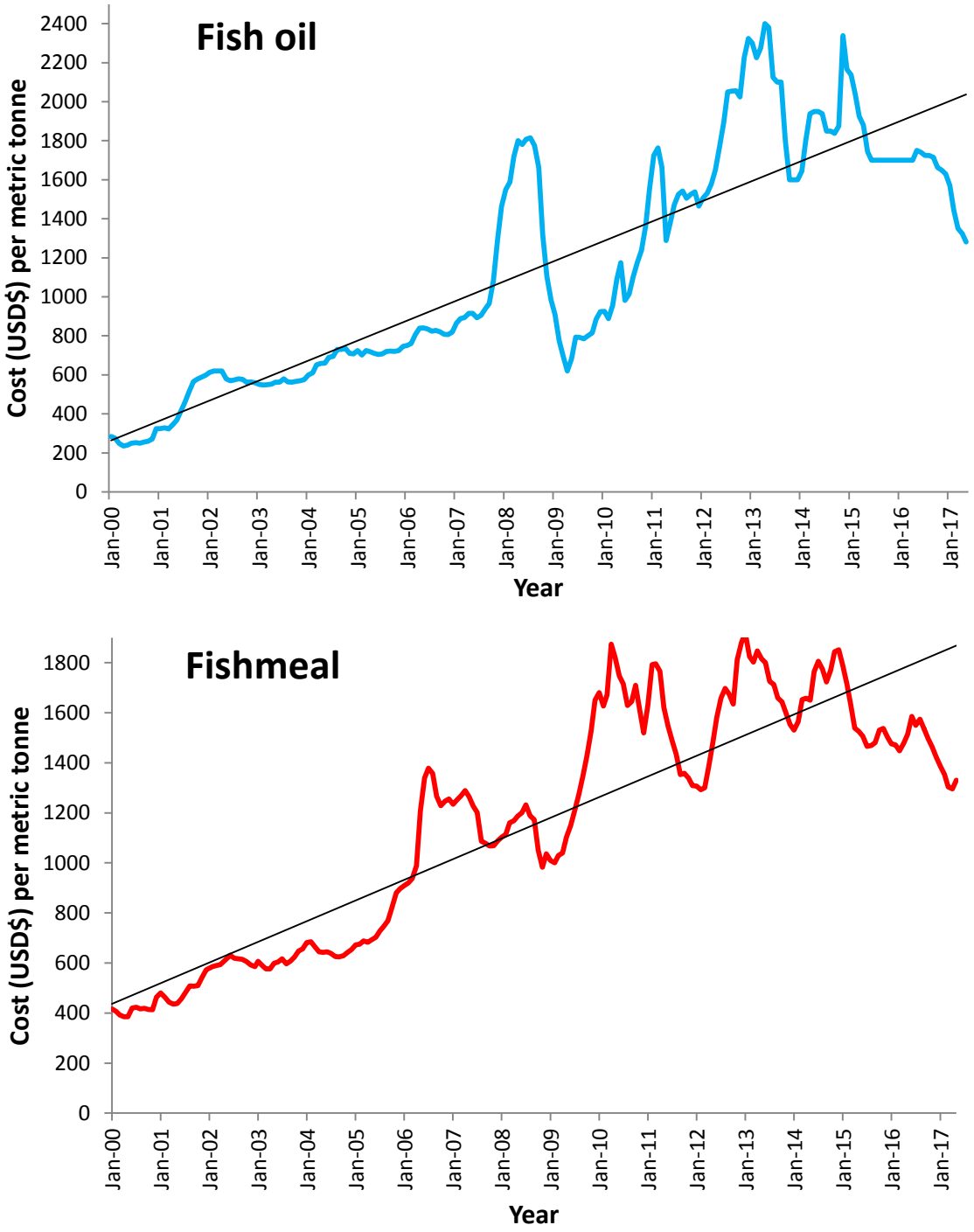


Figure 1.4. Monthly price (USD$) of fish oil per metric tonne from January 2000 to May 2017 (blue line), and monthly price of fishmeal (65% protein) per metric tonne from January 2000 to May 2017 (red line). The black line present on both graphs depicts a linear trend line for each commodity during the time period displayed. Data on fishmeal and oil collected from FAO via personal communication (GLOBEFISH and OilWorld, 2015) and an online database (FAO, 2017h). Similar data published at IndexMundi (2015) and FAO (2016).

Table 1.3. Fishmeal inclusion rates in aquaculture feeds for main groups of finfish produced. A significant decrease in inclusion rate is seen for most culture species groups.

Species / group	Fishmeal inclusion in aquaculture feed (%)	
	1995	2008
Fed carp	10	3
Tilapias	10	5
Catfish	5	7
Salmons	45	25
Trout's	40	25
Eels	65	48
Marine fish	50	29
Miscellaneous freshwater fish	55	30
Milkfish	15	5

Data modified from Sea fish summary report produced on the FAO state of world fisheries 2012 report (Green, 2012, FAO, 2012).

1.2.4 Farmed species and species of interest

Many species are now produced by aquaculture globally; in 2014 there were a total of 580 species and/or species groups registered in FAO statistics. This included 362 (including hybrids) species of finfish, 104 species of molluscs, 62 species of crustaceans, 6 species of amphibians and reptiles, 9 species of other aquatic invertebrates and 37 species of aquatic plants (freshwater and marine). Finfish are clearly the most prevalent aquaculture species group produced (62 %)(FAO, 2016), therefore finfish species are focused on here. Of the top 10 most produced aquaculture species in 2015, eight were fish, including six carp species (Table 1.4, (FAO, 2015d)). Discussed below are fish species of interest and of consideration for inclusion in this study.

Common carp (*Cyprinus carpio*) was the third most produced fish species worldwide in 2015 (FAO, 2017a) therefore having commercial significance as a food species (FAO, 2012, Peteri, 2015, Kottelat and Freyhof, 2007), as well as substantial status as a recreational species for angling (Rapp et al., 2008, Kottelat and Freyhof, 2007) and it is also an analogue for koi carp (*Cyprinus carpio haematopterus*), an ornamental variety of carp, believed to originate from *C. rubrofruscus* (often referred to as *C. c. haematopterus*) or a hybrid of both *C. rubrofruscus* and *C. carpio* (Froese and Pauly, 2015, Kottelat and Freyhof, 2007), which is a valuable and popular pet fish in the global ornamental trade. Common carp have been subject to research due to the economic importance and value of the species so the existing literature is well developed.

Rainbow trout (*Oncorhynchus mykiss)*, formerly known as *Salmo gairdneri* (Kottelat and Freyhof, 2007) are an important aquaculture species in itself, especially in Europe (Cowx, 2005, Kottelat and Freyhof, 2007). As a carnivorous diadromous salmonid species, it is also considered a sound analogue of Atlantic salmon (*Salmo salar*) the tenth most produced species in 2015 (FAO, 2017a). Due to the smaller size of *O. mykiss* and availability in the UK, it is more feasible to source and house during trials than *S. salar*. Similar to common carp, as a result of its economic significance and value, there is a vast research base for rainbow trout.

Nile tilapia (*Oreochromis niloticus*) is a cichlid species native to parts of Africa, but now widely distributed globally as an aquaculture species. Several species are now cultured, although, *O. niloticus* is the predominant species. This species is omnivorous; however, it relies heavily on plant material, and is most commonly fed a diet resembling that of a herbivore. With the use of mono-sex populations, commercial tilapia culture has developed in over 100 countries (Rakocy, 2005). Tilapia as a species is the second most prevalent aquaculture species worldwide; with *O. niloticus* the most important tilapia species and the fourth most produced finfish species globally in 2015 (Table 1.4).

European Sea bass (*Dicentrarchus labrax*) has seen increased production in recent years. It is also considered to be a sound representative of marine finfish aquaculture as the majority of marine species are carnivorous. *D. labrax* are eurythermic (5 - 28 °C) and euryhaline (3 % to full strength sea water) (Bagni, 2005), thus housing this species in an inland research facility becomes feasible. This, combined with a local producer of *D. labrax* being present for sourcing stock allows inclusion of this marine species. See Figure 1.5 for annual quantity of fish (thousand tonnes) produced from 1950 – 2015 for the four species described, *C. carpio*, *O. mykiss, O. niloticus,* and *D. labrax*.

Table 1.4. Top ten most produced aquaculture species worldwide in 2015.

Species	Common name	Production volume (million tonnes)
Ctenopharyngodon idellus	Grass carp	5.82
Hypophthalmichthys molitrix	Silver carp	5.13
Cyprinus carpio	Common carp	4.33
Ruditapes philippinarum	Japanese carpet shell	4.05
Oreochromis niloticus	Nile tilapia	3.93
Penaeus vannamei	White leg shrimp	3.88
Hypophthalmichthys nobilis	Bighead carp	3.40
Carassius carassius	Crucian carp	2.91
Catla catla	Indian carp	2.76
Salmo salar	Atlantic salmon	2.38

Data collected from FAO statistical dataset FishstatJ (FAO, 2017a). Data excludes cupped oysters nei (not elsewhere included), as this consists of multiple species grouped together.

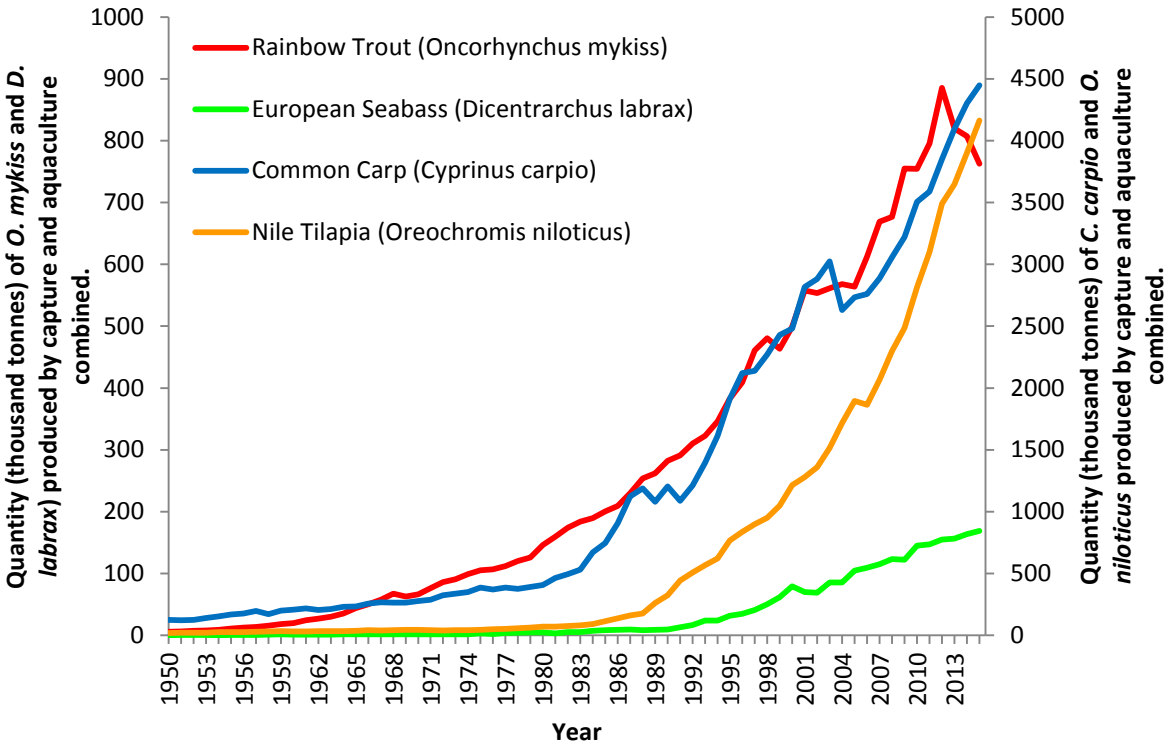


Figure 1.5. Annual worldwide quantity (thousand tonnes) of fish produced from capture and aquaculture combined from 1950 – 2015 for the target food fish species of this research; common carp (*C. carpio*), Rainbow trout (*O. mykiss*), Nile tilapia (*O. niloticus*) and European sea bass (*D. labrax*). Data collected from FishStatJ (FAO, 2017a).

Zebrafish (*Danio rerio*) are not produced as a food product, however, they will be used in this study due to their importance and accessibility as a research model. Fish are second only to mice in numbers of animals used in regulated experimental research, in 2013 that was 12.5 % (501 841 individuals) of the total animals used (UAR, 2014). The total number of fish involved in non-regulated research and kept as brood stock can be speculated to vastly exceed this figure. *D. rerio* a member of the *Cyprinidae* family, therefore related to *C. carpio*, is a tropical fish, 2.5 – 4 cm in length, native to the Himalayan region (Froese and Pauly, 2015, Talwar and Jhingran, 1991). Zebrafish possess several characteristics which render it an ideal model species; being a vertebrate their organ structure is similar to humans, fertilization is external, coupled with fast development and transparent embryos this enables monitoring of development from early stages, generation time is short

with fish reaching maturity at three months of age, fecundity is very high; a single pairing can produce 200 - 300 offspring weekly, they also spawn with onset of dawn, easily manipulated using artificial techniques (Tavares and Lopes, 2013, NC3R's, 2014). As zebrafish are small in size and robust in nature they require less space to house, are cheaper and are easier to care for and maintain. For the same reasons that render this species an ideal model for medical research, this species presents a useful model in aquaculture research, feeds can be trialed on small scale with high throughput of replicates while minimizing space and cost requirements. The previously described species will enable the results of this research to relate to and possibly impact a broad spectrum of industries.

1.3 Fish nutritional requirements

Manufacturers require a full understanding of the nutritional requirements of an aquaculture species to formulate diets that achieve maximum utilisation and result in maximum growth and health, yet achieve economic and environmental targets. A nutrient can be defined as “a fully characterized (physical chemical, physiological) constituent of a diet, natural or designed, that serves as either (i) a significant energy yielding substrate, (ii) a precursor for the synthesis of macromolecules and/or compounds needed for normal cell differentiation, growth, renewal, repair, defence and/or maintenance, (iii) a required signalling molecule, cofactor and/or determinant of normal molecular structure/function and/or (iv) a promoter of cell and organ integrity”(Young, 2000). Primarily nutrients are released from food during digestion then absorbed through the intestinal tract; some are products of metabolism of the original constituents. Nutrients can be classified based on the amounts they are required in; macronutrients include carbohydrates, lipids and protein while micronutrients includes minerals and vitamins (Lall and Dumas, 2015).

1.3.1 Proteins and amino acids

Proteins are complex macromolecules composed of carbon (C, 50 %), nitrogen (N, 16 %, range 12 - 19 %), oxygen (O, 21.5 %), and hydrogen (H, 6.5 %), while occasionally phosphorus (P) and sulphur (S) (Craig and Helfrich, 2009, Tacon, 1987).

Protein differs from other macromolecules of biological importance as they can comprise of up to 100 amino acids, giving greater compound variability's and ranges to composition and structure shape.

Protein is primarily used for growth if the availability of carbohydrates and lipids are adequate (Craig and Helfrich, 2009). Proteins are made up of amino acids; over 200 amino acids occur naturally with 20 being commonplace. Of the 20 common proteinogenic (protein building) amino acids, 10 are considered 'essential' amino acids (EAA) across all fish species, which cannot be synthesised within the body so they must be gained through diet. These are: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine, the most commonly deficient EAA in feed materials are lysine and methionine (Craig and Helfrich, 2009). It is recognised that two of the non-essential amino acids: cystine and tyrosine are synthesised within the body from methionine and phenylalanine, respectfully (Takeuchi et al., 2002, Lall and Dumas, 2015, Li et al., 2008). The required dietary inclusion of these essential amino acids is therefore dependant on the concentration of the two non-essential amino acids (Tacon, 1987).

Measurement of tissue and feed protein content is determined using nitrogen content; referred to as crude protein (CP) (N x 6.25), based upon the average nitrogen content of protein being 16 %. Dietary protein requirements were first investigated in the Chinook salmon (*Oncorhynchus tshawytscha*) by DeLong et al. (1958) using techniques originally developed for terrestrial species. The method of calculating the protein requirements for fish species has not changed much since 1958 except for the use of maximum tissue protein retention or nitrogen balance in preference to weight gain as the criterion of requirement (Ogino, 1980). The standard measurement of required dietary protein is expressed as a fixed dietary percentage or as a ratio of protein to energy (Tacon, 1987). Research shows protein requirement as a proportion of diet decreases as fish reach maturity (Lall and

Dumas, 2015), protein requirements are highest during life stages which experience fastest growth, once mature the growth rate slows.

Dietary EAA requirements can be determined using the carcass deposition method pioneered by Ogino (1980a). Dietary EAA requirement for a given species is determined from the daily EAA tissue deposition value of individual amino acids within the fish carcass; for this analysis fish are fed the same diet which contains a whole protein source of high biological value. The Ogino method enables all ten EAA's to be assessed simultaneously due to the fish being fed the same diet rather than running 10 separate experiments. EAA requirements can be established for species from first feeding fry through to adult brood stock with no loss of precision within the analysis. The protein source is a whole protein of high biological value and feeding regimes can be controlled to ensure amino acid requirements are ascertained during optimal growth (Tacon, 1987); many fish species express inferior growth rates when fed diets consisting of free amino acids as opposed to protein-bound amino acids or whole proteins (Lall and Anderson, 2005).

Protein quality of ingredients is fundamental to formulating good quality, high performance diets. Protein quality of ingredients is assessed by comparing the EAA composition of the ingredient with the EAA profile of the target species; the closer the EAA patterns match, the higher the quality of the ingredient (Tacon, 1987). Protein sources are graded based on EAA profile, the outcome is determined by the EAA in greatest deficit when compared to the target profile, known as the most limiting amino acid. The concept of a limiting EAA is explained by what is referred to as 'Liebig's law of the minimum', a theory first developed by Carl Sprengel (1828), later popularised by Justen Von Liebig (1840), Liebig (1855), therefore it can also be known as the Sprengel-Liebig law of the minimum (Ploeg et al., 1999). During diet formulation addition of crystalline amino acids can increase the amount of such limiting amino acids to create an ideal EAA profile. However, as mentioned briefly, some species of fish display sub-optimal growth and reduced feed conversion ratios

(FCRs) when fed diets containing free or crystalline amino acids compared to whole protein or protein-bound amino acids (Lall and Anderson, 2005). It is reasonable to conclude, therefore, the optimum diet would consist of multiple protein sources to achieve the desired EAA profile of the target fish species rather than one single protein source with amino acid supplementation.

1.3.2 Lipids and fatty acids

Lipids take the form of fats, which are semi-solid, and oils, which are liquid at room temperature. Lipids are energy rich (9.5 kcal/g) compared to other nutrients: protein (5.6 kcal/g) and carbohydrates (4.1 kcal/g), and are an important source of adenosine triphosphate (ATP). They also serve as transporters for lipid soluble vitamins. Lipids can, therefore, be utilised in the diet to spare protein for growth rather than metabolism. Naturally occurring fats and oils within foodstuffs and body deposits of most species of animals take the form of triglycerides which are esters of fatty acids and glycerol. Over forty fatty acids occur in nature. The base structure of a fatty acid can be represented as: CH_3 (CH_2)n COOH where n represents the number of repeated CH_2 units that are in the chain. Linolenic acid can be abbreviated as '18:3 n-3' where the number preceding the colon is the number of carbon atoms in the chain, the number following the colon represents the number of double bonds in the chain and the number after the n- is the location of the first double bonded carbon atom. Fatty acids that consist primarily of an unbranched carbon chain with no double bonds between the carbon atoms are referred to as saturated fatty acids (SFA), those with a single double bond are mono-unsaturated fatty acids (MUFA) and those with more than one double bond are poly-unsaturated fatty acids (PUFA). Highly unsaturated fatty acids (HUFA) have four or more double bonds and the carbon chain exceeds 20 carbon atoms. The PUFAs include three groups: linolenic (omega 3 fatty acid, n-3), linoleic (omega 6 fatty acid, n-6) and oleic (n-9) (Tacon, 1987).

The fatty acid profile of fish tissue is strongly influenced by the lipid profile of what is consumed in the diet. The predominant PUFA/HUFA in the tissue of both

freshwater and marine fish is the linolenic (n-3) series with n-6 levels being much lower. It has been reported that freshwater species of fish retain higher levels of n-6 fatty acids than marine fish because their diet contains terrestrially derived components that are rich in n-6 fatty acids (Tacon, 1987). Elongation and desaturation of the carbon chain in a fatty acid reduces the melting point (Owusu-apenten, 2005). The high levels of n-3 series HUFA in fish tissue is believed be due to this effect (Halver, 1980). The structure of n-3 series fatty acids allows for a greater degree of desaturation, required to enable greater membrane flexibility, fluidity and permeability at low temperatures. Fish lack the enzymes to completely synthesise PUFA or HUFA of the n-3 and n-6 series *de novo* (Henderson, 1996), so these must be provided preformed via the diet, making them essential fatty acids (EFA); See Table 1.5 for EFA's.

Table 1.5, Essential dietary omega fatty acids of the n-3 and n-6 series required by fish, modified from Tacon (1987).

Unsaturated fatty acid	**Structure**	**Abbreviation[1]**
Linoleic acid	$CH_3(CH_2)_4CH=CH(CH_2)_7COOH$	18:2 n- 6
Linolenic acid	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COOH$	18:3 n- 3
Arachidonic acid	$CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$	20:4 n- 6
Eicosapentaenoic acid (EPA)	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$	20:5 n- 3
Docosahexaenoic acid (DHA)	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH =CHCH_2CH=CH(CH_2)_2COOH$	22:6 n- 3

[1] Number of carbon (C) atoms in the chain: number of double bonds and position of the first double bond counting from the methyl (CH_3) end of the fatty acid.

Freshwater fish (depending on the species), with the exception of strict carnivores, are able to elongate and further desaturate PUFA into the corresponding HUFA; 18:2 n-6 into 20:4 n-6 and 18:3 n-3 into 20:5 n-3 or 22:6 n-3 (Lall and Dumas, 2015), through an enzymatic pathway which, although complex, is now well understood

(Buzzi et al., 1996, Buzzi et al., 1997); Figure 1.6. Marine fish, however, have low Δ5 desaturase activity or lack it entirely so cannot synthesise long-chain EFA's; therefore they require them as dietary lipids (Sargent et al., 2002). Generally cold freshwater fish such as salmonids have an exclusive requirement for n-3 series fatty acids (18:3 n-3, 20:5 n-3 and 22:6 n-3), while warm freshwater fish can vary. Carp species require both n-3 and n-6 series fatty acids whereas Tilapia species require only n-6 series fatty acids. Carnivorous marine species have lost the ability to chain elongate and further desaturate 18:3 n-3 into the corresponding HUFA. This has likely arisen due to a diet naturally rich in HUFA; these species must therefore be supplied with dietary HUFA (22:6 n-3 or 22:5 n-3) (Kanazawa, 1985).

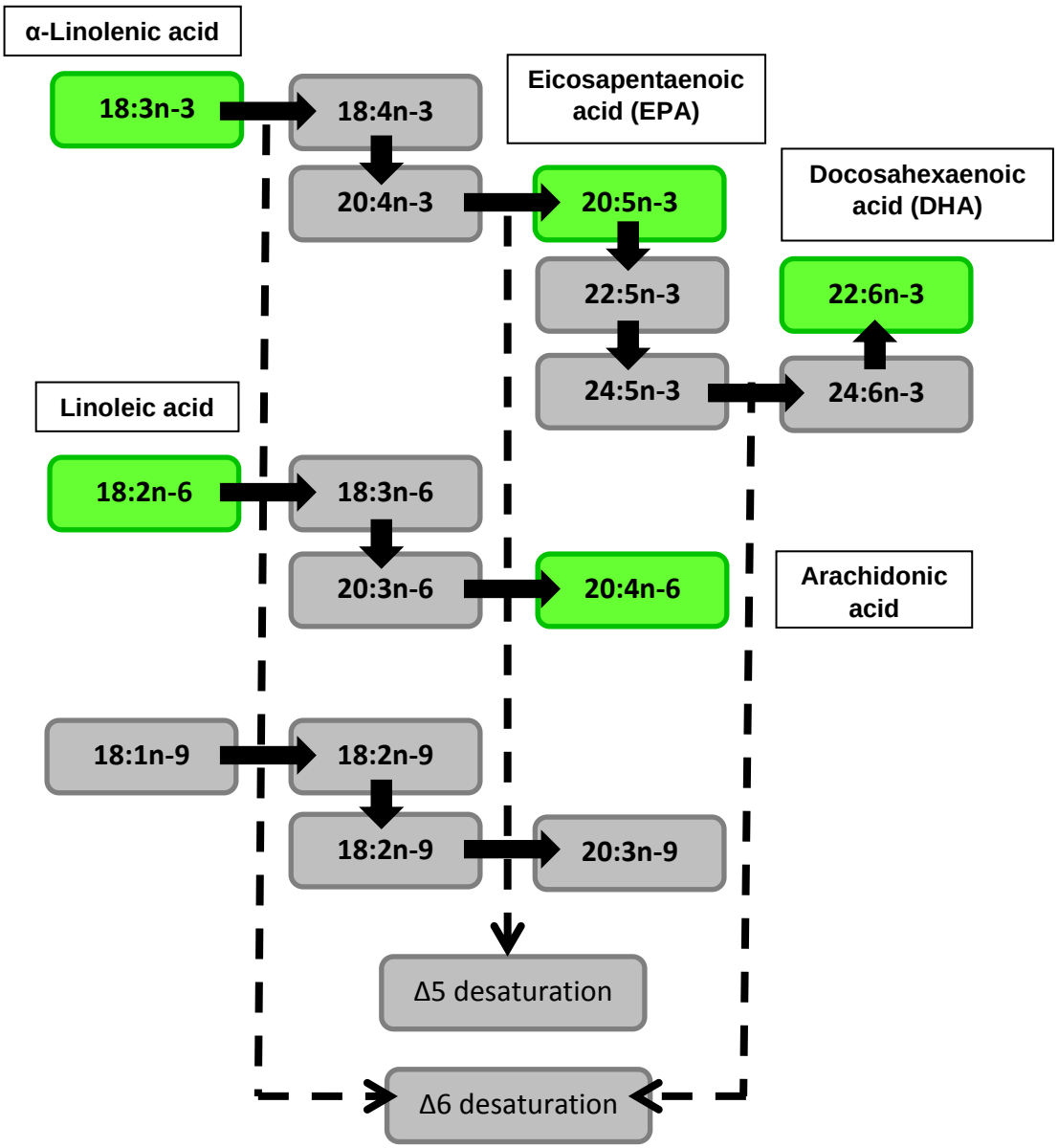


Figure 1.6, modified from Sargent et al. (2002). Biosynthesis pathways within fish of C_{20} AND C_{22} PUFA/HUFA from n-3, n-6 and n-9 C_{18} precursors. Essential fatty acids are labelled and presented in green. Fatty acid chain elongation reactions are represented by downward vertical arrows. Fatty acid desaturations are represented by horizontal arrows. The upward vertical arrow represents peroxisomal chain shortening. The perforated vertical lines show desaturase activity.

1.3.3 Carbohydrates and fibre

Carbohydrates represent the third most abundant organic compounds in the animal body after proteins and lipids. This includes glucose, fructose, sucrose, lactose,

starch, glycogen, chitin and cellulose. For many terrestrial animals carbohydrates serve as a crucial source of metabolic energy (adenosine triphosphate - ATP), in fish however, no absolute dietary requirements have yet been established. This is largely due to the ability of fish to synthesize carbohydrates (glucose) from protein and lipid sources (known as gluconeogenesis); for that reason fish are able to satisfy energy requirements from protein and lipid catabolism alone so carbohydrates are often considered non-essential dietary nutrients. However, carbohydrates can provide an inexpensive source of energy for non-carnivorous species and can spare dietary protein for growth ('protein sparing') (Tacon, 1987). Certain fish species also exhibit reduced growth when fed diets free of carbohydrates (Wilson, 1994).

Warm water omnivorous and herbivorous fish species, including common carp (*C. carpio*), channel catfish (*I. punctatus*), Nile tilapia (*O. niloticus*), and eel (*A. japonica*), have been shown to utilize carbohydrates as energy sources with excess energy stored as body lipids, these species can as a result tolerate high dietary carbohydrate levels (Chiou and Ogino, 1975, Degani et al., 1986, Robinson and Wilson, 1985, Anderson et al., 1984). In contrast, carnivorous fish species have a limited capability to hydrolyse or digest complex carbohydrates due to weak amylolytic activity in their digestive tracts (Spannhof and Plantikow, 1983). Carnivorous species, such as trout, possess limited capability for starch digestion (Singh and Nose, 1967, Bergot and Breque, 1983).

Utilization of dietary carbohydrate by fish has also been demonstrated to vary with complexity or chemical structure, along with the physical state of the carbohydrate source used; cooked or gelatinized starches generally having greater digestibility than native or raw starches (Pieper and Pfeffer, 1980, Robinson and Wilson, 1985, Anderson et al., 1984, Spannhof and Plantikow, 1983, Bergot and Breque, 1983, Wilson and Poe, 1987, Furuichi and Yone, 1982, Buhler and Halver, 1961, Akiyama et al., 1982).

Dietary fibre consists of fibrous carbohydrates, such as cellulose. In many fish, fibre is believed to pass through the stomach and small intestine relatively unchanged. This is due to resistance against chemical treatment (including acid digestion in the stomach), along with intestinal cellulose activity of bacteria present within the fish being weak or entirely absent (Stickney and Shumway, 1974). This plausibly pertains more to those species which have evolved to consume a diet consistent of animal protein. Crude fibre has, therefore, been considered a non-essential dietary component which in excess may contribute to adverse health effects (Anderson et al., 1984, Poston, 1986, Bromley and Adkins, 1984).

1.3.4 Vitamins, minerals and ash

1.3.4.1 Vitamins

Vitamins are a heterogeneous group of organic compounds vital for growth and maintenance. Most vitamins are synthesized in the animal body in insufficient quantities to meet requirements or not at all, even though they are required only in trace amounts. Vitamins are chemically distinct from one another. Approximately 15 have been isolated so far from biological material.When fed diets deficient in distinct vitamins, animals do display morphological and physiological changes. Vitamins are classified as water-soluble or fat-soluble, information regarding vitamin functions and utilisation within the fish body is reviewed by Tacon (1987).

1.3.4.2 Minerals and ash

Discounting the organically bound elements hydrogen, carbon, oxygen and nitrogen, there are a further 21 inorganic mineral elements essential for the correct function of the animal body (table 1.6). These inorganic mineral elements can be classified as macroelements and microelements based on the quantities required. The function of minerals within the body is diverse, including: forming structural components of the soft tissues and skeletal structures, maintenance of osmotic pressure within the body, transfer of nerve impulse and therefore muscle

contraction, and use in the acid-base equilibrium within the body. Minerals also serve as constituents of many enzymes, vitamins, hormones, and respiratory pigments, in conjunction with being cofactors in metabolism, catalysts and enzyme activities.

Table 1.6. Essential inorganic mineral elements required by fish for correct body function.

Macro elements		Microelements		
Cations	Anions			
Calcium (Ca)	Phosphorus (P)	Iron (Fe)	Manganese (Mn)	Nickel (Ni)
Magnesium (Mg)	Chlorine (Cl)	Zinc (Zn)	Cobalt (Co)	Silicon (Si)
Sodium (Na)	Sulphur (S)	Copper (Cu)	Fluorine (F)	Molybdenum (Mo)
Potassium (K)		Tin (Sn)	Vanadium (V)	
		Iodine (I)	Chromium (Cr)	

Information taken from Tacon (1987).

Fish living in aquatic environments can absorb minerals from the surrounding water through their gills, fins, and skin. Therefore, quantifying dietary mineral requirements is more complex than it is for terrestrial animals. Marine fish live in a hypertonic environment and suffer desiccation as water is lost through the gills. In response, marine species drink small regular amounts of water, actively pumping the excess salt into the external environment via the gills or kidneys (released in urine) (Cowey and Sargent, 1972). The mineral requirements can partially be satisfied via drinking (NRC, 1983), combined with absorption and ingestion. In contrast, freshwater species living in a hypotonic environment gain water through their gills, fins and skin. They respond to the loss of urinary salts by drinking very little or no water, actively pumping salt across the gills from the external water into their plasma. As a result it is understood that freshwater fish demand higher dietary vitamin levels than marine species (Cowey and Sargent, 1972). Dietary mineral requirements relate closely to the mineral content of the water the fish is housed

in. Ash contains minerals, both essential and non-essential elements and ash content is typically low as higher inclusion levels reduces diet digestibility.

1.3.5 Energy

Energy is required for cellular metabolism, growth, reproduction and activity. Fish acquire chemical energy from catabolism and oxidation of carbohydrates, proteins and lipids. All forms of energy are convertible into heat energy, consequently energy is customarily expressed as a unit of heat, the calorie (cal), which is the amount of heat required to raise the temperature of one gram of water by one degree centigrade or joule (J) where 4.184 J = 1 cal (Tacon, 1987). The chemical energy of food can be measured by combustion in a bomb-calorimeter. The resulting heat output is measured compared with a standard, giving the gross energy value, also known as the calorific value. This method has been used to estimate the mean gross energy values of carbohydrates (4.1 kcal/g or 17.2 kJ/g), proteins (5.6 kcal/g or 23.4 kJ/g) and lipids (9.5 kcal/g or 39.8 kJ/g) (Cho et al., 1982).

Fish are ectotherms, body temperature is regulated environmentally by the surrounding water not internally, with few exceptions: Pacific bluefin tuna (*Thunnus orientalis*) (Kitagawa et al., 2006) and opah (*Lampris guttatus*) (Wegner et al., 2015), therefore maintenance energy requirements are low (Cho and Kaushik, 1985). Fish are also able to maximise energy release from protein catabolism, obtaining 10 - 20 % more energy; this is because they excrete ammonia, the final product of protein catabolism, directly into an aquatic environment instead of converting it into less toxic substances prior to excretion (Brett and Groves, 1979). .

1.3.6 Species specific nutritional requirements

The nutritional requirements of the target study species must be known In order to formulate a nutritionally balanced diet. A list of these requirements has been compiled and summarised for the species of interest in Appendix 1.

1.3.7 Species functional morphology – gut structure and function, digestive enzymes and adsorption, and regulation

1.3.7.1 Structure and function

The morphology of the intestinal tract can determine the digestive capability of the species. The digestive tract or 'gut' is a tubular structure beginning at the mouth and terminating at the anus. The gut structure can be considered in terms of each section; the mouth which consists of the oral (buccal) and gill (branchial, pharyngeal) cavities, the foregut which encompasses the oesophagus, the stomach, the pylorus, and the structure posterior to the pylorus known as the pyloric region (pyloric caeca present here in some species), and the hindgut which begins posteriorly to the pyloric region, this comprises the mid intestine and distal intestine (Buddington and Kuz'mina, 2000, FAO, 1980).

Fish capture and assess food items in the mouth; once food is recognised, the food is manipulated from the buccal cavity into the pharynx, and here water is passed over the gills while food items are transferred into the oesophagus. Fish chew food to kill or initiate breakdown of the food item, their teeth structure relates to dietary constituents with predatory fish having conical teeth with distal pointing tips to aid capture and retention, whereas species which feed primarily on plant matter possess flattened molar-like teeth for grinding food items. Some species use non-mandibular teeth, pharyngeal teeth are present on the upper and lower pharyngeal bones, these interlock during chewing actions, creating the 'pharyngeal mill' that is used to grind food items. Most species of Cyprinidae and Cobitidae possess such pharyngeal teeth, common carp (*C. carpio*) with the most developed (FAO, 1980, Buddington and Kuz'mina, 2000). Some fish are capable of producing mucous during chewing to aid ingestion of rough food items. The oesophagus can be considered the start of the alimentary canal; it is a muscular passageway from the mouth to the stomach in most species, with the exception of stomachless species (Buddington and Kuz'mina, 2000). Some fish species lack a true secretory stomach, in this

circumstance the oesophagus connects directly to the intestines. There are four distinct morphological classifications of fish in terms of the presence and structure of their stomachs: stomachless (cyprinids), straight stomach with elongated lumen (*Esox*), U-shaped stomach with elongated lumen (Salmo), and T or Y-shaped stomachs (*Alosa*). The morphology of the stomach has evolved in response to size of dietary items. Species which feed frequently on small items generally possess straight stomachs or no true stomach at all, whereas species that ingest larger items infrequently possess more complex structures. The Y-shaped stomach is ideally suited for larger items of food. The main purpose of a stomach is to store and breakdown food; three regions within the stomach can be defined: the cardiac region is non-secretory and is primarily used to store food, the fundic and pyloric regions are secretory, which secrete hydrochloric acid and pepsin (FAO, 1980). The stomach walls are thicker and more well developed than other regions of the alimentary canal, more so in the pyloric region, this can be used to physically grind the stomach content (known as chyme) mixed with stomach secretions using the rough inner lining before passage through the pyloric sphincter (known as the pylorus) into the intestine (FAO, 1980, Buddington and Kuz'mina, 2000).

The intestine is the prime section of the alimentary canal where food digestion and nutrient absorption takes place. The structure of this section corresponds with feeding habit and intestine length is a distinguishing variable. Most carnivorous species possess relatively short intestines, some as short as 20 % of body length, while herbivorous species possess the longest intestines, up to 20 times the body length (Helfman et al., 2009). As intestinal length increases it becomes more folded and/or coiled to fit within the coelomic cavity (FAO, 1980). Fish, in contrast to other vertebrates, do not have a colon or large intestine but some do possess a distal intestine, as is the case with salmonids, which can be identified by a greater diameter, change in coloration and change in mucosa structure. Despite this distinction, the function throughout the intestine remains characteristic of small intestine. Increased intestinal length often results in a thinner profile normally accompanied by thinner mucosa tissue. This enables improved digestion and

absorption through increased intestinal surface area, increased transit distance and increased transit time. Other methods of increasing surface area, usually seen in fish with shorter intestines, include thicker intestinal tracts with thicker mucosa tissue with complex intricate folding. Some species such as sharks, rays, and some primitive fish, for example coelacanths, have a spiral valve, which is an internal epithelial fold within the distal intestine with the appearance of a spiral staircase or corkscrew. The most researched intestinal structure that appears to have evolved purely for the purpose of increased intestinal surface area is the pyloric caecum. Pyloric caecum are closed end diverticulum located at the anterior end of the intestine just posterior to the pylorus, they vary between species in: number (one to thousands), size, shape and appearance (FAO, 1980, Helfman et al., 2009). They are unique to fish; they can be present as individual structures (salmonids), as a single mass joined by connective tissue (tuna) or as a single organ (sturgeons). Histological examination of the pyloric caeca has revealed the same structure and function as that of the adjacent proximal intestine.

1.3.7.2 Food hydrolyses and absorption.

Despite the morphological variations of the intestinal tract between species, its function remains the same: to digest food items and absorb nutrients. Ingested material is hydrolysed into base components: proteins into amino acids or small polypeptide chains, lipids into fatty acids in addition to glycerol and digestible carbohydrates into simple sugars. These nutrients are then absorbed into the blood stream by passage across the gut wall. Material hydrolyses is achieved using aqueous secretions produced directly from the intestinal wall in conjunction with those of the liver/gall bladder and pancreas delivered to the intestinal tract through connective ducts. Digestive secretions include enzymes and other components required for altering or maintaining the chemical environment within sections of the alimentary canal, done to achieve optimum enzyme activity and consequently digestion. Digestive enzyme effectiveness is regulated by temperature and pH; enzymes denature around 50-60 degrees Celsius, beyond the lethal temperature of the host, whereas enzymes operate within a limited pH range, as low as 2 pH units.

Digestion by enzymes is, in fish species which possess a true stomach, initiated by an acid phase, stomachless fish lack the acid phase digestion (FAO, 1980, Buddington and Kuz'mina, 2000).

For fish species with an acid phase, the low pH within the stomach is maintained by secretion of hydrochloric acid (HCL). The production of HCl in teleosts is presumed to mimic that of mammals: NaCl reacts with H_2CO_3 producing $NaHCO_3$ and HCl, the blood providing both input materials. Provision of chloride ions in a chloride-poor environment has been suggested as a possible explanation for the evolution of stomachless fish. Teleost fish possess just one type of secretary cell responsible for HCL and enzyme release, whereas mammals possess two, one cell type for each secretion. Very little is known regarding enzyme expression within fish, however, some research has been done. The enzyme pepsin is the major gastric enzyme in all vertebrates. Pepsin in not produced by stomachless fish, however, peptic activity has been shown in other fish species: Salmo, Oncorhynchus and Ictalurus among others, the optimal pH ranges for maximal proteolytic activity have been reported for salmon (1.3 - 3.5) and Ictalurus (3 - 4). Together with the secretion of HCL and enzymes, mucus is secreted in the stomach, providing mucus production exceeds removal this forms a protective layer for the stomach epithelium, preventing the stomach wall being digested, under stressful conditions mucus production may slow or fail entirely leading to erosion or perforation of the gut in extreme conditions (Buddington and Kuz'mina, 2000, FAO, 1980).

In the mid-intestine there are two sources of secreted enzymes, the secretary cells of the intestinal epithelium and the pancreas. The intestinal epithelium is folded and the secretary cells responsible for both mucus and enzymes develop in the troughs of the folds, migrating to the peak of the ridge, nearest the lumen for expulsion of produce. The pancreas, in most fish species, is a diffuse tissue located throughout the peritoneal cavity. It consists of exocrine and endocrine tissue, secretions from which enter the proximal intestine and, if present, the pyloric

caeca, via the common bile duct. The exocrine pancreatic secretion is an alkaline solution consisting of water, bicarbonate and other ions in conjunction with a multitude of enzymes. Enzyme activity has been identified within the pancreas and intestine evident of each type of enzyme; however, there is speculation about which enzymes are responsible. Trypsin appears to be the leading protease but most studies have concentrated on testing for proteolytic activity and reporting tryptic activity and, to date, the enzyme has not been isolated. Tryptic activity has been shown in the intestine of several species of varied intestinal morphologies, seriola and a puffer species are stomachless fish which rely on tryptic activity as the primary protease enzyme. Other species where tryptic activity has been measured include perch, tilapia, rainbow trout, grass carp, and Chinook salmon. In several of these cases it was shown that tryptic activity increased significantly when gut contents entered the intestine. This suggests the release of enterokinase by the intestinal epithelium; in mammals this activates the pancreatic trypsin once it enters the intestine.

Fatty acids are essential dietary components for fish; therefore at least some form of lipase enzyme must be present to hydrolyse lipids. Lipolytic activity has been identified in carp, killifish, and goldfish, esterase (another lipase) activity has also been shown in rainbow trout. Carbohydrases have been more extensively researched due to the lack of ability of salmonids to utilise large carbohydrates. Amylase, a widespread starch-digesting enzyme, activity has been found in goldfish, bluegill sunfish, rainbow trout, perch, tilapia, Pacific salmon, cod, eel and flounder. Common carp have been found to possess several carbohydrases: amylase, glucosidases, maltase, sucrase, lactase, melibiase, and cellobiase. Some of these are also present in other species however there is far less information compared to that for common carp. The lack of ability of carnivorous fish species to utilise large carbohydrate appears not to be due to lack of enzymes.

Once the food constituents have been hydrolysed into the base nutrients they must be absorbed across the intestinal wall. Nutrient uptake takes place mostly within the anterior intestine including the pyloric caeca if present (Nordrum et al., 2000). The posterior region of the intestine has less nutrient uptake capability and greater phagocytotic activity (Buddington and Diamond, 1987, Ezeasor and Stokoe, 1981). The vertebrate intestine is formed of several distinct tissue layers with different functions. The epithelium is the barrier lining the lumen that separates the interior and exterior mediums. It consists predominantly of a layer of absorptive columnar enterocyte cells, oxyntic secretary cells, along with mucus-secreting goblet cells and endocrine cells, which together with the succeeding lamina propria, forms the mucosa. The epithelium of fish is expanded via folding into a structure that closely resembles mammalian villi; however, fish 'villi' lack the crypts seen in the mammalian system (Jutfelt, 2006). The surface area of the epithelium is further expanded by microvilli at the apical (luminal) surface of the enterocytes. This provides greater area for digestion by membrane-bound enzymes and greater absorption (Clements and Raubenheimer, 2006). The apical surface of the epithelium as a whole is referred to as the brush border membrane (BBM).

Nutrients cross the epithelium by diffusion or by active transport. The majority of lipid uptake in fish takes place in the pyloric caeca and anterior intestine (Vernier, 1990); lipophilic substances are able to cross the lipid bilayers of the epithelium. Dietary lipids consist of triglycerides, phospholipids and cholesterol, of which triglycerides form the major component, the remaining components contributing only a small percentage (Olsen and E. Ringö, 1997). Hydrolysed triglycerides and phospholipids are absorbed by the epithelial enterocyte cells in the form of free fatty acids glycerol and 2-monoglycerides. Lipid absorption is understood to transpire in an analogous process (Olsen and E. Ringö, 1997, Oxley et al., 2006) to that of mammals (Thomson et al., 1993). The outcome of absorbed free fatty acids within to epithelial enterocytes is re-esterification with glycerol, partial acyl glycerols, and lysophospholipids which reform triacylglycerols and phosphoglycerides (Sargent et al., 1989), for review of lipid metabolism see Tocher

(2003) and Sheridan (1988). Absorption of proteins and carbohydrates, once hydrolysed, is completed by active transport which requires energy. This is provided by hydrolysis of ATP by Na^+/K^+ATPase (Collie and Ferraris, 1995). A select number of membrane bound transporters have been isolated in fish, glucose is transported by a transporter protein with functional and genetic similarity to that of the mammalian glucose transport system (SGLT1 in the BBM) (Buddington et al., 1997, Collie and Ferraris, 1995). Amino acids are transported as free amino acids, small peptides or larger proteins; amino acid transport within the mammalian system is conducted by Na+-dependant or independent transporters, each corresponding to certain types of amino acids (Thomson et al., 2001, Silk et al., 1985, Ray et al., 2002).

Peptide transporters are responsible for the selective transport of dipeptides and tripeptides across enterocyte membranes (Chen et al., 2005). One such oligopeptide transporter, PepT1 (SLC15A1), exists in the epithelium of the small intestine in mammals (Fei et al., 1994, Liang et al., 1995). PepT1 along with PepT2 (SLC15A2) have both been isolated in the intestines of multiple fish species: Zebrafish, *D. rerio* (Verri et al., 2003), Ice fish, *Chionodraco hamatus* (Maffia et al., 2003), Atlantic cod, *Gadus morhua* (Amberg et al., 2008), Common carp, *C. carpio* (Ostaszewska et al., 2009), and Rainbow trout, *O. mykiss* (Ostaszewska et al., 2010),(Thamotharan et al., 1996a, Thamotharan et al., 1996b, Maffia et al., 1997, Verri et al., 2000, Romano et al., 2006, Goncalves et al., 2007, Hakim et al., 2009, Sangaletti et al., 2009, Terova et al., 2009). The protein and nucleotide sequences of PepT1 are conserved amongst teleost fish (Ostaszewska et al., 2009). Mammalian PepT1 expression can be regulated by several hormones: insulin (Meredith and Boyd, 2000), epidermal growth factor (Nielsen et al., 2001), leptin (Buyse et al., 2001) and thyroid hormone (Ashida et al., 2002). Similar regulation of PepT1 transporter expression within fish intestinal epithelium may be conducted by the hormones leptin, gastrin and cholecystokinin (CCK) (Ostaszewska et al., 2010).

