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The protein requirement of juvenile silver trevally (*Pseudocaranx georgianus*) to optimise growth in hatchery environments.

A thesis presented in partial fulfilment of the requirements for the degree of

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Abstract

Aquaculture is a growing primary industry in New Zealand. Currently the industry is comprised of three main species: Greenshell^{TM} mussels, Pacific oyster, and King Salmon. The introduction of a white fleshed fish presents obvious commercial opportunity and production gains for New Zealand aquaculture. Silver trevally provides this opportunity and has the potential to further develop the industry. When developing a new species for aquaculture an understanding of their nutritional requirements at the different life stages is required. This thesis investigates the protein requirement of juvenile silver trevally.

Silver trevally (67.5±12.0g) were randomly assigned to 12 tanks, 15 fish per tank. Four iso-energetic diets ranging in crude protein (CP) content from 30 to 60% CP were fed, in triplicates, for 12 weeks. Growth, including specific growth rate (SGR), did not significantly differ between diets. Feed efficiency was lowest in fish fed the 40% CP diet compared with the other three diets. Protein retention was highest in fish fed the lowest protein diet. Condition indices in silver trevally were unaffected by the protein content of the diet. Overall, this experiment was inconclusive on the ideal protein level in the diet.

A palatability trial was carried out to determine if feed intake varied among diets. For comparison a commercial pellet from Ridley's (50% CP) and a gel diet (20.4% CP) used by Plant & Food Research was also included in this trial. Twenty-four fish from the growth trial were allocated to two tanks for the palatability trial. Four behavioural responses were observed: the food item was ignored; fish approached the food but did not ingest; the fish took the food into their mouths before spitting it out; and the food was ingested. The 60% CP experimental diet, a commercial pellet, and a gel diet had significantly higher rates of intake than the other diets, with the 30% CP diet having the lowest rate of complete ingestion. The 60% CP and gel diet had the lowest rate of food being ignored. The most palatable diets were the 60% CP diet and the gel diet.

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Abbreviations

ADC – apparent digestibility coefficient

ADG – average daily gain

BSA – Bovine serum albumin

CF – condition factor

CL – crude lipid

CP – crude protein

DHA – docosahexaenoic acid

DM – dry matter

EPA – eicosapentaenoic acid

FCR – feed conversion ratio

FD – freeze dried

FE – feed efficiency ratio

GE – gross energy

Hb - haemoglobin

HSI – hepatosomatic index

n-3 HUFA – n-3 highly unsaturated fatty acids

Ind – indicator

IPF – intraperitoneal fat

LGR – length growth rate

PER – protein efficiency ratio

PFR – Plant & Food Research

PR – protein retention

PUFA – polyunsaturated fatty acids

SGR – specific growth rate

VSI – viscerosomatic index

Chapter One

1. Introduction

Globally aquaculture is a growing industry and has the potential to supply a large proportion of the world's seafood demands. China makes up more than 60% of the worlds aquaculture production (FAO, 2016a) while New Zealand contributes very little. Aquaculture is defined as the farming of aquatic organisms, such as fish, molluscs, and crustaceans. Marine aquaculture, as opposed to fresh water aquaculture, makes up 96% of the total aquaculture production (FAO, 2016b).

Of the all the primary industries, aquaculture is the fastest growing worldwide (Aquaculture New Zealand, 2015a). Internationally, fish is one of the most traded food items (FAO, 2016a). Fish consumption has increased worldwide over the past two decades (FAO, 2016a). Aquaculture has allowed for this increase in fish consumption as fisheries production has remained relatively static since the 1980s (FAO, 2016a). Globally, in 2014, aquaculture supplied 39% of seafood available for consumption; this was a significant increase from 7% in 1974 (FAO, 2016a). This was also the first time that aquaculture surpassed fishing in terms of fish supply. It is predicted that by 2025 over half the seafood consumed worldwide will be farm produced (New Zealand Goverment, 2012). To meet the demands of the growing population, aquaculture is needed to make up the deficit that fishing cannot supply.

Fish provide nutritional and health benefits when included in the human diet. This is because it is easily digested and contains essential amino acids in an ideal balance, essential fatty acids (such as omega-3 fatty acids), vitamins, and minerals (FAO, 2016a). Fish protein is considered to be a high quality protein (Sidhu, 2003). This is due to the high levels of essential amino acids, usually at a higher than other foodstuffs, especially lysine, which is often considered a limiting amino acid (Usydus & Szlinder-Richert, 2012). In 2013, seafood made up 17% of the total animal protein consumed worldwide (FAO, 2016a). Fish contains high levels of minerals and vitamins. Of the bioavailable vitamin B-12 in humans, 42% is contributed from fish meat (Watanabe, 2007). Vitamin D3 content in higher in fish than that of most other food items (Usydus & Szlinder-Richert, 2012). Selenium, calcium, phosphorus, fluorine, and iodine are also high in fish products (Usydus & Szlinder-