1.3.7.3 Regulation of feed intake

CCK is synthesized in multiple sites around the mammalian and avian body: in regions of the brain (CCK - 8), gut neurons and by endocrine cells (CCK - 8, - 33, - 39) of the epithelium of the anterior small intestine (Reidelberger, 1994, Denbow, 1994, Figlewicz et al., 1996). CCK has also been detected in the brain and anterior intestinal epithelium of several species of fish (Ostaszewska et al., 2010, Himick and Peter, 1995, Himick et al., 1993, Holmquist et al., 1979, Vigna et al., 1985, Sankaran et al., 1987). CCK type A receptors have been located in several tissues, across multiple species, known to be involved in food intake regulation (Morley, 1995). CCK is a hormone that plays a role consistent with that of short term satiety (Moran, 2009, Bail and Boeuf, 1997), peripheral or intraventricular injections of CCK into the central nervous system (CNS) of some fish incited a reduced volume of food intake or delayed response to food presentation (Himick and Peter, 1994). The role of CCK as a satiety hormone in fish is debated however, agreement on some actions has been established; CCK secretion from epithelial endocrine cells in the anterior intestine, including pyloric caeca if present, is stimulated upon the arrival of chyme, plasma CCK then reduces gastric transit and emptying time, the stomach distends activating the vagal afferent neurons which in turn inhibits the brain feeding system (Bail and Boeuf, 1997). Inhibition of gastric emptying stimulated by CCK has been demonstrated in Rainbow trout (Olsson et al., 1997). The major nutrients that stimulate CCK secretion in vertebrates are ingested protein and fats, particularly their hydrolysed products (Liddle, 1994), this has been demonstrated for fish, specifically yellowtail (*Seriola quinqueradiata*) (Murashita et al., 2008). Measurement of CCK production by intestinal endocrine cells can therefore be used as an indicator of short term satiety response to any alternative protein or lipid source, method of analyses as described by Daly et al. (2012).

1.4 Potential sustainable ingredients

1.4.1 Ingredient suitability

Fishmeal and fish oil have been staple ingredients within the aquaculture industry as they represent ideal nutritional profiles, approximating to those required by

most target species produced (NRC, 2011, Tacon and Metian, 2015). The quality of any alternative protein or oil source is therefore assessed in comparison to these. Other factors affecting ingredient suitability include: physical form, material composition, nutrient bio-availability, palatability, stability during storage, and toxic or anti-nutritional factors (ANFs) (FAO, 1980, GLENCROSS et al., 2007). Research into alternative protein and lipid sources for use in aquaculture feeds has been ongoing for some years, testing animal derived materials: meat meals, bone meals, feather meals, blood meals (Millamena, 2002, Nogueira et al., 2012), and poultry by-products (Saadiah et al., 2010, Parés-Sierra et al., 2014). Proteins and oils derived from single celled organisms such as algae (Patterson and Gatlin, 2013, Kiron et al., 2012), fungi and bacteria, both as whole cell material and extracted oils have been investigated along with alternative marine resources, predominantly derived from krill (Naylor et al., 2009). However, the majority of attention has been given to plant materials including: soy protein and soymeal (Sevgili et al., 2015), wheat gluten meal (Bonaldo et al., 2015), corn gluten meal (Güroy et al., 2013), copra and palm kernel meals (Obirikorang et al., 2015), pistachio and almond nut meals (Barrows and Frost, 2014), lupin seed meal (Aliro S. Borquez et al., 2011), duckweed (El-Shafai et al., 2004), pea, canola and rapeseed meals (Hernández et al., 2013, Ranjan and Athithan, 2015, Obirikorang et al., 2015). There has been a shift in recent years towards protein concentrates which have higher protein levels compared to raw material, which can improve nutritional properties. However, the cost of concentrates are also higher. Vegetable concentrates include soy protein concentrate (Li et al., 2015, Zhao et al., 2010a), potato protein concentrate (Tusche et al., 2011a, Tusche et al., 2011c), rice protein concentrate (Guroy et al., 2013), canola protein concentrate (Thiessen et al., 2004), pea and narrow-leaf lupin protein concentrates (Carter and Hauler, 2000), and rapeseed protein concentrate (Slawski et al., 2012).

Animal derived materials, or processed animal proteins (PAPs), commonly possess higher protein levels and a more complete amino acid profile (Naylor et al., 2009), however, PAPs were subjected to a total ban from being used in animal feed in

2001, except for fishmeal for use in fish and non-ruminant feeds (REGULATION (EC) No 999/2001) (Commission, 2001), following an outbreak of bovine spongiform encephalopathy (BSE). BSE, known as mad cow disease, is transmitted via BSE-contaminated meat and bone meal in feed (Wilesmith et al., 1988). It is now thought very unlikely such transmission can occur between non-ruminant species; therefore the ban was eased in 2013 to permit the use of non-ruminant sourced PAPs for use in aquaculture species (COMMISSION REGULATION (EU) No 56/2013) (Commission, 2013), consequently non-ruminant PAPs (pig and poultry) are now utilised in some aquaculture feeds (Axmann et al., 2015). Despite the use of animal derived meals yielding adequate growth and no negative health implications, consumer acceptance remains a barrier to their wider exploitation (Ghosh et al., 2016). Traditional PAPs such as feather meals, blood meals and bone meals have not been included in this study. There has been an increasing trend in recent years for the production and consumption of insects and other invertebrates as food, for both humans and for agriculture diets. There are now many companies around the world producing insects or products derived from insects on ever growing scales. Therefore insect and invertebrate PAPs will be included in this study.

Culture of certain algae species has now become a stable process with the products being incorporated into the pharmaceutical industry, human food chain and agricultural feed industry, including aquaculture and ornamental aquatic feeds (Hasan and Chakrabarti, 2009). Algal cultures composed of single or multiple species can provide high quality feed ingredients with a protein content of 30 - 40 %, carbohydrates (5 - 15 %), and lipids (10 - 20 %) (Fujii et al., 2010). Inclusion of algae into fish feeds is of particular interest as high levels of omega-3 fatty acids (EPA and DHA) can be obtained (Lane et al., 2014). The greatest potential in algal sources is as a substitute oil source, heterotrophically grown algae and fungi have been used with promising success (Harel et al., 2002). Inclusion of one microorganism group, thraustochytrids, has shown initial success for complete fish oil replacement in Atlantic salmon during the pre-smolt grow-out phase (Miller et al., 2007). Microalgae are cultured and used in aquaculture mainly for shrimp and larval fish

production (Spolaore et al., 2006). Although cultured algal materials are promising alternatives, the cost of producing such cultures on a commercial scale are prohibitive (FAO, 1996, Ochsenreither et al., 2016).

Plant derived meals, as with many other alternative meals, can possess comparable protein levels as fishmeal, although they frequently possess lower levels, accompanied by more deficient EAA profiles, lacking first most in methionine and lysine (Nunes et al., 2014). Plant materials may also include high levels of fibre and starch (non-soluble carbohydrates) which reduce overall digestibility, along with presence of anti-nutritional factors (ANFs) (Naylor et al., 2009). Plant derived materials are already used extensively in the aquaculture feed industry. Protein sources include: barley, canola, corn, cottonseed, peas/lupins, soybeans and wheat (Naylor et al., 2009). Soybean concentrate and wheat gluten are the most substantially utilised (FAO, 2014). Plant oils in use consist of: sunflower, linseed, canola/rapeseed, soybean, olive and palm oils (Naylor et al., 2009, Bendiksen et al., 2011).

ANFs are substances which exert effects opposing optimum nutrition (Kumar, 1991) and the presence of ANFs in certain plant feed sources limits their use in fish feeds. ANFs include protease inhibitors, phytates, glucosinolates, saponins, tannins, lectins, oligosaccharides and non-starch polysaccharides, phytoestrogens, alkaloids, antigenic compounds, gossypols, cyanogens, mimosine, cyclopropenoid fatty acids, canavanine, antivitamins, and phorbol esters (Francis et al., 2001). Kumar (1991) reviews the physiological effects and remedial techniques for many ANFs on farm animals and Francis et al. (2001) review effects on fish. Heat treatment is the most commonly applied technique for reducing ANF levels and to improve bioavailability of macro and micro-nutrients in plant materials, as many are heat liable. Microbial fermentation; research using rumen microbial activity in farm species has also yielded good results in reducing ANFs (Kumar, 1991, Francis et al., 2001).

Insect meals are now attracting a great deal of interest; a part of a natural diet for many fish species, they are an obvious choice. Insect production techniques are now well established and the nutritional profiles of many insects are good with high protein levels and lipid levels. However, relatively little research has been conducted compared to that into different vegetable sources (Sing et al., 2014, ST-Hilaire et al., 2007b).

1.4.2 Plant and single cell materials

At the start of the project our partner, Skretting (a Nutreco company), the largest aquaculture feeds producer in the UK & Ireland, helped to identify a number of materials for investigation. These where: Natto, Rapeseed meal (RSM), Potato protein concentrate (PPC), and an Algal material.

Natto is a traditional soybean product in Japan, created by fermentation of whole soybeans with the bacterium *Bacillus subtilis* (Leejeerajumnean et al., 2001). Soybean meal is a by-product of oil extraction from soybean (*Glycine max*) beans, a species of legume native to East Asia. Soybean meal is high in protein (43 - 53 %), with a good balance of amino acids. Levels of lysine, tryptophan, threonine and isoleucine are high, amino acid and protein digestibility values are also high, as a result soybean meal is currently the most widely used animal feed component (Heuzé et al., 2015b). Soybean meal is also considered the most pertinent protein source in aquaculture, after fishmeal, as a result of its low cost and ready availability (Brown et al., 2008). Soybean meal does contain ANFs: Protease (trypsin) inhibitors, lectins, phytic acid, saponins, phytoestrogens, antivitamins and allergens (Francis et al., 2001), the majority of which are heat liable and should be destroyed with heat treatment. A common adverse health effect seen with soybean meal use in Atlantic salmon diets is enteritis (Baeverfjord and Krogdahl, 1996). Of the total phosphorus content in soybean meal, approximately 60 - 70 % is bound to phytic acid, this is unavailable to fish and also reduces the absorption of other micronutrients (Wilcox et al., 2000, Heuzé et al., 2015b). Natto contains high oil and protein levels; with

soybean meal used extensively already, therefore Natto (full fat soybean post heat treatment then fermentation) could also be a successful feed ingredient.

RSM (*Brassica*) is a UK crop and again is a by-product of rapeseed oil extraction, the protein content is approximately 41 - 43 % (dry matter basis) and the amino acid profile resembles that of soybean meal, lysine is lower, however, methionine content is higher (INRA et al., 2015). RSM contains high levels of micronutrients including calcium, phosphorus, and niacin which are particularly beneficial for growth (Rutkowski, 1971). ANFs present in RSM include protease inhibitors, glucosinolates, phytic acid, tannins, erucic acid, and sinapine. Glucosinolates have been shown to inhibit thyroid metabolism in terrestrial species, leading to enlargement and goiter, they also possess a strong harsh taste making them quite unpalatable (INRA et al., 2015). Tannins are phenolic compounds which bind to protein reducing availability (Bell, 1993). Heat treatment is recommended to reduce levels of tannins and glucosinolates (Francis et al., 2001).

PPC is a by-product of the starch industry; protein content ranges between 75 - 85 % with a high quality amino acid profile, especially high in lysine, methionine and cystine. However, significant levels of solanidine glycoalkaloids are present, of which α-solanine and α-chaconine are the best known. These ANFs are bitter-tasting substances associated with natural defence mechanisms against insects; they are present near the peal of the potato, during processing they are incorporated throughout the resulting PPC giving levels of 1500 - 2500 µg/g glycoalkaloid. Glycoalkaloids are believed to be the cause of reduced palatability, feed intake and consequential poor performance seen in studies on fish, their metabolites may also adversely affect gastrointestinal and liver tissues. Reduction of glycoalkaloids is difficult as they are thermally stable, thus heat treatment has little effect. Feeds trials using a low glycoalkaloid PPC has shown feasible inclusion in rainbow trout (*O. mykiss*) diets, thus supporting the conclusion the adverse

health effects is due to glycoalkaloid levels (Tusche et al., 2011a, Refstie and Tiekstra, 2003, Tusche et al., 2011c, Xie and Jokumsen, 1997).

Schizochytrium is a genus of unicellular protists which has been assigned to the same group as kelp and multiple species of micro-algae: the stramenopiles, *schizochytrium* is therefore considered to be micro-algae. Certain species of *Schizochytrium* produce high levels of DHA; one newly isolated species found in decaying Kandelia candel leaves in a mangrove habitat in Hong Kong, *Schizochytrium mangrovei*, is a prime example with 32 - 39 % of its total fatty acids being DHA (Jiang et al., 2004). Inclusion of DHA-rich oil produced from schizochytrium micro-algae into animal feeds has been tested with rats, the results of which showed no negative health implications (Hammond et al., 2001). Such high levels of desirable omega-3 fatty acids, along with good safety test results, have led to the commercial scale production of certain species. New Horizons Global LTD is a Northern Ireland company in the Biotechnology sector; the company have isolated and produce a species of *schizochytrium* micro-algae (NHG S-002), which has been certified by the Food Safety Authority of Ireland (FSAI), for inclusion into food products with the statement “DHA-rich oil from the micro-algae Schizochytrium sp.” or alternately “Oil from the micro-algae Schizochytrium sp.”. With the strong potential of this micro-algae product being able to provide the omega-3 fatty acids required in fish feeds as an alternative to fish oil, this material was chosen. Details of production of this micro-algae product are retained by the company; the material will be referred to, in this research, as “NH Algae”. The NH Algae material will be tested in the form it was supplied.

Natto was selected as a soybean meal product which undergoes both heat treatment and fermentation during production. RSM and PPC were chosen as UK industry waste by-products, incorporation of these into fish feeds would provide a new industrial use for an otherwise waste material and improve the sustainability of the aquaculture feed end product.

Eminate Ltd., possessed manufacturing capability for Natto; this was produced using standard methods outlined above. RSM and PPC materials were subjected to heat treatment (autoclaved) and fermentation by Eminate Ltd in an attempt to reduce ANFs and improve protein bioavailability. Eminate Ltd carried out nutritional analyses and material production for testing in fish feeds. Due to the confidential nature of this fermentation process the details of the process cannot yet be given.

1.4.3 Invertebrate protein sources

The use of insects and other invertebrates as a source of feed has gained interest in recent years. Considerable research has been carried out in this field, including use in aquaculture feeds e.g. Henry et al. (2015). Insects and other invertebrates form part of a natural diet for many species of freshwater and marine fish species (Howe et al., 2014, Whitley and Bollens, 2014), they often possess high quality nutritional profiles rich in amino acids, lipids, vitamins and minerals (Huis, 2013). Protein levels can be high, ranging between 50 - 82 % (dry matter basis, DM) (Rumpold and Schluter, 2013a) making it comparable to fishmeal. In addition, commercial production has already been established for several species. Insects are a sustainable food source as they can be grown in large quantities using little land area, water and energy, thus resulting in a small ecological footprint (Oonincx and deBoer, 2012). They can also be grown on low quality organic waste materials (Huis, 2013). Many species express antifungal and microbial properties that may benefit the shelf life of an end product (Zhao et al., 2010b).

The primary reason for use of fishmeal in fish feeds is high protein content and well balanced essential amino acid profile (Nguyen et al., 2009, NRC, 2011, Oliva-Teles, 2012), fishmeal is specifically abundant in the amino acids lysine, methionine and leucine, which are often limited (Hall, 1992). Insects generally have well balanced amino acid profiles too, species such as those of the order Diptera are considered similar to the profiles found in fishmeal (Barroso et al., 2014). Two species that have

attracted interest have well balanced profiles in which most essential amino acids exceed the requirements of many fish species: silkworms (*Bombyx mori*) and mealworms (*Tenebrio molitor*) (Hossain et al., 1997, Barroso et al., 2014, Finke, 2002, Finke, 2007, Barker et al., 1998, Rumpold and Schluter, 2013c, Yi et al., 2013, Longvah et al., 2011). These species are considered two of the most promising alternatives for fishmeal (Henry et al., 2015). The use of insects may also aid fish oil reduction. Terrestrial insects are considered to have too little omega 3 and 6 fatty acids to meet the high levels required by fish species, however, these lipid profiles may be improved by dietary manipulation (Ogunji et al., 2008) as shown by St-Hilaire et al. (2007a) using black soldier fly larvae (*Hermetia illucens*).

A few potential problems have been highlighted with the use of insects in fish feeds. First, bioaccumulation of toxins such as insecticides and heavy metals can be overcome by commercial rearing of insects using controlled and monitored feed substances (Spiegel et al., 2013). Second, production on a commercial scale would inevitably be required to support the volumes required if insects were utilised in commercial aquaculture feeds. Mass production of insects worldwide is already being established through the silk, fishing bait and pet food industries (Huis, 2013, Veldkamp et al., 2012, Schabel, 2010, Rumpold and Schluter, 2013c, Ji et al., 2013, FAO, 2013, Kroeckel et al., 2012). The most prominent factor, often given as the reason for adverse health impacts observed when insects are incorporated into diets, is the level of chitin (Lindsay et al., 1984, Longvah et al., 2011, Köprücü and Özdemir, 2005, Alegbeleye et al., 2012). Chitin is a mucopolysaccharide composed of 2-acetamido-2-deoxy-β-D-glucose through a β (1→4) linkage (Kumar, 2000). Digestion of chitin is achieved using three enzymes: chitinase, chitobiase and lysozyme, all of which are present in carnivorous and omnivorous fish, both freshwater (Lindsay et al., 1984, Jeuniaux, 1993) and marine species (Fänge et al., 1979, Danulat and Kausch, 1984, Kono et al., 1987, Clark et al., 1988, Fines and Holt, 2010, Kurokawa et al., 2004). Chitinase is found in the stomach and chitobiase is present in the intestine of fish (Jeuniaux, 1993). Dietary inclusion of chitin for fish species, which naturally feed on crustaceans, insects or benthic invertebrates may

be possible, even beneficial. The 1% inclusion of chitin in the diets of *C. carpio* showed no effects on growth. It did, however, increase the innate immune response (Gopalakannan and Arul, 2006).

The silk moth, *Bombyx mori*, was domesticated from the wild silk moth, *B. mandarina,* for the production of silk. 90 % of global silk produced today is from *B. mori* (Heuzé et al., 2015a). The silkworm caterpillar is the larval form of the *B. mori* silk moth, when the fifth instar is reached and the larva is ready it spins a cocoon over a period of three to four days, from a single strand of silk (approximately 300 to 900 meters or 1000 to 3000 ft in length) in which to pupate. Once complete, under normal conditions, the pupae will develop and after three weeks release proteolytic enzymes creating a hole in the cocoon, thus enabling the moth to emerge (Datta and Nanavaty, 2007, Jintasataporn, 2012). This does, however, damage the silk strand, cutting it into several smaller strands. To prevent damage, the cocoons are harvested and boiled killing the pupae inside (Datta and Nanavaty, 2007, Jintasataporn, 2012) and allowing extraction of the single intact silk strand. This renders the pupae a by-product (Swarts, 2011). For every 1 kg of silk, 2 kg of dry pupae (8 kg wet) are produced (Patil et al., 2013). Silk culture (sericulture) has been practised in China for 5000 years (Barber, 1992, Goldsmith et al., 2004). The silk industry has expanded vastly, China now accounting for approximately 80 % of global production. The industry is now so vast that China's annual production of dry silkworm pupae is approximately 200 000 tonnes (Dong and Wu, 2010). China consumed 1.34 million tonnes of fishmeal in 2014 (IndexMundi, 2016); utilisation of silkworm pupae could, therefore, have significant impact on fishmeal consumption. The waste pupae are often discarded or used as fertiliser (Wei et al., 2009). They degrade rapidly due to the high water content, producing a foul odour. This odour has been attributed to the compounds (flavenoids and terpenoids) present in the mulberry leaf diet of the silkworm and is believed to be associated with palatability issues (Rao, 1994, Finke, 2002). Drying and grinding can extend shelf life of the spent pupae (Usub et al., 2008, Jintasataporn, 2012) and in doing so they remain available for several secondary markets: oil can be extracted for industrial products

(Trivedy et al., 2008) while the meal can be utilised for chitin extraction (Suresh et al., 2012). Silkworm pupae are also consumed as a human food item in many of the Asian silk producing countries: China (Zhi-Yi, 1997), Japan (Mitsuhashi, 1997), Thailand (Yhoung-Aree et al., 1997), India (Longvah et al., 2011). They are also a suitable feed source for livestock because of the nutritional profile (Trivedy et al., 2008). Silkworm meal and oil have been tested in the diets of several fish species to replace fishmeal and oil up 100 %. Many of these diets yield equal or improved growth compared to fishmeal diets: Rohu, *Labeo rohita* (Hossain et al., 1997, Begum et al., 1994), common carp, *C. carpio* and other species of cyprinids (Kim, 1974, Nandeesha et al., 1990, Rahman et al., 1996, Jeyachandran and Raj, 1976, Jayaram et al., 1980, Rangacharyulu et al., 2003), putitor mahseer, *Tor putitora* (Sawhney, 2014), walking catfish, *Clarias batrachus* (Venkatesh et al., 1986), Japanese sea bass, *Lateolabrax japonicas* (Ji et al., 2010), rainbow trout, *O. mykiss* (Dheke and Gubhaju, 2013), chum salmon, *Oncorhynchus keta* (Akiyama et al., 1984), and olive flounder, *Paralichthys olivaceus* (Lee et al., 2012). Ground dried silkworm pupae have also been shown to be accepted by *C. carpio* (Nandeesha et al., 2000). In contrast to these findings, Nile tilapia, *Oreochromis niloticus*, showed reduced growth at low (5 %) silkworm pupae dietary inclusion (Boscolo et al., 2001). Snakeskin gourami, *Trichopodus pectoralis*, show equal growth at 50 % fish meal replacement and reduced growth at higher replacement levels (Jintasataporn et al., 2011). Jian carp (*C. carpio var. Jian*) is a newly developed strain of *C. carpio* which showed similar growth response to dietary inclusion of silkworm pupae as *T. pectoralis*, fishmeal replacement above 50 % resulted in reduced growth (Ji et al., 2013). Silkworm pupae have a high quality nutritional profile, are relatively abundant, are a cheap commodity and have shown promising results in previous fish dietary trials across multiple species.

The yellow mealworm (*Tenebrio molitor*) is the larval form of the darkling beetle (Tran et al., 2015). This is a pest of food stores, affecting mainly grains and flour (Ramos-Elorduy et al., 2002). This does make them easy to feed and rear artificially. Adults contain quinones rendering them unusable as a feed source, however, the

larvae are high in protein, high in lipids and low in ash making them a high quality feed item (Makkar et al., 2014). Mealworms are produced on an industrial scale as animal feed (Veldkamp et al., 2012) and are usually fed to birds, reptiles, batrachians, Callitrichidae and fish (Tran et al., 2015). Mealworms are considered highly palatable to fish (Henry et al., 2015) and dietary inclusion of dried mealworm has shown good results. Inclusion up to 26 % (60 % fishmeal replacement) yielded equal or improved growth in African catfish compared to fish fed fishmeal diets but higher rates of inclusion: 35 - 43 % (80-100 % fishmeal replacement) produced reduced growth (Ng et al., 2001). Mealworms have also been used in feed for carnivorous fish species; at 50 % dietary inclusion for rainbow trout (Gasco et al., 2014a), and at 25 % inclusion for gilthead seabream, *Sparus aurata*, and European sea bass, *D. labrax* (Gasco et al., 2014b, Piccolo et al., 2014). As with silkworm pupae, mealworms (*T. molitor*) are readily available, relatively cheap, and possess a high quality nutritional profile.

Earthworms are a specific group of invertebrates belonging to the Oligochaetes; within the phylum Annelida. Worldwide there has been over 3000 earthworm species identified with 27 found in the UK. Earthworms can be classified into one of four ecotypes: (i) compost earthworms, found in areas rich in rotting vegetation such as compost, (ii) epigeic earthworms live on the soil surface amongst leaf litter, (iii) endogeic earthworms live in and feed on soil, making horizontal burrows as they move around, and (iv) anecic earthworms, which make permanent vertical burrows into which they drag leaves into to feed on (ESB, 2015). Earthworms are traditionally used as fishing bait. In the UK the recreational fishing industry had an estimated value of £1.16 billion in 2005, of this expenditure £833 million can be attributed to fishing trip specific costs which encompasses bait (Sen et al., 2011, Mawle and Peirson, 2009). Commercial production of many bait species, including earthworms, has developed to meet the high demand. The lob worm, a term used throughout the angling community to describe a large earthworm, is highly desired. The most common species of large earthworm living in gardens is *Lumbricus terrestris*, however, this is an anecic species, which creates deep vertical burrows in

the soil and commercial culture in standard shallow trays is not feasible. The two species that are commercially produced are composting red wrigglers (*Eisenia fetida*, formerly spelt: *foetida*), and epigeic European night crawlers (*Eisenia hortensis* or *Dendrobaena veneta*). Of these the European night crawler grows larger, but the red wriggler is common worldwide, reproduces faster and, being a composting species, can be reared on a wide variety of feed items including waste stream materials. *E. fetida* has been analyzed as a potential protein source for inclusion into fish feeds as an alternative to fishmeal, on dry matter basis. Protein content is high, between 54.6 - 71 %, with an amino acid balance close to that of fishmeal (Zhenjun et al., 1997, Dynes, 2003). Inclusion of dried earthworm meal, made from *E. fetida*, at low levels into diets of rainbow trout showed no negative health implications (Stafford and Tacon, 1985). Fish fed earthworm diets showed improved feed efficiency (Velasquez et al., 1991). Research into other earthworm species has also been conducted with promising results for earthworm meal being used in place of fishmeal to some extent (Pucher et al., 2014, Dedeke et al., 2010). A method for earthworm meal production has been published by Istiqomah et al. (2009), modified from Edwards (Edwards, 1985), which will be emulated for earthworm meal production during this research. The species of choice for investigation will be, for the reasons given above, the red wriggler (*E. fetida)*.

Chapter 2

2.0 Fish perception of food

2.1 Feed detection and acquisition

A feed must be appealing to the target fish to encourage consumption. An understanding of the physiological capabilities which enable fish to perceive feed items is vital. Feed must be located, "captured" then consumed. Teleost fish possess several sensory capabilities employed in obtaining feed items in natural habitats. Sensory systems can include vision, hearing, current and pressure detection, electroreception, magnetoreception, olfaction and gustation (Hara and Zielinski, 2007). Many species of fish display a vigorous feed response in intensive culture. Gustation is the sensory system which enables feed assessment once feed items have been attained, therefore taste will be focused on during this research due to the importance of feed achieving a positive response. Olfaction has also been discussed here as a key sense for feed detection over greater distances, such as those found in pond culture systems.

2.2 Visual and physical sensory systems.

Teleost fish comprise the largest group of vertebrates, greater than 25 000 species, which span the globe (Fernald, 2000), thus a wide range of evolutionary differences are seen between species. The eye structure in fish is superficially similar to that of terrestrial vertebrates (Fernald, 2000, Kapoor and Khanna, 2004), however, certain fish species focus by moving the lens, as opposed to changing its shape (Kapoor and Khanna, 2004). Rod and cone cells are present, rod cells detect intensity of light and cone cells detect colour, as in other vertebrates which have colour vision; most vertebrates possess colour pigments within the cone cells: rhodopsin, formed when opsin combines with vitamin A_1 (11-*cis*-retinal) based retinal. A second form, porphyropsin, created when opsin combines with vitamin A_2 (11-*cis*-3-dehydroretinal) based retinal, is found in the cone cells of fish, amphibians and aquatic reptiles (Fernald, 2000). The wavelength absorption specificity of opsins (both rhodopsin and porphyropsin) is determined by its interaction with the retinal

molecule. This produces different cone cell types which contain varied photopigments each with an optimal sensitivity to different light wavelengths. The human retina contains three cone cell types, trichromatic vision, referred to as short (optimal absorbance of 419 nanometres, blue), medium (optimal absorbance of 531 nm, green) and long (optimal absorbance of 559 nm, red); human rod cells have an optimal absorbance of 496 nm (Purves et al., 2001). Fish colour pigments generally match the colour spectrum environment in which they live. Fish generally also have trichromatic vision, with the exception of few species which have a fourth type of cone cell and tetrachromatic vision. Adult zebrafish (*D. rerio*) also respond to ultra violet (UV) cues (Nava et al., 2011, Risner et al., 2006) and juvenile rainbow trout (*O. mykiss*) and brown trout (*Salmo strutta*) can detect UV light until two years of age, tetrachromatic vision only returns afterwards, in *O. mykiss*, when mature and during spawning periods (Coughlin and Hawryshyn, 1994, Bowmaker and Kunz, 1987). Other species which have been shown to detect UV light include goldfish (Neumeyer, 1992, Neumeyer and Arnold, 1989) and carp species (Hawryshyn and Harosi, 1991). Guppies (*Poecilia reticulate*) have been found to express six different cone types (Kapoor and Khanna, 2004). Research suggests that vision is a dominant sensory system in species living in clear water environments with complex eye structure and advanced visual capabilities, whereas fish living in murky or muddy environments may rely more on other senses. Those fish with developed eyes are fully capable of perceiving colour of prey or food items.

Studies testing environmental colour preference and food item colour preference have been conducted for several species. These highlight preferences of particular species for specific colours under specific environmental conditions. Rainbow trout (*O. mykiss*) possess tetrachromatic vision with cones that express optimal absorbance of 365 nm (UV), 434 nm (indigo), 531 nm (green), and 576 nm (yellow) (Sabbah et al., 2013). *O. mykiss* display a preference for environmental colour to be green or blue (Luchiari and Pirhonen, 2008), with a background colour of pale greenish-blue. *O. mykiss* prefer feed items coloured as follows: blue, red, black, orange, brown, yellow, and green, however, with other background colours feed

items of highest contrast were preferred. Specific colour combinations also result in higher consumption (Ginetz and Larkin, 1973). Zebrafish (*D. rerio*) possess tetrachromatic vision with cones that express optimal absorbance of 360 - 362 nm (UV), 415 - 420 nm (violet-indigo), 480 - 520 nm (blue-green), and 570 - 600 nm (yellow-orange) (Robinson et al., 1993, Hughes et al., 1998, Risner et al., 2006). Zebrafish show an aversion to a blue environment relative to red, green and yellow. No significant difference was seen between the other three colours (Avdesh et al., 2012, Avdesh et al., 2010). Zebrafish prefer red feed items (Spence and Smith, 2008).

High density culture systems are highly competitive environments; as a result a behavioural feeding response in a large fish shoal can be initiated by movement patterns of a minority of individuals. Fish are capable of hearing; they can also detect vibrations and water currents surrounding them. Sound and vibration are detected by the inner ear, the lateral line systems (mechanoreceptor, cilia hair cells encapsulated in jelly like capula which respond to water movement), otoliths (dense bones inside the skull act as an accelerometer) and, in some fish, the swim bladder (couples with the inner ear via three bones, Weberian Ossicles, and acts as a pressure gradient sensor) (Popper et al., 2003). These sensory mechanisms, alongside visual stimuli, enable fish to detect the movements of other fish and respond accordingly. Once a feed response is initiated in one or two individuals, often the surrounding fish also respond.

2.3 Chemosensation

Chemosensation (smell and taste) is the detection of chemical stimuli in the external environment via specific chemicals binding to chemoreceptors; essential for vertebrate survival and reproduction. Chemical sensory systems evolved 500 million years ago making them the most ancient of the sensory systems (Hara, 1994g). Chemoreception plays a crucial role in detection of and discrimination between food and toxins, predator avoidance, mating and territoriality (Prasad and

Reed, 1999). Two types of chemoreception are described here. Olfaction is the detection of odorants and pheromones in the nasal cavity of the olfactory system, once a chemical is detected information is transmitted to the central nervous system (CNS) via the neurones of cranial nerve I (smell). Gustation is the detection of tastants, typically with the tongue. Once a chemical is detected by gustatory epithelial cells information is transmitted to the CNS via neurones of cranial nerves VII (facial), IX (glossopharyngeal), and X (vagal) (Shi and Zhang, 2009, Hara, 1994g).

2.3.1 Olfaction

There are two anatomically distinct olfactory organs; the main olfactory epithelium (MOE) and the vomeronasal organ (VNO). It is now thought both the MOE and VNO detect ordinary odorants and pheromones (Shi and Zhang, 2009). Fish, living in an aquatic environment, often rich with chemical stimuli, have evolved highly developed chemosensory systems. The olfactory epithelium, or mucosa, lines the floor of the nasal cavity..

There are a series of known physiologically important odorants for teleost fish; amino acids, polyamines and nucleotides (all food signalling molecules), bile acids, steroids and prostaglandins (pheromones) and other, so far unidentified, alarm substances (Korsching, 2009). The olfactory thresholds for amino acids have been electrophysiologically determined across 30 species of fish. The values consistently range from 10^{-9} to 10^{-7} mol^{-1} throughout the species examined for the most stimulatory amino acids, which approximates free amino acid levels found in natural waters (Hara, 1994a, Hara, 1994g). As total amino acid level in surrounding water increases, so does the apparent threshold for individual amino acids (Caprio, 1982). Olfactory response to amino acids is conserved across fish species; it has not become species-specific (Hara, 1994a). Olfactory response will not be investigated here.

2.3.2 Gustation

Fish have developed and express very species-specific responses to the following substances: amino acids, betaine, nucleotides and nucleosides, amines, sugars and other hydrocarbons, organic acids and alcohols, each acting as a stimulant, indifferent or deterrent substance depending on the species. Amino-acids are the most studied chemical stimuli in fish taste ability and preference. Electrophysiological analyses has determined thresholds for the most stimulatory amino acids to range between 10^{-10} M to 10^{-6} M dependant on species (Hidaka et al., 1976, Caprio, 1978). Table 2.1 shows the electrophysiological response to amino acid, betaine and inosine monophosphate (IMP) stimuli for the species of interest during this project except for *D. labrax* as this research has yet to be conducted.

Table 2.1. Stimulatory effectiveness (in relation to L-Alanine in percent) of taste stimuli: amino acids, betaine and IMP, as determined using electrophysiological analyses for the four target fish species of this study. Stimuli concentration used is 1mM unless stated otherwise.

Taste stimuli	Stimulatory effectiveness at pH 6.4-7.2 (Mean ± SD)			
	Zebrafish, *Danio rerio.*[1]	Common Carp, *Cyprinus carpio.*[2]	Rainbow Trout, *Oncorhynchus mykiss.*[3]	Tilapia, Oreochromis niloticus.[4]
L-Alanine (standard)	100	100	100	100 ± 6.3
L-Arginine	16 ± 3	0 [a]	0	157.3 ± 22.5
L-Asparagine	-	0	0	-
L-Aspartic acid	1 ± 0.1	43.0 ± 16.9 [b]	0	-
L-Cysteine	48 ± 8	83.4 ± 17.4	0	-
L-Glutamic acid	10 ± 2	76.4 ± 25.1 [c]	0	110.2 ± 17.9
L-Glutamine	-	0	0	144.9 ± 6.6
Glycine	36 ± 5	52.1 ± 12.4	0	-
L-Histidine	16 ± 5	47.4 ± 17.7	0	161.5 ± 171
L-Hydroxyproline	-	47.9 ± 11.9	127.5 ± 26.5	-
L-Isoleucine	-	0	0	-
L-Leucine	8 ± 0.1	0	108.1 ± 48.9	-
L-Lysine	9 ± 0.1	0 [d]	0	-
L-Methionine	-	0	0	154.6 ± 13.2
L-Phenylalanine	-	0	61.6 ± 36.9	-
L-Proline	99 ± 3	107.1 ± 10.6	183.7 ± 30.5	51.7 ± 6.9
L-Serine	53 ± 4	46.3 ± 12.1	0	154.9 ± 18.0
L-Threonine	-	0	0	-
L-Tryptophan	-	0	-	102.6 ± 21.4
L-Tyrosine	49 ± 19	-	-	-
L-Valine	1 ± 0.1	0	0	-
Betaine	5 ± 2	68.9 ± 24.1	130.2 ± 69.2	44.6 ± 12.6
Inosine monophosphate (IMP)	9 ± 4	-	-	-
L-alpha-amino-beta-guanidinopropionic acid	-	-	265.2 ± 1014	-
L-Argininic acid	-	0	166.0 ± 61.0	-

Grey highlights non-essential amino acids, green highlights essential amino acids.
[1] (Yasuoka and Abe, 2009). Data estimated from a bar graph. Figure presented is mean ± STerror.
[2] (Marui et al., 1983d). [a] L-Arginine-HCL. [b] L-Aspartate-Na. [c] L-Glutamate-Na. [d] L-Lysine-HCL.
[3] (Marui et al., 1983a).
[4] (Yacoob et al., 2001).
- No data presented.

The mammalian gustatory system is capable of discriminating five basic tastes; bitter, salty, sour, sweet and umami (umami is stimulated by amino acids) (Lindemann, 2001, Zhang et al., 2003). Stimulation of sweet and umami receptors identifies nutrients, eliciting an appetitive response, bitter receptors identify noxious and toxic stimuli, provoking aversive responses (Shi and Zhang, 2009, Zhang et al., 2003). Taste receptor cells are small neuroepithelial cells located throughout the oral cavity and concentrated in distinct regions, taste papillae, of the tongue and palate epithelium (Zhang et al., 2003, Behrens and Meyerhof, 2009), receptor cells are contained within taste buds in varying numbers, dependant on species, along with precursor and support cells (Lindemann, 1996). Two families of G-protein coupled receptors (GPCR's) are selectively expressed in subgroups of taste receptor cells; T1Rs and T2Rs (Hoon et al., 1999). The T1R receptor family consists of three GPCR's distantly related to metabotropic glutamate and V2R vomeronasal receptors (Zhao et al., 2003, Hoon et al., 1999, Nelson et al., 2001). These are T1R1, T1R2 and T1R3 which combine generating two heteromeric receptors which mediate sweet and umami taste (Zhang et al., 2003). T1R1 is always expressed with T1R3 to form a heteromeric receptor which detects L-amino acids and monosodium L-glutamate (MSG); umami taste (Nelson et al., 2002, Shi and Zhang, 2009, Zhao et al., 2003). T1R2 is also always expressed with T1R3 forming a broad heteromeric sweet receptor (Nelson et al., 2001, Li et al., 2002, Zhao et al., 2003), capable, in humans and mice, of detecting all classes of sweet compounds; artificial sweeteners, D-amino acids, natural sugars and intensely sweet proteins (Zhao et al., 2003). Without coexpression of two T1R receptor types no response to a sweet or umami stimuli is achieved (Vigues et al., 2009). The T2R receptor family comprises ~30 taste specific GPCRs, remotely related to opsins (Zhao et al., 2003), which detect bitter tastants (Behrens and Meyerhof, 2009). T2Rs may also act as heteromeric receptors in order to accommodate the wide array of bitter tastants (Zhang et al., 2003).

Fish species are more sensitive than mammals to water soluble chemical compounds, nerves associated with taste respond to compounds including L-amino

acids, nucleic acids, fatty acids, alkaloids, organic and inorganic acids, and salts (Hara, 1994g, Yasuoka and Abe, 2009). Taste buds also constitute a major part in teleost gustatory systems. A fish orthologue of a common effecter enzyme, phospholipase C-β2 (fPLC-β2), is expressed in fish taste buds (Yasuoka et al., 2004), as in mammalian taste buds, which enables manipulation and visual expression through fluorescence of taste bud cells using mfPLC-β2 promoter (Yasuoka and Abe, 2009). In teleost fish, unlike mammals were taste buds are restricted to the oropharyngeal region, taste receptors are distributed in the oral cavity and pharynx (pharyngeal epithelia), lips, gill arches and rakes, appendages (barbells and fins) and the body surface depending on species (Hara, 1994a, Hara, 1994g, Yasuoka and Abe, 2009, Ishimaru et al., 2005). The number and distribution of taste buds is highly variable between fish species; salmonids for example lack external taste buds, however, taste buds are present in high density, 30 per mm^2, within regions of the palatal organ surrounding the teeth (Marui et al., 1983a, Hara, 1994a). Cyprinids and siluroids on the other hand show an abundance of external taste buds (Hara, 1994g). Cyprinids in particular possess external taste buds all over the body and fins, of the 10 species studied by Gomahr et al. (1992), minnow (*Phoxinus phoxinus*) express the highest density on any region of the body; up to 297 ± 75 taste buds per mm^2 in the gular region, the density of oral taste buds in cyprinids is between 300 - 400 mm^{-2} (Osse et al., 1997). Information gathered by taste buds is transferred to the CNS via the same cranial nerves as mammals; VII, IX and X (Yasuoka and Abe, 2009). Nerve VII constitutes the extra oral taste system associated with feed detection, nerves IX and X constitute the oro-pharyngeal taste system, associated with feed assessment and ingestion (Kanwal and Caprio, 1983). Taste bud and taste nerve organisation along the anterior-posterior axis are predominantly conserved between fish species and are comparable with mammals (Puzdrowski, 1987, Kotrschal, 2000). Teleost taste buds are bulbiform in shape, they vary in size (45 - 75 μm in height and 30 - 50 μm in width), and they contain receptor, support and basal cells mirroring mammalian taste buds. Unlike olfactory receptors, which are neurons, gustatory receptors are specialised epidermal cells with single or two apical processes (microvilli) (Hara, 1994g, Hara, 1994a). The number of taste receptors per taste bud varies substantially amongst fish species

from as little as five in *Pomatoschistus* (*Gobiidae*) to as many as 67 in *Corydoras* catfish (*Callichthyidae*) (Jakubowski and Whitear, 1990).

Homology-based analyses of model fish genome databases in search of candidate taste receptors revealed two receptor families; T1Rs and T2Rs, which show significant similarity to corresponding mammalian taste receptors (Ishimaru et al., 2005). Four T1R and six T2R receptors were first identified in the puffer fish (*Fugu rubripes*) (Venkatesh et al., 2000). This result was used to search zebrafish (*D. rerio*) and medaka fish (*Oryzias latipes*) databases, four T1R and seven T2R receptors were located in *D. rerio,* five T1R and one T2R receptors were found in *O. latipes* (Yasuoka and Abe, 2009, Ishimaru et al., 2005). As bioinformatics has revealed homologous T1R, T2R and fPLC-β2 genes in fish and mammals (Ishimaru et al., 2005, Yasuoka et al., 2004, Go, 2006, Shi and Zhang, 2006), and fPLC-β2 has been shown to be co-expressed with both T1R or T2R receptors (Ishimaru et al., 2005, Asano-Miyoshi et al., 2001), it is reasonable to suggest common mechanisms for taste reception among vertebrates (Oike et al., 2007). Fish T1R1s demonstrate the greatest degree of amino acid identity with mammalian T1R1s (39 - 43 %, with 56 - 67 % identity between fish species), subsequently followed by fish T1R3s with mammalian T1R3s (34 - 37 %, with 51 - 58 % between fish species), and fish T1R2s showing equal identity to mammalian T1R1s and T1R2s (31 - 34 %, with 42 - 62 % between fish species) (Ishimaru et al., 2005). A CLUSTAL W phylogenetic analyses carried out by Ishimaru et al. (2005) suggested fish T1R1 and T1R3 receptors are orthologs of mammalian T1R1s and T1R3s while fish T1R2 receptors are not orthologs of mammalian T1R2s; fish T2Rs were not orthologous to mammalian T2Rs (13 - 22 % identity). This, therefore, suggests each fish T1R2 member is species-specific. Due to the presence of multiple T1R2 receptors fish may have wider responsiveness in receptor cells than mammals do (Ishimaru et al., 2005).

Heteromeric receptors are formed between fish T1R1 and T1R3; they are also formed between T1R2 and T1R3 receptors, mimicking the mammalian gustatory

system. Contrasting mammalian systems fish have a small number of taste receptors, which express individual T1R gene members (Ishimaru et al., 2005), suggesting a more complex gustatory system (Hashiguchi et al., 2007). Furthermore, fish possess fewer T2R receptors than mammals (Yasuoka and Abe, 2009). Analogous to mammals, fish T1R1/T1R3 heteromers respond to L-amino acids. Unlike mammals', fish T1R2/T1R3 heteromers do not respond to sugars or other mammalian sweeteners (Yasuoka and Abe, 2009). In zebrafish and medaka fish they have been shown to respond to L-amino acids; amino acids which exhibit sweet or umami taste to mammals (Oike et al., 2007, Yasuoka and Abe, 2009). Mammal taste responses to L-amino acids showed a potentiated response with addition of purine nucleotides such as inosine monophosphate (IMP) (Hellekant and Ninomiya, 1991, Yoshii et al., 1986). Interestingly, fish receive IMP, they also receive betaine, a compound known to also elicit taste responses in certain fish species (Valentincic and Caprio, 1997, Kiyohara and Hidaka, 1991, Marui and Kiyohara, 1987), however, no such potentiated response was observed in the majority of fish to either compound (Yasuoka and Abe, 2009, Oike et al., 2007), with a few exceptions where they act as stimulants (Kasumyan and Doving, 2003). Fish T2R receptors responded to two tastants which are perceived as bitter to mammals; denatonium benzoate and quinine chloride (Yasuoka and Abe, 2009, Chandrashekar et al., 2000). These findings suggest fish share some taste modalities with mammals.

2.4 Palatability

Study of fish gustatory systems began with morphological techniques in the 19th century, this advanced into electrophysiological methods (Kasumyan and Doving, 2003), that analyse stimulation of gustatory receptors, which gave insight into what fish are capable of tasting. Electrophysiological analyses revealed how several tastants elicit a response within varying taste receptors across several fish species (Hara, 1994a, Hara, 1994g, Yasuoka and Abe, 2009, Oike et al., 2007, Caprio, 1975). However, this method is highly invasive and does not present a complete overview of fish gustation response. Studies principally focus on only one cranial nerve, the facial (VII) nerve of the extra oral system, which innervates the front palate and

external taste receptors, thus omitting other taste receptors involved in gustatory testing of feed items which are innervated by nerves IX and X, the oro-pharyngeal system (Marui et al., 1983a, Marui et al., 1983d, Kasumyan and Morsi, 1996). Electrophysiological analyses show a physiological response to a tastant, and the intensity of the response, however, they do not determine the nature of that response, if that tastant is perceived positively or negatively.

Alternative research in the field has involved interpretation of how fish perceive tastants and taste stimuli (Kasumyan, 1997) based on evaluation of non-invasive behavioural responses of fish to a substance; otherwise known as the palatability of a substance or taste preference. A method using agar gel as a solid transporter for potential tastants was developed by Mearns et al. (1987) and expanded upon by Alexander Kasumyan and partners (Kasumyan and Sidorov, 1993a, Kasumyan and Sidorov, 1993b, Kasumyan and Sidorov, 1995a, Kasumyan and Sidorov, 1995b, Kasumyan and Morsi, 1996, Kasumyan and Morsi, 1997, Kasumyan, 2004). This method can distinguish between olfaction and gustation responses when applied using anosmiated fish (Kasumyan and Morsi, 1996). It also presents a quantitative index of palatability in percent which may be applied across species.

Of the classic taste modalities: sweet, sour, bitter, salty and umami, only bitter and umami receptors have been identified in fish. Historic testing of taste preference in fish to basic taste sensations consisted of the following substances: sucrose (sweet), acetic acid (sour), quinine (bitter), and sodium chloride (salty). Out of 36 [sic] species of fish tested (Kasumyan and Doving, 2003), sucrose proved to be a stimulant for 15 species; predominantly herbivorous or omnivorous species such as grass and common carp, dace, roach, guppy, black molly and platy (Kasumyan and Morsi, 1996, Kasumyan, 1997, Kasumyan and Morsi, 1997, Andriashev, 1944, Kasumyan and Nikolaeva, 1997, Kasumyan and Nikolaeva, 2002), sucrose proved indifferent to 18 mainly carnivorous species and was a deterrent for one species; puffer (Hidaka, 1982). Taste preference testing of acetic and other acids has been

conducted for 37 [sic] fish species (Kasumyan and Doving, 2003): in 16 of which, primarily acipenserids and many cyprinids, it was a deterrent. For 15 species, primarily salmonids and poecilids, acids acted as stimulants, however, a further eight species of fish were indifferent to citric acid. Quinine was found to be a feed deterrent in all fish species examined, in contrast, calcium chloride was found to be a deterrent to three species, a stimulant to seven species and indifferent for 17 species. Sodium chloride has proved to either be indifferent or a stimulant in different fish species (Kasumyan and Doving, 2003). From these early studies, focused on classic taste substances, which examined gustation in fish, results have been gathered that suggest fish possess mechanisms capable of detecting other taste modalities. They simply have not yet been identified. Chemical substances can be categorised into several types depending on their effects on the feed response; 'Incitants', prompt capture of food items by means of the extraoral taste system, 'Suppressants', mediated again by the extraoral taste system, decrease the rate at which food items are grasped, 'Stimulants', promote feeding and ingestion of food items, usually upon first capture of the item; this is controlled by the oral taste system, 'Deterrents', mediated by the oral taste system, also evoke food rejection and abandonment. 'Enhancers' are substances which on their own may not provoke a response but potentiate the response to other substances; this is also mediated by the oral taste system. Finally substances can be indifferent; failing to evoke a response at all (Kasumyan and Doving, 2003).

Amino acids can be efficient incitants, stimulants (Hidaka, 1982, Adams et al., 1988, Mackie, 1982, Mackie and Mitchell, 1983, Mearns et al., 1987, Lamb and Finger, 1995, Jones, 1989) or deterrents to specific fish (Kasumyan and Doving, 2003). The taste preferences of each species is highly specialised, see Figure 2.1 for the taste preference profile of *C. carpio*. The most important and required dietary amino acids, the essential amino acids (EAA) (Millkin, 1982), are not necessarily always palatable as is the case with *C. carpio*. The time frame between feeding events also influences the behavioural response to taste stimuli. It has been shown in *C. carpio* that as the time frame since last feed increases, the discrimination of feed item

taste diminishes; the number of previously deterrent stimuli decreases and the fish ingests a wider range of feed items (Kasumyan and Sidorov, 2010). As fish produced in aquaculture are typically fed multiple times daily under high stocking or naturally feed throughout the day under low stocking their taste preference profiles will remain unchanged.

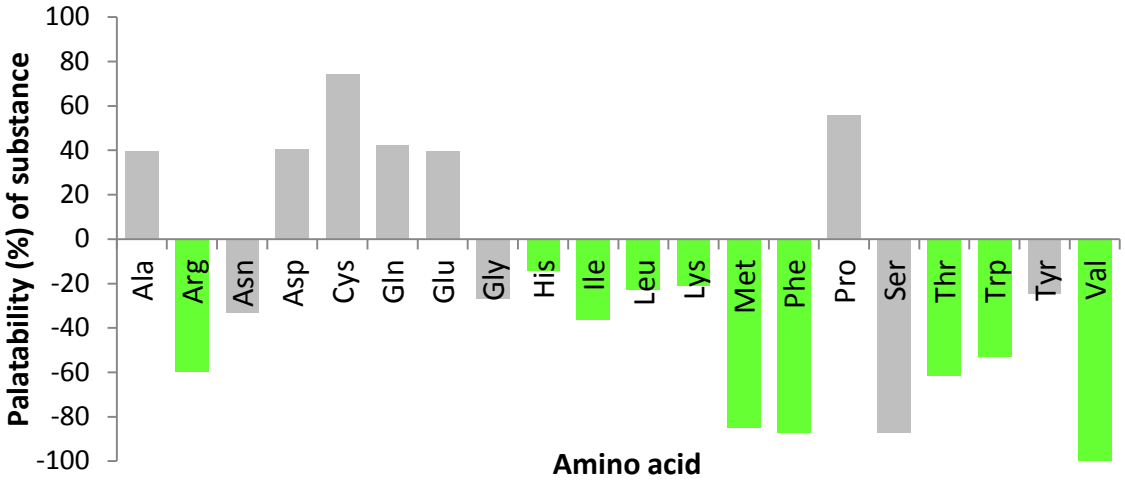


Figure 2.1. Index of palatability (%) in Common carp (*Cyprinus carpio*) to non-essential (grey bars) and essential (green bars) amino acids. Data modified from (Kasumyan and Morsi, 1996). Amino acid concentration: L-Tyrosine (0.001M), L-Tryptophan, L-Isoleucine, L-Leucine, L-Glutamic acid and L-Aspartic acid (0.01M), all remaining amino acids (0.1 M).

Palatability of a feed or feed ingredient is important, influencing acceptance and success of its use. The agar gel method of determining substance palatability provides the most informative taste response data; however, this method can only test individual stimuli, such as crystalline amino acids for example. Therefore, due to lack of established methods for testing palatability of whole ingredients, many studies investigating feed ingredients simply use feed intake of the completed diet to assess taste response (Solomon et al., 2017, Houlihan et al., 2001, Jobling, 2016).

In this study the agar gel method (Kasumyan and Morsi, 1996, Kasumyan, 2004) was used but modified to attempt to establish a method of testing palatability response to powdered feed ingredients across multiple species.

2.4.1 Experimental procedure:

2.4.1.1 Subjects and housing

Experiments were conducted sequentially using three fish species: 13 Carp (*C. carpio*), sourced from Rodbaston College Aquaculture, measuring 9 cm, 16 Tilapia (*O. niloticus*), sourced from Stirling University, measuring 12 cm, and six Rainbow Trout (*O. mykiss*), sourced from Kilnsey Park Estate trout farm, measuring 30 cm. Carp and Tilapia were housed individually in 20 L tanks, maintained at 25 ± 1 ^{o}C, on a central system. Trout were housed individually in 100 L tanks on a separate central system, maintained at 14 ± 1 ^{o}C. The 20 L tanks possess self-cleaning outflow pipes, both sizes of tanks had an inflow enter through the lid; a vertical spray bar was used in the 100L tanks to provide necessary flow for trout. All tanks had all sides except the front panel covered by vinyl, preventing influence from behaviour of neighbouring conspecifics, tank lids had one end covered by vinyl (providing cover), and a hole at the other for introduction of feed items/experimental pellets. Cool white (6500 K) LED lights were situated above the tanks. Air was supplied to tanks via a compressor, delivered through porous ceramic aquarium air stones, aiding suspension of experimental pellets.

2.4.1.2 Experimental agar gel pellets

Pellets, 2 mm square, were cut from agar gel (2 %), using a stainless steel cutting wheel with multiple blades. Agar powder was mixed into water (2 %) containing the dye, then autoclaved using a Prestige ™ Medical Series 2100 clinical autoclave at 126 ^{o}C for 10 minutes, sterilising the gel in the process. The gel fully dissolved leaving a clear coloured liquid gel, any tastant was mixed into the liquid gel at 75 g/L between 40 - 50 ^{o}C, before the gel started setting below 40 ^{o}C. After addition of tastant, except controls which only contain the dye, the liquid gel was immediately

mixed thoroughly then poured into 7 cm square weigh boats (20 ml mix per boat, 2 ml deep gel), then allowed to set before transferred to a 5 ^{o}C fridge to fully cool. Once cooled, the gel was cut into 2 mm square pellets for use in experiments.

Using ponceau 4R red stain (E124 ponceau 4R granulate 80 %) at 5 µM/L produced a pale pink gel, in order to achieve the 'bright red' colour described by Kasumyan and Morsi (1996), after testing increased concentrations, a dye concentration of 50 µM/L was used (Figure 2.2). Upon testing gel production with addition of tastants, macerated Chironomidae larvae and mealworm meal, the gel colour altered with addition of tastant material (Figure 2.2), therefore, addition of a second blue dye, indigotine (also known as indigo carmine) was tested, experimental pellets are required to be visually indistinguishable from one another. Addition of indigotine (E132 indigotine 85 %) at increasing concentrations produced increasingly darker purple gels which remained most similar in colour with addition of macerated chironomidae larvae and mealworm meal, (Figure 2.2). ImageJ was used to measure the mean RGB value for a consistent section of each photo of the gels, with increasing Indigotine added 10 - 50 µM/L, the lowest difference between sample pellets was achieve by 50 µM/L red and 50 µM/L blue dye (55.1, 46.2, 50.0, 35.8 and 27.9). A final dye concentration of 50 µM/L ponceau 4R red and 50 µM/L indigotine was used. Stock dye concentrate solutions of 1 mM were made, with RO water, and used for each dye, 0.75559 g/L ponceau 4R, 0.54865 g/L indigotine.

The final experimental pellets (Figure 2.3) consisted of control, containing only the dye, and the following tastants at 75 g/L: macerated Chironomidae larvae, Danish fishmeal, silkworm meal, earthworm meal, mealworm meal; insect mix (25 g/L of each silkworm, earthworm and mealworm meals), rapeseed meal, Natto, PPC, NH Algae, and MSG. As mentioned in the gustation section above, fish T1R1 and T1R3 heteromeric receptors detect umami; therefore MSG was included in this trial for initial taste testing before further investigation of its use as a feed attractant. Some tastant materials presented a more profound change in appearance in the gel, the

NH Algae is much paler in colour than the other tastants used, it also clumped when mixed into the liquid gel, slow addition and constant mixing produced optimum results, however the pellets remained more distinct than other pellets. All experimental pellets were kept at +5 ^{o}C for no more than 48 hours, any longer and gel pellets lost colour.

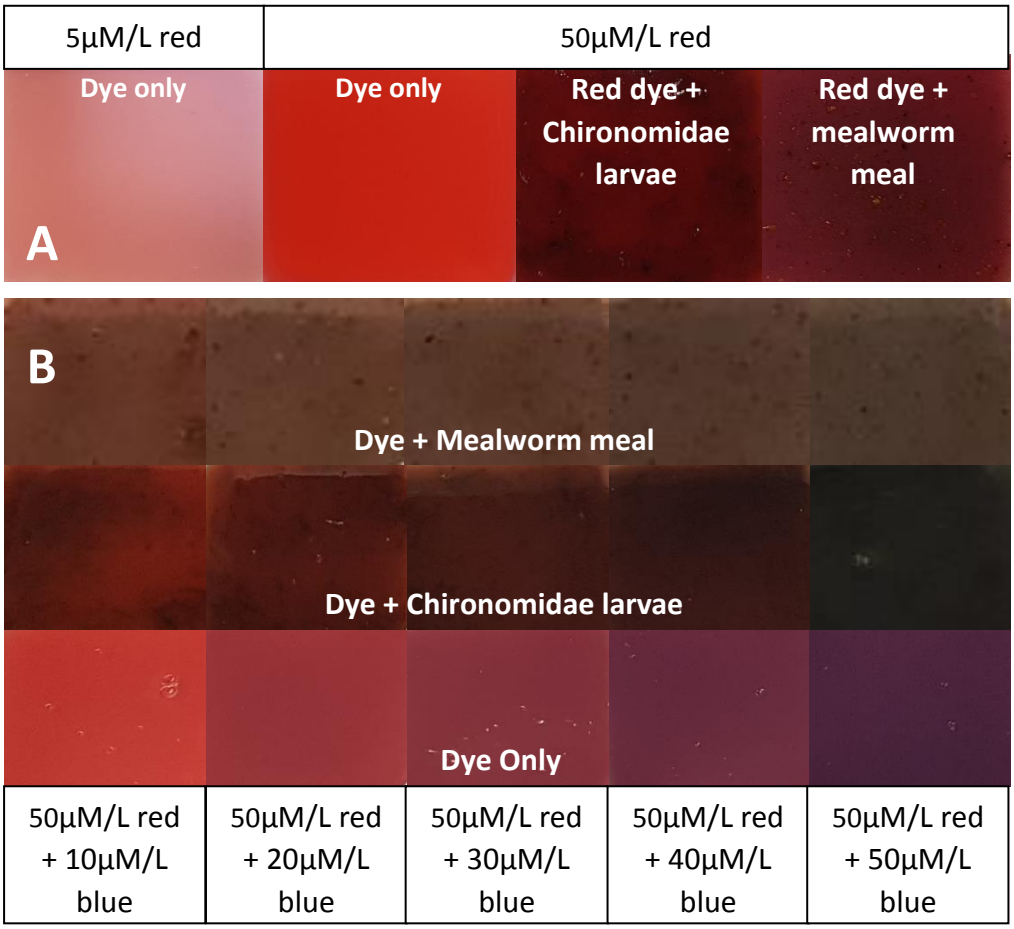


Figure 2.2. Experimental Agar gel pellets. **A)** Gel containing original dye concentration of 5 µM/L 4R red, new dye concentration of 50 µM/L, and 50 µM/L red stained gel with addition of macerated Chironomidae larvae or mealworm meal. **B)** Experimental gels containing tastants described in **A** with increased addition of indigotine dye, 10 µM/L to 50 µM/L.

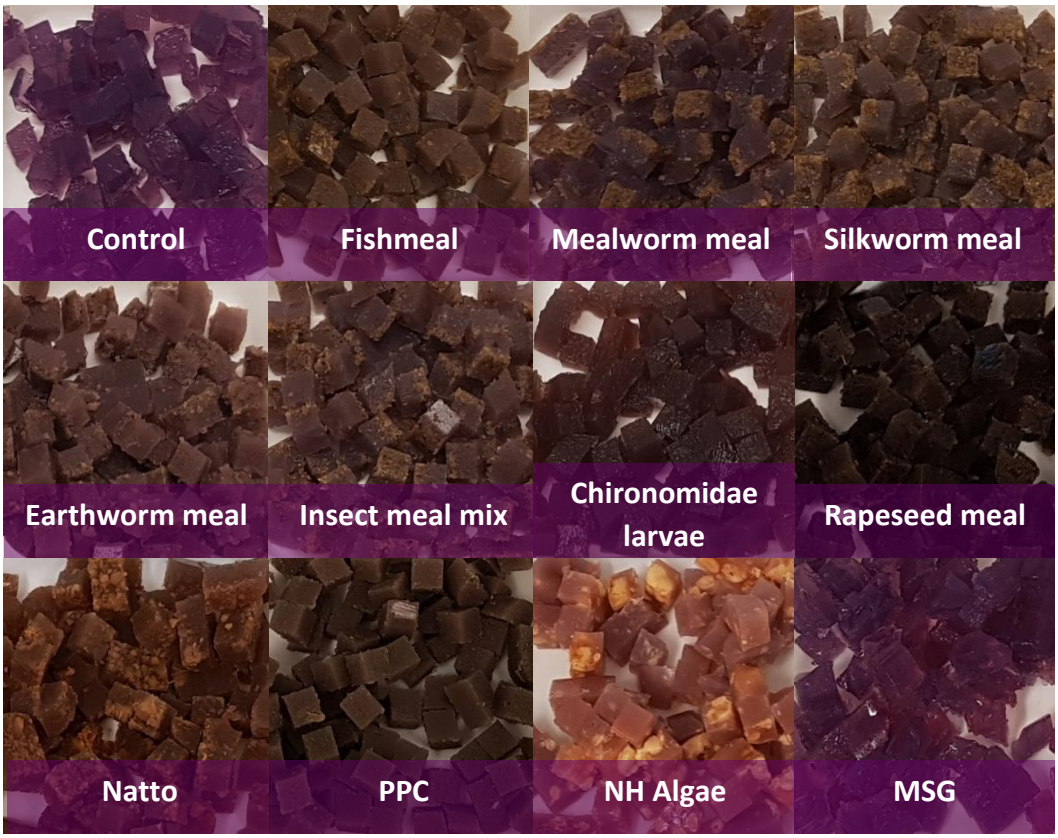


Figure 2.3. Experimental agar gel pellets containing each tastant under investigation here. Control pellets contained only dye, 50 µM/L ponceau 4R red mixed with 50 µM/L indigotine.

2.4.1.3 Behavioural observations

Experimental observations were conducted by repeating the method of Kasumyan and Morsi (1996). Fish were housed individually from time of acquisition and fed commercial pellets individually several times daily until trained to catch pellets upon entering the tank. Fish were then fed frozen fresh chironomidae larvae for three days, introducing the experimental gel pellets containing macerated chrionomidae larvae during the last two days. Fish successfully caught 100 % of the gel pellets.

Each experimental observation involved the use of a single experimental gel pellet, a one minute observation began the moment the fish caught the pellet, recording: 1) total number of times the pellet was caught; 2) the duration the pellet was held

in the mouth upon first catch (seconds); 3) total duration the pellet was held within the mouth culminated for the whole observation (seconds); 4) consumption rate of the pellet, was the pellet swallowed or rejected. From this data, percentage of eaten pellets was calculated for each tastant including the control. The index of palatability was also calculated described by Kasumyan and Morsi (1996);

$$Ind_{pal} = ((\underline{R - C)/(R + C)}) \times 100$$

Where Ind_{pal} = Index of palatability of substance; R = consumption of pellets with substance (%); C = consumption of control pellets (%).

Whether the fish swallowed the pellet was determined on the basis of it ceasing of characteristic masticatory jaw movements, and the continuation of rhythmic opercula movements, often accompanied with foraging behaviour, searching for the next food item. Pellet retention time within the oral cavity was recorded using a hand held stop watch of the summing type.

No recording was made for any occurrences whereby the experimental pellet was not caught by the fish for over a one minute period (such instances were rare). Experimental pellets containing each tastant where randomly assigned to five separate fish per species, each fish was assigned controls, chironomidae larvae, plus multiple tastants; in total carp and tilapia received between 5-6 substances per fish, whilst trout received between 10-11 substances per fish (due to smaller total number of fish used). Control pellets and those containing tastants were given in random sequence and alternated with those containing chironomidae larvae. Any rejected or ignored pellets were removed from the tank post experiment. A time interval of at least 15 minutes was given between experiments using the same specimen.

2.4.1.4 Statistical analyses

Observational data for each tastant pellet were grouped for analyses. Kolmogorov-Smirnoff tests were used to test for normal distribution of data for number of pellet catches and pellet retention time, both first catch and total. Students t-tests or Mann-Whitney U tests were used to test for differences between pellets; control vs each tastant, again for fishmeal vs each other tastant. Data gathered for pellet consumption and index of palatability is not suitable for statistical analyses.

All data was first tested comparing each tastant with the control in order to establish and compare palatability for each ingredient tested. The data was then tested again comparing each tastant with fishmeal, this determined the taste preference of the alternative materials in relation to the current industry standard material which is widely used in aquaculture feeds. Consumption of pellets and index of palatability express the taste preference of the fish for each tastant. Number of pellet catches and pellet retention times indicate pellet assessment and maceration, which can be indicative of a positive or negative response relative to the control. A tastant with high consumption, in combination with a single pellet catch and low retention time, indicates a highly stimulant ingredient, prompting rapid ingestion, whereas pellets achieving very low consumption, indicates an ingredient which is less attractive that the control.

2.4.1.5 Results

Each species was found to express varied taste preferences to the array of tastants presented to them, Tables 2.2 and 2.3 for carp, Tables 2.4 and 2.5 for trout, Tables 2.6 and 2.7 for tilapia. No direct comparisons of the index of palatability can be made between species of fish; each species consumed a differing amount of control pellets, therefore providing different possible maximum palatability figures. Consumption rate and the order in which the tastants ranked in palatability can be used to compare taste preference between species.

Table 2.2. Taste response of Carp (*Cyprinus carpio*) to feed ingredients / tastants.

Tastant	Concentration (g/l)	Consumption of pellets (%)[a]	Index of palatability (%)[a]	Number of pellet catches[b] (/min)	Pellet retention time (seconds ±SEM)[b]		Number of tests
					First catch	Whole experiment	
Earthworm meal	75	100	66.67	1 ± 0***	14.5 ± 1.1***	14.5 ± 1.1**	50
Danish Fishmeal	75	100	66.67	1 ± 0***	16.4 ± 1.1***	16.4 ± 1.1***	51
Mealworm meal	75	100	66.67	1 ± 0***	18.6 ± 2.0***	18.6 ± 2.0***	51
Insect meal mix	75	100	66.67	1 ± 0***	20.0 ± 1.1***	20.0 ± 1.1***	50
Silkworm meal	75	100	66.67	1 ± 0***	25.4 ± 1.7***	25.4 ± 1.7***	50
Natto	75	100	66.67	1 ± 0***	51.7 ± 1.9***	51.7 ± 1.9***	50
NH Algae	75	100	66.67	1.04 ± 0.04***	33.5 ± 2.2***	33.9 ± 2.3***	50
Rapeseed meal	75	100	66.67	1.1 ± 0.05***	35.0 ± 2.5***	37.8 ± 2.2***	50
Chironomidae larvae	75	78	59.18	1.74 ± 0.1***	22.0 ± 0.9***	26.2 ± 1.0***	326
PPC	75	76	58.33	1.5 ± 0.22***	46.7 ± 2.2***	49.8 ± 2.0***	50
MSG	75	10	-33.33	2.38 ± 0.4	7.2 ± 1.9	9.5 ± 2.1	50
Control	-	20	0	2.61 ± 0.23	8.6 ± 1.0	12.3 ± 1.2	130

[a] This data expresses the taste preference of the fish for each tastant pellet in relation to the control (containing only dye; 50µm/L ponceau 4R red mixed with 50µm/L indigotine).

[b] This data indicates pellet assessment and maceration compared with the control.

Significant differences are indicated with asterisk: * < 0.05, ** < 0.01, *** < 0.001.

Table 2.3. Taste response of Carp (*Cyprinus carpio*) to feed ingredients / tastants.

Tastant	Concentration (g/l)	Consumption of pellets (%)[a]	Index of palatability (%)[a]	Number of pellet catches[b] (/min)	Pellet retention time (seconds ±SEM)[b]		Number of tests
					First catch	Whole experiment	
Earthworm meal	75	100	0	1 ± 0	14.5 ± 1.1	14.5 ± 1.1	50
Danish Fishmeal	75	100	0	1 ± 0	16.4 ± 1.1	16.4 ± 1.1	51
Mealworm meal	75	100	0	1 ± 0	18.6 ± 2.0	18.6 ± 2.0	51
Insect meal mix	75	100	0	1 ± 0	20.0 ± 1.1*	20.0 ± 1.1*	50
Silkworm meal	75	100	0	1 ± 0	25.4 ± 1.7***	25.4 ± 1.7***	50
Natto	75	100	0	1 ± 0	51.7 ± 1.9***	51.7 ± 1.9***	50
NH Algae	75	100	0	1.04 ± 0.04	33.5 ± 2.2***	33.9 ± 2.3***	50
Rapeseed meal	75	100	0	1.1 ± 0.05*	35.0 ± 2.5***	37.8 ± 2.2***	50
Chironomidae larvae	75	78	-12.36	1.74 ± 0.1***	22.0 ± 0.9	26.2 ± 1.0**	326
PPC	75	76	-13.64	1.5 ± 0.22**	46.7 ± 2.2***	49.8 ± 2.0***	50
MSG	75	10	-81.82	2.38 ± 0.4***	7.2 ± 1.9***	9.5 ± 2.1***	50
Control	-	20	-66.67	2.61 ± 0.23***	8.6 ± 1.0***	12.3 ± 1.2***	130

[a] This data expresses the taste preference of the fish for each tastant in relation to fishmeal.

[b] This data indicates pellet assessment and maceration compared with fishmeal.

Significant differences are indicated with asterisk: * < 0.05, ** < 0.01, *** < 0.001.

Table 2.4. Taste response of Rainbow Trout (*Oncorhynchus mykiss*) to feed ingredients / tastants.

Tastant	Concentration (g/l)	Consumption of pellets (%)[a]	Index of palatability (%)[a]	Number of pellet catches [b] (/min)	Pellet retention time (seconds ±SEM)[b]		Number of tests
					First catch	Whole experiment	
Rapeseed meal	75	100	12.99	1 ± 0**	4.5 ± 0.4	4.5 ± 0.4**	50
Danish Fishmeal	75	100	12.99	1.02 ± 0.02**	4.8 ± 0.3	5.0 ± 0.3*	50
Mealworm meal	75	100	12.99	1.02 ± 0.02**	5.7 ± 4.3	5.8 ± 0.6	51
Earthworm meal	75	100	12.99	1.04 ± 0.04**	5.9 ± 0.5	6.1 ± 0.5	50
NH Algae	75	100	12.99	1.5 ± 0.11*	8.2 ± 0.9*	11.2 ± 1.0***	50
Silkworm meal	75	94	9.94	1.28 ± 0.11	6.6 ± 0.6	7.9 ± 0.7	50
Insect meal mix	75	94	9.94	1.10 ± 0.06	5.6 ± 0.6	6.1 ± 0.7	50
PPC	75	84	4.35	1.76 ± 0.3	8.6 ± 0.8**	11.0 ± 1.0**	50
Chironomidae larvae	75	82	3.14	1.03 ± 0.01	6.7 ± 0.2	7.0 ± 0.2*	310
MSG	75	78	0.65	1.38 ± 0.17	8.5 ± 0.8**	10.0 ± 1.0*	50
Natto	75	76	-0.65	1.62 ± 0.18	7.9 ± 0.9	9.9 ± 1.0*	50
Control	-	77	0	1.23 ± 0.08	5.7 ± 0.6	6.9 ± 0.7	60

[a] This data expresses the taste preference of the fish for each tastant pellet in relation to the control (containing only dye; 50µm/L ponceau 4R red mixed with 50µm/L indigotine).

[b] This data indicates pellet assessment and maceration compared with the control.

Significant differences are indicated with asterisk: * < 0.05, ** < 0.01, *** < 0.001.

Table 2.5. Taste response of Rainbow Trout (*Oncorhynchus mykiss*) to feed ingredients / tastants.

Tastant	Concentration (g/l)	Consumption of pellets (%)[a]	Index of palatability (%)[a]	Number of pellet catches[b] (/min)	Pellet retention time (seconds ±SEM)[b]		Number of tests
					First catch	Whole experiment	
Rapeseed meal	75	100	0	1 ± 0	4.5 ± 0.4	4.5 ± 0.4	50
Danish Fishmeal	75	100	0	1.02 ± 0.02	4.8 ± 0.3	5.0 ± 0.3	50
Mealworm meal	75	100	0	1.02 ± 0.02	5.7 ± 4.3	5.8 ± 0.6	51
Earthworm meal	75	100	0	1.04 ± 0.04	5.9 ± 0.5	6.1 ± 0.5	50
NH Algae	75	100	0	1.5 ± 0.11***	8.2 ± 0.9**	11.2 ± 0.9***	50
Silkworm meal	75	94	-3.09	1.28 ± 0.11*	6.6 ± 0.6	7.9 ± 0.7**	50
Insect meal mix	75	94	-3.09	1.10 ± 0.06	5.6 ± 0.6	6.1 ± 0.7	50
PPC	75	84	-8.7	1.76 ± 0.3**	8.6 ± 0.8**	11.0 ± 1.0***	50
Chironomidae larvae	75	82	-9.89	1.03 ± 0.01*	6.7 ± 0.2	7.0 ± 0.2	310
MSG	75	78	-12.36	1.38 ± 0.17*	8.5 ± 0.8**	10.0 ± 1.0***	50
Natto	75	76	-13.64	1.62 ± 0.18**	7.9 ± 0.9	9.9 ± 1.0**	50
Control	-	77	-12.99	1.23 ± 0.08**	5.7 ± 0.6	6.9 ± 0.7*	60

[a] This data expresses the taste preference of the fish for each tastant in relation to fishmeal.

[b] This data indicates pellet assessment and maceration compared with fishmeal.

Significant differences are indicated with asterisk: * < 0.05, ** < 0.01, *** < 0.001.

Table 2.6. Taste response of Nile Tilapia (*Oreochromis niloticus*) to feed ingredients / tastants.

Tastant	Concentration (g/l)	Consumption of pellets (%)[a]	Index of palatability (%)[a]	Number of pellet catches[b] (/min)	Pellet retention time (seconds ±SEM)[b]		Number of tests
					First catch	Whole experiment	
Earthworm meal	75	100	17.65	1.32 ± 0.14	8.5 ± 1.3	10.6 ± 1.4	50
NH Algae	75	98	16.67	1.02 ± 0.02**	10.3 ± 1.4	10.9 ± 1.5	50
Insect meal mix	75	94	14.63	1.32 ± 0.10	9.8 ± 1.8	14.6 ± 2.4	50
Danish Fishmeal	75	94	14.63	1.10 ± 0.05	15.8 ± 2.0**	16.4 ± 2.0*	50
Mealworm meal	75	88	11.39	1.22 ± 0.09	11.4 ± 1.8	13.7 ± 2.0	50
Silkworm meal	75	84	9.09	2.02 ± 0.28**	6.1 ± 1.2**	10.4 ± 1.3	50
Rapeseed meal	75	84	9.09	2.40 ± 0.32***	15.0 ± 1.9**	26.2 ± 2.2***	50
Chironomidae larvae	75	77	4.76	1.53 ± 0.08	6.2 ± 0.4**	9.2 ± 0.6*	335
Natto	75	66	-2.94	1.70 ± 0.22	9.8 ± 1.7	13.1 ± 2.0	50
PPC	75	58	-9.38	5.22 ± 0.71***	14.1 ± 2.1	27.3 ± 1.9***	50
MSG	75	26	-45.83	1.68 ± 0.31	5.5 ± 1.4***	7.8 ± 1.8***	50
Control	-	70	0	1.55 ± 0.15	9.0 ± 0.7	10.6 ± 0.8	161

[a] This data expresses the taste preference of the fish for each tastant pellet in relation to the control (containing only dye; 50µm/L ponceau 4R red mixed with 50µm/L indigotine).

[b] This data indicates pellet assessment and maceration compared with the control.

Significant differences are indicated with asterisk: * < 0.05, ** < 0.01, *** < 0.001.

Table 2.7. Taste response of Nile Tilapia (*Oreochromis niloticus*) to feed ingredients / tastants.

Tastant	Concentration (g/l)	Consumption of pellets (%)[a]	Index of palatability (%)[a]	Number of pellet catches[b] (/min)	Pellet retention time (seconds ±SEM)[b]		Number of tests
					First catch	Whole experiment	
Earthworm meal	75	100	3.09	1.32 ± 0.14	8.5 ± 1.3**	10.6 ± 1.4*	50
NH Algae	75	98	2.08	1.02 ± 0.02	10.3 ± 1.4	10.9 ± 1.5*	50
Insect meal mix	75	94	0	1.32 ± 0.10*	9.8 ± 1.8**	14.6 ± 2.4	50
Danish Fishmeal	75	94	0	1.10 ± 0.05	15.8 ± 2.0	16.4 ± 2.0	50
Mealworm meal	75	88	-3.3	1.22 ± 0.09	11.4 ± 1.8	13.7 ± 2.0	50
Silkworm meal	75	84	-5.62	2.02 ± 0.28***	6.1 ± 1.2***	10.4 ± 1.3*	50
Rapeseed meal	75	84	-5.62	2.40 ± 0.32***	15.0 ± 1.9	26.2 ± 2.2**	50
Chironomidae larvae	75	77	-9.94	1.53 ± 0.08*	6.2 ± 0.4***	9.2 ± 0.6***	335
Natto	75	66	-17.5	1.70 ± 0.22**	9.8 ± 1.7**	13.1 ± 2.0*	50
PPC	75	58	-23.68	5.22 ± 0.71***	14.1 ± 2.1	27.3 ± 1.9***	50
MSG	75	26	-56.67	1.68 ± 0.31*	5.5 ± 1.4***	7.8 ± 1.8***	50
Control	-	70	-14.63	1.55 ± 0.15	9.0 ± 0.7**	10.6 ± 0.8*	161

[a] This data expresses the taste preference of the fish for each tastant in relation to fishmeal.

[b] This data indicates pellet assessment and maceration compared with fishmeal.

Significant differences are indicated with asterisk: * <0.05, ** <0.01, *** <0.001.

Chironomidae larvae were expected to prove highly palatable and achieve 100% consumption, as chironomidae larvae extract did when testing taste preference of amino acids in carp performed by Kasumyan and Morsi (1996), however, consumption ranged between 77 % - 82 %, with other tastants being consumed more frequently.

MSG was detected by all three species of fish, eliciting an umami taste response, as in humans, when consumed alone it was perceived negatively (BEAUCHAMP et al., 1998) by the carp and tilapia, and was perceived equally to the control in trout, further testing of MSG added to other taste materials is recommended to establish effect as an 'enhancer'.

The carp showed little variation in taste response to most of the remaining tastants presented to them here, consuming 100 % of eight tastants, with only chironomidae larvae and PPC showing little reduction in attractiveness. Some tastants were consumed equally, although, the time fish spent assessing each tastant varied, some pellets were caught multiple times during observations, some only once. This method of assessment does not determine if increased retention time is due to the taste of the material or the texture, therefore any tastants achieving equal consumption rate and palatability scores can only be deemed equal in taste. Pellet retention time was taken into consideration in order to rank those tastants which achieved equal palatability scores, the tastant with the lowest retention time ranking higher as pellets were consumed faster. The top three ranking tastants, in order, were earthworm meal, Danish fishmeal and mealworm meal, all showing no difference ($P > 0.05$) in pellet catches or retention time. The other tastants: insect meal mix, silkworm meal, Natto, NH Algae and Rapeseed meal, were equally palatable although consumed at slower ($P < 0.05$) rates. PPC scored the lowest index of palatability (58.33 %) excluding MSG.

An interesting result was found for trout, a carnivorous species. Fermented rapeseed meal, a vegetable material, ranked as the most palatable ingredient followed by fishmeal, mealworm meal and earthworm meal with no differences ($P > 0.05$) in pellet retention times. NH Algae pellets also achieved equal palatability as those listed previously, although pellets were caught more times per observations ($P < 0.001$) and retention time was higher ($P < 0.001$). With the exception of the rapeseed meal, rainbow trout show a distinct preference towards the invertebrate meals over vegetable sources. Natto scored the lowest index of palatability (-0.65 %) excluding MSG.

Nile tilapia show more sensitive taste preferences than the other two species tested, only one tastant, earthworm meal, being consumed 100 % of the time, followed by NH Algae (98 %), insect meal mix (94 %), Danish fishmeal (94 %) and mealworm meal (88 %). The insect meal mix achieved equal palatability to fishmeal and mealworm meal showed slight reduction in palatability although there was no difference ($P > 0.05$) in pellet retention time. Both earthworm meal and NH Algae showed higher palatability and reduced pellet retention, indicating improved taste response. PPC scored the lowest index of palatability (-9.38 %) excluding MSG.

Of the species tested here, the largest literature base on taste response is available for carp. Individual amino acids have been shown to elicit varied taste preferences (Kasumyan and Morsi, 1996), amino acid profile may therefore influence taste response to each protein source material. Plotting amino acid preferences against the stimulatory effectiveness of each amino acid on the taste receptors innervated by the facial nerve (VII) (Marui et al., 1983d) (Figure 2.4), demonstrates that cystine and proline elicit the strongest stimulatory response. However, without further research to establish stimulatory effectiveness of the glossopharyngeal (IX) and vagal (X) nerves, no conclusions can be drawn as to which amino acid would elicit the strongest deterrent response; no stimulation of receptors is shown by previous studies, yet the fish do show a behavioural response. It is subsequently difficult to

fully explore the effects of individual amino acids on overall taste response to a more complex material.

Initial comparison of the amino acid profile of each tastant with the palatability responses achieved here by carp, is evidence that taste response of individual amino acids may have little bearing on the overall taste response to the more complex materials. PPC for example contains the highest amount of Proline (% material) (Table 2.8), yet was the least palatable of the complex materials tested. In a complete diet the taste profile of each ingredient may also be masked, as the response to single amino acids has been here, by other dietary components present at higher inclusion rates, the tastants were tested at concentration of 7.5%. Therefore, application of this method to complete diets may not be feasible, without further development; the data gathered would cost considerable time and effort and may gain no more insight into palatability response to a complete diet than simply quantifying the amount of feed consumed. In contrast, this method may prove applicable for testing of attractants, with the use of lower, more relevant, concentrations.

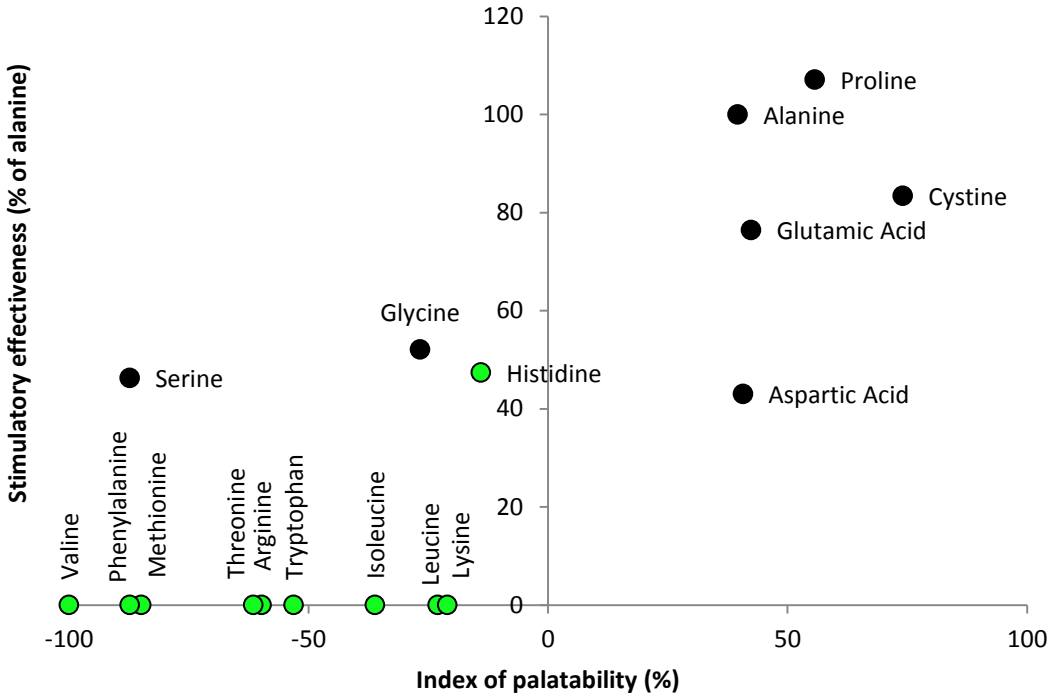


Figure 2.4. Stimulatory effectiveness (in relation to L-Alanine in percent) of taste stimuli: essential (green points) and non-essential (black points) amino acids, presented in Table 2.2 (Marui et al., 1983d), plotted against Index of palatability (%) results for amino acids presented in Figure 2.3 (Kasumyan and Morsi, 1996) in carp.

Table 2.8. Amino acid profile (% material) of each protein source material / tastant tested during palatability observation trials.

Amino acids % material (DM)		Feed Material								
		Fish-meal	Meal-worm meal	Silk-worm meal	Earth-worm meal	Insect meal mix	Rape-seed meal	Natto	NH Algae	PPC
Essential	Arginine	4.26	1.90	1.98	3.80	2.56	1.86	1.87	1.43	3.03
	Histidine	1.57	1.25	1.23	1.31	1.27	1.21	1.34	0.50	1.38
	Isoleucine	2.91	2.09	2.06	3.09	2.41	1.62	1.98	0.84	3.44
	Leucine	5.01	2.67	2.69	4.21	3.19	3.07	3.61	1.43	6.48
	Lysine	5.49	2.06	2.68	3.86	2.87	2.45	2.89	1.07	4.86
	Methionine	1.94	0.48	1.28	1.09	0.95	0.93	0.73	0.35	1.40
	Phenylalanine	2.87	1.39	2.07	2.28	1.91	1.95	2.50	0.71	4.09
	Threonine	2.94	1.40	1.63	2.23	1.75	1.79	1.65	0.90	3.57
	Tryptophan	0.73	-	-	-	-	-	-	-	-
	Valine	3.45	2.33	2.18	2.65	2.39	2.17	2.29	1.05	4.07
Non-essential	Alanine	-	3.03	2.33	2.67	2.68	2.01	1.71	-	3.02
	Aspartic Acid	-	2.96	3.88	4.80	3.88	2.98	4.32	-	7.64
	Cystine	0.60	0.65	0.97	0.99	0.87	0.83	0.62	0.32	0.89
	Glutamic Acid	-	4.16	4.06	6.69	4.97	6.34	7.86	-	7.05
	Glycine	-	1.66	1.65	2.28	1.86	1.88	1.53	-	3.13
	Proline	-	2.48	1.72	1.56	1.92	2.25	2.62	-	3.00
	Serine	-	1.60	1.73	2.34	1.89	1.48	1.54	-	3.40
	Tyrosine	1.92	2.37	2.19	1.65	2.07	1.32	1.46	0.55	2.93

No data provided by supplier for non-essential amino acids (except Cystine and Tyrosine) for fishmeal and NH Algae. Tryptophan was not tested for in all other materials when analysed.

The three species here: common carp, rainbow trout and Nile tilapia accepted all tastants, except MSG. Earthworm meal and Mealworm meal proved equally as palatable as fishmeal, with earthworm meal ranking higher than fishmeal for *C. carpio* and *O. niloticus*. NH Algae proved equally palatable although differences in assessment time ($P < 0.05$) were seen, likely due to the nature of the material clumping in the gel. In general a preference was seen towards the insect meals. Fish are often housed in high density in aquaculture; therefore, competition between them might render minor differences in taste almost irrelevant, as long as feed is acceptable and not highly repellent, such as the MSG alone. All the tastants except MSG proved palatable enough to warrant further investigation in formulated fish feeds.

Limitations did present during testing of this method. Kasumyan and Morsi (1996) stated their agar-gel pellets containing tastants were kept for no longer than seven days, while those containing chironomidae extract were kept for no longer than four days. Here, with addition of powdered materials, the agar-gel pellets could only be used within 48 hours, they lost colour and became pale beyond that time period. The gel pellets were stained, aiming to achieve a consistent appearance, although testing was conducted to establish the concentrations of dye and resulting purple colouration of the pellets used here, slight colour variation still remained, most apparent with the NH Algae pellets (Figure 2.3). This provides opportunity for fish to learn to recognise those pellets and respond without catching the pellet. Elimination of such opportunities would increase the robustness of the protocol.

This method was applied to two additional species of fish, zebrafish (*Danio rerio*), in which the gel pellets, when cut as small as possible and remain intact, remained too large for the adult sized fish to swallow. Pangasius catfish (*Pangasius hypophthalmus*) were also tested. However, they consumed the tastant pellets, including those containing chironomidae larvae, but completely ignored the control pellets, therefore, without control observation data it was not possible to calculate an index of palatability. In both cases the trials were abandoned.

This pilot study indicates that testing of whole feed ingredients with this method is not of value, however, this may be suitable for testing dietary attractants, included in diets solely for their attractive properties. Possible improvements for application of this method for assessment of attractants include: testing lower concentrations in the gel pellets, testing acceptance of pellets stained other colours or black to completely disguise tastant pellets and remove the opportunity for fish to express a preconditioned response without assessing the pellet. Application to a larger number of species would also provide opportunity to further develop and establish

this behavioural observation technique as a robust method of assessing the palatability of attractants.

2.5 Satiety response to protein source ingredients

2.5.1 CCK detection and quantification

CCK is a hormone that plays a role consistent with that of short term satiety (Moran, 2009, Bail and Boeuf, 1997). There is some evidence of similar feed response effects in fish (Himick and Peter, 1994) where it has been identified as a anorexigenic factor in all of the species studied to date. It acts as an appetite-inhibitor and stimulating release of digestive enzymes in the gastro intestinal tract (Volkoff, 2016). It has also been demonstrated that protein and fats, along with their hydrolysates, are major nutrients which stimulate CCK secretion in fish (Liddle, 1994, Murashita et al., 2008). Assessment of CCK secretion, therefore, may provide a useful tool for identifying if individual feed ingredients influence satiety response and feeding intake. Also if feeding to satiety is the feeding method used, as is often the case in aquaculture, the amount of feed consumed is further utilised as an indicator of palatability, with higher consumption amounts being equated to a more palatable diet. In such a scenario, if satiety response is influenced by diet composition then palatability will also be influenced by that same satiety response.

Rainbow trout (*O. mykiss*) is one of few species of fish that are known to express CCK and it is the only species to express three different CCK-8 peptides: CCK-N, CCK-L and CCK-T. All three of these are expressed in the pyloric caeca and mid intestine (Jensen et al., 2001). CCK-8 has also been shown, in this species, to slow down gastric emptying (OLSSON et al., 1999). Therefore, Rainbow trout was selected to assess change in CCK secretion in response to different tastant stimuli. Testing was conducted by blood sampling, following feeding with pellets containing each feed material, and by *in vitro* intestinal tissue sample exposure to protein hydrolysates manufactured from each protein source material. Hypothesis: there will be a

difference in the amount of CCK released by *O. mykiss* in response to various feed materials compared to unfed fish.

2.5.2 Manufacturing protein hydrolysates

2.5.2.1 Hydrolyses

All chemicals used here are of analytical grade. Water refers to ultra-pure Milli-Q water (resistivity of 18.2 MΩcm^{-1}).

The gastro-intestinal tract of *O. mykiss* consists of four distinct regions: a stomach, pyloric caeca, mid-intestine and distal intestine. The stomach produces HCL, providing an acid phase of digestion, the pH of which, in 30 cm fish, is 2.23 (± 0.54). The conditions within the pyloric region onwards are alkali, pH of 7.64 (± 0.29), relying on trypsin activity for digestion (Yasumaru and Lemos, 2014, Golchinfar et al., 2011). In order to carry out in vitro tissue exposure trials, the protein source material had to be enzymatically hydrolysed using a method that replicates natural digestion. A method based on previous publications was developed to achieve this (Adamson and Reynolds, 1996, Muzaifa et al., 2012, Kim et al., 2007). Pepsin from porcine gastric mucosa 1200 - 2400 U/mg (Sigma-Aldrich: 77151, powder) and trypsin from bovine pancreas ≥7500 BAEE units/mg (Sigma-Alrdrich:T9201 powder) were used in sequence.

Danish fishmeal, mealworm meal, silkworm meal, earthworm meal, insect meal mix, Natto, and PPC were used as protein sources. Only small amounts of each hydrolysate were required, 14 g of each source material, insect meal mix sample consisted of 4.66 g of mealworm meal and 4.67 g of each silkworm and earthworm meals, was mixed with Milli-Q water at a ratio of 1: 5 (source material: water), then blended using a Polytron (Ystral of Reading, Berkshire, UK) on setting five with the small probe for two minutes. The pH of the solution was tested using a Fisher brand Hydrus 300 pH reader coupled with a Fisher brand FB68793 pH probe, and adjusted

to 2.0 using concentrated HCL. The solution was then heated to 50 ^{o}C using a water bath (Grant low temperature circulator LTD-6). Once up to temperature the pH was retested and adjusted if required. Pepsin was added (1 % protein equivalence basis) to each solution (table 2.9). Solutions were incubated (Tenovus Luckham R300 Incubator Shaker) at 50 ^{o}C for two hours. The pH was tested every 30 minutes and any adjustments made to maintain the pH at 2.0. After two hours pepsin was inactivated by placing solutions in a water bath at 90 ^{o}C for 10 minutes, then allowing to cool back to 50 ^{o}C.

Table 2.9. Test material protein content and amount of enzymes, pepsin and trypsin, used to hydrolyse 14 g samples.

Test material	Protein content (% DM)	Protein content per 14 g sample (g)	Quantity of enzyme used for 1 % protein equivalence (g)
Danish fishmeal	71.2	9.968	0.100
Natto	42.26	5.916	0.059
PPC	84.41	11.817	0.118
Mealworm meal	55.98	7.837	0.078
Silkworm meal	57.58	8.061	0.081
Earthworm meal	73.44	10.282	0.103
Insect meal mix	62.34	8.728	0.087

The pH was retested and adjusted to 8.0 using concentrated NaOH, trypsin was added (1 % initial protein equivalence basis), (table 2.9). Solutions were incubated for a further two hours, retesting the pH every 30 minutes and adjusting to maintain the pH at 8.0. After two hours the trypsin was deactivated again by placing solutions in a water bath at 90 ^{o}C for 10 minutes. Samples were then allowed to cool before being centrifuged at 1732 g (3000 rpm) at 4 ^{o}C for 10 minutes (Sorvall® RC5C plus centrifuge, HS-4 swing out rotor). Silkworm meal hydrolysate solution was further centrifuged at 10 490 g (8000 rpm) at 4 ^{o}C for 10 minutes (Sorvall® RC5C plus centrifuge, HB-6 swing out rotor), in order to pelletise the larger particles to achieve a solution of equal consistency with the other solutions. The supernatant was decanted then frozen and stored in a -80 ^{o}C freezer until they were freeze dried

at the University of Nottingham Sutton Bonington Campus. The dried hydrolysates were used for further experiments.

In order to assess the extent of protein hydrolysis 1 ml samples of hydrolysis reactions were collected throughout the procedure, prior to addition of pepsin, then every 30 minutes thereafter:

1. Original material once heated to 50 ^{o}C.
2. 30 minutes after addition of pepsin.
3. 60 minutes after addition of pepsin.
4. 90 minutes after addition of pepsin.
5. 120 minutes after addition of pepsin.
6. 30 minutes after addition of Trypsin.
7. 60 minutes after addition of Trypsin.
8. 90 minutes after addition of Trypsin.
9. 120 minutes after addition of Trypsin.

These samples were placed into a water bath at 90 ^{o}C for 10 minutes after collection to deactivate enzyme activity and freeze dried along with final samples for further analyses.

2.5.2.2 SDS-PAGE analyses and protein assays.

Reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE analyses) was carried out on each of the protein hydrolysates manufactured, to test if the protein was successfully hydrolysed before using them for in vitro tissue exposure experiments.

SDS-PAGE gels were prepared based on the method of Laemmli (1970) and Frederick M. Ausubel (1988) using the Bio-Rad Mini-PROTEAN® 3 system, hand casting and running two 0.75 ml mini gels each time. The running gel (14 % acrylamide) was produced by mixing 2.74 ml ddH_2O (Milli-Q), 4.66 ml acrylamide

(30 % v/v stock solution), 2.5 ml running gel buffer (1.5 M tris/HCL pH 8.8), 0.05 ml SDS (20 % w/v solution), 0.05 ml Ammonium Persulphate (APS, 20 % w/v solution) and 0.005 ml N,N,N',N'-tetramethylethane-1,2-diamine (TEMED), then pipetted into the gel frame. 0.05 ml of butan-2-ol-saturated water was pipetted on top of the gel to prevent it shrinking and it was left to polymerise for 30 minutes at room temperature. Once the gel had polymerised, the butan-2-ol-saturated water was removed with filter paper and the stacking gel (3 % acrylamide), 6.4 ml ddH_2O (Milli-Q), 1 ml acrylamide (30 % v/v stock solution), 2.5 ml running gel buffer (0.5 M tris/HCL pH 6.8), 0.05 ml SDS (20 % w/v solution), 0.05 ml APS (20 % w/v solution) and 0.0010 ml TEMED, was added then left to polymerise for a further 30 minutes.

Protein concentrations of the hydrolysate samples were established based on the Bradford (1976) method using Bio-Rad Protein Assay Dye Reagen and monitoring the absorbance at 595 nm. Porcine γ-globulin (1.5 mg/ml) was used as the standard in 800 µl Milli-Q water for producing a standard curve with concentrations 0 - 24 µg/µl. Dried hydrolysate samples were re-suspended in SDS lysis buffer at 10 %, 30 % for silkworm meal and PPC, as lower concentrations proved insufficient. 1 µl of re-suspended hydrolysate sample was diluted in 800 µl Milli-Q water. 200 µl of protein dye was added to all samples before incubating at room temperature for 10 minutes. Absorbance at 595 nm was read on a Hitachi U-2000 Spectrophotometer, protein concentrations were interpolated from the standard curve. Due to the low protein level achieved for the initial sample collected for the silkworm hydrolysate sample set, a 1 mm thick gel was produced to allow a larger load volume.

The polymerised gels were transferred into a buffer chamber, with the combs gently removed, filled with tank buffer (1.44 % (w/v) glycine, 0.3 % (w/v) tris base and 0.1 % (w/v) SDS). The gels were loaded with a molecular weight marker ladder (ThermoFisher scientific: PageRuler™ Prestained Protein Ladder, 10 to 180 kDa) then the nine hydrolysate protein samples, at 50 µg protein per well, one gel per protein source. The gel was run at 16 mA/gel until the dye front reached the bottom

of the gel. The spacer plates were detached; the stacking gel was carefully removed and discarded before submerging the front plate in Milli-Q water and detaching the running gel from it. The polyacrylamide gel was then placed in fixing solution (1: 1: 5 – acetic acid: methanol: ddH_2O) and agitated on a rocking platform (Hoefer red rocker) for two hours. The gel was then gently washed with Milli-Q water and placed in either staining solution: 50 % methanol (v/v), 0.05 % Coomassie brilliant blue R-250, 10 % (v/v) acetic acid and 40 % ddH_2O, or storage solution (7 % (v/v) aqueous acetic acid) and stored at 4 ^{o}C until later stained. Gels were agitated in the staining solution for four hours, rinsed off with milli-Q water then placed in de-staining solution (5 % methanol, 7 % acetic acid, 88 % ddH_2O) and agitated for four hours, replacing the de-staining solution after two hours. De-staining of the gel was successful in the pathway of the ladder, less so for the hydrolysate samples. Figure 2.5 shows the gel pattern achieved for earthworm meal, a strong band can be seen in lane B, the original material, at 180 kDa. This initial high molecular weight band is no longer present following 30 minutes of incubation with pepsin (lane 1), five new clear bands appear in lane 1 at 25, 40, 65, 100 and 130 kDa; these remain throughout the pepsin stage of hydrolysis (lanes 1 – 4). Almost complete degradation of the protein is achieved following 30 minutes of incubation with trypsin (lane 5), only one thin band remains at 25 kDa throughout the trypsin stage of hydrolysis (lanes 5 – 8). SDS-PAGE patterns for all test materials show similar patterns, with varying numbers and clarity of bands. This indicates proteins were successfully hydrolysed into polypeptides and amino acids with progressively smaller molecular weights following incubation with each enzyme. The chosen method of protein hydrolyses was successful in application, the final dried protein hydrolysates can therefore be utilised for in vivo tissue exposure experiments.

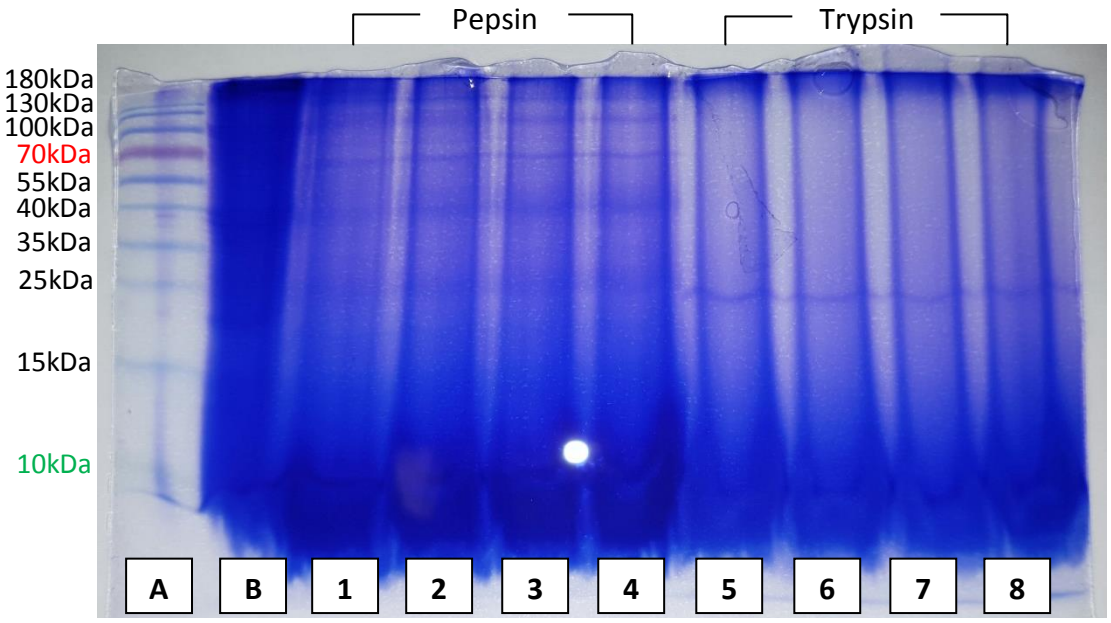


Figure 2.5. SDS-PAGE patterns of hydrolysates of earthworm meal produced by 1 % (protein equivalence basis) enzymatic treatments.; earthworm meal (1:5 with Milli-Q water) was incubated at 50 ^{o}C for 30, 60, 90 and 120 minutes with pepsin, then further incubated for 30, 60, 90 and 120 minutes with trypsin. Lane A: molecular weight marker ladder (ThermoFisher scientific: PageRuler™ Prestained Protein Ladder, 10 to 180 kDa), Lane B: original earthworm meal solution once heated to 50 ^{o}C, Lanes 1 – 4: incubated at 50 ^{o}C for 30, 60, 90 and 120 minutes with pepsin, Lanes 5 – 6: further incubated for 30, 60, 90 and 120 minutes with trypsin.

2.5.3 Tissue exposure sample collection

Rainbow trout (*O. mykiss*) were group housed at 15.5 ± 0.5 ^{o}C; fish were not fed for 24 hours prior to sample collection. The method of sampling intestinal CCK secretion was based on that of Daly et al. (2012) in mice. Fish were humanely euthanised, UK Home Office Approved Schedule 1 method of concussion, and destruction of the brain. The intestinal tract was swiftly dissected out and excess visceral fat was removed except surrounding the pyloric caeca. A 2 cm section of mid-intestine was taken posterior to the pyloric region, cut longitudinally and washed in saline (0.9 % sodium chloride); the serosa was then gently removed by scraping with a scalpel. Successful removal of the serosa without damaging to the circular muscle layer beneath was confirmed via haematoxylin – eosin staining. The 2 cm section was transferred to 0.5 ml of Hanks Balanced Salt Solution (HBSS) and

incubated in a water bath for one hour at 15.5 ^{o}C; tissue was then transferred into 0.5 ml of fresh Hanks Balanced Salt Solution (HBSS) with added dipeptidyl peptidase-4 (DPP-IV) inhibitor at 20 µl/ml for the control samples, protein hydrolysate was added at 1 % (w/v protein equivalence) for each treatment and further incubated for one hour. Samples were then centrifuged at 95 g (1000 rpm) at room temperature for 20 seconds (Hettich Mikro 20) to remove cell debris, the supernatant was decanted and snap frozen in liquid nitrogen for storage at -80 ^{o}C for further analyses.

2.5.4 Manufacturing experimental pellets

The protein source materials being tested were manufactured into highly concentrated pellets in order to be presented and consumed by the subject fish prior to collecting blood samples. Each pellet mix consisted of 95 % protein material, Danish fishmeal, Natto and PPC, and 5 % carboxymethyl cellulose (CMC) as a binding agent, the CMC content is 10 times higher than in normal pelleted feeds to ensure the single ingredient in use forms a pellet. One kilogram of each batch of tastant pellets was produced. Ingredients were mixed using a Hobart mixer for 30 minutes, water was added until moist enough to hold shape when compressed, but crumble when pressured to do so. The mixture was then passed through a Kenwood pro 1600 mincer with a 3 mm die, then manipulated manually into pellets before being dried overnight in a nine shelf Excalibur food dehydrator at 50 ^{o}C. Pellets were stored at 4 ^{o}C until used.

2.5.5 Collecting blood samples

Four groups of 30 cm fish were fed *ad libitum* until sated, determined by lack of feed response when presented with feed pellets, after 30 minutes one fish was humanely euthanised (UK Home Office Approved Schedule 1 method of concussion, and destruction of the brain), 1 ml blood samples were extracted from the caudal vein, using a 25 gauge needle (sterile BD Microlance^{TM} 3), ventrally just posterior of the anal fin (Houston, 1990, Congleton and LaVoie, 2001), aspirating into a 2 ml sterile syringe. Blood was transferred into a BD Microtainer® K2EDTA tube and

placed in ice. A second fish was sampled immediately after. Each sample took up to 5 minutes per fish; no more than two fish were sampled from each group each day, allowing 24 hours before feeding again, ensuring a consistent time period from feeding to sample collection. Control samples were collected without feeding. Blood samples were then centrifuged at 10 490 g (3000 rpm) at 4 ^{o}C for 10 minutes (Sorvall® RC5C plus centrifuge, HB-6 swing out rotor); plasma was decanted and frozen in liquid nitrogen before storing at -80 ^{o}C for further analyses.

2.5.6 CCK detection using assay kit

A Sincere^{TM} Biotech fish cholecystokinin (CCK) ELISA Kit was used to determine CCK concentrations of the samples collected, as described above. Manufacture instructions were followed; in brief, duplicate standards were run in CCK concentrations from 15.6 pg/ml to 500 pg/ml, these were aliquoted along with experimental samples into the 96 well plate coated with fish CCK monoclonal immobilised antibodies. The plate was incubated for 90 minutes at 37 ^{o}C. The wells are washed before addition of biotinylated fish CCK antibody, which binds to the CCK present in the standards and test samples, and further incubated for 60 minutes at 37 ^{o}C. Unbound antibodies are washed away before addition of the Avidin-Biotin-Peroxidase Complex, which binds to the affixed antibody, the plate was then incubated for another 30 minutes at 37 ^{o}C. Wells were washed, 3,3',5,5'-tetramethylbenzidine (TMB) colour developing reagent solution was added to each well before incubating for up to 30 minutes at 37 ^{o}C. The solution in each well was initially yellow, once the appropriate samples turn blue during incubation the TMB stop solution was added. The absorbance of each solution in each well was read at 450 nm, a standard curve created from the standards was used to calculate the CCK concentration of each sample.

2.5.7 Results

Initial testing of CCK released by 1 cm mid-intestine control tissue samples incubated in HBSS solution alone, without the first one hour incubation step, concurred with the literature (Barrenechea et al., 1994), CCK secretion within the

mid-intestine was highest, although most varied, posterior to the pyloric region; CCK is released in the anterior end of the mid-intestine, decreasing towards the distal intestine (Figure 2.6). Coupled with some individuals possessing a mid-intestine of only 3 cm in length, only the first 2 cm of mid-intestine was used for future samples, to ensure consistent tissue type was used and to give maximum CCK secretion potential. As CCK secretion varies with each progressive sample taken along the intestine, only one sample per fish was collected.

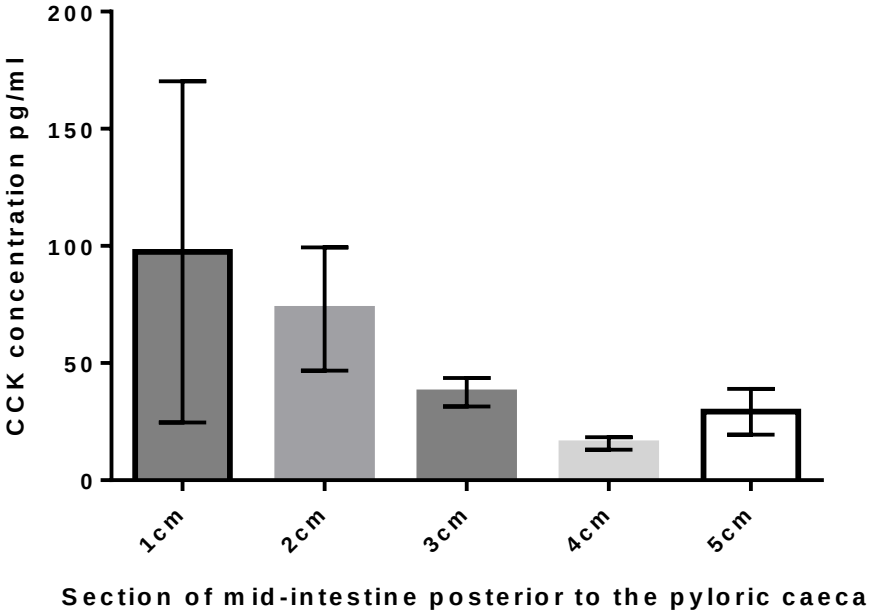


Figure 2.6. Mean (± SEM) concentration of CCK released (pg/ml) from 1 cm mid-intestine samples, collected from rainbow trout (*O. mykiss*), in response to incubation in HBSS solution for one hour at 15.5 ^{o}C. Large errors of the mean are due to small sample size of three.

The results gathered from the blood and tissue sampling methods, for testing CCK secretion in response to each feed protein source, showed successful detection of CCK, albeit the blood sampling results differed from those of the tissue sampling method. CCK concentrations varied considerably between the control fish, increasing the standard deviation and reducing the likelihood of any significant changes between test groups from being identified. Blood samples were collected

from fish housed in groups; this may have resulted in larger or more dominant fish consuming more feed than smaller or subordinate individuals, this could account for the large variation seen between the fish post feeding. Using *in vitro* tissue exposure sampling removes this factor by testing the same 2 cm section of intestinal tract exposed to equal amounts of protein hydrolysates (protein equivalence); yet large variation was still seen between individual fish. From the tissue exposure samples, no significant differences were found between test groups (Figure 2.7). When blood sampling was used there was a significant increase in CCK released when fish were fed Natto compared to the control group (increase of 70.6 %, $P = 0.046$), no other significant differences were found. Fish fed fishmeal showed an increase of CCK release by 57.9 % ($P = 0.057$), fish fed PPC pellets also showed an increase in CCK release of 62.2 % ($P = 0.197$), when compared to the control group. This shows the presence of a baseline level of CCK present when fish have not fed for a period of up to 24 hours. It also shows that CCK release increases within 30 minutes of feeding taking place; however, with no differences ($P > 0.05$) between tastant pellets there is no indication that the amount of CCK released (the severity of the response) is influenced by the different ingredients.

Recommendations for further study and refinement of tissue exposure sampling includes varied incubation periods, attempts to sample pyloric caeca, and testing with varying age and size of fish. Sampling the first 2 cm section of mid-intestine ensured the same region is used, this did not account for the variation in intestinal diameter seen between fish during the trial. Samples with greater diameter will potentially have a greater surface area of epithelial tissue and therefore may possess greater capacity to secrete CCK than samples with a small surface area. Collecting sample weight would provide the data necessary to generate CCK secretion results as pg/ml/g of sample, accounting for variation in sample weight and potentially surface area. Recommendations for further study using blood sampling include housing of fish individually, training to catch feed pellets upon entry to the tank, and then collecting blood after consumption of set amounts of feed per body weight for each fish. This would ensure all fish sampled have fed

equally, thus any variation would be due to physiological response of the fish not quantity of feed consumed. Sampling at increasing time periods from feed ingestion to sampling of blood would indicate longevity of the satiety response. If CCK levels within the blood remain elevated for longer for one feed material over another, this may indicate a more undesirable attribute for an aquaculture feed as fish will feed less frequently.

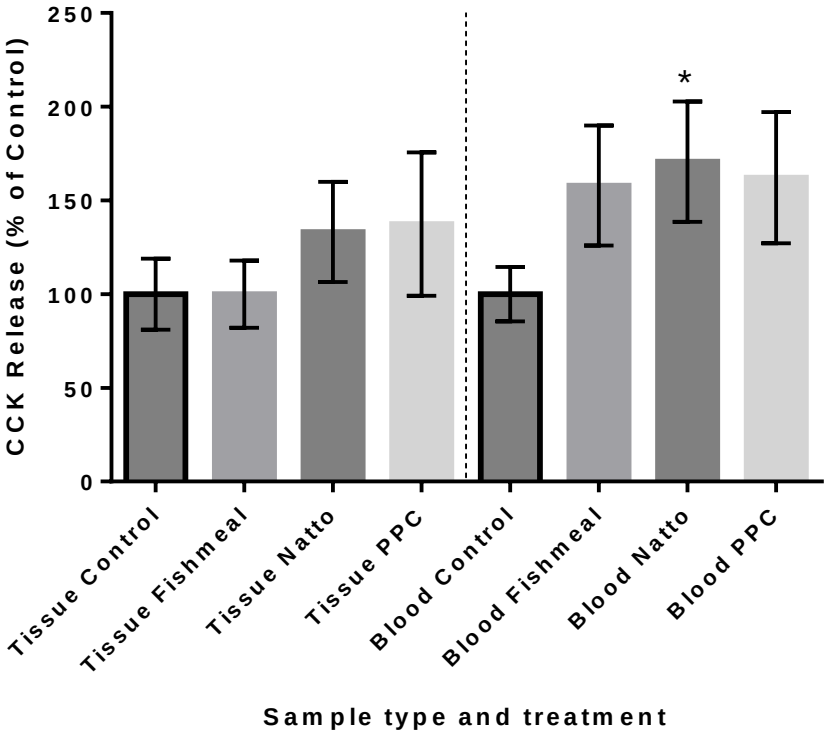


Figure 2.7. Mean (± SEM) concentration of CCK released (% of control) by 2 cm anterior mid-intestinal tissue samples exposed to protein hydrolysates (1 % protein equivalence basis) in HBSS solution *in vitro*. Controls remained in HBSS solution only. *In vivo* blood samples were collected following feeding until sated with protein source materials. Control fish were not fed.
* Significantly different from control, P > 0.05.

Chapter 3

3.0 Fishmeal and fish oil replacement with dietary inclusion of vegetable materials - growth and performance

The trials reported in this chapter were conducted as part of the 'Fish Food Innovation' (FFINN) project and was therefore funded by industry. Skretting, who helped identify test materials, design the fish feeds and provided some feed materials, New Horizons Global LTD provided the Algae material, Eminate Ltd manufactured and provided the remaining test materials, fermenting ingredients with the aim of increasing bioavailability and reducing anti-nutritional content and Robert Bristow, helped to run the zebrafish palatability and performance trial. The ingredients discussed in the chapter where tested specifically to find out if i) they are acceptable to fish when incorporated into diets, ii) fish grow sufficiently feeding on such diets.

3.1 Nutritional profiles of alternative vegetable and algae materials

Nutritional and anti-nutritional analysis was carried out on each of the vegetable materials by Skretting before and after fermentation, NH Algae was analysed as the raw material provided only (Tables 3.1 A + B). These analyses were used by Skretting to formulate isonitrogenous and isoenergetic diets with the maximum amount of fishmeal being substituted by each test material. Diets were formulated for zebrafish (*D. rerio*) (Table 3.2), rainbow trout (*O. mykiss*) (Table 3.3) and European sea bass (*D. labrax*) (Table 3.4). During each feed trial the control diet was formulated to industry standard using fishmeal and oil to emulate current aquaculture feeds for the target species.

Table 3.1 A. Proximate analyses and amino acid profile for alternative feed ingredients: Rapeseed meal, Natto and PPC pre and post autoclave treatment and fermentation. Analyses of NH Algae material was conducted on the material as is supplied.

Diet component		Vegetable material						Algae material
		Rapeseed meal		Whole Soya bean (Natto)		Potato Protein concentrate (PPC)		NH Algae
		Initial material	Post fermentation	Initial material	Post fermentation	Initial material	Post fermentation	As supplied
%	Moisture	9	4.9	8.6	5.1	10.6	19.2	3.3
Proximate analyses (% DM)	Crude Protein	34.5	40	34.1	40.1	70.4	68.2	16.4
	Crude lipid	4.1	6.3	21.5	27.4	3.3	3.1	26
	NFE	-	30.7	-	23.08	-	31.19	22.7
	Fibre	12.6	16.7	8.9	5.3	1.2	0.9	1
	Ash	6.1	7.8	4.8	5.3	3.8	2.6	12.8
	Energy content (MJ/kg DM)	-	15.96	-	20.14	-	14.24	21.1
Essential amino acids (% protein)	Arginine	5.86	4.64	7.16	4.66	4.99	4.45	8.7
	Histidine	2.67	3.01	2.67	3.34	2.10	2.02	3.02
	Isoleucine	3.77	4.06	4.31	4.93	4.94	5.04	5.15
	Leucine	6.78	7.68	7.42	9.00	9.56	9.50	8.69
	Lysine	5.48	6.12	6.19	7.21	7.19	7.13	6.55
	Methionine	1.97	2.32	1.47	1.82	2.05	2.06	2.13
	Cystine	2.41	2.09	1.64	1.55	1.35	1.31	1.98
	Phenylalanine	3.77	4.87	4.78	6.25	5.67	5.99	4.32
	Tyrosine	2.49	3.30	3.17	3.64	4.35	4.29	3.33
	Threonine	4.46	4.46	4.08	4.11	5.51	5.24	5.46
	Valine	4.70	5.42	4.43	5.72	5.60	5.97	6.4
Non-essential amino acids (% protein)	Alanine	4.32	5.01	4.31	4.25	4.57	4.43	-
	Aspartic Acid	7.19	7.45	11.47	10.76	12.17	11.21	-
	Glutamic Acid	16.87	15.86	18.04	19.59	10.77	10.34	-
	Glycine	4.99	4.70	4.22	3.81	4.63	4.59	-
	Proline	5.71	5.62	4.55	6.54	4.47	4.40	-
	Serine	4.38	3.71	5.22	3.84	5.36	4.99	-

Table 3.1 B. Anti-nutritional factor analyses for alternative feed ingredients: Rapeseed meal, Natto and PPC pre and post autoclave treatment and fermentation. Analyses of NH Algae material was not carried out.

Diet component		Vegetable material					
		Rapeseed meal		Whole Soya bean (Natto)		Potato Protein concentrate (PPC)	
		Initial material	Post fermentation	Initial material	Post fermentation	Initial material	Post fermentation
Anti-nutritional factors (ANFs)	Chaconine (mg/kg)	-	-	-	-	581.66	680.69
	Glucosinolates (umol/g)	23.19	0.42	-	-	-	-
	Glucosinolates (mg/kg)	10000.00	186.12	-	-	-	-
	Lectins (mg/g)	-	-	-	-	< 0.05	n/a
	Progoitrin (umol/g)	13.19	0.36	-	-	-	-
	Progoitrin (mg/kg)	5770.33	151.42	-	-	-	-
	Sinapine (g/100g)	1.12	0.49	-	-	-	-
	Solanine (mg/kg)	-	-	-	-	570.47	816.83
	Soya saponin (g/kg)	-	-	6.39	22.72	-	-
	Tripsin inhibitor activity (TIA) (mg/g)	-	-	19.91	1.26	3.24	1.98
	VMO (umol/g)	0.08	-	-	-	-	-
	VMO (mg/kg)	7.14	-	-	-	-	-
	Water soluble protein (WSP) (g/100g)	3.63	21.24	10.07	31.61	2.68	10.77

Table 3.2. Experimental diets formulated by Skretting for Zebrafish (*D. rerio*) to incorporate Rapeseed meal, Natto, PPC and NH Algae as partial fishmeal replacements compared to a control diet.

Zebrafish (*D. rerio*) diets					
Nutrient, % in diet (DM)	**Control**	**Rapeseed meal**	**Natto**	**PPC**	**NH Algae**
Dry matter	92.00	94.61	93.12	89.67	93.71
Crude protein	40.00	40.00	40.00	40.00	40.00
Crude oil	8.00	17.13	12.41	9.21	15.71
Crude fibre	0.27	3.35	2.06	0.56	0.42
Ash	7.49	7.28	6.00	4.64	9.80
Gross energy	18.83	20.78	19.91	19.08	20.43
Digestible energy	16.61	16.60	16.63	16.60	16.60
Lysine	2.58	2.50	2.50	2.50	2.54
Methionine	0.96	0.95	0.95	0.95	0.95
Ingredient					
Rapeseed meal	-	34.38	-	-	-
Natto	-	-	40.26	-	-
Potato Protein Concentrate	-	-	-	25.18	-
NH Algae	-	-	-	-	25.00
Peruvian prime fishmeal	47.08	25.00	20.00	20.00	41.18
Corn starch	38.80	15.19	26.48	36.40	17.79
Wheat gluten	10.00	10.00	10.00	10.00	10.00
Rapeseed oil	2.83	12.58	-	5.74	4.72
Lysine (77%)	-	0.57	0.72	0.10	-
Pluvirel (70%)	-	0.03	0.06	0.06	-
DL-Methionine (98%)	-	0.14	0.28	0.12	0.01
Monoammonium Phosphate	-	0.80	0.90	1.10	-
Trout vitamin premix	0.9	0.90	0.90	0.90	0.90
Fish mineral premix	0.40	0.40	0.40	0.40	0.40

Table 3.3. Experimental diets formulated by Skretting for Rainbow trout (*O. mykiss*) to incorporate Rapeseed meal, Natto, PPC and NH Algae as partial fishmeal replacements compared to a control diet.

Rainbow trout (*O. mykiss*) diets					
Nutrient, % in diet (DM)	**Control**	**Rapeseed meal**	**Natto**	**PPC**	**NH Algae**
Dry matter	92.59	94.33	93.28	90.45	94.00
Crude protein	42.00	42.31	42.00	42.00	42.00
Crude oil	16.00	19.45	16.00	16.00	20.00
Crude fibre	0.25	2.66	1.66	0.52	0.42
Ash	6.77	6.53	5.48	4.22	8.92
Gross energy	20.98	21.69	20.95	20.96	21.71
Digestible energy	18.91	18.00	18.08	18.60	18.00
Lysine	2.41	2.40	2.40	2.42	2.40
Methionine	0.95	0.94	0.94	0.94	0.94
Ingredient					
Rapeseed meal	-	26.70	-	-	-
Natto	-	-	31.34	-	-
Potato Protein Concentrate	-	-	-	23.36	-
NH Algae	-	-	-	-	25.00
Peruvian prime fishmeal	43.56	27.50	22.50	20.00	36.35
Corn starch	28.81	13.80	22.45	27.69	11.69
Wheat gluten	16.00	16.00	16.00	15.00	17.00
South American fish oil	5.38	7.30	2.56	6.15	4.48
Rapeseed oil	5.38	7.30	2.56	6.15	4.48
Lysine (77%)	0.02	0.42	0.61	0.09	0.12
Pluvirel (70%)	-	0.01	0.61	0.05	-
L Arginine (98%)	-	-	0.04	-	-
DL-Methionine (98%)	-	0.08	0.20	0.08	0.02
Monoammonium Phosphate	-	0.04	0.27	0.58	-
Trout vitamin premix	0.45	0.45	0.45	0.45	0.45
Fish mineral premix	0.4	0.4	0.4	0.4	0.4

Table 3.4. Experimental diets formulated by Skretting for European sea bass (*D. labrax*) to incorporate Rapeseed meal, Natto, PPC and NH Algae as partial fishmeal replacements compared to a control diet.

European sea bass (*D. labrax*) diets					
Nutrient, % in diet (DM)	**Control**	**Rapeseed meal**	**Natto**	**PPC**	**NH Algae**
Dry matter	91.41	93.59	93.20	89.27	93.20
Crude protein	42.51	44.00	43.02	43.31	43.01
Crude oil	16.00	17.94	18.00	16.00	19.00
Crude fibre	0.24	2.37	2.41	0.56	0.45
Ash	7.25	5.70	6.02	4.35	7.90
Gross energy	20.72	21.52	21.34	20.84	21.58
Digestible energy	18.50	17.90	17.90	18.20	17.90
Lysine	2.41	2.40	2.40	2.59	2.40
Methionine	0.97	0.89	0.89	0.90	0.89
Ingredient					
Rapeseed meal	-	23	-	-	-
Natto	-	-	49.13	-	-
Potato Protein Concentrate	-	-	-	30	-
NH Algae	-	-	-	-	23.67
Danish fishmeal	47.04	22.73	20	20	30.22
Corn starch	26.88	14.46	14.83	24.84	11.95
Wheat gluten	15.67	25	11.25	11.83	25
South American fish oil	9.71	12.75	3.04	11.98	7.89
Lysine (77%)	-	0.77	0.48	-	0.53
Vitamin C (35%)	-	0.24	-	-	
Pluvirel (70%)	-	0.06	0.07	0.07	0.02
DL-Methionine (98%)	-	0.06	0.18	-	0.01
Monoammonium Phosphate	-	0.23	0.32	0.58	
Trout vitamin premix	0.3	0.3	0.3	0.3	0.3
Fish mineral premix	0.40	0.4	0.4	0.4	0.4

3.2 Feed trial methodology

All feed trials were conducted at the Institute of Integrative Biology, University of Liverpool. Research on alternative materials to fishmeal and fish oil has been ongoing for many years, there are a number of indicators widely accepted and used for assessment of new diet formulations and incorporation of novel or alternative ingredients. The indicators used to assess growth and efficiency during the trials in this chapter, specifically aimed at assessing growth, include Feed Conversion Ratio (FCR), calculated as follows:

$$FCR = \frac{\text{Total feed intake (kg)}}{\text{Weight gain (kg)}}$$

Total Feed Intake (TFI) = total feed given.

Weight gain = weight at end of study period – weight at start of study period.

(NRC, 2011)

FCR indicates the efficiency at which feed is converted into animal biomass (Aquatext, 2014). Protein is an expensive feed component; efficient use within aquafeeds is therefore an important aspect of feed formulation and manufacturing. Protein efficiency ratio (PER) is the ratio of weight gain to protein consumed:

$$PER = \frac{\text{fish wet weight gain (g)}}{\text{dry weight of protein fed (g)}}$$

(NRC, 2011)

Specific growth rate (SGR), the growth achieved per day during the feeding period on the diet in question:

$$SGR\ (\%) = 100 \times (\ln W_2 - \ln W_1) \times (t_2 - t_1)^{-1}$$

Where:

Ln = natural log

W_1 = Initial weight

W_2 = Final weight

t_1 = Starting time point (day one)

t_2 = End time point (final day number) (Korkmaz and Cakirogullari, 2011)

Further indicators can be used to analyse fish condition. The Fulton type condition factor (K) is a common measurement in fisheries science that uses the relationship of fish weight and length as an indicator of condition. K factor was first utilized as a measure of fish condition by Friedrich Heincke (1908), Thomas Wemyss Fulton refined "K" for several fish species (Fulton, 1902, Fulton, 1904), this became known as 'Fulton's condition factor' (Ricker, 1975). The formula is usually followed by a scaling factor to achieve a figure close to one (Nash et al., 2006) . The formula below incorporates an example scaling factor ($x10^5$) used for salmonids:

$$k= (M/L^3) \times 10^5$$

Where:

M = mass (g)

L = length (mm)

(Nash et al., 2006, Kerambrun et al., 2011, Barnham and Baxter, 2003, Snyder et al., 2004)

Histological analyses of the intestinal tract and liver will also be carried out, along with calculation of liver, gastro-intestinal tract and spleen indices:

Hepato-somatic Index (HSI) = (Liver mass (g)/mass of fish (g)) x100

Intestinal-somatic Index (ISI) = (Intestine mass (g)/mass of fish (g)) x100

Splenic-somatic Index (SSI) = (Spleen mass (g)/mass of fish (g)) x100

(Sadekarpawar and Parikh, 2013)

3.2.1 System and trial parameters

During each trial, each system was maintained with the following water quality parameters, Ammonia (NH_4); 0mg/l, Nitrite (NO_2); 0mg/l, Nitrate (NO_3); <20mg/l and pH; 7.0, unless stated otherwise. Fish were exposed to 12:12 (Day: Knight) light cycles throughout their entire housing period.

3.2.2 Weighing daily feeds

During a trial, fish were fed strictly controlled diet rations. Feeds for zebrafish were measured using a five point decimal place Kern 770-60 laboratory weigh balance (linearity ±0.03mg) and were measured to three decimal places, into a corresponding Sarstedt 1.5mL micro tube. Feeds for all other species were weighed using a Sartorius BP2100 S weigh balance, to two decimal places, and placed in appropriately sized tubs. The scales were zeroed for every tube, before addition of the feed, to ensure precision in the measurements.

3.2.3 Weighing fish

Fish were weighed one day prior to the trial start to establish an initial mass. This was conducted by experienced personnel using an appropriately size tank with the base blacked out using polyurethane sheeting, filled to a depth sufficient to contain the fish. This was used in conjunction with an appropriate weigh balance. All fish in a tank were moved to a holding tank; while the fish were situated here the housing tank was cleaned as required. Fish were weighed by catching in a net, lifting proud of the water, then removing excess water from the net by dabbing it on a paper towel; the net providing fish with protection from dehydration and preventing damage to the mucosal layer. Removing the excess water reduces the effect of unintentional addition of water to the weigh tank thus improving accuracy of measurement. Fish were then placed in the weigh tank and the weight recorded to two decimal places, before returning fish to the housing tank. Fish were weighed weekly, repeating this process, in order to record growth and maintain the desired feed ration throughout the trial period.

3.2.4 Assessing growth and performance

In order to assess the performance of the diets, fish weight gain, FCR, PER and SGR were calculated, as described above in section 3.2. All fish in each tank were humanely euthanized (UK Home Office Approved Schedule 1 method of concussion, and destruction of the brain), by a fully trained researcher. Fish length was measured from the tip of the snout to the caudal peduncle once euthanized. This along with fish weight was used to calculate the K Factor using an appropriate scaling factor (given below). An increased K factor score is considered to be a good outcome, however, as K factor is a ratio of weight (g) to length (mm), it is often miss-interpreted as those with higher scores being healthier, in reality fish with a higher score are simply fatter, therefore caution is advised when considering the K factor results.

3.2.5 Tissue sampling

A number of fish from each tank were humanely euthanised (UK Home Office Approved Schedule 1 method of concussion, and destruction of the brain) and dissected. The coelomic cavity was opened via a midline incision. The spleen, liver and intestinal tract were removed and weighed (Kern 770-60 laboratory weigh balance (linearity ±0.03mg), weights recorded to 3 decimal places), this data was used to calculate organ indices: as described above (section 3.2). Once weighed the spleen was discarded. The intestinal tract was divided if required (into four sections; stomach, pyloric caeca, mid-intestine and distal intestine.); 5mm sections of each region (if applicable) were collected. These samples, along with a 5mm section of the liver were fixed in 10% NBF for processing into slides for histological analyses, via wax embedding, cross sectioning and H&E staining. Samples once fixed were processed by the Veterinary Pathology Diagnostic Service Department, University of Liverpool, Leahurst. Photographs were taken of each slide using a LEICA ICC50 HD microscope with an appropriate magnification for intestinal and for liver tissue. ImageJ computer software was used to analyse the digital photographs, determining villi length (L) and width (w). ImageJ was also used to assess photographs of the liver slides, determining the area within a photograph of liver

tissue that was occupied by colour. Photos were cropped to only include liver tissue without other structures present. Subsequently the photos were converted to 32-bit grey scale and analysed for area (in pixels), mean, mode, minimum and maximum grey value. The colour threshold was then altered to a value equal to the mean grey value plus 10%; this highlighted the stained tissue only, ignoring any unstained gaps. The image was then analysed again for area. Information generated was used to calculate the percentage of sample stained and unstained by the H&E process, the unstained areas indicating fat deposition.

3.2.6 Palatability assessment

In order to assess feed palatability, observations were made on 1 % body weight feed rations; the remaining daily ration was offered later in the day. Observing the time taken for each tank to consume a set quantity of feed provides a simple measure of feed interaction, both palatability and presentation will affect how the fish perceive and interact with the feed pellets. A diet that is consumed more rapidly indicates a preference for that diet, an improved palatability. Although this simple measure does not specify if this preference is due to taste, pellet characteristics or time period the pellet takes to sink, all of which may influence such interaction.

3.2.7 Statistical analyses

Statistical tests for difference for were carried out with 95% confidence levels ($P = <0.05$) between the fishmeal control diet and each of the alternative diets (PPC, Rapeseed meal, Natto and NH Algae) for all measures calculated. Kolmogorov Smirnoff tests for normality were used, and then either Students T-test was used for parametric data, or Mann Whitney-U test's for non-parametric data.

3.3 Zebrafish (*D. rerio*) feed trials

3.3.1 Trial specific methodology

Zebrafish trials were carried out first. Their small size (requiring small amounts of feed ingredient), gregarious nature and relatively simple husbandry make them ideal for this kind of first-pass trial to rule out any materials which may prove unsuitable for further investigation before manufacturing larger quantities for trials with commercially relevant species.

A 42 day feed trial was conducted in collaboration with Robert Bristow, who performed most of the day to day tasks and data analyses. 50 fish, six months of age, were used; of which all were bred in house, an ideal life stage due to fast growth rate. The fish were housed in groups of ten in five identical 3 L Aquatic Habitats tanks kept under the conditions described in section 3.2.1 at 28^{o}C. The tank design removes waste reducing intervention and disturbance. The tanks were blacked out on three sides and base (the front open for welfare checks) to ensure fish could not see their neighbours, which may have influenced their behavioural responses. Fish were allowed to acclimatise to the tanks for a period of two weeks; an industry standard flake food (Tetramin flakes) was used throughout that time period. Isonitrogenous and isoenergetic diets with 40 % protein and 16.6 - 16.63 % digestible energy were formulated by Skretting to include control, Natto, Rapeseed meal, PPC and NH Algae diets (Table 3.2). Each vegetable based diet has reduced fishmeal content compared to the control: Natto (57.52 % reduction), Rapeseed meal (46.90 % reduction), Potato protein (57.52 % reduction) and NH Algae (12.53 % reduction). These diets were then manufactured at the Institute of Integrative Biology Aquarium's research facility at the University of Liverpool, following the methods described in Appendix 2 to be aesthetically similar as possible with pellets appropriate for the size of fish. Fish were fed 4 % bodyweight daily, split into two 2 % feeds 8:30 am – 10:30 am and 13:00 pm – 14:00 pm. Fish were fed the full 4 % feed ration at once on weekends.

Fish were weighed individually (as described in section 3.2.3 using a 1.5L tank containing approximately 2 cm of water) one day prior to the trial start to establish an initial mass. Weight gain, FCR and SGR was used to assess growth. Five fish from each diet were humanely euthanized (UK Home Office Approved Schedule 1 method of concussion and destruction of the brain). Length was measured in order to calculate the K Factor using a scaling factor of 10^5. Fish were dissected under a dissection microscope (Olympus SZ51), the intestinal tract, spleen and liver were removed and weighed. These organ weights were used to calculate the intestinal-somatic index, hepato-somatic index and splenic-somatic index respectfully. No histological analyses too place due to the small sample size making fixation and processing difficult.

In order to assess feed palatability, visual observations were made on the first feed of the day; the percentage of feed remaining in the tank at 10 second intervals for two minutes was recorded. Recordings were scaled from 0 % - 100 % at 10 % increments. An inter-observer reliability test was conducted to ensure the accuracy of the observational method; one observer conducted all the feed observations to further ensure consistency. A buoyancy test was also conducted on each diet using the same method as used to assess fish palatability; however, percentage of diet floating was measured at 20 second time intervals. Buoyancy observations were conducted in tanks without fish in. This test was conducted as diet presentation was suspected as a vital component to the response behaviour of the fish. Each of the five tanks was allocated a colour marker which corresponded to each of the trial diets. Markers were allocated to the diets by a non-associated researcher to ensure a blind test preventing observer bias with diets being revealed after the data was analysed.

3.3.2 Results

All diets yielded a significant increase in body weight after the 42 days ($P < 0.05$). Fish fed the control diet grew significantly more than all other diet groups,

achieving an 89 % gain in weight from 0.28 ± 0.05 to 0.53 ± 0.16, followed by NH Algae (78 %, P = 0.043) from 0.23 ± 0.02 to 0.41 ± 0.08, then Natto (42 %, P = 0.000) from 0.24 ± 0.04 to 0.34 ± 0.05, PPC (36 %, P = 0.001) from 0.25 ± 0.02 to 0.34 ± 0.05 and Rapeseed meal (28 %, P = 0.004) from 0.29 ± 0.04 to 0.37 ± 0.07. An FCR of 1.90 was calculated for the control diet, NH Algae (2.05), Natto (4.11), PPC (4.53) and Rapeseed meal (5.76). The control group also achieved the highest SGR (1.54), followed by NH Algae (1.46), Natto (0.84), PPC (0.77) and Rapeseed meal (0.62). All fish remained in good condition although the control group achieved a significantly higher condition score (K = 2.23 ± 0.13) than all other diets; PPC (K = 1.86 ± 0.08, P = 0.025), NH Algae (K = 1.84 ± 0.09, P = 0.017), Rapeseed meal (K = 1.81 ± 0.07, P = 0.009) and Natto (K = 1.69 ± 0.03, P = 0.000).

The Inter-observer reliability test carried out on feed observations showed no significant differences between the two observers (P > 0.05) and thus the method of observation is reliable. Using this method, feed observation times were grouped into four time intervals: 0 - 30, 40 - 60, 70 - 90 and 100 - 120 seconds, for statistical analysis. Strong significant differences (P < 0.01) in consumption rates were observed between the control diet and all the alternative diets across all time intervals (Figure 3.1 A). Results from the diet buoyancy test showed that the diets (Control and NH Algae) which were consumed quicker were those that remained on the water's surface for longer (Figure 3.1 B), indicating a more palatable or simply more accessible feed, reflective of the natural feeding behaviour expressed by zebrafish; feeding from the surface and upper region of the water column.

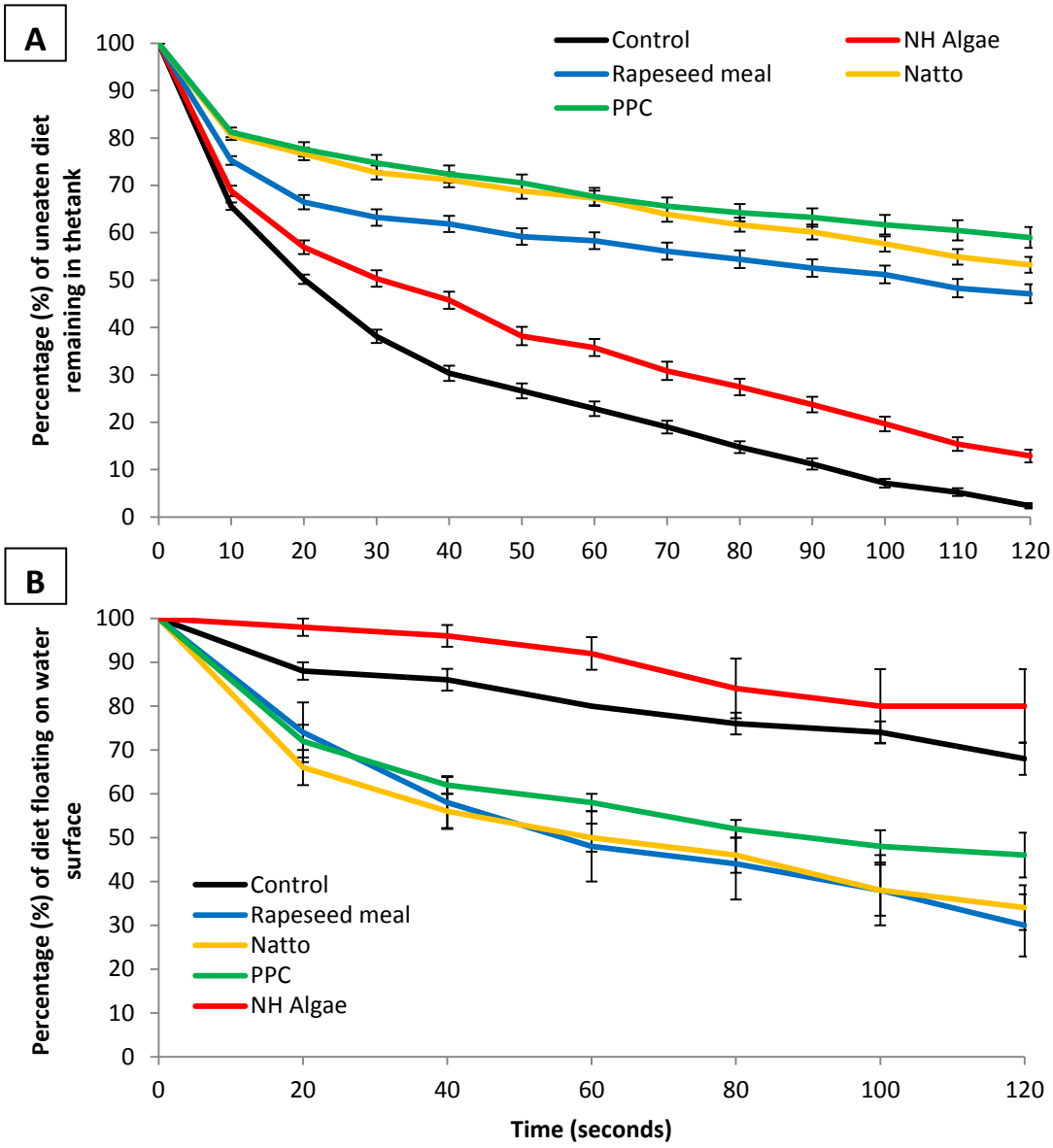


Figure 3.1. Zebrafish trial feed observations, **A**: Palatability, percentage (%) of uneaten diet remaining in the tank every 10 seconds for two minutes. **B**: buoyancy of experimental diets, percentage (%) of diet floating on water surface.

There was a significant decrease in Hepato-somatic index for fish fed the Natto diet compared to those fed the control ($P = 0.022$). There were no other significant differences ($P > 0.05$) between the control diet and any other diets for Hepatic-somatic index, Intestinal-somatic index and Splenic-somatic index (Table 3.5).

Table 3.5**.** The mean (± SEM) Hepatic-somatic index (HSI), Intestinal-somatic index (ISI) and Splenic-somatic index (SSI) achieved from 5 zebrafish obtained from each diet.

Diet	HSI	ISI	SSI
Control	2.12 ± 0.28[a]	1.46 ± 0.34[a]	0.06 ± 0.02[a]
Rapeseed meal	1.31 ± 0.58[a]	1.81 ± 0.20[a]	0.06 ± 0.02[a]
NH Algae	1.23 ± 0.65[a]	2.07 ± 0.45[a]	0.05 ± 0.01[a]
Natto	0.29 ± 0.11[b]	1.03 ± 0.09[a]	0.12 ± 0.06[a]
PPC	0.67 ± 0.20[a]	1.48 ± 0.16[a]	0.04 ± 0.01[a]

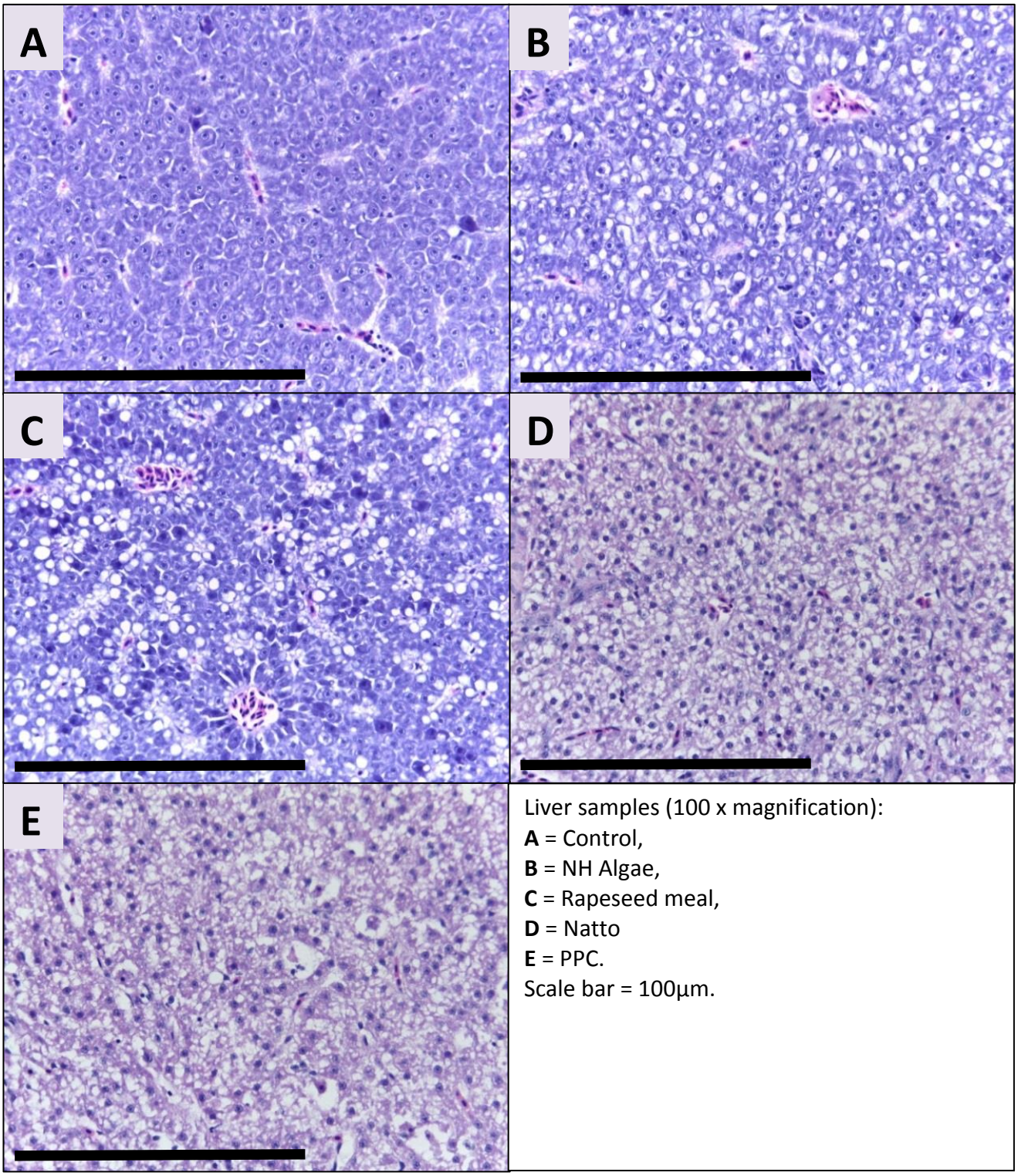


Figure 3.2. Representative photos taken of sectioned and H&E stained liver tissue samples collected after *D.rerio* where fed experimental diets: control, rapeseed meal, Natto, PPC and NH Algae for six weeks.

3.3.3 Discussion

This first trial was conducted to rule out any ingredients which may prove unpalatable or cause negative impacts on fish grow or condition. The results for this initial pilot trial show that diets containing each of the alternative feed ingredients being tested were accepted by zebrafish and they grew during the test period, although only the NH Algae fed fish grew at a rate near to that achieved by the control diet. In order to further assess these alternative ingredients they will now be tested in commercial species.

3.4 Rainbow trout (*O. mykiss*) performance trial

3.4.1 Trial specific methodology

Rainbow trout (*O. mykiss*) was selected as a freshwater carnivorous species with commercial importance. A feed trial was conducted over 42 days investigating the same alternative protein sources.

3.4.1.1 Subjects and husbandry

44 fish per diet, total of 220 fish were used for this trial. All 44 fish per diet were housed in single tanks measuring 67.5 x 46.5 x 38 cm (LxWxH) filled to 30 cm (holds 94 L). The tanks were divided to confine the fish to an area measuring 25 x 46.5 x 30 cm (LxWxH), 34L. This created an average stocking density of 31.5 kg/m^3, a stocking density of 20 - 30 kg/m3 is recommended for optimal welfare, feed Intake and reduction of aggressive interactions (Stevenson, 2007). Outflows were combined into a single fluidised biofilter system with a flow through of approximately 10 % water exchange per hour. The systems were maintained with a temperature of 12 ^{o}C under the conditions described in section 3.2.1.

3.4.1.2 Diets and feeding regime

Isonitrogenous and isoenergetic diets with 42 - 42.13 % protein and 18 - 18.91 % digestible energy were formulated by Skretting to include a control, Natto, Rapeseed meal, PPC and NH Algae (table 3.3). The fishmeal content in each diet was reduced as follows: Natto (48.34 % reduction), Rapeseed meal (36.86 % reduction),

Potato protein (54.08 % reduction) and NH Algae (16.55 % reduction). Diets were manufactured at the University of Liverpool (see Appendix 2 for method). Fish were fed 4 % body weight per day. The diet was split into four 1 % feeds at approximately 9:00 am, 12:00 pm, 2:00 pm and 5:00 pm. The 9:00 am feed was used to carry out consumption rate observations once waste had been collected for the previous day.

3.4.1.3 Growth and performance assessment indicators

Fish were weighed individually one day prior to the trial start to establish an initial mass. Pre-trial and post-trial weights, along with feed intake were used to calculate FCR, other indices used include: SGR, PER, K factor (with a scaling factor of 10^3). Hepatic and splenic somatic indices were also measured as described in section 3.2. Intestinal somatic index was not measured as fish were fed prior to sampling.

3.4.1.4 Tissue sampling

From each diet 10 fish were dissection as described in section 3.2.5. The intestinal tract was dissected into four sections for this species; stomach, pyloric caeca, mid-intestine and distal intestine. In addition to the pre-described sampling, second 5 mm sections were also collected for each section and cut open to produce a flat section of tissue which was fixed as flat as possible. Slides were photographed under x40 magnification for liver samples, and x100 magnification for intestinal samples.

3.4.1.5 Statistical analyses

All data was analysed as described in section 3.2.7. As FCR, SGR and PER is calculated per tank, with single tanks per diet there are no replicates for this data, therefore statistical analyses cannot be carried out for these measures.

3.4.2 Results

3.4.2.1 Growth and performance.

Fish were weighed pre-trial and post-trial. No significant differences were seen pre-trial between the control and each alternative diet. No difference ($P > 0.05$) remained between the control and both Natto and NH Algae. Fish fed with PPC and Rapeseed meal diets gained less weight ($P < 0.001$) than the control fish, (Figure 3.2).

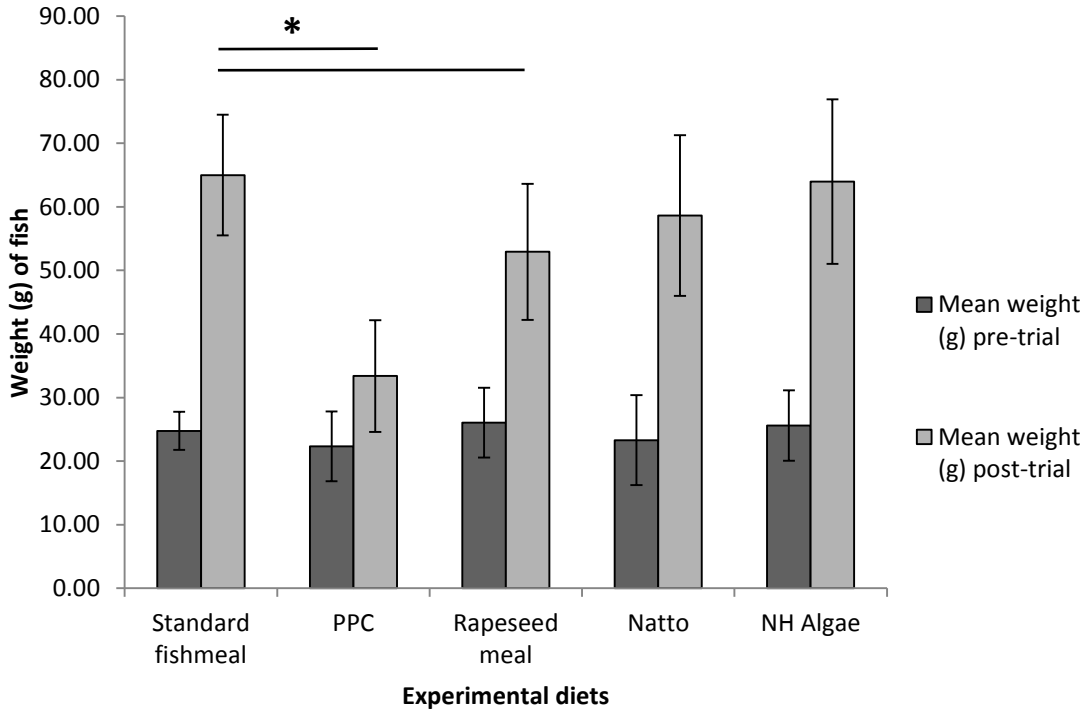


Figure 3.2. Mean (±SD) weight (g) of fish (*O. mykiss*) fed trial diets manufactured with reduced fishmeal substituted with vegetable and algal proteins, pre-trial and post-trial (42 days).

* Indicates a significant difference ($P<0.05$).

Feed Conversion ratio (FCR) (Figure 3.3), Specific Growth Rate (SGR) (Figure 3.4), Protein Efficiency Ratio (PER) (Figure 3.5) and Fulton-type condition factor (K) (table 3.5) have been calculated for each diet.

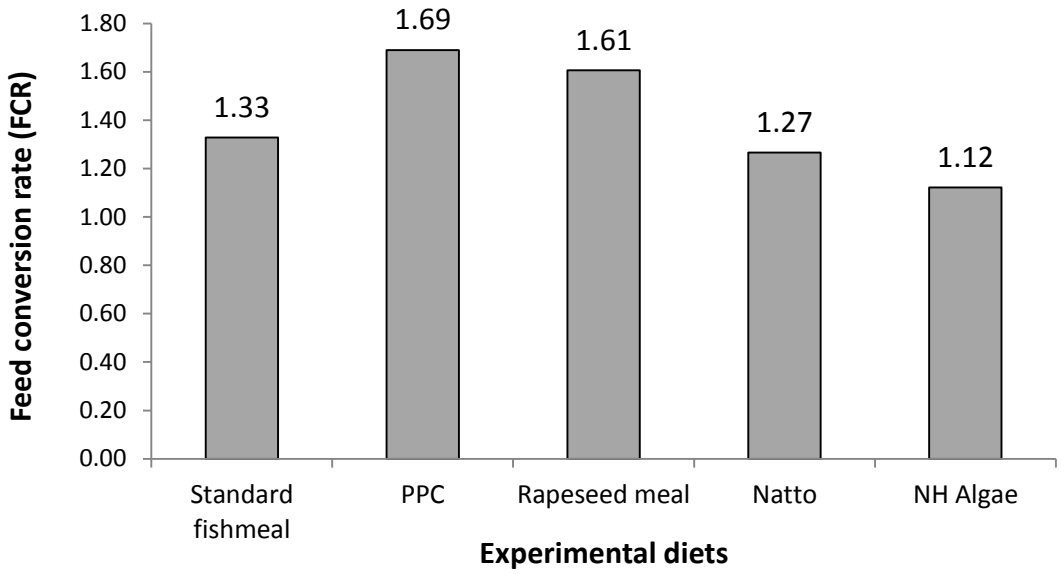


Figure 3.3. Feed Conversion Ratio (FCR); required amount of dry feed (kg) required to produce 1 kg of fish (*O. mykiss*). FCR's of each trial diet manufactured with partial fishmeal substitution for vegetable or algal proteins. Natto and NH Algae are most similar to the Standard fishmeal diet.

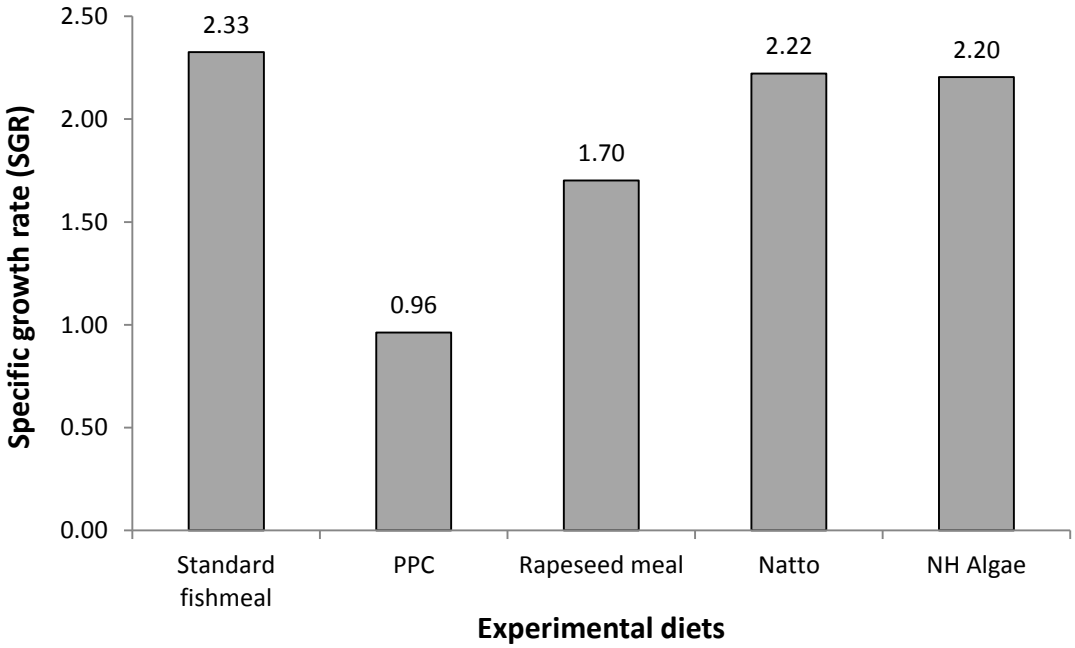


Figure 3.4. Specific Growth Rate (SGR); Percentage (%) of body weight gained per day. SGR's displayed by fish (*O. mykiss*) fed each trial diet manufactured with partial fishmeal substitution for vegetable or algal proteins. Natto and NH Algae are most similar to the Standard fishmeal diet.

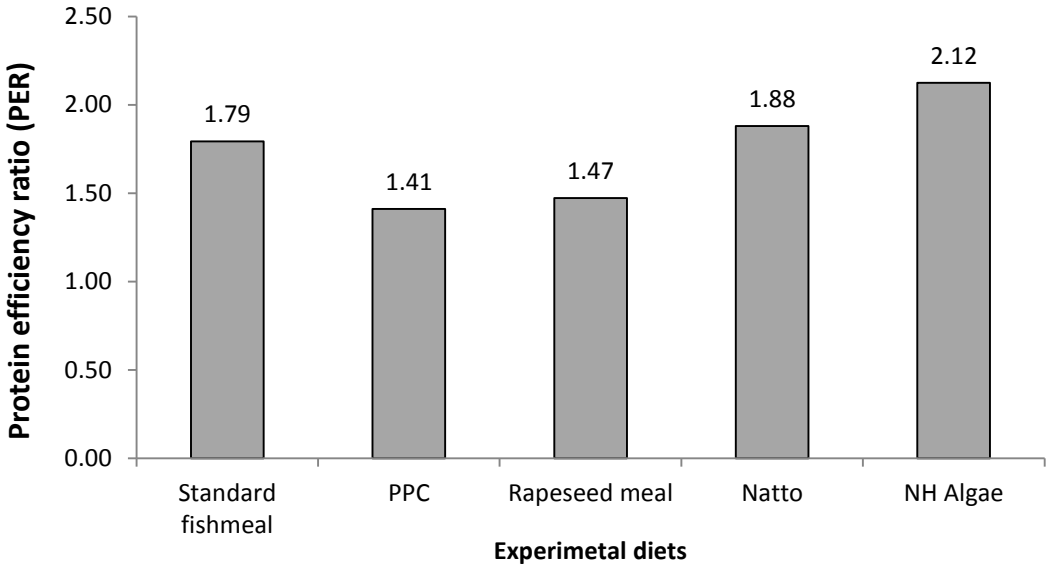


Figure 3.5. Protein Efficiency Ratio (PER); amount of fish (kg) produced from 1kg of protein fed. PER's displayed by fish (*O. mykiss*) fed each trial diet manufactured with partial fishmeal substitution for vegetable or algal proteins. Natto and NH Algae are most similar to the Standard fishmeal diet.

Table 3.5. Fulton-type condition factor (K). K values observed for fish fed each experimental diet: standard, PPC, Rapeseed Meal, Natto and NH Algae.

Diet	K factor observed	K value comments
Standard (control)	1.7	**1.60** - **Excellent** condition, trophy class fish. **1.40** - A **good**, well-proportioned fish. **1.20** - A **fair** fish, acceptable to many anglers. **1.00** - A **poor** fish, long and thin. **0.80** - **Extremely poor** fish, big head and narrow, thin body.
PPC	1.6	
Rapeseed Meal	1.6	
Natto	1.5	
NH Algae	1.6	

K value comments from Barnham and Baxter (2003).

No differences ($P > 0.05$) in organ indices were seen between the control diet and any alternative diets (Table 3.6).

Table 3.6. The mean (± SD) Hepatic-somatic index (HSI), Intestinal-somatic index (ISI) and Splenic-somatic index (SSI) achieved from five European sea bass obtained from each diet.

Diet	HSI	SSI
Control	1.24 ± 0.23[a]	0.22 ± 0.09[a]
Rapeseed meal	1.20 ± 0.14[a]	0.17 ± 0.09[a]
NH Algae	1.31 ± 0.09[a]	0.21 ± 0.10[a]
Natto	1.29 ± 0.15[a]	0.21 ± 0.11[a]
PPC	1.35± 0.18[a]	0.23 ± 0.11[a]

Data in each column which do not share a letter are significantly different (P > 0.05).

3.4.2.2 Histological analyses

Intestinal tract samples were taken from three sections; pyloric caeca, mid-intestine and distal intestine. For each section villi length and width was measured and surface area (μm^3) was calculated. Results from statistical analyses revealed no significant changes in villi structure within the distal intestine between the control group and all other diet groups. Fish fed the Rapeseed meal diet developed longer villi in the pyloric caeca and mid-intestine. Fish fed the Natto diet developed longer villi in the pyloric caeca while they grew shorter in the mid-intestine. Fish fed the PPC diet experienced reduced villi length in both regions (Figure 3.7). Villi in the pyloric caeca of fish fed the PPC and NH Algae diets were thinner than the control group, so too were the villi in the mid-intestine of fish fed the Natto diet (Figure 3.8). See Figure 3.9 for representative photos of H&E stained samples of intestinal tissue.

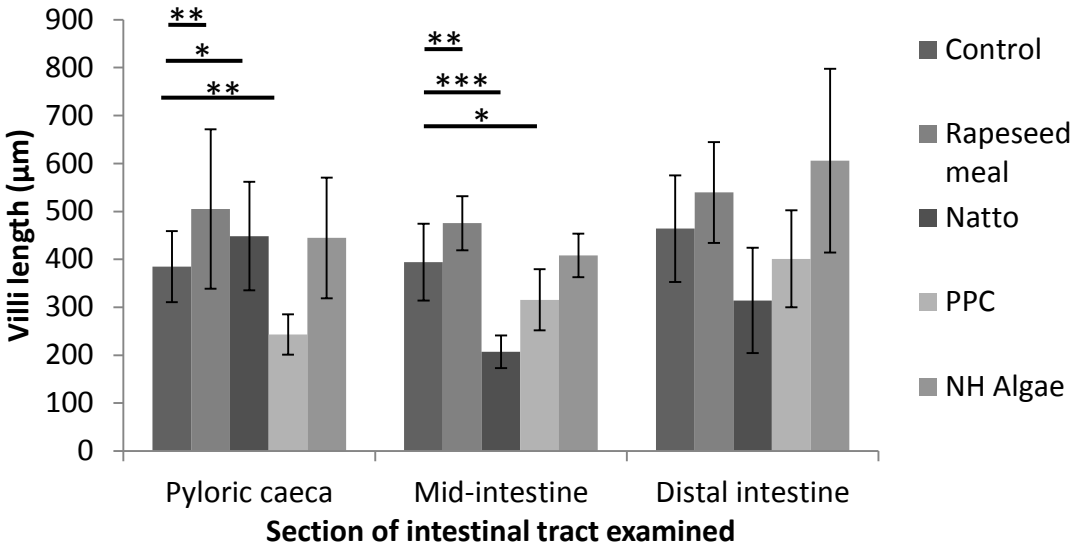


Figure 3.7. Villi length (µm ± SD) within the pyloric caeca, mid-intestine and distal intestine of rainbow trout fed experimental diets for six weeks: Control, Rapeseed meal, Natto, PPC and NH Algae. Significant differences are indicated with asterisk: * <0.05, ** <0.01, *** <0.001.

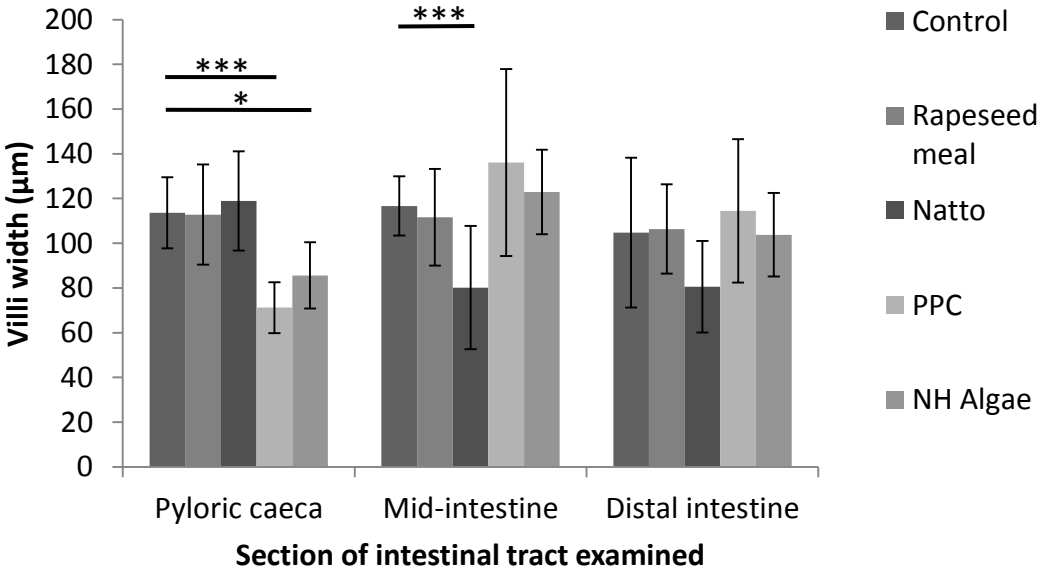
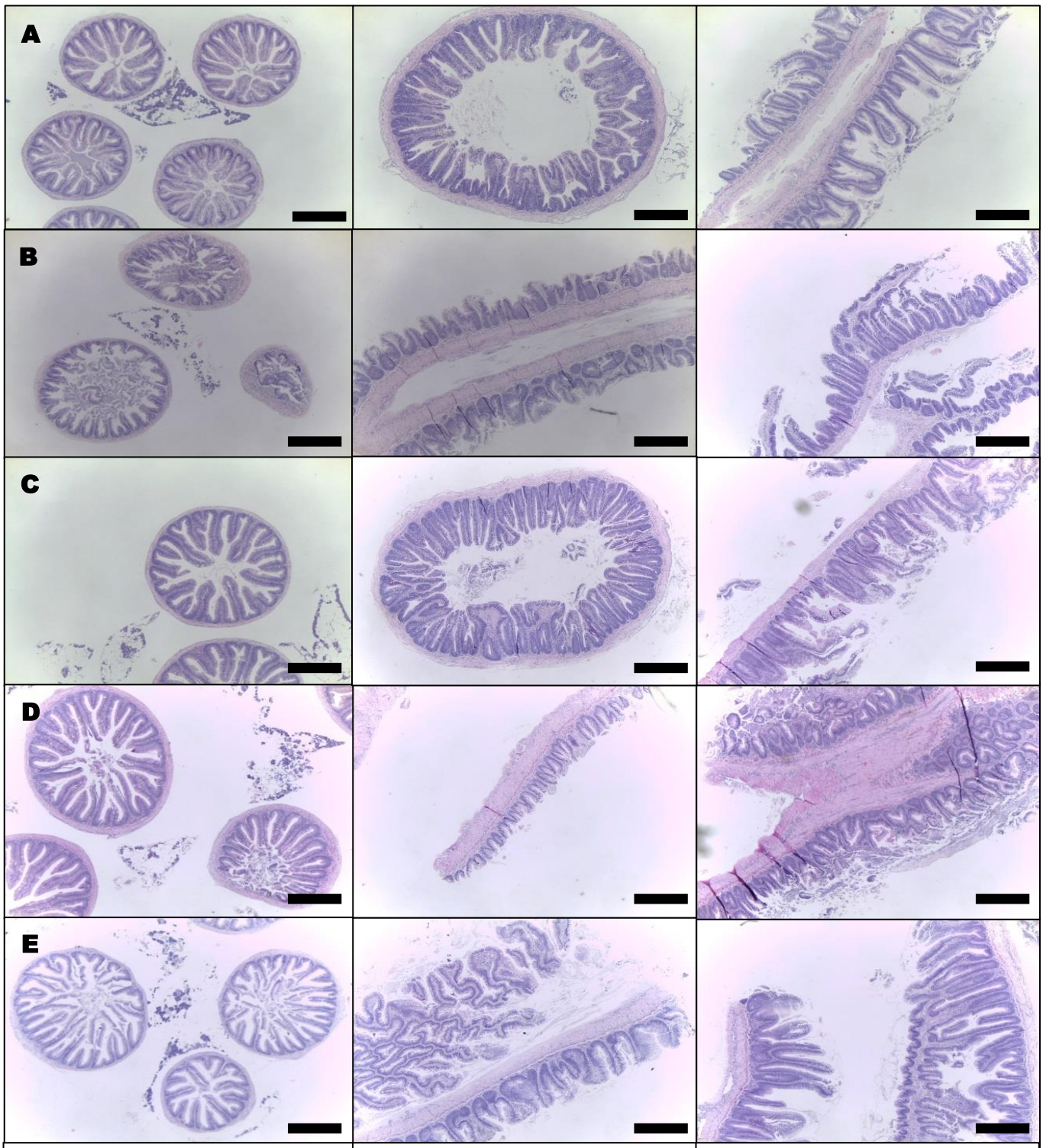


Figure 3.8. Villi width (µm ± SD) within the pyloric caeca, mid-intestine and distal intestine of rainbow trout fed experimental diets for six weeks: Control, Rapeseed meal, Natto, PPC and NH Algae.
* Indicates a significant difference (P < 0.05).



Tissue samples collected from the pyloric caeca (sectioned whole), the mid and distal intestine (sectioned whole and flat). Above are representative photos (40 x magnification) for:
A – Standard (control) diet, **B** – PPC diet, **C** – Rapeseed Meal diet, **D** – Natto diet, **E** – NH Algae diet.
Scale bar = 500 µm

Figure 3.9. Representative photos of sectioned and H&E stained intestinal samples collected from rainbow trout fed for six weeks on experimental diets: Control, Rapeseed meal, Natto, PPC and NH Algae.

Liver samples were analysed for percentage of sample stained and unstained. The PPC (72.2 ± 7.1 %) and Natto (70.6 ± 2.7 %) fed fish express lower ($P < 0.05$) percentages of stained tissue compared to those fed the control diet (79.7 ± 2.9 %), there was no difference for fish fed the Rapeseed meal (76.8 ± 1.7 %) and NH Algae (75.7 ± 2.7 %) diets. See Figure 3.10 for representative photos of sectioned and H&E stained liver samples for fish fed each experimental diet.

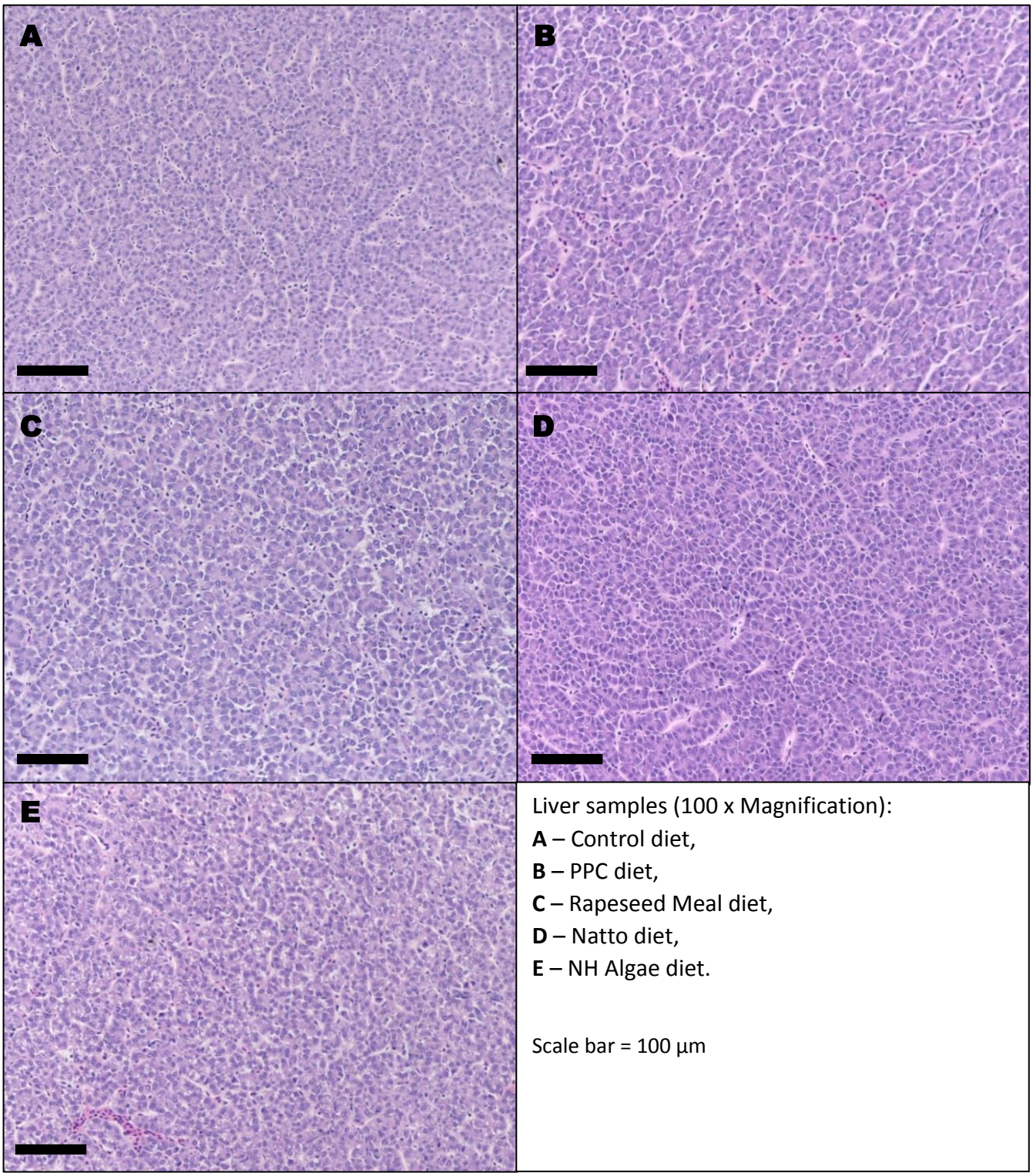


Figure 3.10. Representative sample of photos taken of H&E slides produced from liver tissue samples collected from fish (*O. mykiss*) fed for six weeks on experimental diets: Control, Rapeseed meal, Natto, PPC and NH Algae.

3.4.2.3 Palatability

Five weeks of data for consumption rate of 1 % body mass in feed were analysed. There was no difference between the control (19.21 seconds) and the NH Algae (18.74 seconds, $P = 0.995$) diets. The three remaining diets; PPC (1086.18 seconds), Rapeseed meal (43.15 seconds), and Natto (22 seconds) were all consumed slower ($P < 0.05$) than the control.

3.4.3 Discussion

The aim in these trials is for the alternative diets to perform equally with the control diet. K factor shows that all the fish fed each diet remained in good condition, even with the lower performance seen with the PPC diet. Fish fed the Natto and NH Algae diets presented similar FCR, SGR and PER. Fish fed the Rapeseed meal diet displayed reduced performance and failed to grow comparably in weight. Fish fed the PPC diet also failed to grow comparably, displaying reduced performance when assessing FCR, SGR and PER. Therefore the PPC and Rapeseed meal diets would take considerably longer for fish to reach market size, culminating in greater costs.

Assessment of villi structure through the separate regions of intestinal tract revealed no changes ($P>0.05$) in the distal intestine; all changes were observed within the pyloric and mid-intestine, as described above. Reduction in villi length can be an indicator of 'non-infectious' subacute enteritis. Further signs of enteritis include i) loss of the normal supranuclear vacuolization of the absorptive cells, ii) widening of the central stroma within the villi and iii) a notifiable infiltration of inflammatory cells in the lamina propria (Rašković et al., 2011). An analysis of these additional parameters is recommended to fully assess if enteritis has resulted in those fish with shorter villi. Liver analyses showed reduction ($P<0.05$) in percentage of tissue stained for the PPC and Natto diets. Consequently a significant increase in the percentage of unstained area within a sample is also true. This unstained area was not consistent with fatty metamorphosis, lipidosis and there are no visual signs

of necrosis. Liver cells appear normal with no signs of cellular swelling (Mumford et al., 2007). Examination by a fish pathologist may highlight a cause for the change.

Palatability testing showed the NH Algae diet was consumed at an equal rate to the control. Despite the rapeseed meal and Natto diets being consumed slower ($P < 0.05$), it is unlikely this would be of significant influence in a commercial setting as the feed is still consumed quite rapidly. The PPC diet was consumed at a much slower rate, an average of 18 minutes, if this was used in a commercial setting the feed would likely be lost as waste, either buried in silt in pond culture or washed away in race way systems. Such a diet would not be desired by industry, this combined with performance data provides sufficient evidence not to pursue this ingredient for use in rainbow trout.

3.5 European Sea bass (*D. labrax*) performance trial

3.5.1 Trial specific methodology

European sea bass (*D. labrax*) was selected as a marine carnivorous species with increasing commercial importance; an industrial collaborating partner also expressed significant interest in this species and was able to supply stock for study. A feed trial was conducted over 42 days investigating the same alternative protein sources: Control, Rapeseed meal, Natto, PPC and NH Algae.

3.5.1.1 Subjects and husbandry

A total of 150 fish were used, 30 fish per experimental diet. All fish were held in one recirculation system consisting of five identical tank measuring 102.5 x 102.5 x 42 cm (LxWxH) filled to 35 cm (holding 360 L). The total volume of the system is approximately 2000 L including the filter system. Fish were randomly assigned to a tank, 30 fish per tank, creating an average stocking density of 8.36 kg/m^3. All 30 fish per diet were housed in one tank, juvenile sea bass are gregarious, and this enabled a more natural behavioural repertoire. The system was maintained at a

temperature of 25 ± 1 ^{o}C. Weekly 10 % water changes were carried out to maintain water quality parameters were subsequently maintained at Ammonia (NH_3): 0mg/l, Nitrite (NO_2): <0.3mg/l, Nitrate (NO_3): <50mg/l and pH: 8.

3.5.1.2 Diets and feeding regime

Five isonitrogenous and isoenergetic diets with 42 - 44 % protein and 17.9 - 18.5 % digestible energy were formulated to include one control and four with fishmeal partially substituted for vegetable and algal protein sources: Rapeseed meal, Natto, PPC, and NH Algae (Table 3.4). The experimental diets were formulated with the following amount of fishmeal substituted: Natto (57.49 % reduction), Rapeseed meal (51.68 % reduction), Potato protein (57.49 % reduction) and NH Algae (35.75 % reduction). Diets were manufactured at the University of Liverpool (see Appendix 2 for method). Fish were fed to satiation four times per day (approximately 9:00 am, 12:00 pm, 2:00 pm and 5:00 pm). Satiation was determined when the fish failed to respond to introduction of feed and feed pellets were allowed to settle on the bottom of the tank. A maximum of 4 % body weight of feed was given per day to prevent overfeeding. Each day the 4 % ration was measured (± 1 g) for each tank, fish were fed from this throughout the day, and any food remaining after the final feed was weighed, calculating the exact amount of food given per tank per day. This method of feeding was chosen due to the fish failing to consume a full 4 % feed during preliminary testing. Quantification of the amount of feed eaten, when fed to satiation, can be used as an indicator of palatability. Increased feed intake is indicative of increased palatability and acceptability (GLENCROSS et al., 2007).

3.5.1.3 Growth and performance assessment indicators

Fish were weighed (± 1g) individually one day prior to the trial start to establish an initial mass. After 42 days fish were individually caught and euthanized. Performance was measured the same as described in the previous trial (section 3.4), using weight gain, FCR, SGR, PER, organ indices. Condition was assessed using K factor, (with a scaling factor of 10^5).

3.5.1.4 Tissue sampling

Five fish per diet were dissected tissue sampling and analyses was carried out as described in section 3.2.5, the intestinal tract was sampled only as mid-intestine.

3.5.1.5 Statistical analyses

All data was analysed as described in section 3.2.7. As FCR, SGR and PER is calculated per tank, with single tanks per diet there are no replicates for this data, therefore statistical analyses cannot be carried out for these measures.

3.5.1.6 Palatability

Fish were fed to satiation to a maximum of 4 % body weight. Satiation was determined when the fish failed to respond to introduction of feed and feed pellets were allowed to settle on the bottom of the tank. Fish that consumed greater quantities of feed are deemed to find that feed more palatable. Fish consuming lower quantities of feed are deemed to find that feed less palatable. The consumption of feed as percentage of body weight was calculated for each day of the trial.

3.5.2 Results

3.5.2.1 Growth and performance

No significant differences ($P > 0.05$) in weight were seen pre-trial between the control and each alternative diet. There was still no difference ($P > 0.05$) between the control and each alternative diet post-trial except PPC. Fish fed with the PPC diet weighed less ($P < 0.05$) than the control fish, (Figure 3.11). Fish fed the control diet consumed 67.53 g of feed per fish, growing by 34.8 % (36.2 g weight gain). Fish fed the Rapeseed meal diet consumed 85.97 g per fish, gaining the most weight at 44.5 % (45.7 g). Fish fed the NH algae diet consumed 71.67 g per fish and also grew by 44.4 % (41.0 g weight gain). Fish fed the Natto diet consumed 86.13 g per fish but grow less at 20.7 % (21.7 g). Fish fed the PPC diet consumed very little feed

(38.2 g per fish) and displayed very poor performance as they lost 7.5% weight (7.3 g).

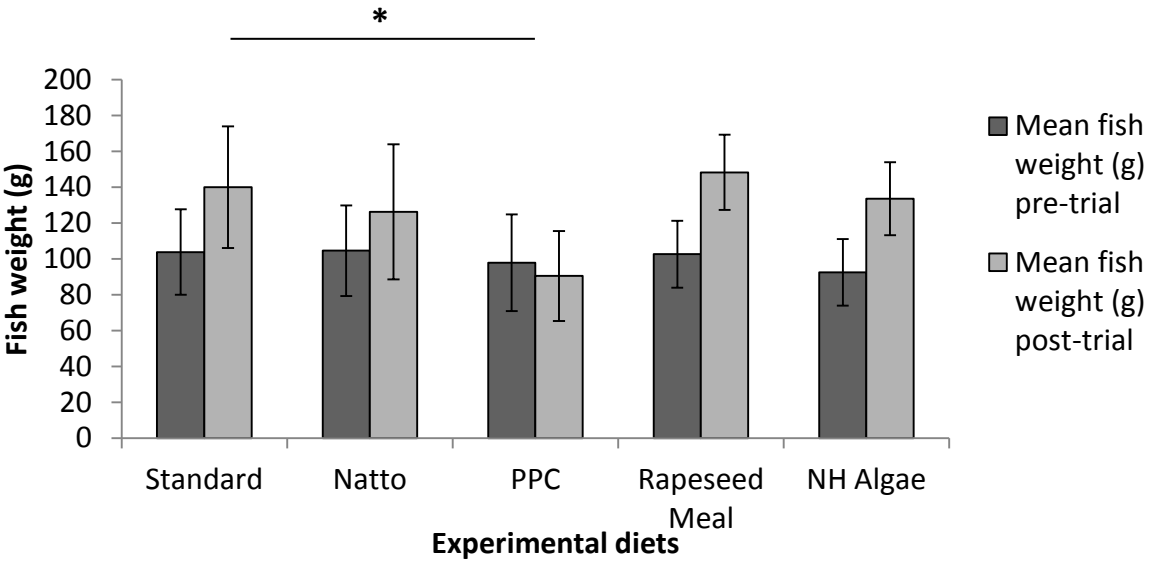


Figure 3.11. Mean (± SD) weight (g) of fish (*D. labrax*) fed trial diets: standard, Natto, PPC, Rapeseed meal and NH Algae, pre-trial and post-trial (42 days).
* Indicates a significant change between diets post-trial (P<0.05).

FCR (Figure 3.12), SGR (Figure 3.13), and PER (Figure 3.14) shows the Rapeseed Meal and NH Algae diets achieved similar values as the Standard fishmeal diet, displaying slight improvement. The Natto diet achieved a much higher FCR value, therefore being much less efficient than the control. Fish fed the PPC diet reduced slightly in weight although this was not significant (P > 0.05) and so the FCR, SGR and PER are negative values, reflecting very poor performance. Condition (K) scores revealed that all diet groups including the PPC group remained in excellent condition (Table 3.7), despite the loss of weight seen.

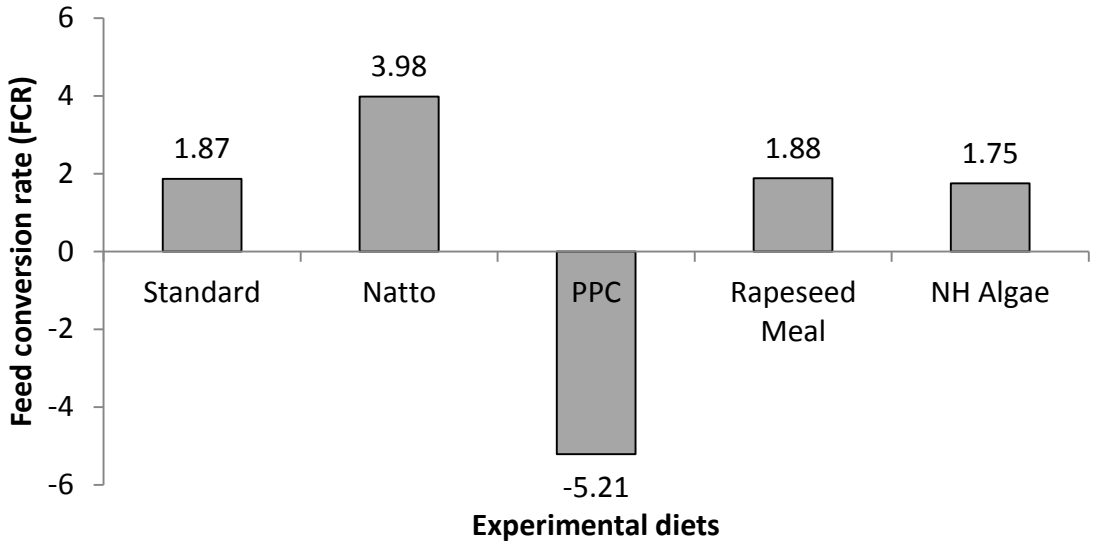


Figure 3.12. Feed Conversion Ratio (FCR); required amount of dry feed to produce 1 kg of fish.

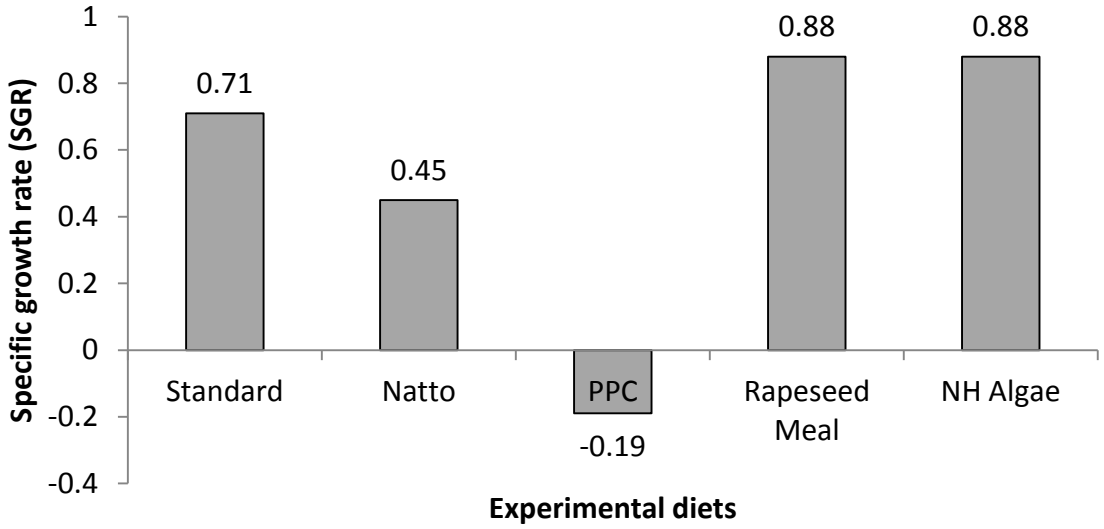


Figure 3.13. Specific Growth Rate (SGR); Percentage of body weight gained per day.

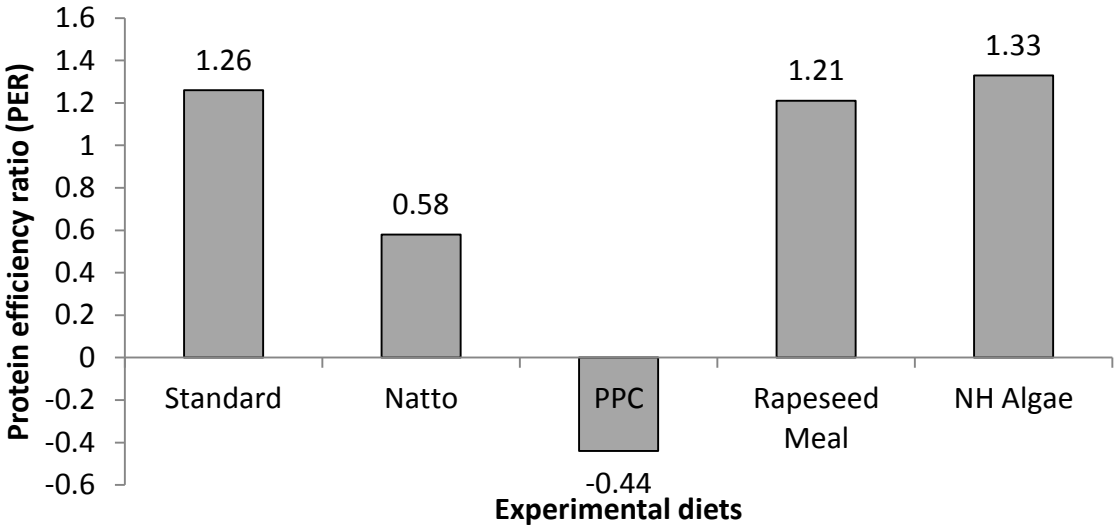


Figure 3.14. Protein Efficiency Ratio (PER); amount of fish produced from 1 kg of protein in diet.

Table 3.7. Fulton-type condition factor (K). K values observed for fish fed each experimental diet: standard, Natto, PPC, Rapeseed Meal, and NH Algae.

Diet	K factor observed	K value comments
Standard (control)	1.87	**1.60** - **Excellent** condition, trophy class fish. **1.40** - A **good**, well-proportioned fish. **1.20** - A **fair** fish, acceptable to many anglers. **1.00** - A **poor** fish, long and thin. **0.80** - **Extremely poor** fish, big head and narrow, thin body.
Natto	1.62	
PPC	1.74	
Rapeseed Meal	1.74	
NH Algae	1.94	

Fish fed the Natto diet showed a significant increase ($P < 0.001$) in Intestinal-somatic index. Fish fed the PPC diet showed a significant decrease ($P < 0.05$) in hepatic-somatic Index. No other differences in organ indices were seen between diets (Table 3.8).

Table 3.8. The mean (± SD) Hepatic-somatic index (HSI), Intestinal-somatic index (ISI) and Splenic-somatic index (SSI) achieved from five European sea bass obtained from each diet.

Diet	HSI	ISI	SSI
Control	2.08 ± 0.27[a]	1.88 ± 0.12[a]	0.07 ± 0.01[a]
Rapeseed meal	2.07 ± 0.18[a]	1.96 ± 0.11[a]	0.08 ± 0.01[a]
NH Algae	1.72 ± 0.34[a]	2.03 ± 0.18[a]	0.06 ± 0.01[a]
Natto	1.64 ± 0.22[a]	2.36 ± 0.19[b]	0.08 ± 0.01[a]
PPC	1.33± 0.53[b]	1.99 ± 0.10[a]	0.09 ± 0.01[a]

Data in each column which do not share a letter are significantly different (P > 0.05).

3.5.2.2 Histological analyses

Villi length and width was measured. Villi length increased (P < 0.05) in fish fed the Natto, PPC and NH Algae diets compared to the control fish (Figure 3.15). There were no differences (P > 0.05) in villi width found between the control and each experimental diet (figure 3.16). See Figure 3.17 for representative photos H&E stained intestinal samples for fish fed each experimental diet.

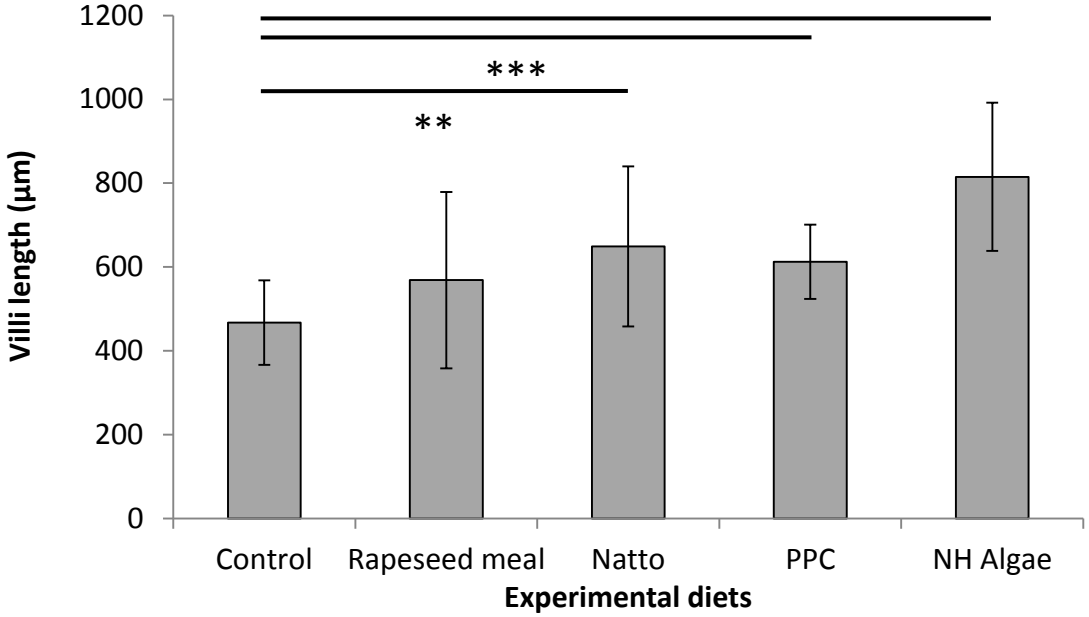


Figure 3.15. Average length (±SD) of villi (µm) found in the mid-intestine of *D. labrax* after six weeks being fed five experimental diets: control, Rapeseed meal, Natto, PPC and Algae.
Significant differences are indicated with asterisk: * <0.05, ** <0.01, *** <0.001.

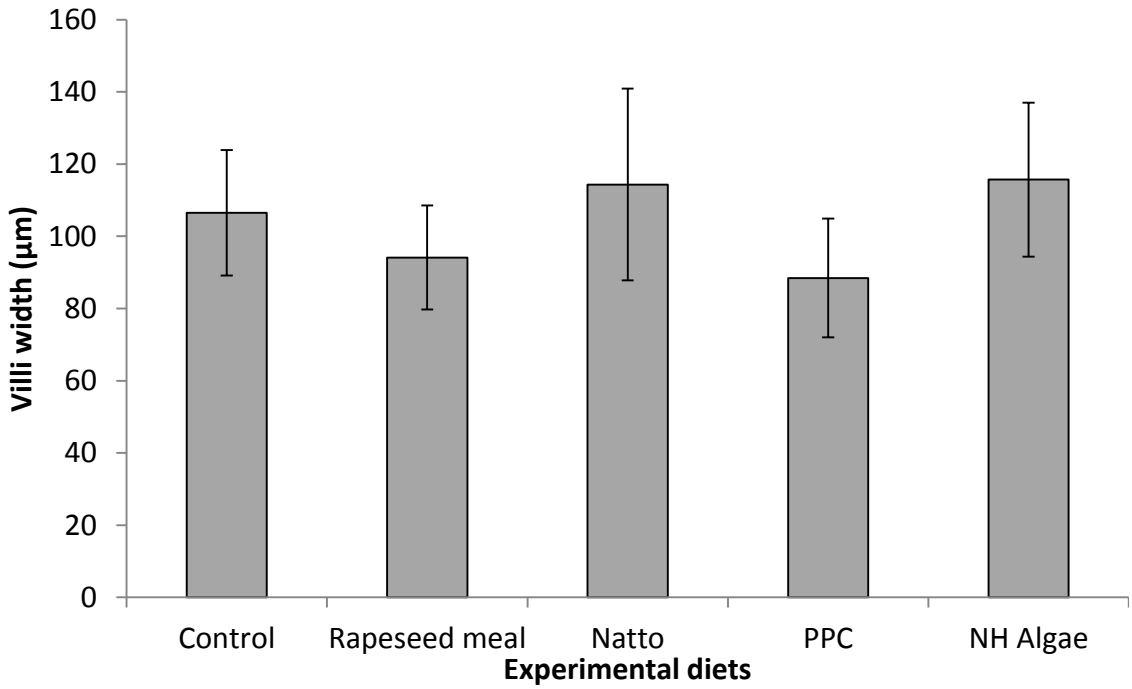


Figure 3.16. Average width (± SD) of villi (µm) found in the mid-intestine of *D. labrax* after six weeks being fed five experimental diets: Control, Rapeseed meal, Natto, PPC and NH Algae.

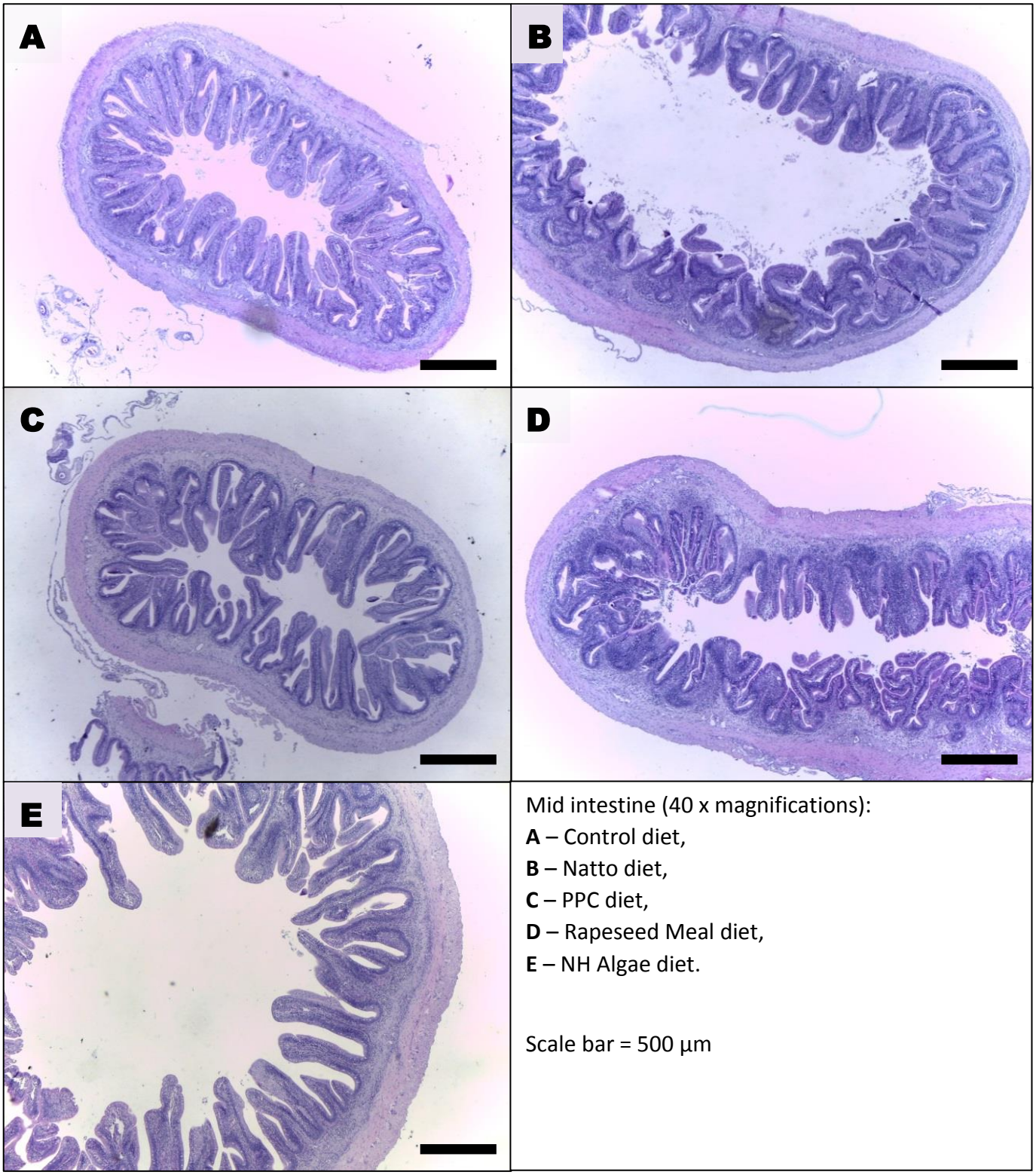


Figure 3.17. Representative H&E stained intestinal samples for *D. labrax* post six week feed trial being fed Control, Rapeseed meal, Natto, PPC and NH Algae diets.

Liver sample analyses revealed no significant differences ($P > 0.05$) in percentage of stained tissue between the control diet (35.22 %) and all alternative diets: Rapeseed meal (39.27 %), Natto (40.72 %), PPC (38.72 %) and NH Algae (39.25 %). See Figure 3.18 for representative photos of H&E stained liver samples for fish fed each experimental diet.

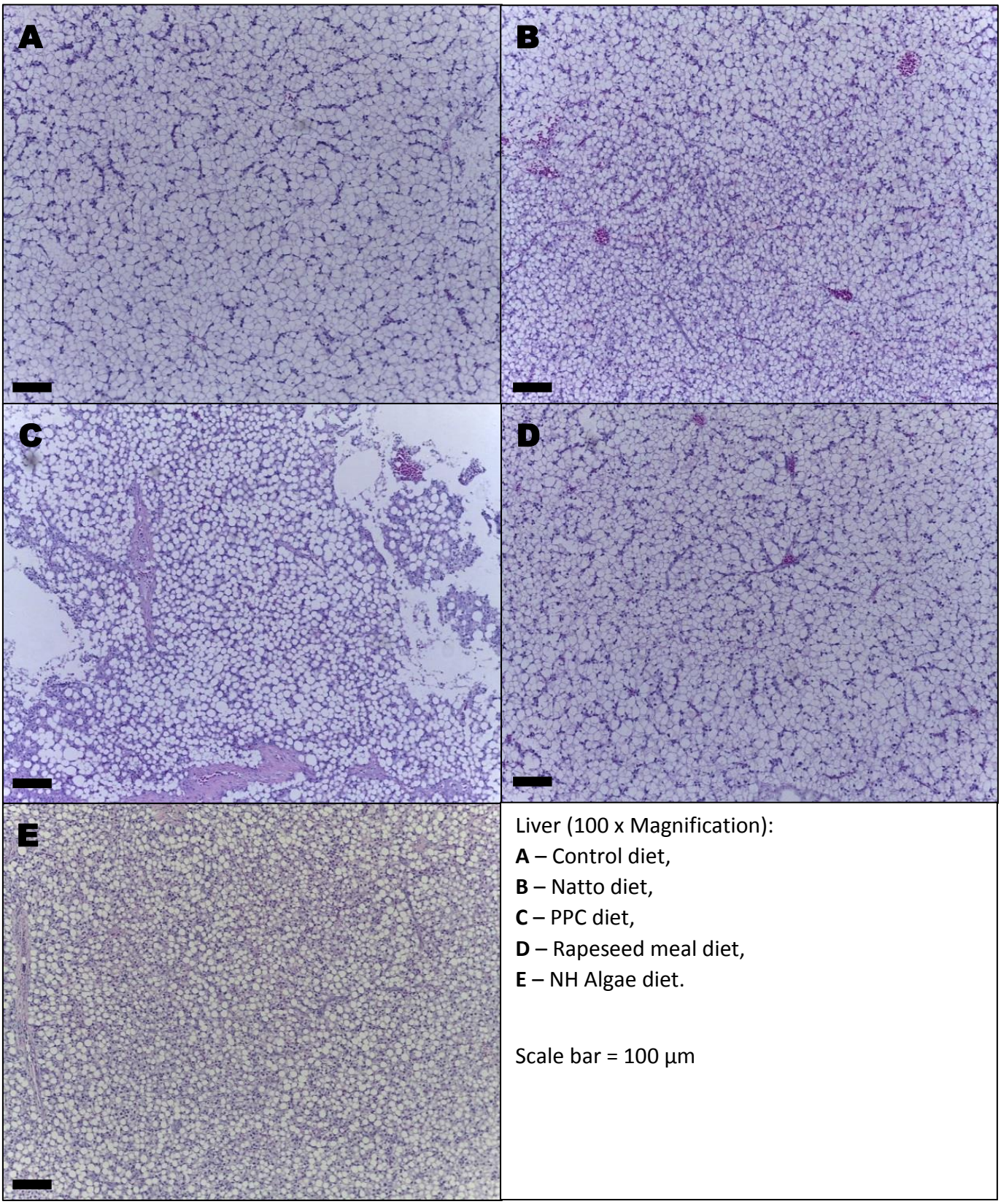


Figure 3.18. Representative photos taken of sectioned and H&E stained liver tissue samples collected from (*D. labrax*) after six weeks feeding on experimental diets: Control, Rapeseed meal, Natto, PPC and NH Algae. Lipid deposition is high across all diets.

3.5.2.3 Palatability

There was no difference between the amount of feed consumed on the control diet (1 %) and either the Rapeseed meal diet (1.64 %, P = 0.213) or NH Algae diet (1.66 %, P = 0.142) diet. Despite the lack of a significant difference the fish fed the two alternative diets did consume approximately 60 % more feed than the control fish, suggestive of improved taste response. Natto was consumed more (1.81 %, P = 0.034) indicating increased palatability, and PPC was consumed less (0.72 %, P = 0.000) indicating reduced palatability.

3.5.3 Discussion

It can be seen that all the fish remained in 'excellent' condition, as described by K factor. However, the fish fed the PPC diet reduced slightly in weight and did not grow. This does bring into doubt the use of K factor as a useful indicator within aquaculture. Fish fed all the other experimental diets grew during the six week trial period. Post-trial there was no difference between the control diet and the Rapeseed meal, Natto and NH Algae diets (P > 0.05). However, the growth achieved by the Rapeseed meal and NH Algae fed fish was higher at 44 % compared with 34.8 % for the control, therefore given a longer trial period there may have been significant differences seen between the diets.

This result is reflected by the FCR and SGR for each diet. Again Rapeseed meal and NH Algae achieved similar results to the control diet. Fish fed the Natto diet reached comparable size to the control group with no difference (P>0.05) in final weight, however, weight gain, FCR, SGR, PER and percentage of feed consumed per day all show reduced efficiency. Fish fed this diet would take longer to reach market size, they would also consume greater amounts of feed, culminating in greater costs. The PPC diet showed very poor performance through the lack of feed consumption, loss of weight, and the resulting poor performance indicators.

There were no significant differences between the control diet and either the Rapeseed meal or NH Algae diets when assessing organ indices. Fish fed the PPC diet showed a smaller ($P < 0.05$) liver, likely due to the fish utilising lipid reserves as the feed consumption rate was low during the trial. Fish fed the Natto diet showed a larger ($P<0.05$) intestinal tract, considering this alongside the increased feed consumption, FCR, reduced SGR and PER, this indicates a physiological adaption of increasing the area of uptake in order to utilise the poorer quality feed. When investigating villi structure of the mid-intestine, Natto, PPC and Rapeseed meal showed increased villi length ($P < 0.05$) compared to the control. No significant differences were seen in villi width between the control and any alternative diet. There were no reduction in villi length seen in any diet groups compared to the control group, therefore from these limited investigations there are no signs of enteritis as described by (Rašković et al., 2011), although villi length is only one symptom of the condition, further analyses would be required to comprehensively declare there was no enteritis caused. Liver analyses showed no significant changes in percentage of tissue stained for all alternative diets compared to the control. Consequently no change in the percentage of unstained area within a sample is also true. This unstained area was consistent with liver fat deposition.

The results of this trial provide clear evidence that a prolonged feeding period using the PPC diet with *D. labrax* would undoubtedly compromise the health of any subjects due to loss of weight, therefore also compromising welfare of the fish.

3.6 Conclusion, are these vegetable materials suitable for dietary inclusion?

After testing the potential feed ingredients: Rapeseed meal, Natto, PPC and NH Algae compared to the fishmeal based controls, utilised in the formulations in Tables 3.2 to 3.4, the NH Algae diets performed the most similar to the control diets across all three test species: zebrafish, rainbow trout and European sea bass. In each case the diets containing the algal material achieved this without causing observable alterations to the condition of the fish. This may be due to the NH Algae

diets having the lowest reduction levels of fishmeal in all three species. The Natto diet also performed well when fed to rainbow trout, it also proved to be very palatable to European sea bass. The Rapeseed meal diet achieved equal efficiency and performance when fed to European sea bass as the control. The PPC diets performed poorly in all three test species, proving unpalatable to rainbow trout and more so to European sea bass: where fish lost weight during the trial period due to minimal feed consumption.

When evaluating the diets, which achieved equal growth and performance, for fish oil reduction, the rainbow trout diets show fish oil was reduced by 16.73 % in the NH Algae diet and by 52.42 % in the Natto diet. In the European sea bass diets fish oil was reduced in the NH Algae diet by 18.74 %, however, fish oil had to be increased in the Rapeseed meal diet by 31.31 %. No fish oil was used in the zebrafish diets.

Therefore, the results from these feed trials indicate that the NH Algae material is suitable as an alternative feed ingredient. However, this material contains the lowest protein content of all the alternative ingredients (at only 16.5 %, table 3.1A), which resulted in the lowest level of fishmeal reductions, along with high lipid content, it therefore lends itself to be seen more as a lipid source to aid fish oil reduction than a protein source.

Defatted soybean meal is already widely used within aquaculture feeds, at <15 % in trout diets (Hardy, 2017), here the Natto material performed similarly to the control when fed to trout, used at an inclusion rate of 31 %. Although the trout trial results were positive, the Natto diets performed poorly compared to the control when fed to the other species tested. The fermentation method applied in the production of Natto appears to have enabled higher inclusion rate of a soybean product in the diet of trout. Soy protein concentrate has in recent years become a material desired for its higher protein levels; this is likely to be the chosen soy bean product due to

its superior nutritional quality. Natto therefore will likely not be considered as an aquaculture ingredient in its current state. The Rapeseed meal diets displayed mixed results across the three species tested, performance was poor in the zebrafish, good in the trout, although lower than the control, and equal to the control in the sea bass trial. For the commercial species this material managed to replace between 37 % (trout) to 52 % (sea bass), if the price of this material after fermentation is considerable lower than fishmeal this material could be of consideration for further testing and use in aquaculture feeds. The Natto diet was identified as the most palatable feed in the sea bass trial, while the Rapeseed meal diet was the most palatable diet in the trout trial. Perhaps these two materials will be valued greater as dietary stimulants for certain species, used at low inclusion levels to improve the taste of other diets.

The PPC diets performed poorly in all trials compared to the controls. Raw PPC material has been shown to reduce growth of trout by 88 % at an inclusion level of 20 % (Xie and Jokumsen, 1997), here an inclusion level of 23 % of fermented PPC resulted in a 48 % reduction in growth. This indicates that fermentation has improved the quality of the material, however, these results are attained from separate studies and therefore trial parameters differed. Dedicated research would be required to establish if this effect is true. From analyses of the ANF's (Table 3.1B), it was shown that most of the ones identified increased following fermentation, also, levels of Chaconine and Solanine were high even before treatment. The ANF's may have contributed to the poor performances seen here (Xie and Jokumsen, 1997). As growth and performance of all fish tested reduced and the ANF's increased with processing, the final PPC product is not recommended for further use as a feed ingredient in aquaculture feeds.

The success of each of these materials for inclusion into fish feeds is also highly influenced by cost of material and efficiency. The NH Algae material achieved equal or improved performance across all three species tested here, it has therefore been

recommended for further research and inclusion into aquaculture feeds. However, the production cost of algal materials is still quite high at present, until the cost of algal products, such as the NH Algae used here, becomes comparable or reduces below that of fishmeal, there will be little incentive to include such materials.

The trials carried out in this chapter also identified other issues with methodology. The ability of K factor to assess condition of fish is in doubt for the purpose of assessing performance in this circumstance, as shown in section 3.5 (sea bass trial) where fish remained in excellent condition yet lost weight. Using this measure fish would only be deemed in poor condition beyond a weight loss acceptable in aquaculture production. K factor will therefore no longer be used in the next chapter; it will be replaced with whole body composition analyses. The histology slides collected here failed to provide sufficient good quality samples of intestinal tract to enable stereological analyses of the surface area of the epithelium. Efforts will be made in further trials to produce sufficient numbers of slides to enable stereological analyses for improved assessment.

Chapter 4

4.0 Fishmeal and fish oil replacement with dietary inclusion of insect meals - growth and performance

4.1 Invertebrate meal production and analyses.

Two of the invertebrate species discussed in Chapter 1, 'invertebrate protein sources' were sourced as dried products: spent silkworm pupae (*B. mori*) and mealworms (*T. molitor*), while the earthworms (*E. fetida*) were sourced live, then dried. All three materials where processed into dry meals. The live earthworms were processed using a method inspired by those of Edwards (1985) and Istiqomah et al. (2009). Earthworms were washed, removing substrate and faecal matter, and then kept in the refrigerator (4 ^{o}C) for 12 hours, encouraging intestinal emptying. Worms were then washed a second time, placed in just boiled water for no more than 5 minutes, to kill them, before being oven dried (Genlab DC1000 drying cabinet) at 50 ^{o}C for 10 – 12 hours. Dried worms were then ground (manual hand grinder) into a fine powder.

All three meals once produced where taken to the School of Biosciences (UK), Division of Nutritional Sciences, University of Nottingham: Sutton Bonnington campus (referred to as Nottingham from here on in), where the author carried out nutritional analyses under supervision following the methods described in Appendix 3. All samples which were analysed from here were done so by technicians at Nottingham using the methods in Appendix 3.

Percent (%) carbohydrate (Nitrogen Free Extract – NFE) was calculated for each material tested upon culminating all other results, using the following equation: % Carbohydrate (NFE) = 100 - (% moisture + % crude protein + % crude lipid + % crude fibre + % ash) (Novoa et al., 1994). The nutritional profiles of all three meals can be seen in Table 4.1.

Table 4.1. Nutritional profiles: proximate analyses, amino acid and fatty acid profiles, of each invertebrate meal under investigation.

Diet component		Invertebrate species		
		Silkworm pupae (*Bombyx mori*)	Mealworms (*Tenebrio molitor*)	Earthworms (*Eisenia Fetida*)
Proximate analyses	Dry matter, DM (%)	94.07	94.8	94.73
	NFE (% DM) Carbohydrates	5.51	0	10.23
	Ash (% DM)	4.19	3.76	2.7
	Fibre (% DM)	2.24	9.73	0.24
	Crude Protein (% DM)	57.58	55.95	73.44
	Lipid (crude fat) (% DM)	30.48	30.59	13.39
	Energy content (MJ/kg DM)	27.04	27.12	24.54
Essential amino acids (% protein)	Arginine (ARG)	3.44	3.39	5.17
	Histidine (HIS)	2.14	2.24	1.79
	Isoleucine (ILE)	3.57	3.73	4.21
	Leucine (LEU)	4.68	4.78	5.74
	Lysine (LYS)	4.65	3.69	5.25
	Methionine (MET)	2.23	0.86	1.48
	Cystine (CYS)	1.68	1.17	1.35
	Phenylalanine (PHE)	3.60	2.48	3.10
	Tyrosine (TYR)	3.81	4.24	2.25
	Threonine (THR)	2.84	2.49	3.03
	Tryptophan (TRY / TRP)	-	-	-
	Valine (VAL)	3.79	4.16	3.61
Essential fatty acids (% of total fatty acids).	18:2 n-6 (Linoleic acid).	7.05	36.69	9.07
	18:3 n-3 (α-linolenic acid).	31.95	1.79	0.22
	20:4 n-6 (Arachidonic acid).	-	-	-
	20:5 n-3 (Eicosapentaenoic acid (EPA)).	-	-	1.92
	22:6 n-3 (Docosahexaenoic acid (DHA)).	-	-	-
Non-Essential fatty acids (% of total fatty acids)	12:0 (Lauric acid).	-	0.24	25.99
	13:0 (Tridecanoic acid).	-	-	2.3
	14:0 (Myristic acid).	-	2.37	21.21
	15:0 (Pentadecanoic acid).	0.05	0.2	1.58
	16:0 (Palmitic acid).	23.57	18.21	6.86
	16:1 n-7 (Palmitoleic acid).	1.41	1.6	2.51
	17:0 (Heptadecanoic acid).	-	-	0.98
	18:0 (Stearic acid).	5.04	2.6	10.45
	18:1 n-9 (Elaidic acid).	-	-	0.84
	18:1 n-9 (Oleic acid).	30.93	36.3	5.67
	20:2 n-6 (Eicosadienoic acid).	-	-	5.54
	20:3 n-6 (Dihomo-γ-linolenic acid).	-	-	1.12
	20:3 n-3 (Eicosatrienoic acid (ETE)).	-	-	3.61

– Not detected.

Comparison of the data gathered from analysis of the invertebrate meals created here (Table 4.1) with that gathered from the literature base (Rumpold and Schluter, 2013a), (Finke, 2002), (Zhenjun et al., 1997), (Xiao, 1984), evidence the potential variation between source materials, and therefore the importance of testing each source material prior to use. Each of the three meals under investigation display fairly well balanced amino acid profiles as predicted based on the literature base, they also show high levels of certain desired PUFAs, however, individually each material lacks in specific aspects of the desired elements, when compared to fishmeal which is considered ideal. Silkworm meal is lower in crude protein, amino acids arginine, leucine, lysine, threonine and valine, and devoid of fatty acids EPA and DHA, although it does possess higher energy content, increase in amino acids cystine and tyrosine, along with increased crude lipid with high levels of linoleic and α-linolenic acid levels. The mealworm meal displays a very similar profile to silkworm meal with additional reduction in amino acids methionine and phenylalanine, while displaying sufficient amounts of valine. The earthworm meal in contrast has a similar crude protein and lipid levels to fishmeal, with slightly higher energy content. Amino acid profile shows reduced histidine, lysine, methionine, phenylalanine, threonine and valine, while it is sufficient in others, and excels in cystine levels. There are good levels of linoleic acid and low levels of EPA, although no α-linolenic acid or DHA present. Therefore, providing more reasoning to utilise and investigate these meals as a combined product, mixed based on target nutritional profiles and inclusion of other base dietary ingredients.

From previous studies on fishmeal replacement, a combination of multiple sources are more likely to achieve the desired dietary nutritional profile for each target fish species than using single invertebrate materials (Zhang et al., 2012a, Zhang et al., 2012b, Hu et al., 2013, Hansen et al., 2007, Hansen et al., 2011, Torstensen et al., 2008). In this research the three species discussed above were tested for palatability individually and then combined for testing in diet formulations.

4.2 Diet formulation and manufacture.

Once the nutritional profiles of all three invertebrate meals had been determined, nutritionally balanced experimental feeds were formulated, based on the current knowledge of nutritional requirements, for three target test fish species: zebrafish (*D. rerio*), carp (*C. carpio*) and rainbow trout (*O. mykiss*), see Appendix 1 for nutritional requirements. Diets were formulated using a best fit modeller programme. This programme (DMAF) was created in house by Andrew Fletcher; see Appendix 4 for Andrew's summary of how this works.

Two diets were created for each species; a control diet based on industry formulations, and an alternative feed utilising all three invertebrate meals to completely replace fishmeal and fish oil. The control diets were created with a restricted number of feed ingredients to avoid over complicating the feeds, and to better assess the utilisation of the invertebrate meals; inclusion of which is higher when fewer ingredients are used. Based on previous projects and personal communication (P. Gallimore 2018) with the international aquaculture feed manufacturer Skretting, the ingredients used for control diets were restricted to commonly used materials, fishmeal as the primary protein source, wheat gluten as a secondary protein source which also provides binding properties, corn starch is a source of carbohydrates and acts as a filler material, Rapeseed and fish oils provide lipids and additional energy. The remaining dietary constituents are always added at set levels and consist of vitamin and mineral premixes, added at industry levels (personal communication with P. Gallimore 2018), and a binding agent, CMC, to ensure pellet stability. Supplementation with crystalline amino acids was carried out as necessary.

The zebrafish diets (Table 4.2 and 4.5) were formulated to include 48 % fishmeal and 7.5 % rapeseed oil in the control diet; no fish oil was used. These inclusion levels were based on zebrafish control diets formulated by Skretting for previous

projects. The carp diets (Table 4.3 and 4.5) were formulated to include 14.8 % fishmeal, 4 % rapeseed oil combined with 4 % fish oil in the control diet; the fish meal inclusion level was chosen to replicate the current level used by Skretting (personal communication with P. Gallimore 2018), the oil level was chosen during formulation to achieve the required lipid and energy levels in the diet. Carp can be raised on a diet containing natural feed items with added supplementary feeds, such as grains (commonly wheat), often without the use of fishmeal (FAO, 2015b), therefore wheat gluten as the secondary protein rich ingredient was increased to achieve the desired dietary protein level with the low fishmeal inclusion. The rainbow trout diets (Table 4.4 and 4.5) were formulated to include 51 % fishmeal and 12 % fish oil in the control diet. Current industry fishmeal levels are much lower than this, on occasion as low as 10 % (personal communication with P. Gallimore, 2018), however, other protein source materials are utilised in conjunction with fishmeal in such diets. Due to the limited number of ingredients used here it would not be feasible to achieve such a low inclusion level of fishmeal in a carnivorous diet. Wheat gluten is typically included at > 21 % for rainbow trout, also fishmeal is traditionally included between 48 % for fingerlings and 68 % for fry (FAO, 2015f), therefore an inclusion level of 51 % fishmeal was used with 15 % wheat gluten. If the invertebrate meals successfully replace such a high inclusion of fishmeal, they will be even more applicable to replacing lower levels in future trials.

Table 4.2 – Nutritional profiles of zebrafish diets as predicted by best fit diet modeller programme.

Nutrient		Zebrafish (*D. rerio*)	
		Control	Insect
		Formulation	Formulation
Crude Protein	% DM	46.93	46.96
Crude Lipid		14.50	14.92
Carbohydrates (NFE)		32.73	32.49
Crude Ash		8.62	2.39
Crude Fibre		0.09	2.24
Linoleic Acid	% Lipid	15.73	22.63
Linolenic Acid		5.21	13.72
Arachidonic Acid		0	0
EPA		0	0.3
DHA		6.92	0
Arginine	% Protein	5.23	3.82
Histidine		2.13	2.01
Isoleucine		3.87	3.69
Leucine		6.87	5.49
Lysine		6.01	3.69
Methionine		1.24	1.53
Cystine		1.14	1.57
Phenylalanine		2.60	2.64
Tyrosine		2.60	1.79
Threonine		2.90	2.90
Tryptophan		-	-
Valine		3.41	2.64
Gross Energy	MJ/kg DM	23.06	23.69

Table 4.3 – Nutritional profiles of common carp diets as predicted by best fit diet modeller programme.

Nutrient		Carp (*C. carpio*)	
		Control	Insect
		Formulation	Formulation
Crude Protein	% DM	38.68	38.38
Crude Lipid		12.64	12.54
Carbohydrates (NFE)		43.99	42.90
Crude Ash		3.39	1.96
Crude Fibre		0.19	2.11
Linoleic Acid	% Lipid	20.32	25.87
Linolenic Acid		9.02	13.18
Arachidonic Acid		0	0
EPA		0	0.16
DHA		6.88	0
Arginine	% Protein	3.82	3.80
Histidine		1.96	2.04
Isoleucine		3.30	3.59
Leucine		6.34	5.50
Lysine		4.70	4.90
Methionine		1.86	1.88
Cystine		1.64	1.61
Phenylalanine		3.21	3.23
Tyrosine		2.17	2.19
Threonine		3.52	3.54
Tryptophan		0.52	0.52
Valine		3.21	3.23
Gross Energy	MJ/kg DM	23.20	22.45

Table 4.4 – Nutritional profiles of rainbow trout diets as predicted by best fit diet modeller programme.

Nutrient		Trout (*O. mykiss*)	
		Control	Insect
		Formulation	Formulation
Crude Protein	% DM	48.47	48.44
Crude Lipid		19.88	19.51
Carbohydrates (NFE)		24.41	25.20
Crude Ash		10.23	3.15
Crude Fibre		0.09	2.83
Linoleic Acid	% Lipid	5.51	17.75
Linolenic Acid		11.90	16.11
Arachidonic Acid		0	0
EPA		0	0.34
DHA		14.02	0
Arginine	% Protein	4.86	4.08
Histidine		1.98	2.03
Isoleucine		3.29	3.84
Leucine		6.09	5.10
Lysine		4.92	4.63
Methionine		1.49	1.49
Cystine		1.06	1.42
Phenylalanine		2.56	2.56
Tyrosine		1.73	1.73
Threonine		2.81	2.81
Tryptophan		0.41	0.41
Valine		2.56	2.56
Gross Energy	MJ/kg DM	24.32	23.93

Table 4.5. Recipes of experimental diets for all three species of fish.

Ingredient (% diet)	Zebrafish (*D. rerio*)		Carp (*C. carpio*)		Trout (*O. mykiss*)	
	Control	Insect	Control	Insect	Control	Insect
Danish Fishmeal	48	-	-	-	-	-
Organic Fishmeal	-	-	14.8	-	51	-
Wheat Gluten	15	16	33.5	16.7	15	-
Corn Starch	28.3	29	41.5	40.93	20.8	21.3
Vitamin premix	0.3	0.3	0.3	0.3	0.3	0.3
Mineral premix	0.4	0.4	0.4	0.4	0.4	0.4
Binder (CMC powder)	0.5	0.5	0.5	0.5	0.5	0.5
Arginine (98%)	-	-	-	0.09	-	-
Lysine (77%)	-	-	1	0.79	-	-
Rapeseed Oil	7.5	-	4	-	-	-
South American Fish Oil	-	-	4	-	12	-
Mealworm meal	-	17	-	16.8	-	21.3
Silkworm pupae meal	-	20	-	16	-	31
Earthworm meal	-	16.8	-	7.5	-	25.2
Total	100	100	100	100	100	100

These diets were manufactured in house to be as aesthetically similar as possible. Diets were manufactured at the University of Liverpool (see Appendix 2 for method), pellet size made to be appropriate for the size of fish based on size guides available with Skretting products (Skretting, 2018) and published by FAO (New, 1987).

4.3 Modified and improved trial methodology

All feed trials in this chapter were conducted based on an improved method developed following trials in chapter three. With modifications made to suit each subject species. Previous feed trials (chapter 3) were conducted over a period of six weeks, which was sufficient to achieve significant growth and show differences between diets. However, this was increased to a trial period of 10 weeks to provide opportunity for more subtle differences between diets to be identified and provide

sufficient time for fish to double in weight. During each trial, systems were maintained the same parameters as described in section 3.2.1, unless stated otherwise.

4.3.1 Weighing feeds and fish

Feeds were pre-weighed as described in section 3.2.2. Fish were weighed as described in section 3.2.3. Fish were again weighed weekly, repeating this process, in order to record growth and maintain the desired feed ration throughout the trial period.

4.3.2 Assessing growth and performance

In order to assess the performance of the diets and condition of the fish the same measurements as described in section 3.2 were used: weight gain, FCR, PER, SGR and organ indices. In addition the fishmeal ratio (FMR) was calculated. FMR indicates the quantity of fishmeal required to produce 1 kg of live fish:

$$FMR = FCR \times \frac{\% \text{ Fishmeal in feed}}{100}$$

(Boyd et al., 2007)

FIFO was also calculated, as described above in section 1.2.3. FMR and FIFO will be calculated for all the Insect diets below to be zero, as no fish derived materials were used in those diets; data will be included for control diets to show the potential impact of changing to fish derivative free diets. As concluded in chapter 3 (section 3.6) Fulton's type condition (K) factor will not be used, instead fish will be analyses for whole body composition using the methods described in appendix 3.

4.3.3 Tissue sampling and Palatability assessment

All fish in each tank were humanely euthanized (UK Home Office Approved Schedule 1 method of concussion, and destruction of the brain), by a fully trained

researcher. A number of fish from each tank were dissected and sampled as described in section 3.2.5. Histological analyses was carried out as described in section 3.2.5, further to villi length and width measurements, imageJ was also used to calculate the area (μm^2) of epithelium using stereology cavalieri point counting methods.

Feed palatability is assessed as described in section 3.2.6.

4.3.4 Statistical analyses

Statistical tests for difference were carried out between the Control and Insect diets as described in section 3.2.7. For analyses of FCR, SGR and PER where n = three per diet, a number too small for normality tests, non-parametric Mann Whitney-U tests were used to compare diets.

4.4 Zebrafish (*D. rerio*) growth and performance

4.4.1 Trial specific methodology

A trial was carried out using Zebrafish first to establish if the invertebrate meals would be feasible for inclusion with commercially relevant species. 1000 fish, two months of age, were used; all of which were bred in house at the University of Liverpool. Fish were housed in groups of 100 individuals in 10 identical 9 L Aquatic Habitats Zebrafish tanks (same design as previous Zebrafish trial only the larger size), connected to a 300 L system which was centrally filtered. Water quality was maintained as described in section 4.3. Five tanks were allocated per diet; diets and tanks were marked with corresponding colours by an independent researcher to enable blind testing of the diets, diets were revealed post-trial data collection. During the trial fish kept at 28 ± 1 ^{o}C.

Fish were raised using ZM fry foods (ZM000, ZM100 and ZM200), live newly hatched artemia nauplii then Tetramin flake food. Once allocated to the trial tanks fish were

allowed one week to acclimatise. During this trial fish were fed a daily ration of 4 % bodyweight as in the previous zebrafish trial. All feeds were measured as described previously.

Fish were weighed, as described in section 4.3.1, as a group of 100 fish, too many to weigh individually in one day. A 1.5 L weigh tank was used, filled to an approximate depth of 2 cm, with a Fisher scientific SG – 602 weigh balance. As fish were weighed all together, a holding tank was not required. Performance and health was measured as described in section 4.3.2. . Using a microscope (Olympus SZ51) five fish were dissected, removing the intestinal tract, this was fixed whole due to small size, all five samples per tank were fixed in one wax block. Histological analyses were carried out as described in section 4.3.3, liver samples were too small and delicate to collect then fix and process into slides, therefore no analysis of liver tissue took place. Photographs of the intestinal tissue samples were done using x100 magnification. Organ weights were not collected due to the small size of these fish; therefore organ indices were not calculated. All remaining fish per tank were culled, and then frozen at -80 °C before being transported, on dry ice, to Nottingham for post-trial nutritional analysis. Feed palatability was assessed as described in section 4.3.3. Statistical analyses were also carried out as described in section 4.3.4. Five tanks were used per diet so Kolmogorov Smirnoff tests were used to test for normality.

4.4.2 Results

Two balanced isoenergetic and isonitrogenous diets (Table 4.2): Control and Insect were tested. Throughout the trial, fish fed both diets grew consistently; on week nine fish on the control diet were larger ($P<0.05$) than those on the Insect diet. However, at the end of the trial there was no difference ($P>0.05$) in fish weight between the diets (Figure 4.1). There was no difference ($P>0.05$) found between the two diet groups for SGR. Analysing efficiency measures, FCR and PER both showed

no difference (P>0.05) between the diets (Figure 4.1). The control diet achieved an FMR score of 1.13 ± 0.05 and a FIFO score of 5.02 ± 0.23.

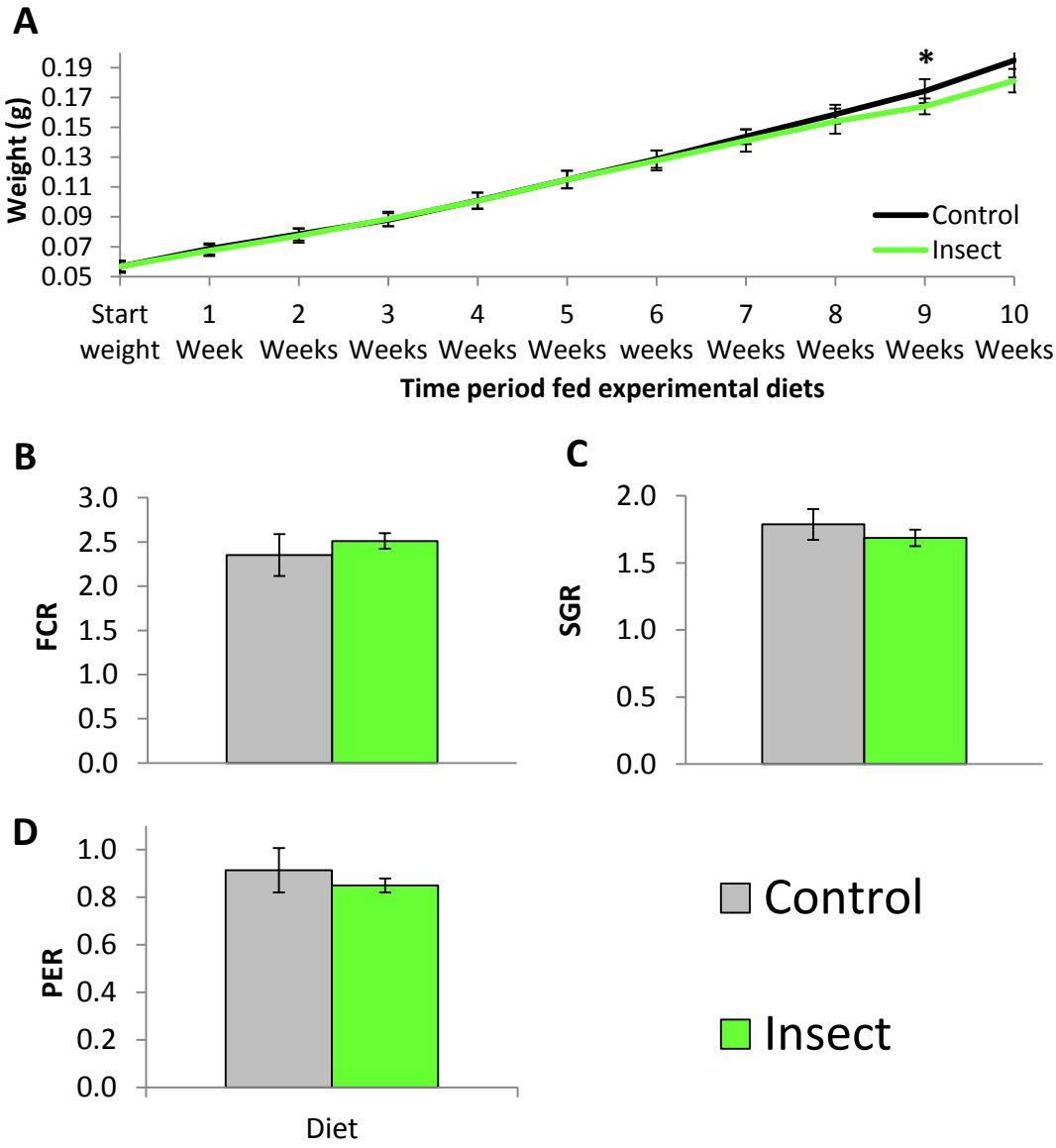


Figure 4.1. Zebrafish (*D. rerio*) growth and performance indicators (± SD) achieved for each experimental diet, control and Insect: **A**) Average fish weight (g), showing growth throughout trial period, **B**) Feed Conversion Ratio (FCR), **C**) Specific Growth Rate (SGR), **D**) Protein Efficiency ratio (PER). Significant differences are indicated with asterisk: * <0.05, ** <0.01, *** <0.001.

Observing the time taken for each tank to consume a 1 % feed ration, the control diet was consumed quicker (111 ± 9 seconds, P = 0.0078) than the insect diet (185 ± 26 seconds), indicating a preference for the control pellets. Despite the significant difference between the diets, the insect diet was still fully consumed by the subject fish, thus must not be distasteful or a deterrent. This result therefore, does not rule out these insect meals as potential fishmeal replacement materials.

Histological analyses revealed no significant differences (P>0.05) in villi length and width between the two diets (Figure 4.2). There was also no difference (P>0.05) in villi surface area per 5µm sample of intestine, control = 2.0 ± 0.44 mm^2 and Insect = 1.79 ± 0.68 mm^2. See Figure 4.3 for representative photos of the H&E stained cross sections of mid-intestine.

Figure 4.2. Zebrafish (*D. rerio*) intestinal villi structure (± SD): length and width, for fish fed each experimental diet. No significant differences were found between the control and insect based diets.

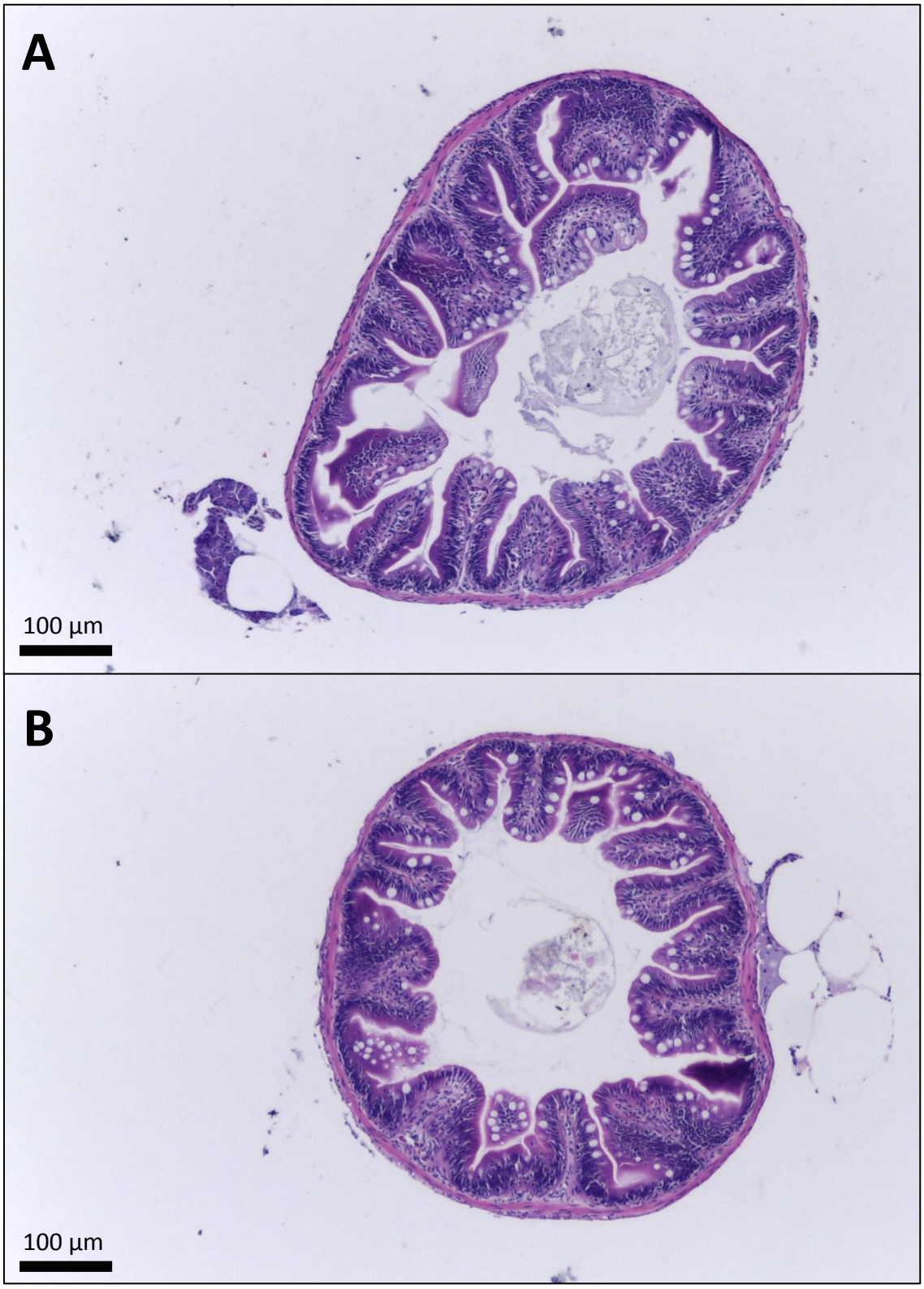


Figure 4.3. Representative photos of the H&E stained cross sections of mid-intestine collected from fish (*D. rerio*) fed two experimental diets: **A** control and **B** Insect, for a period of 10 weeks.

The remaining fish per tank were freeze dried, pooled into one sample per tank, and then ground at Nottingham before being analysed for whole body crude protein, crude lipid, gross energy content, ash, fibre and fatty acid content (Table 4.6). Fish fed the insect based diet displayed significant alterations in all dietary parameters measured (indicated on Table 4.6). Proximate analysis revealed a decrease in water content and crude protein, while crude lipid and gross energy increased. Analysis of essential fatty acids shows fish fed on the insect based diet displayed increased ($P < 0.05$) PUFA's: linoleic and α-linolenic acid, while displaying reduced ($P < 0.05$) HUFA's: EPA and DHA.

Table 4.6. Results (mean ± SD) from post-trial nutritional analysis of zebrafish following 10 weeks feeding on either the control or insect diets; one other unknown fatty acid was recorded, although not included here as it was unidentified.

Nutrient		Control	Insect
Water	%	67.90 ± 0.65[a]	63.52 ± 1.01[b] ****
Crude Protein	% fresh weight	14.03 ± 0.62[a]	12.85 ± 0.57[b] *
Crude Lipid		13.25 ± 0.99[a]	18.72 ± 1.24[b] ****
Linoleic Acid (18:2n-6)	Essential fatty acids (% Lipid)	13.55 ± 0.68[a]	15.41 ± 0.6[b] *
α-Linolenic Acid (18:3n-3)		5.63 ± 0.28[a]	7.64 ± 0.65[b] ***
EPA (20:5n-3)		1.64 ± 0.13[a]	0.73 ± 0.09[b] ****
DHA (22:6n-3)		3.10 ± 0.31[a]	0.96 ± 0.16[b] ****
Myristic (13:0)	Non-essential fatty acids (% Lipid)	1.87 ± 0.12[a]	1.28 ± 0.16[b] *
Palmitic (15:0)		15.99 ± 0.66[a]	19.77 ± 1.24[b] **
Palmitoleic (16:1n-7)		2.51 ± 0.10[a]	1.73 ± 0.11[b] ****
Stearic (18:0)		3.04 ± 0.14[a]	4.9 ± 1.0[b] **
Oleic (18:1n-9)		37.48 ± 0.9[a]	31.63 ± 3.75[b] *
cis-8,11,14-Eicosatrienoic (20:3n-6)		1.0 ± 0.06[a]	0.68 ± 0.03[b] ****
Gross Energy	MJ/kg DM	27.11 ± 0.51[a]	28.41 ± 0.74[b] *

Measures which do not share a letter indicate significant difference, asterisks indicate strength of significance: * <0.05, ** <0.01, *** <0.001, **** <0.0001.

4.4.3 Discussion

This initial trial utilising zebrafish as a model species yielded positive results; providing strong indication that the selected invertebrate meals can be used, at least for the length of time this trial was conducted, to completely replace fish meal as a protein source, at the inclusion levels in this Insect diet, with no reduction in growth or performance when compared to a control diet containing 48 % fishmeal. Although, no level of EPA and DHA were provided via the insect diet, due to the lack of these fatty acids in the insect meals, the fish consequently show considerably lower ($P < 0.0001$) levels of EPA and DHA post-trial than those fed the control after ten weeks feeding on that diet. Zebrafish have functional desaturase Δ5 and Δ6 activity (Hastings et al., 2001), enabling synthesis of such HUFA's by elongating and desaturating α-linolenic acid following the enzymatic pathway described in Figure

1.6. As the insect diet has a higher level ($P < 0.001$) of α-linolenic acid, this could account for the low levels of EPA and DHA detected post-trial in the insect fed fish. These findings indicate that the combined invertebrate meals being investigated provide sufficient linoleic and α-linolenic acid to the fish, although they are not suitable sources of EPA and DHA. EPA and DHA would have to be supplied via other ingredients. Consequently this research investigating the previously described invertebrate meals will be progressed, further testing complete fish material replacement in the diet of an omnivorous fish species of high commercial relevance; carp (*C. carpio*).

4.5 Common carp (*C. carpio*) growth and performance

4.5.1 Trial specific methodology

Following the promising results gathered during the initial zebrafish trial, a second trial was carried out using carp (*C. carpio*). This species is the third most produced aquaculture species globally, and like *D. rerio* belongs to the Cyprinidae family, which represent six of the top 10 most produced aquaculture species (Table 1.4), therefore, is a good commercial candidate species for next phase testing. A total of 90 fish (25.88 ± 4.79 g initial weight) were sourced from Rodbaston Aquaculture (South Staffordshire College). They were housed in groups of 15 individuals in six identical 50 L tanks, connected to a 750 L centrally filtered system. Water quality was maintained as described in section 4.3. Three tanks were allocated per diet; colour coding was done as described in previous trial (section 4.4.1). The system was kept at room temperature (17 ± 1^{o}C).

The fish were allowed to acclimatise to the trial tanks for one week before trial commenced. During the trial fish were fed a ration of 2 % bodyweight in feed daily following a recommended feeding rate for the system temperature (NRC, 2011), prepared as described in section 4.3.1. As with previous trials this ration was split into two feeds per day; one was given between 8:30 am – 10:30 am, the other between 13:00 pm – 15:00 pm. The full feed ration was given at once on weekend days. Fish were weighed as described in section 4.3.1 using a 3 L tank, filled to

approximately 5 cm, with a Sartorius BP2100 S weigh balance. Performance was assessed as described in section 4.3.2. All fish per tank were culled at the end of the trial, six fish per tank were dissected. Intestinal tract samples, extracted centrally, and liver samples were collected and processed as described in section 4.3.3. Histological analyses of the intestine and liver was also carried out as described in section 4.3.3. Photographs were taken at x40 magnification for intestinal tissue samples and x100 magnification for liver tissue samples. Three fish per tank were frozen at -80 ^{o}C before being transported to Nottingham for post-trial nutritional analysis (Appendix 3). Palatability of diets was measured as described in section 4.3.3. Statistical analysis was carried out as described in section 4.3.4.

4.5.2 Results

Two balanced isoenergetic and isonitrogenous diets: Control and Insect, were tested. Throughout the trial, no difference ($P>0.05$) was found in average fish weight between the two experimental diets (Figure 4.4), there was also no difference ($P>0.05$) between diets for FCR, SGR and PER. The Control diet achieved an FMR of 0.26 ± 0.01, and a FIFO of 1.19 ± 0.07.

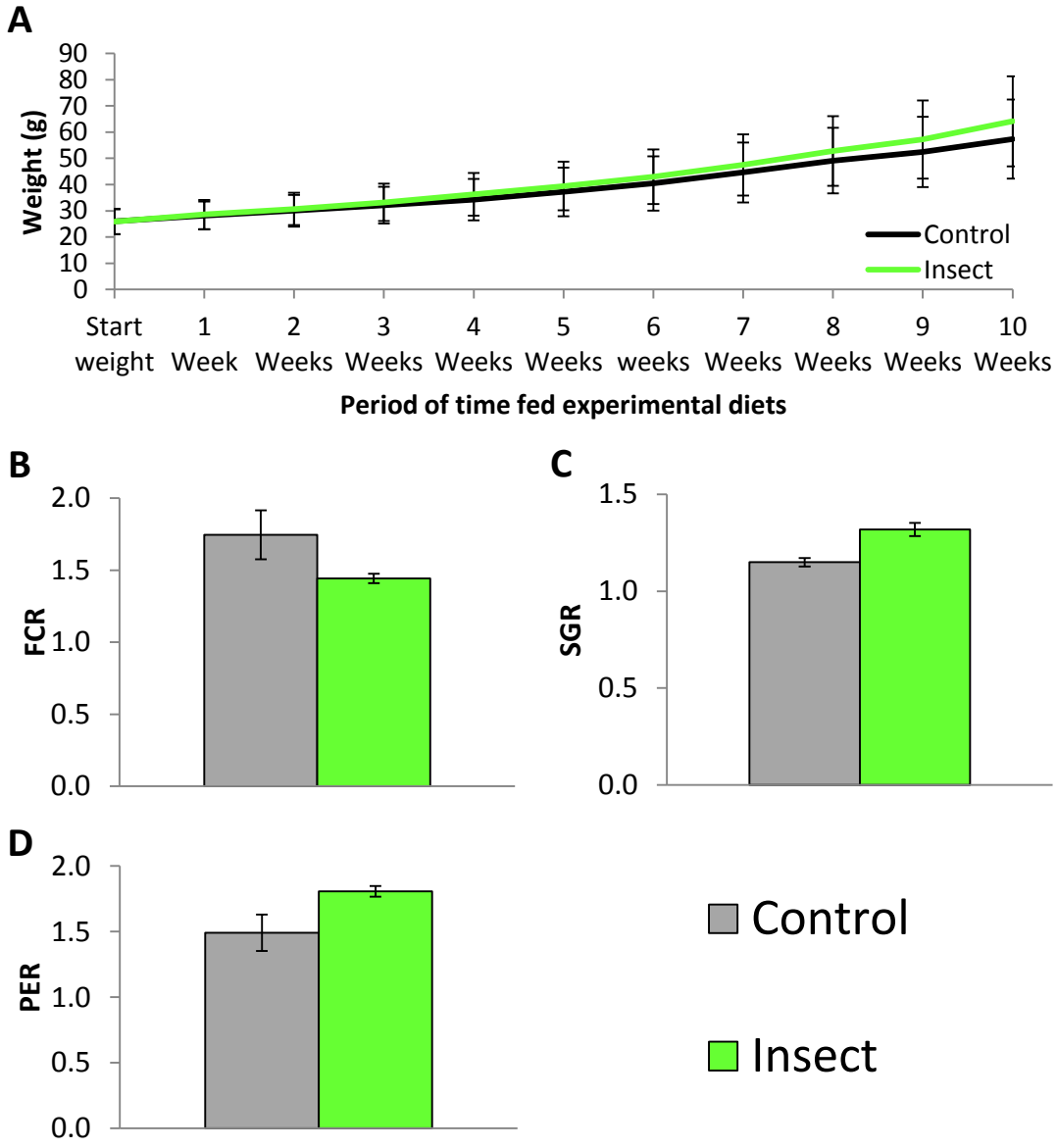


Figure 4.4. Carp (*C. carpio*) growth and performance indicators achieved (± SD) for each experimental diet, control and Insect: **A**) Average fish weight (g), showing growth throughout trial period, **B**) Feed Conversion Ratio (FCR), **C**) Specific Growth Rate (SGR), **D**) Protein Efficiency ratio (PER). No significant (P>0.05) differences were found between diets.

Fish consumed the Control diet slower (28.9 ± 0.5 minutes, $P < 0.0001$) than the Insect diet (2.3 ± 0.2 minutes), indicating a strong preference for the Insect pellets. Histological analyses revealed no differences ($P > 0.05$) between the two diets for villi width (Figure 4.5). Villi length increased ($P < 0.05$) in fish fed the Insect diet (Figure 4.5), despite which there was no difference ($P > 0.05$) in villi surface area per 5µm sample of intestine per gram of fish between the diet groups, control = 0.35 ± 0.13 mm^2 and Insect = 0.31 ± 0.03 mm^2. Photos of histology samples are presented in Figure 4.6. Liver analyses showed fish fed the Control diet had a lower ($P = 0.001$) percentage of stained tissue (70.74 ± 0.68 %) than fish fed the Insect diet (75.02 ± 0.95 %), indicating reduced fat deposition within the liver tissue of fish fed the Insect diet. There were no significant differences found between the diet groups for all three organ indices (Figure 4.5).

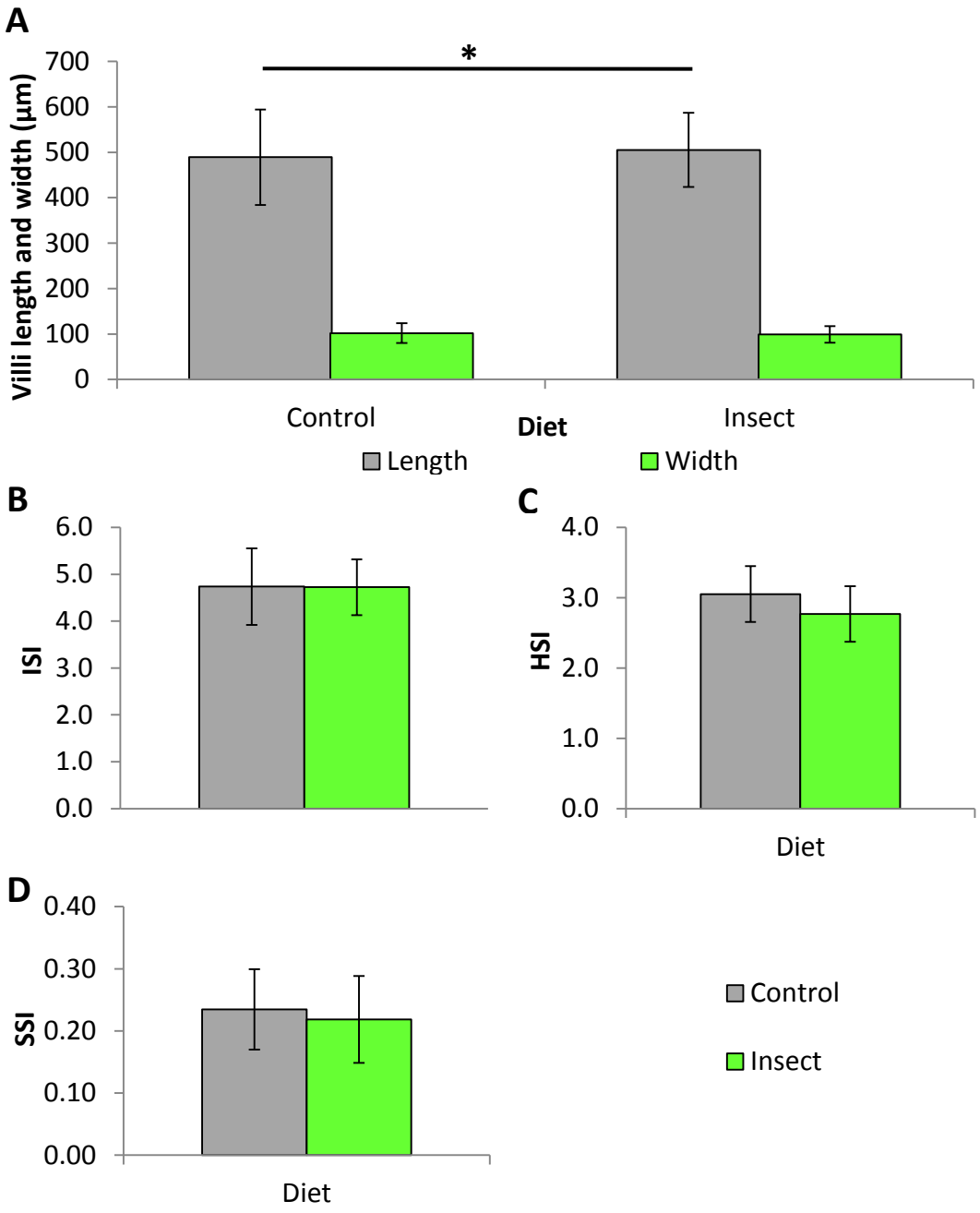


Figure 4.5. Carp (*C. carpio*) organ health indicators: **A**) intestinal villi structure: length and width for fish fed each experimental diet, **B**) Intestinal-somatic index (ISI), **C**) Hepato-somatic index (HSI), **D**) Splenic-somatic index (SSI). Significant differences are indicated with asterisk: * <0.05, ** <0.01, *** <0.001.

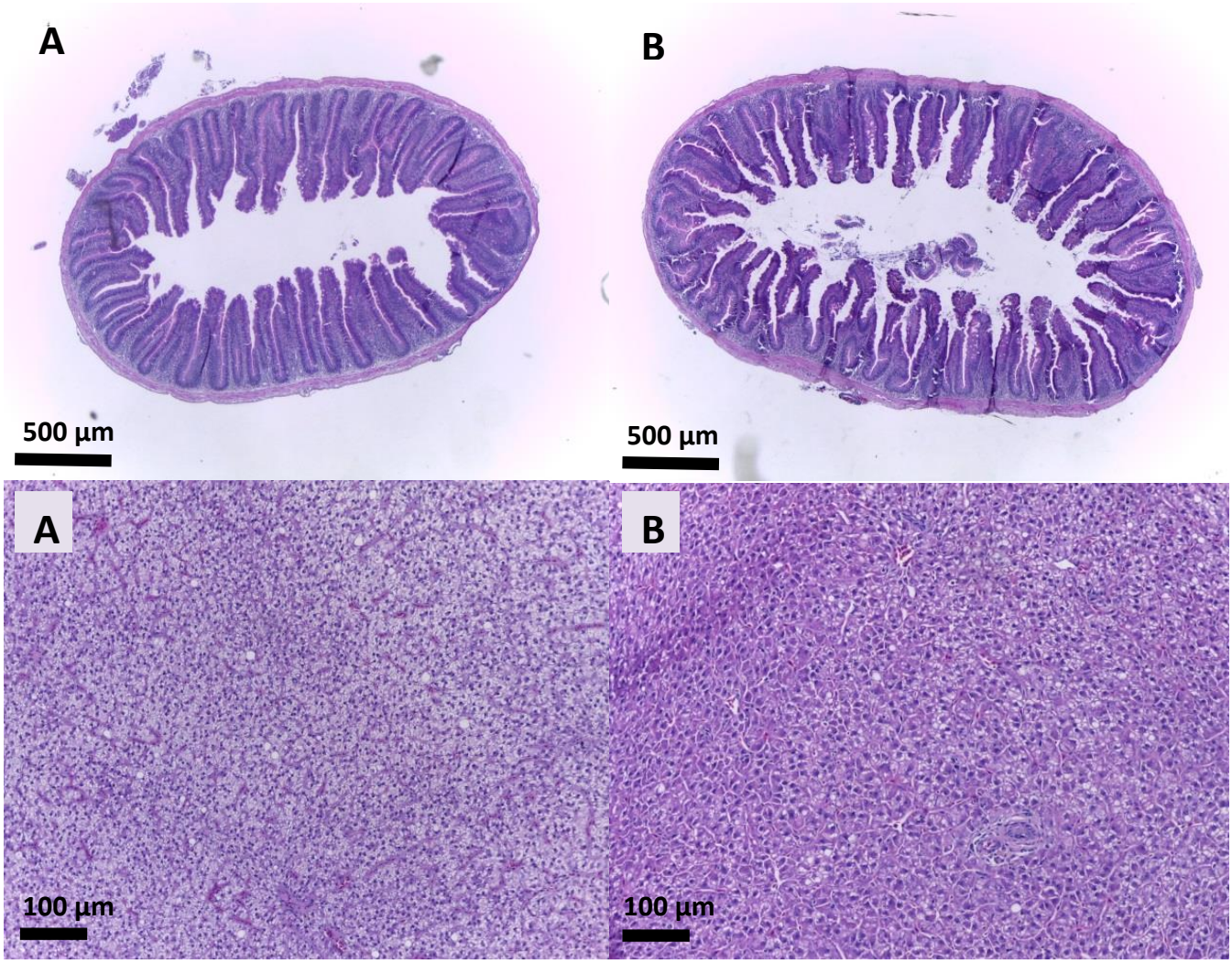


Figure 4.6. Representative photos of the H&E stained cross sections of mid-intestine (x40 magnification) and liver tissue (x100 magnification) collected from fish fed two experimental diets: **A** control and **B** Insect, for a period of 10 weeks.

Samples of fish were analysed at Nottingham (Table 4.7), of the dietary parameters measured there were no differences ($P > 0.05$) found between the two diets for water content, crude lipid, gross energy and some non-essential fatty acids. Fish fed the insect diet increased in crude protein level ($P < 0.05$). The results gathered for essential PUFAs reflects the findings of the zebrafish trial, with significant increases ($P<0.05$) in the insect fed fish of linoleic and α-linolenic acid, while significant decreases ($P<0.05$) in EPA and DHA were also seen. These finding reflect the fatty acid profile of the diets.

Table 4.7. Results (mean ±SD) from post-trial nutritional analysis of carp following 10 weeks feeding on either the control and insect diets; one other unknown fatty acid was recorded, although not included here as it was unidentified.

Nutrient		Control	Insect
Water	%	68.79 ± 2.77 [a]	69.67 ± 1.85 [a]
Crude Protein	% fresh weight	13.34 ± 0.48 [a]	14.02 ± 0.34 [b] **
Crude Lipid		14.74 ± 1.86 [a]	13.41 ± 1.69 [a]
Linoleic Acid (18:2n-6)	Essential fatty acids (% Lipid)	13.23 ± 2.12 [a]	17.16 ± 0.64 [b] ****
α-Linolenic Acid (18:3n-3)		4.74 ± 0.89 [a]	8.83 ± 0.5 [b] ****
EPA (20:5n-3)		3.11 ± 0.51 [a]	1.27 ± 0.18 [b] ****
DHA (22:6n-3)		5.18 ± 0.73 [a]	1.52 ± 0.22 [b] ****
Lauric (12:0)	Non-essential fatty acids (% Lipid)	0.06 ± 0.02 [a]	0.16 ± 0.01 [b] ****
Myristic (13:0)		2.95 ± 0.28 [a]	1.95 ± 0.15 [b] ****
Pentadecanoic (14:0)		0.36 ± 0.03 [a]	0.24 ± 0.02 [b] ****
Palmitic (15:0)		16.74 ± 1.13 [a]	16.25 ± 0.67 [a]
Palmitoleic (16:1n-7)		9.49 ± 1.21 [a]	6.68 ± 0.51 [b] ****
Heptadecanoic (17:0)		0.28 ± 0.06 [a]	0.3 ± 0.03 [a]
Stearic (18:0)		2.85 ± 0.3 [a]	3.57 ± 0.23 [b] ****
Elaidic (18:1)		0.33 ± 0.05 [a]	0.19 ± 0.03 [b] ****
Oleic (18:1n-9)		37.24 ± 1.82 [a]	38.72 ± 0.84 [b] *
γ-Linolenic (18:3n-6)		0.17 ± 0.02 [a]	0.39 ± 0.07 [b] ****
Arachidic (20:0)		0.14 ± 0.02 [a]	0.13 ± 0.02 [a]
cis-11,14-Eicosadienoic (20:3n-3)		0.42 ± 0.05 [a]	0.47 ± 0.05 [b] *
cis-8,11,14-Eicosatrienoic (20:3n-6)		0.37 ± 0.02 [a]	0.52 ± 0.03 [b] ****
Erucic (22:1n-9)		0.67 ± 0.07 [a]	0.33 ± 0.03 [b] ****
DPA (22:5n-3)		1.01 ± 0.19 [a]	0.53 ± 0.10 [b] ****
Gross Energy	MJ/kg DM	28.65 ± 0.77 [a]	28.28 ± 0.82 [a]

Measures which do not share a letter indicate significant difference, asterisks indicate strength of significance: * <0.05, ** <0.01, *** <0.001, **** <0.0001.

4.5.3 Discussion

During this trial, carp feeding on a diet comprised predominantly of invertebrate meals while completely void of fish derived ingredients, achieved equivalent growth and performance when compared to a control diet containing 14.8 % fishmeal and 4 % fish oil. The palatability response to the insect diet was also improved over the

control. During feed rate observations the poor reaction to the control feed is predicted to be as a response to the high inclusion of wheat gluten, which possesses binding properties (Apper-Bossard et al., 2013), the control pellets appeared to be harder, which could account for longer processing of the pellets once captured by the fish before swallowing. Due to the slow consumption rate of the control pellets, a cut-off time period of 30 minutes was implemented; recordings of 30 minutes were taken if this time limit was exceeded. Even though the insect diet performed very well fed to carp, the supply of fatty acids mimicked that seen in zebrafish (trial presented in section 4.4), resulting in increased levels of PUFA's and decreased levels of HUFA's. Having achieved such positive results for a commercial omnivorous species, this research investigating the previously described invertebrate meals will be progressed, testing complete fish material replacement in the diet of a carnivorous salmonid species of high commercial relevance; trout (*O. mykiss*).

4.6 Rainbow trout (*O. mykiss*) growth and performance

4.6.1 Trial specific methodology

With invertebrate based diets performing well in the first commercially relevant omnivorous species, another feed trial was carried out, this time using Rainbow trout (*O. mykiss*). This is a salmonid species with high commercial value in Europe; it is therefore considered a good initial test subject for carnivorous species, the group of fish proving the most reliant on fishmeal and fish oil, showing reduced growth and performance in previous studies with high levels of fish derived ingredient replacement. A total of 60 fish, sourced from Kilnsey fly fishery and trout farm, were used (54.4 ± 7.48 g initial weight). Fish were housed in groups of 10 individuals in six identical 400 L tanks, incorporated into a 3000 L centrally filtered system. The system was maintained as described in section 4.3. Three tanks were again allocated per diet; colour coding was done as described in previous trials (section 4.4.1). The system was kept at 14 ± 1 ^{o}C.

Once allocated to the trial tanks fish were allowed to acclimatise for a period of one week before trial commenced. During the trial fish were fed a daily ration of 2 % bodyweight, following the approximate recommended feed rate for these sized fish at the system temperature (NRC, 2011), prepared and managed the same as in the carp trial above (section 4.5.1). Growth and performance data collection was carried out as described in section 4.3.2. Five fish from each tank were dissected and processed as described in section 4.3.3. Due to the morphology of salmonids the intestinal tract was divided and additional samples were taken; one from the pyloric caeca, at least three caeca alongside one another, and three consecutive samples from the proximal end of the mid intestine. Three fish per tank were analysed post-trial at Nottingham as in the carp trial above. The tanks used to house the fish were dark green in colour and view inside the tanks was from above, making it extremely hard to monitor the amount of feed remaining once added to the tank, further hindered by fish and water movements during feeding. It was therefore not possible to observe the consumption rates of each diet as in previous trials, therefore palatability was not assessed. Statistical analyses was carried out as described in section 4.3.4.

4.6.2 Results

During the trial, fish fed the Insect diet had grown larger ($P < 0.05$) than the control fed fish for week's three to nine, by the final week there was no longer a significant difference between the two diet groups (Figure 4.7). There was no difference ($P>0.05$) between diets for FCR, SGR and PER. The Control diet achieved an FMR of 0.74 ± 0.05 and an FIFO score of 3.30 ± 0.23.

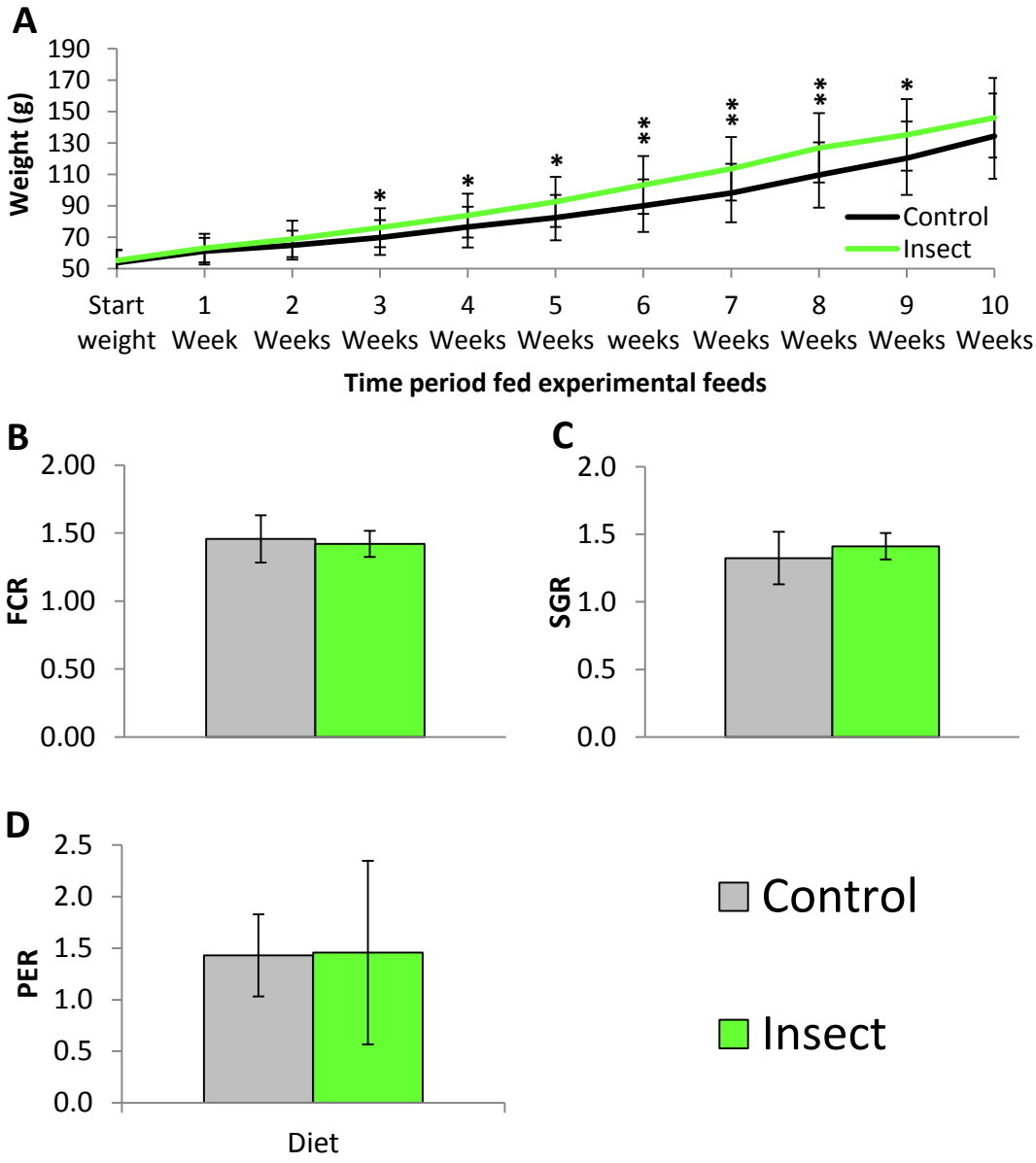


Figure 4.7. Trout (*O. mykiss*) growth and performance indicators achieved for each experimental diet, control and Insect: **A**) Average fish weight (g), showing growth throughout trial period, **B**) Feed Conversion Ratio (FCR), **C**) Specific Growth Rate (SGR), **D**) Protein Efficiency ratio (PER). Significant differences are indicated with asterisk: * <0.05, ** <0.01, *** <0.001.

Histological analyses showed no differences between the two diets ($P = 0.272$) for villi length in the pyloric caeca, although villi width decreased ($P \leq 0.001$) in fish fed the Insect diet. In the mid-intestine there was a significant increase in villi length and width ($P < 0.000$) for fish fed the Insect diet (Figure 4.8). There was no differences ($P>0.05$) in villi surface area per 5µm sample of intestine per gram of fish between the diet groups: control mid-intestine = 0.22 ± 0.05 mm^2, control pyloric caeca = 0.16 ± 0.05 mm^2 and Insect mid-intestine = 0.20 ± 0.04 mm^2, insect pyloric caeca = 0.13 ± 0.03 mm^2. Photos of histology samples are presented in Figure 4.9. There was no difference ($P = 0.163$) in percentage of stained liver tissue between the Control (70.99 %) and Insect (69.25 %) diets, indicating no fatty change within liver tissue (Figure 4.9). There were no differences ($P > 0.05$) found between the diet groups for all three organ indices (Figure 4.8).

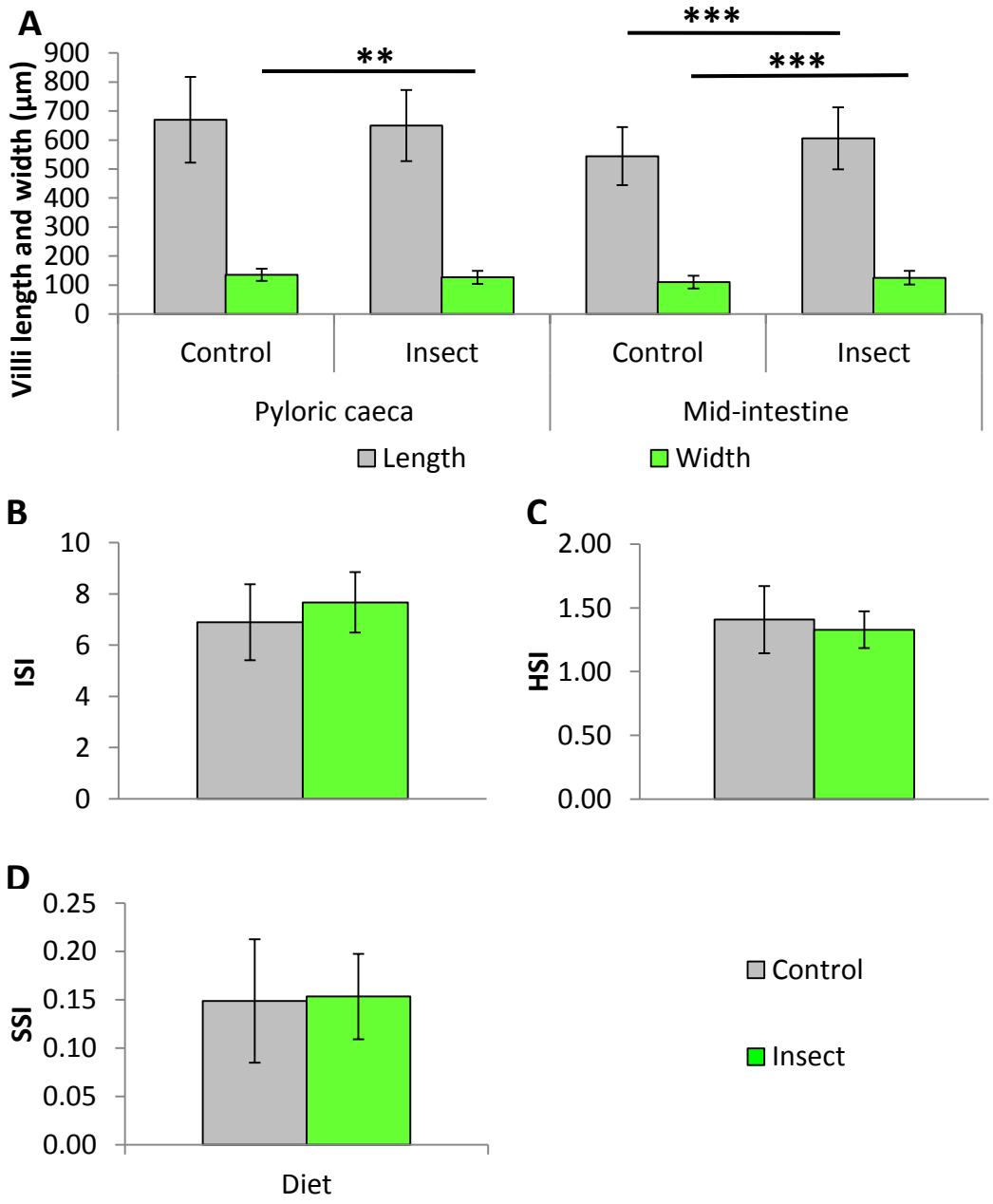


Figure 4.8. Trout (*O. mykiss*) organ health indicators: **A**) intestinal villi structure: length and width of two sections: Pyloric caeca and mid-intestine, for fish fed each experimental diet, **B**) Intestinal-somatic index (ISI), **C**) Hepato-somatic index (HSI), **D**) Splenic-somatic index (SSI). Significant differences are indicated with asterisk: * <0.05, ** <0.01, *** <0.001.

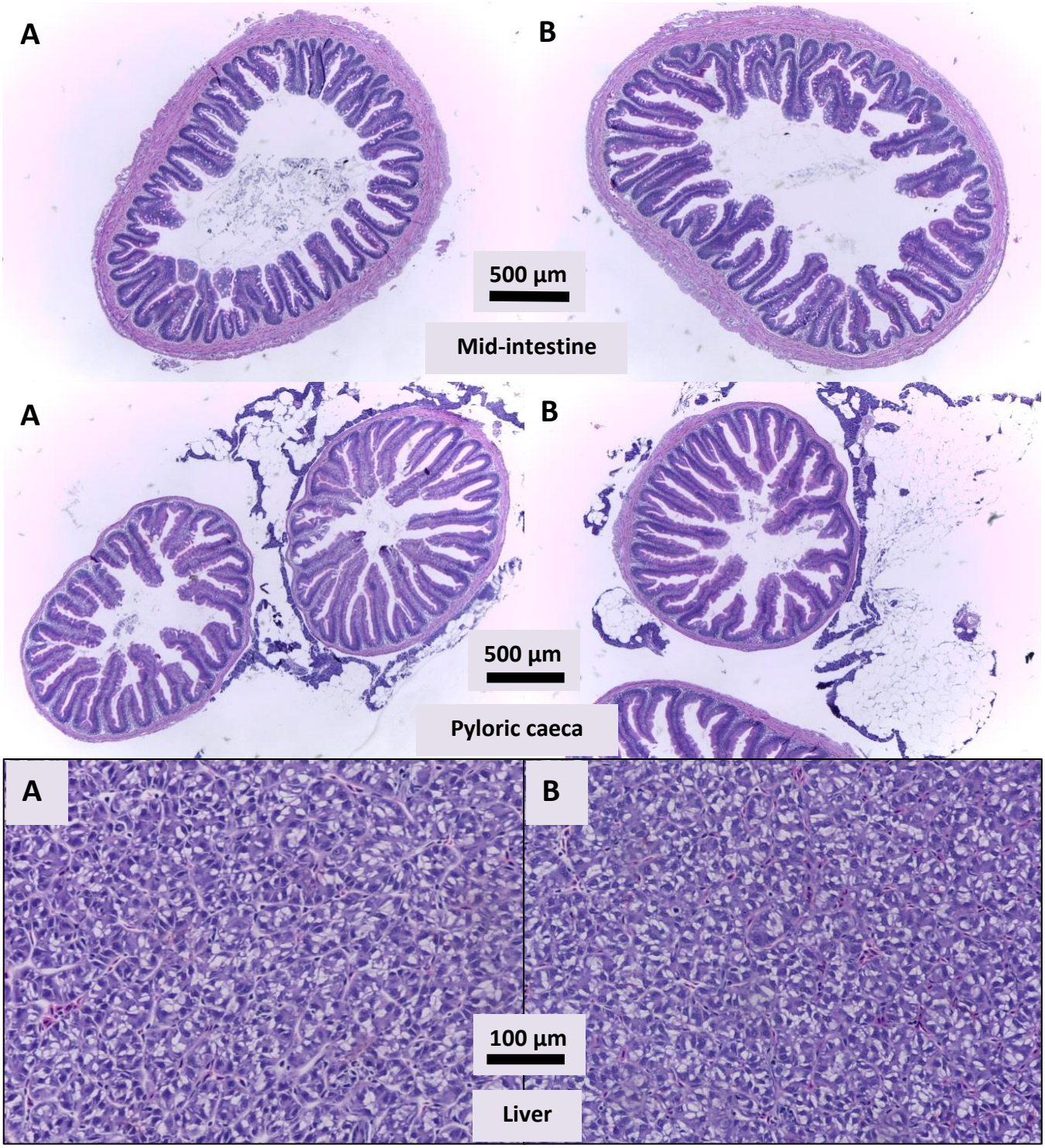


Figure 4.9. Representative photos of the H&E stained cross sections of trout (*O. mykiss*) mid-intestine, pyloric caeca and liver tissue collected from fish fed two experimental diets: **A** control and **B** Insect, for a period of 10 weeks.

Of the nutritional parameters measured (Table 4.8) there were no differences ($P > 0.05$) found between the two diets for water content, crude Protein, DHA and some non-essential fatty acids, there was an increase ($P < 0.05$) in crude lipid level and gross energy. The results gathered for essential PUFAs mostly reflects the findings of the previous trials, with significant increases in the insect fed fish of linoleic and α-linolenic acid, while a decrease ($P < 0.05$) in EPA was also seen. The results gathered here indicate that the alternative invertebrate meal complex was sufficient in replacing fishmeal and provided sufficient DHA, however, the data for fatty acid composition showed high variation between fish in the control group for DHA, believed to be the cause of the statistical outcome. From these findings it is recommended that the meals investigated here would not supply sufficient EPA or DHA, therefore the fatty acid requirements of such carnivorous fish species would have to be met by other ingredients.

Table 4.8. Results (mean ± SD) from post-trial nutritional analysis of trout following 10 weeks feeding on either the control and insect diets; one other unknown fatty acid was recorded, although not included here as it was unidentified.

Nutrient		Control	Insect
Water	%	71.66 ± 1.01 [a]	70.2 ± 2.9 [a]
Crude Protein	% fresh weight	16.16 ± 0.56 [a]	15.31 ± 1.84 [a]
Crude Lipid		10.23 ± 0.93 [a]	11.74 ± 1.03 [b] **
Linoleic Acid (18:2n-6)	Essential fatty acids (% Lipid)	10.06 ± 1.89 [a]	10.56 ± 8.87 [b] ****
α-Linolenic Acid (18:3n-3)		6.26 ± 0.3 [a]	7.03 ± 5.03 [b] ****
EPA (20:5n-3)		3.06 ± 2.07 [a]	0.63 ± 0.24 [b] *
DHA (22:6n-3)		6.97 ± 5.15 [a]	2.42 ± 0.58 [a]
Lauric (12:0)	Non-essential fatty acids (% Lipid)	0 [a]	0.86 ± 0.9 [b] ***
Myristic (13:0)		5.69 ± 0.97 [a]	1.49 ± 1.15 [b] ****
Pentadecanoic (14:0)		0.49 ± 0.09 [a]	5.13 ± 8.57 [b] ****
Palmitic (15:0)		19.21 ± 3.12 [a]	11.44 ± 7.72 [b] **
Palmitoleic (16:1n-7)		6.87 ± 0.97 [a]	1.89 ± 1.27 [b] ****
Heptadecanoic (17:0)		0.36 ± 0.2 [a]	2.06 ± 2.7 [a]
Stearic (18:0)		4.66 ± 0.89 [a]	3.18 ± 2.65 [a]
Elaidic (18:1)		0.37 ± 0.06 [a]	13.23 ± 22.65 [b] ****
Oleic (18:1n-9)		29.36 ± 3.84 [a]	31.95 ± 14.34 [b] ****
γ-Linolenic (18:3n-6)		0.21 ± 0.08 [a]	3.94 ± 6.19 [b] **
cis-11,14-Eicosadienoic (20:3n-3)		0.73 ± 0.06 [a]	1.01 ± 0.33 [b] ****
cis-8,11,14-Eicosatrienoic (20:3n-6)		0.31 ± 0.12 [a]	0.79 ± 0.43 [b] ***
DPA (22:5n-3)		0.95 ± 0.59 [a]	1.16 ± 1.46 [b] **
Gross Energy	MJ/kg DM	25.66 ± 0.87 [a]	26.84 ± 0.73

Measures which do not share a letter indicate significant difference, asterisks indicate strength of significance: * <0.05, ** <0.01, *** <0.001, **** <0.0001.

4.6.3 Discussion

Trout feeding on a diet comprised of invertebrate meals while completely void of fish derived ingredients, showed equal growth and performance when compared to a control diet containing 51 % fishmeal and 12 % fish oil, with no differences (P > 0.05) in the efficiency parameters measured: FCR, SGR and PER. For a number of weeks of the trial the fish fed the alternative diet containing insect meals had grown

significantly more ($P < 0.05$) than the control although this difference was no longer present at the final week of the trial. Fish fed the insect diet displayed some morphological changes in the intestinal villi although remained in a healthy condition, no signs of enteritis presented during this trial. As with the two previous species, the insect based diet provided sufficient levels of PUFA's linoleic and α-linoleic acid but failed to provide EPA and DHA at high enough quantities.

4.7 Conclusion, are these insect meals suitable for dietary inclusion?

An experimental diet was deemed to be a success if it 1) elicited a feed response, being accepted and consumed by the fish, 2) achieved comparable growth rates to, or exceeded, that of the control diet, and 3) did not elicit deleterious changes to the health state of the test subject. Following the three feed trials carried out here, there is strong evidence for use of mealworm meal (*Tenebrio molitor*), silkworm pupae meal (*Bombyx mori*) and earthworm meal (*Eisenia fetida*), to be considered for future use within aquaculture feeds designed for fish species. When these three materials were utilised together, without fish derived materials, to formulate nutritionally balanced diets specifically for target species, the resulting feed successfully replaced fishmeal in feed for one lab and two commercial fish species. The insect derived feeds yielded comparable growth compared to fish derived feeds without signs of any deterioration in fish health. However, in contrast to fish derived diets, these diets did not provide EPA and DHA. EPA and DHA would therefore have to be provided separately. While many fishmeal and fish oil replacement trials focus on individual ingredients as alternatives, we show that ingredients from multiple sustainable insect sources are more likely to succeed as alternatives to fish derived materials in aquaculture feed if used in combination. Although these invertebrate ingredients proved largely successful here, the feed industry would not consider them viable if the cost of material was not competitive with current materials (unless valued more for certain characteristics). Cost of the formulated feeds was calculated (Table 4.9) based upon current ingredient costs.

Prices for ingredients used commercially were provided by industrial partners (personal communication with Skretting, P. Gallimore 2018) and ABagri (I. Lupatsch 2018). Prices for the specific invertebrate meals used were not available as these are not yet commercially used. Instead, prices published by Allaboutfeed (Koeleman, 2017) for other insect meals were used. Prices range considerably depending on the species being produced, from €3 - €15 (£2.68 - £13.41 with current exchange rate), therefore, cost of diets has been calculated twice based on 'low' cost and 'high' cost of insect meals (Table 4.9). Cost of diet was then used to calculate cost of production of fish (Table 4.10) using the FCR values achieved during trials above (section 4.4 – 4.6).

Table 4.9. Cost of experimental diets used in zebrafish, carp and trout feed trials, testing invertebrate meals, based on ingredient costs (correct at time of collection, November 2017). Two costs were calculated per insect diet: low and high, according to the price range published for multiple insect meals.

Ingredient	Ingredient price (£/kg)	Cost within diet (£/kg)								
		Zebrafish Control	Zebrafish Insect (low)	Zebrafish Insect (high)	Carp Control	Carp Insect (low)	Carp Insect (high)	Trout Control	Trout Insect (low)	Trout Insect (high)
Fishmeal	1.65	0.79	-	-	0.24	-	-	0.84	-	-
Wheat gluten	1.75	0.26	0.28	0.28	0.59	0.29	0.29	0.26	-	-
Corn starch	0.18	0.05	0.05	0.05	0.07	0.07	0.07	0.04	0.04	0.04
Vitamin premix	7.1	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Mineral premix	2.5	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
CMC binder	1.9	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Rapeseed oil	0.67	0.05	-	-	0.03	-	-	-	-	-
Fish oil	1.8	-	-	-	0.07	-	-	0.22	-	-
Insect meals (low)	2.68	-	1.44	-	-	1.08	-	-	2.08	-
Insect meals (high)	13.41	-	-	7.21	-	-	5.40	-	-	10.39
Arginine	10	-	-	-	-	0.01	0.01	-	-	-
Lysine (77%)	1.8	-	-	-	0.02	0.01	0.01	-	-	-
Total cost of diet (£/kg)		**1.20**	**1.81**	**7.59**	**1.06**	**1.51**	**5.83**	**1.40**	**2.16**	**10.47**

- Ingredient not used.

Table 4.10. Cost of producing fish (zebrafish, carp and trout) using experimental diets, based on diet cost (Table 4.9), multiplied by FCR. Calculated based solely on feed costs, other costs associated with aquaculture have not be accounted for.

Species	Diet	Cost of diet (£/kg)	FCR	Cost to produce 1kg of fish (£)
zebrafish	Control	1.20	2.35	2.82
	Insect (low)	1.81	2.51	4.54
	Insect (high)	7.59	2.51	19.05
Carp	Control	1.06	1.74	1.84
	Insect (low)	1.51	1.44	2.17
	Insect (high)	5.83	1.44	8.40
Trout	Control	1.40	1.46	2.04
	Insect (low)	2.16	1.42	3.07
	Insect (high)	10.47	1.42	14.87

The production volumes of insect meals are still very low relative to other ingredient such as the volume of fishmeal and wheat gluten. Therefore the price per kilogram is still high in comparison. Table 4.10 shows how diets consisting largely of insect meals, using these current costs, would be more expensive than the controls used in these formulation (Table 4.5). As the insect meals used also failed to provide EPA and DHA, these fatty acids would have to be supplied with other ingredients, if another ingredient rich in these oils, likely to be expensive, were added the new formulation would likely increase further in cost. The diet formulations used in these trials are simplified, using fewer ingredients and thus with higher inclusion levels of the main materials than desired to replicate modern aquaculture diets. Therefore, based on the economics alone, insect meals are not yet likely to be considered for inclusion in aquaculture on a large scale, until a time when the price per kilogram of insect meals has dropped to be more comparable with other feed protein meals (such as fishmeal). As the insect production industry continues to develop and annual production volumes increase moving forward, the price of insect meals is expected to decrease.

The environmental impact of producing the insect species is expected to be low (Oonincx and Boer, 2012, Salomone et al., 2017). Research on life cycle assessment (LCA) of insect species is very limited (Halloran et al., 2016), although Oonincx and Boer (2012) calculated the environmental impact of producing mealworm larvae to be lower than other protein sources: chicken, pork and beef. However, Oonincx and Boer (2012) assessed production of live larvae, not dry meal. Also, by comparing mealworm production to chicken, pork and beef implies human consumption of the mealworms. For use as an aquaculture feed ingredient, production of mealworm meal (or any other insect meal) needs to be compared with other similar feed ingredients to truly assess the environmental impact of production. Thevenot et al. (2018) more recently compared LCA for production of mealworm meal with that of fishmeal and soybean meal for use in animal feed. They found lower environmental impact than that found by Oonincx and Boer (2012) for production of live larvae, due to energy supply method and mealworm diet used. However, processing the larvae into meal increased the environmental impact to such an extent that it increased to more than that of production of fishmeal and soya bean meal. As highlighted by (Thevenot et al., 2018), insect production is in its infancy, with much less information available compared to production of other ingredients, thus as improvements to the production method occur there is potential to improve the environmental impacts of mealworm production, or other insect meals.

The REGULATION (EC) 999/2001 which regulates provision of processed animal proteins (PAPs) has recently been further amended in July 2017, REGULATION (EU) 2017/893 (Commission, 2017) now permits the use of an approved list of insect species for production of PAPs for use in aquaculture feeds: Black Soldier Fly *(Hermetia illucens)*, Common Housefly (*Musca domestica*), Yellow Mealworm *(Tenebrio molitor)*, Lesser Mealworm (*Alphitobius diaperinus*), House cricket *(Acheta domesticus)*, Banded cricket *(Gryllodes sigillatus)* and Field Cricket *(Gryllus assimilis)*. The feed materials used to raise such insects destined for the PAP market is also restricted to 'feed grade' substrates of vegetable origin alongside some limited animal origin materials: fishmeal, non-ruminant blood products, eggs, milk and

derived products, honey and rendered fats. Although this regulation limits the feedstuff that can be used for production of insect derived PAPs, this is a very promising step towards being able to supply alternative protein materials, which are of sufficient quality for production of fish as a human food protein source. This amended regulation provides another pathway for utilisation of food production and processing waste and by-products, in 2015 an estimated five million tonnes of food wastage material was used for animal feed across all EU countries, predicted to be 50 – 80 % of total food waste from manufacturing ((EFFPA), 2015, FUSIONS, 2016). Use of waste stream material will also be subject to food safety regulations before being viable for use, if certain waste stream materials are deemed viable nutritionally and legally, the use of such material has potential to improve the sustainability of producing the insect meal.

This recent amendment also presents a new opportunity within Europe for a new industry producing insect PAPs. This would not only generate jobs within Europe, with many social and economic benefits, but may also reduce European dependency on imported protein materials such as soy protein (Visser et al., 2014), which are utilised frequently within aquaculture feeds. Insects also provide the opportunity to increase productivity and nutrient recovery from food waste stream materials, if permitted and suitable for use. Insects are efficient at converting lower quality material, which are unsuitable for direct use in aquaculture feeds, such as plant materials, into insect meals which are high in nutritional content, higher in protein levels with more complete amino acid profiles, and acceptable to many aquaculture species, especially carnivorous species.

Overall, the benefits gained from inclusion of insect meals into aquaculture feeds and the development of the production industry, necessary to support such demand for materials, could be far reaching if price of insect meals drop to be comparable with other protein meals. Initial experimental results gathered here strongly suggests that invertebrate meals can successfully be included in

aquaculture feeds without compromising fish health or welfare standards during production while achieving sufficient growth and performance. Further research: investigating a greater variety of insect species, listed in REGULATION (EU) 2017/893, and the combinations of these, more aquaculture species and the impacts of such materials on fish health over longer periods of time, throughout full productions cycles and life stages.

Chapter 5

5.0 Overall discussion and conclusion

The aim of this PhD research project was to investigate the issue of feeding the aquaculture industry as we move into a challenging future, identifying and assessing alternative sources of feed ingredients. As demand for aquaculture produce increases, the demand for aquaculture feed also increases. With the limited supply of fish derived ingredients the cost of fishmeal and fish oil will continue to rise. Alternative, competitive, ingredients which may contribute towards reduction or complete replacement of fishmeal and fish oil are therefore desired.

The fish feeding trials carried out during this PhD showed that some of the selected vegetable meals are of sufficient quality to partially replace fishmeal and the insect meals are of sufficient quality to become alternatives to fishmeal. Many previous studies which have investigated fishmeal reduction or replacement focus on single materials. Investigation of the vegetable sources here was done so in a similar manner with influence from industry. The NH Algae diets were accepted by all of the species trialled. The Natto and Rapeseed meal diets were also accepted well by rainbow trout and European sea bass. During the zebrafish trial all experimental diets performed significantly ($P < 0.05$) poorer than the control, with the NH Algae diet performing best out of the test diets. Two experimental diets performed equally with the control feed when fed to rainbow trout, the NH Algae diet successfully reduced fishmeal by 17 % while the Natto diet reduced fishmeal inclusion by 48 %. The NH Algae diet also performed equally to the control while reducing fishmeal inclusion by 36 % in diets for European sea bass, so too did the Rapeseed meal diet, while reducing fishmeal inclusion by 52 %. The NH Algae diets performed well across all three species; however, in those where the Natto or Rapeseed meal diets were also successful, they reduced fishmeal at a higher amount.

Of these three materials, NH Algae and Natto would have to be produced de novo, while raw Rapeseed meal is a waste stream material. The industrial production of the NH Algae material is relatively limited currently, so scaling up production would require some investment which would increase the cost of the product. Natto and Rapeseed meal indicate that fermentation of these materials is a viable option to improve their suitability for use in the aquaculture feed industry. Natto, however, is a soy bean product. Soy protein concentrate is already used throughout aquaculture feeds, thus Natto would only truly be viable if it proved cost-effective compared to current soy materials. In Europe, the supply of soya bean products is reliant on imports, so more susceptible to price fluctuations. The raw Rapeseed meal on the other hand, being a waste stream/ by-product of the rapeseed oil industry, suggests a more reliable ingredient source. Incorporation of this material into aquaculture feed would reduce the waste and reclaim lost nutrients from another industry. Further analysis are required to assess the environmental impact of autoclaving and fermenting the material, although inclusion of the fermented Rapeseed meal into fish feeds (for specific species) would provide a new supply of protein for the future, which is predicted to cost much less than fishmeal. Although fermentation appears to have improved the Natto and Rapeseed meal materials, it did not improve the raw PPC material, increasing anti-nutritional factors (chaconine, solanine and water soluble protein) and being accepted poorly. The PPC diets also achieved poor performance across all species tested. The fermented PPC material is not recommended for further investigation or use in fish feeds until technology overcomes its nutritional obstacles.

The insect meals investigated were used in combination and contributed to the complete removal of fishmeal in feeds of three fish species namely the zebrafish, common carp and rainbow trout. All three species achieved equal growth to the control feeds. Overall the insect diets achieved desirable growth and performance in fish trials, although they did not generate sufficient levels of EPA and DHA in fish tissue, despite supplying an abundant amounts of linoleic and α-linolenic acid. These results suggest that fishmeal replacement is more likely to be achieved by

combining multiple alternative materials, creating a product with a more complete nutritional profile than presented by individual ingredients. It also paves the way for further research using invertebrate meals to do just this. As discussed previously, the regulation of insect use in fish feeds now recognises their value as feed ingredients and enables the use of certain species. There is great potential for insects and other invertebrates to successfully be included in aquaculture feeds in the not too distant future.

Replacement of fish oil was only partially achieved during the trials discussed in Chapter 3. The rainbow trout Natto diet achieved the highest reduction of 52 %. Fish oil replacement is not feasible with the invertebrate meals investigated during this study, as they each lack essential HUFAs.

Ultimately, any material considered for inclusion into fish feeds will be assessed based on nutritional profiles and cost first and foremost. The study has identified several materials which, if used appropriately, possess adequate nutrition to be included in certain fish feeds. Although nutritionally possible, the cost of each material will influence the likelihood of inclusion. Costs of the ingredients tested in Chapter 3 were not available as cost of the fermentation process would need to be calculated based on scale production. Published costs of insect meals varies considerably depending on species and form, as a commercial price could not be sourced (provided by a feed manufacturer), lowest and a highest published costs were used for calculating the range if diet costs in Chapter 4. These are predicted to reduce in the future as production of insect meals increases. Cost of ingredients varies with several factors, if each alternative material can be made available at a cost competitive with fishmeal they will be viewed more favourably by manufactures, especially in cases where increased efficiency in production of fish is possible, the feed market will favour diets which reduce production cycle periods and maximise profits as long as health of the fish and quality of the final product are not compromised.

Fish taste response is recognised as an important factor following market and economy when designing aquaculture feeds. However, thorough methods for assessing fish taste response are very scarce; and limited to electrophysiological response of taste receptors or behavioural observations (Kasumyan and Doving, 2003). As previously discussed, both are limited in their application. Electrophysiological analyses currently fail to measure the response of two thirds of the gustatory receptors involved in feed assessment, while behavioural observations have yet to be applied to complex substances such as whole feed ingredients or completed diets. The data produced by these two methods could be used to further advance our understanding of fish taste responses, once technology has advanced enough to analyse the stimulatory effectiveness of all three taste receptors involved in gustation, and the data can be used to generate complete taste response profiles such as that presented in Figure 2.6 (created with currently available data, missing stimulatory effectiveness of cranial nerves IX and X), believed to be the first time such data have been combined and presented. Here the method of behavioural observation (Kasumyan and Morsi, 1996) was modified and applied to assess feasibility for assessing whole feed ingredients. Levels of CCK released following exposure to feed was also measured as a novel approach to assess satiety response to alternative feed ingredients.

As discussed in Chapter 2, the inclusion of whole feed ingredients into agar-gel pellets for observational assessments provides unique issues. Although physical properties of the pellets prevented application of this method to some species in question, it was successfully applied to three test species. Comparing the index of palatability results gathered here in Chapter 2, along with the amino acid profiles of each test material, and with the results from published electrophysiological studies, which identified the most stimulatory amino acids, shows how the taste response is not based on one aspect of the feed item alone but the whole profile of stimulatory components. Necessitating such a method of analysing taste response to whole feed ingredients or completed feeds, therefore warrants further development of

this method. Use of the behavioural observation method used here did show that the insect meals investigated here are widely accepted across species, achieving equal palatability to fishmeal, while the vegetable meals showed more species-specific responses, suggesting more specific and restricted application would be appropriate.

When comparing the data from each assessment of taste preferences of zebrafish, rainbow trout and European sea bass towards the experimental diets, using consumption rate of diet, there is no common pattern of preference. The Rapeseed meal diet was preferred over the control by the European sea bass, and yet it was less palatable to zebrafish and rainbow trout, even though palatability testing of the Rapeseed meal ingredient with rainbow trout showed equal palatability with fishmeal in earlier behavioural observation trials (table 2.6). The Natto diets show similar variation; this was the most preferred diet of the European sea bass and was the second to least palatable for zebrafish. It was consumed significantly slower than the control by rainbow trout but it was still accepted well, although the Natto ingredient was the least palatable in earlier trials (table 2.6). The results of these trials and those of others (Kasumyan and Doving, 2003), show how variable taste responses can be between fish species to specific stimuli. Feed palatability is very important to achieve acceptance, although the volume of food consumed by a fish may not only be due to taste, it may also be linked with digestibility and availability of the feed. Fish feed to meet an energy demand (Cho and Kaushik, 1985).

The results from the palatability observations of carp, a coarse fish species, and rainbow trout, a game fish species, indicate that those ingredients which proved as palatable as fishmeal may have further application beyond aquaculture. Primarily the fish bait industry, an industry driven by product attractiveness to fish, valued at a much greater annual worth than the aquaculture feed industry. Earthworm meal, for example, may prove to act as an attractant in carp pellets or boilies, while Rapeseed meal could be utilised in trout pellets. Such bait products attract a higher

retail value and therefore greater commercial return or profit. As the taste response to each ingredient has been evidenced, by consumption rate vs palatability observation data, to be different from that of the response gathered to a complete diet containing the ingredient, the observation method of assessing palatability may also be better employed for analysing taste response of certain species to ingredients used in such bait products as attractants. For such an application of ingredients further work is recommended to establish a dose-response curve to each material for each target species.

Similarly, the measurement of CCK release post-stimulation, with feed or simulated feed, offers scope for refinement and improvement of this novel method. Initial results showed significant increase in the satiety hormone post-stimulation with feed when analysing blood samples. The two methods investigated both showed high variation between individual fish, potentially masking significant findings, sources of this variation and modifications to improve the data collecting have been discussed earlier. This pilot study was therefore only partially successful in that initial results show potential. This method, if it can be developed and refined further, has the strength to identify which feeds elicit the shortest satiety response and therefore which diet will be consumed more often throughout the day, potentially allowing increased total food consumption on the farm vs a diet which elicits a prolonged satiety response.

This study provides further evidence, across all the trials, of the variability between fish species, in taste responses and ability to utilise feeds. Zebrafish have been used here as an initial model for feed trials, testing feeds which require small amounts of test material to rule out unsuitable materials prior to larger scale testing of other species. This has proved useful in most cases, the European sea bass showing a distinct poor taste response to the fermented PPC diet being the exception. It can also be recommended, following this work, that zebrafish be considered for such low cost preliminary trials in future projects. Although with such variation between

species, each target species of any alternative feed ingredients should be trialled to ensure they accept and can use it.

Overall this project has introduced a novel method of assessing satiety response and a modified method of assessing palatability response in fish to whole ingredients. It has also investigated several vegetable, algal and invertebrate meals, including waste stream materials and materials from new industries, some of which were subjected to novel fermentation techniques to improve the nutritional quality. Others were tested as provided, as alternative ingredients to fishmeal and fish oil for use in aquaculture feeds. Certain materials have been identified as unsuitable and others as having great potential although further work is needed.

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Appendix 1

Dietary nutritional requirements of fish

Dietary nutritional requirements for fish species of interest here: Common carp (*Cyprinus carpio*), Rainbow trout (*Oncorhyncus mykiss*), Zebrafish (*Danio rerio*), European sea bass (*Dicentrarchus labrax*), Nile tilapia (*Oreochromis niloticus*), and Channel catfish (*Ictalurus punctatus*). Dietary protein levels for all fish species, except zebrafish, are given for fingerling sized fish onwards depending on availability of information, younger fry require higher protein levels, protein requirements decrease as fish mature. Nutritional requirements are based on a dry extruded pelletized diet.

Nutrient level		Common carp (*C. carpio*)	Rainbow trout (*O. mykiss*)	Zebrafish (*D. rerio*)	European sea bass (*D. labrax*)	Nile Tilapia (*O. niloticus*)	Channel catfish (*I. punctatus*)
Moisture %		12[A]	10[S]	≤10[AL]	≤10[AL]	≤10[AL]	≤10[AL]
Crude protein, % min		38.5[B,C]	40 – 42[T,U]	37 (max growth) - 47[AM]	50[AR]	34[AR]	36[AR]
Amino acids, % min of protein	Arginine	3.8[D]	3.5[D]	5[AN]	3.9[AS]	4.2[AZ]	4.3[BG]
	Histidine	1.4[D]	1.6[D]	2.1[AN]	1.6[AW]	1.7[AZ]	1.5[BH]
	Isoleucine	2.3[D]	2.4[D]	3.5[AN]	2.6[AW]	3.1[AZ]	2.6[BH]
	Leucine	4.1[D]	4.4[D]	6.2[AN]	4.3[AW]	3.4[AZ]	3.5[BH]
	Lysine	5.3[D]	5.3[D]	6.4[AN]	4.4[AT]	5.1[AZ]	5.1[BI]
	Methionine	1.6[D]	1.8[D]	2.9[AN]	1.9[AU]	2.7[AZ]	2.3[BJ]
	Cystine	0.8[D]	0.9[D]		0.9[AU]	0.5[AZ]	-
	Phenylalanine	2.9[D]	3.1[D]	6.1[AN]	2.6[AW]	3.8[AZ]	2.1[BK]
	Tyrosine	2[D]	2.1[D]			1.8[AZ]	2.5[BK]
	Threonine	3.3[D]	3.4[D]	3.4[AN]	2.6[AV]	3.8[AZ]	2.2[BL]
	Tryptophan	0.6[D]	0.5[D]	-	0.6[AW]	1[AZ]	0.5[BL]
	Valine	2.9[D]	3.1[D]	4[AN]	2.9[AW]	2.8[AZ]	3[BH]
Crude lipid, %		7 min[E]	17 = fry 21 = max[S]	8-16[AO]	15-18[DO]	10-15[DQ]	6 max[DN]
Essential Fatty acids, % min diet	Linoleic acid (18:2n-6)	1[F]	-	4[AN]	-	0.5[BA]	-
	Arachidonic acid (20:4n-6)	-	-		-	-	-
	Linolenic acid (18:3n-3)	1[F]	1[V,W,X]	1.4[AN]	-	-	1 – 2[BM]
	Eicosapentaenoic acid, EPA (20:5n-3)	or 0.5-1[F]	or 1[V,W,X,Y]		1[AX]	-	-
	Docosahexaenoic acid, DHA (22:6n-3)					-	-

Nutrient level	Common carp (*C. carpio*)	Rainbow trout (*O. mykiss*)	Zebrafish (*D. rerio*)	European sea bass (*D. labrax*)	Nile Tilapia (*O. niloticus*)	Channel catfish (*I. punctatus*)
Carbohydrate, % max	ideal = 26 max = 40[F]	12 – 25[SZ]	Ideal = 25, (15-35)[AP]	15[DP]	40 [DQ]	20[DR]
Crude fibre, % max	2[A]	3[Z]	-	2-4 [DO]	8-10[DQ]	50[DR]
Ash %	10[A]	-	-	-	-	-
Major minerals, %						
Calcium, % max	≤0.028[G]	0.24[AA]	-	-	0.35-0.43[BB]	1.5[BN]
Available phosphorus, % min	0.6–0.7[G]	0.70[AB]	-	0.65[AY]	0.8-1[DQ]	0.8 [BN]
Magnesium, % min	0.04–0.05[H]	0.05[AC,AD]	-	-	0.06-0.08 [BC]	0.04[BO]
Added dietary supplements						
Trace minerals, mg/kg min						
Iron	15[A]	60[S]	-	-	60[DQ]	30 [BP]
Zinc	15–30[I]	150[AE,AF]	-	-	30 [BD]	20 [BQ]
Manganese	12–13[J]	12–13[J]	-	-	7[BE]	2.4 [BR]
Copper	3[J]	3[J]	-	-	4[BF]	1.5-5 [BS, BT]
Cobalt	0.45[A]	-	-	-	-	-
Iodine	0.9[A]	1–5[AG]	-	-	1[DQ]	-
Chromium	-	≤1.0[AH]	-	-	139.6[DQ]	-
Selenium	0.45[A]	0.07–0.38[AI]	-	-	0.4[DQ]	0.25 [BU]
Vitamins, µg/kg min						
Vitamin A	1200–6000[K]	600-750[AJ]	-	31000[BY,BZ]	1760-2090[CA]	300-600[CB]
Vitamin D_3	75[A]	40-60[BV]	-	60[DO]	9.35[CC]	6.25-25[CD,CE,CF]

Nutrient level		Common carp (*C. carpio*)	Rainbow trout (*O. mykiss*)	Zebrafish (*D. rerio*)	European sea bass (*D. labrax*)	Nile Tilapia (*O. niloticus*)	Channel catfish (*I. punctatus*)
Vitamins, mg/kg min							
Vitamin E		100-300[L]	50–100[AK]	500[AQ]	50[DO]	50-100[CG]	25-50[CH,CI]
Vitamin K		36[A]	1[S]	-	10[DO]	4.4[DQ]	10[DM]
Thiamine (B1)		0.5[BW]	10–12[AJ]	-	1[DO]	4[DQ]	1[CJ]
Riboflavin (B2)		7[M]	20–30[AJ]	-	4[DO]	5[CK]	6-9[CL,CM]
Pyridoxine (B6)		5.4[N]	10–15[AJ]	-	3[DO]	3-16.5[CN,CO]	3[CP]
Pantothenic acid (B5)		30–50[O]	40–50[AJ]	-	20[DO]	10[DQ]	10-15[CQ,CR]
Nicotinic acid (B3)		28[P]	120–150[AJ]	-	10[DO]	26[CS]	7.4-14[CT,CU]
Biotin (B7 or H)		1[Q]	1–1.2[AJ]	-	0.15[DO]	0.06[CV]	0.1[DM]
Folic acid (B9 or M)		3[A]	6–10[AJ]	-	1[DO]	0.82[CW]	1-1.5[CX,CY]
Vitamin B12		0.01[A]	0.02[S]	-	0.01[DO]	-	-
Vitamin C		45-354[BX]	20-500[CZ,DD,DE,DF]	-	5-200[DG,DH,DI]	50-420[DJ,DK]	50[DL]
Choline		4000[Q]	50-813[CZ,DA]	-	1000[DO]	1000[DB]	400[DC]
Inositol		440[R]	200–300[AJ]	-	300[DO]	400[DQ]	-

[A](Csengeri and Majoros, 2004), [B](Nose, 1979), [C](Ogino and Saito, 1970), [D](Ogino, 1980a), [E](Takeuchi and Watanabe, 1977b), [F](Sen et al., 1979), [G](Ogino and Takeda., 1976), [H](Ogino and Chiou, 1976), [I](Ogino and Yang, 1979), [J](Ogino and Yang, 1980), [K](Aoe et al., 1968), [L](Watanabe and Takashima, 1977), [M](Takeuchi et al., 1980), [N](Ogino, 1965), [O](Ogino, 1967), [P](Aoe et al., 1967), [Q](Ogino et al., 1970), [R](Aoe and Masuda, 1967), [S](FAO, 2015f), [T](Austreng and Refstie, 1979), [U](Satia, 1974), [V](Castell et al., 1972), [W](Watanabe, 1974), [X](Yu and Sinnhuber, 1972), [Y](Takeuchi and Watanabe, 1977a), [Z]((NRC). 1993), [AA](Arai et al., 1975), [AB](Ogino, 1978), [AC](Knox et al., 1981), [AD](Knox, 1983), AE(Ketola, 1978), [AF](Ketola, 1979), [AG](NRC, 1983), [AH](Tacon and Beveridge, 1982), [AI](Hilton et al., 1980), [AJ](Halver, 1972), [AK](Watanabe, 1981), [AL](Hardy and Barrows, 2002), [AM](Fernandes, 2014), [AN](Kaushik et al., 2011), [AO](O'Brine et al., 2015), [AP](Robison et al., 2008), [AQ](Mehrad et al., 2012), [AR](NRC, 2011), [AS](Tibaldi et al., 1994), [AT](Tibaldi and Lanari, 1991), [AU](Tulli et al., 2010), [AV](Tibaldi and Tulli, 1999), [AW](Kaushik, 1998), [AX](Coutteau et al., 1996), [AY](Oliva-Teles and Pimentel-Rodrigues, 2004), [AZ](Santiago and Lovell, 1988), [BA](Takeuchi et al., 1983), [BB](Shiau and Tseng, 2007), [BC](Dabrowska et al., 1989), [BD](Eid and Ghonim, 1994), [BE](Lin et al., 2008), [BF](Shiau and Ning, 2003), [BG](Robinson et al., 1981), [BH](Wilson et al., 1980), [BI](Wilson et al., 1977), [BJ](Harding et al., 1977), [BK](Robinson et al., 1980), [BL](Wilson et al., 1978), [BM](Satoh et al., 1989), [BN](Andrews et al., 1973), [BO](Gatlin et al., 1982), [BP](Gatlin and Wilson, 1986a), [BQ](Gatlin and Wilson, 1983), [BR](Gatlin and Wilson, 1984b),[BS](Murai et al., 1981), [BT](Gatlin and Wilson, 1986b), [BU](Gatlin and Wilson, 1984a), [BV](Barnett et al., 1982), [BW](Aoe et al., 1969), [BX](Gouillou-Coustans et al., 1998), [BY](Villeneuve et al., 2005a), [BZ](Villeneuve et al., 2005b), [CA](Hu et al., 2006), [CB](Dupree, 1970), [CC](Shiau and Hwang, 1993), [CD](Brown, 1988), [CE](Andrews et al., 1980), [CF](Lovell and Li, 1978), [CG](Satoh et al., 1987), [CH](Murai and Andrews, 1974), [CI](Wilson et al., 1984), [CJ](Murai and Andrews, 1978), [CK](Lim et al., 1993), [CL](Serrini et al., 1996), [CM](Murai and Andrews, 1978), [CN](Lim et al., 1995), [CO](Shiau and Hsieh, 1997), [CP](Andrews and Murai, 1979), [CQ](Murai and Andrews, 1979), [CR](Wilson et al., 1983), [CS](Shiau and Suen, 1992), [CT](Andrews and Murai, 1978), [CU](Ng et al., 1997), [CV](Shiau and Chin, 1999), [CW](Shiau and Huang, 2001), [CX](Duncan and Lovell, 1991), [CY](Duncan et al., 1993), [CZ](McLaren et al., 1947), [DA](Rumsey, 1991), [DB](Shiau and Lo, 2000), [DC](Wilson and Poe, 1988), [DD](Halver et al., 1969), [DE](Hilton et al., 1978), [DF](Sato et al., 1982), [DG](Saroglia and Scarano, 1992), [DH](Merchie et al., 1996), [DI](Fournier et al., 2000), [DJ](Soliman et al., 1994), [DK](Abdelghany, 1996), [DL](Andrews and Murai, 1975), [DM](FAO, 2015a), [DN](Li et al., 2004), [DO](FAO, 2015c), [DP](John W. Tucker, 1998), [DQ](FAO, 2015e), [DR](National research Council, 1973).

Certain nutritional requirements given here for Nile tilapia have been established for hybrids, Oreochromis niloticus x O. aureus and O. niloticus x O. mossambicus.

Amino acid requirements given for *D. rerio* are predicted from whole body composition levels.

Dash = not determined.

Appendix 2

Method of diet manufacturing

For each diet the desired amounts of all ingredients required were mixed using a Hobart food mixer until thoroughly mixed. For the zebrafish diets water was added until the mixture achieved dough like consistency. The dough mixture was then spread thinly on trays and dried for 24 hours at 50 ^{o}C using a nine shelf Parallexx Excalibur food dehydrator. Once dry the diet was crushed with a grinder by hand and processed through a series of sieves with apertures of 425 µm and 850 µm. The desired pellet size fell between the two. For both the carp and trout diets, once the ingredients were mixed thoroughly water was added until the mixture could be compressed into a firm ball by hand but still crumbled again when pressured to do so. The moist mixture was then cold extruded through a Buffalo CD400 mincer with 3 mm die attached. As the diet was extruded, the string like material was manipulated by hand into fairly consistent 3 - 4 mm pellets. The pellets were then dried (Genlab DC1000 drying cabinet) at 50 ^{o}C for 24 hours. All diets were stored at +4 ^{o}C until used.

Appendix 3

Methods used to analyse nutritional profiles; proximate analyses and fatty acid profile, of samples

Material energy content was determined using a Parr 6300 bomb calorimeter connected to a Parr 6520 water recirculation system. The attached oxygen cylinder pressure was set at 400 psi. Once the system was switched on, the jacket and bucket had heated up to 30 ^{o}C, a pre-test (with no crucible or sample) was ran. Then three standards (one gram Benzoic acid tablets standardised for bomb calorimetry, 26.454 MJ/kg, Parr Instrument Co, item No: 3415) were tested in sequence before analysing samples, a further standard was tested following the final sample. Each test sample was weighed (A & D Instruments Ltd GR-200-EC) to 1 g and compacted into a crucible, once in place the samples are ignited using ignition thread (4" 10 g ~ 1000, Parr Instrument Co, item No: 845DD2).

Material protein content was analysed using a Thermo Scientific FlashEA® 1112 N/Protein Analyser in conjunction with EAGER software. A leak test was conducted before running any analyses. All test samples, standards and the bypass sample (aspartic acid), and the blank samples (sucrose, analytical grade) were weighed to 50 mg (A & D Instruments Ltd GR-200-EC). The bypass sample was analysed first, the result used to set the expected peak and retention time was set at 50 seconds for all remaining tests, then all remaining samples were processed, two blanks, four standards, two known and two as unknowns, ensuring accuracy of equipment, followed by all the test samples.

Amino acids were determined for samples (10 mg nitrogen equivalence) as briefly described; following oxidation for 16 – 18 hours in the fridge then chemical hydrolyses at 110 ^{o}C for 24 hours. the hydrolysates were then centrifuged at approximately 3000 rpm for 2 minutes before filtering the supernatant through a 0.22 µm filter and processing through the amino acid analyser.

Lipid content of each sample was analysed using rapid Soxhlet extraction using a Gerhardt Soxtherm. The machine was switched on along with the air pump, water supply and water booster pump, water is circulated at 2.5 L/m to ensure condensers are cool enough, preventing solvent loss. Each glass flask was oven dried for one hour at 100 - 103 ^{o}C prior to starting, then allowed to cool in a glass desiccator. Three boiling stones (Gerhardt type A), were added to each flask before weighing (A & D Instruments Ltd GR-200-EC), then 155 ml of petroleum ether (Fisher Scientific Petroleum ether 40 - 60 ^{o}C) was added under a fume hood. Test samples were weighed to an amount which contained no more than 0.5 g of fat, based on information in the literature base (table 1.8), 1 g in this case. Samples were contained within a folded piece of filter paper (Fisher brand 110 mm, QL125), and placed inside a cellulose thimble (Fisher brand cellulose extraction thimble, 33 x 80 mm), capped with cotton wool. Each thimble was placed inside a holder, inside a glass flask. The six flasks were pushed into place on the Soxtherm, programme one was started, during which, the solvent was heated to 150 ^{o}C by hot plates, evaporating, leaving the now extracted lipids in the glass flask. Once the programme was complete and all the solvent has evaporated, repeat the programme if any solvent remains in flask, the thimbles and holders were removed and the glass flasks once again oven dried at 100 - 103 ^{o}C for one hour, and then cooled in the desiccator before weighing again, the crude fat content (%) of that sample can now be calculated.

The extracted lipid samples were further analysed to determine the fatty acid profile of each sample by applying a direct method for fatty acid methyl ester (FAME) synthesis. Samples were dissolved in 1 ml of hexane before being transferred into screw top methylation tubes, were 0.7 ml 10 M potassium hydroxide and 5.3 ml methanol was added. Samples were mixed (Fisher brand whirl mixer) for 10 seconds, then incubated at 55 ^{o}C in a water bath (Grant Instruments) for 90 minutes, samples were mixed for a further five seconds every 20 minutes during incubation. Following this first incubation, sample tubes were cooled down

in cold water and ice for 10 minutes. Then 0.58 ml of 12 M sulphuric acid was added, samples were mixed and incubated at 55 ^{o}C as before, the 10 minute cool down was also repeated. Once cool, 3 ml of hexane was added, the sample then vortex mixed for 30 seconds before being centrifuged at 1200 g (2500 rpm) at room temperature for 5 minutes. The top hexane layer was transferred using a glass pipette to a solvent resistant tube, a 1 ml sample was also transferred into a gas chromatograph (GC) crimp vial, and samples were frozen at -30 ^{o}C for further analyses.

GC analyses were carried out on the 1 ml hexane samples (Perkin Elmer Clarus 500 Gas Chromatograph) utilising a varian capillary column CP-Sil 88 for FAME, column length: 100 m, column width: 0.25 mm. Gas flow for air was 450 ml/min, and hydrogen was 45 ml/min, the temperature set point was 250 ^{o}C. This FAME analyses along with fibre analyses was conducted by technicians within the lab.

Ash is determined using the AOAC official method 942.05, which involves ignition of the feed ingredient at 600 ^{o}C for two hours, the unburnt material is ash (Thiex et al., 2012). Fibre content was analysed using the Gerhardt Fibrebag method. Crude fibre is determined following removal of the starch and sugars by digestion in acid, and removal of the protein by digestion in alkali.

Appendix 4

Diet modelling software programme DMAF

DMAF was created by Andrew fletcher during his study of a master's degree, it was very kindly provided for use during part of this PhD study. The programme has been described by Andrew as follows:

"DMAF is a tool to aid in the design of a diet. The user provides a nutritional analysis of the diet required (Diet Required), together with a nutritional analysis of each of the ingredients to be used in formulating the diet. DMAF provides a recommended recipe for 1 kg of the combination of ingredients which gives the closest match to the nutritional requirements of 1 kg of the Diet Required. The program provides the capability to set the amounts of one, several or all of the ingredients as a fixed constituent of the diet to be formulated. The nutrient contribution of these fixed ingredients to the diet is calculated and removed before modelling the amounts required of the remaining, variable ingredients (if any). The recipe recommended by the program (the Model Diet) is the combination of the fixed ingredient amounts and the amounts calculated for the variable ingredients."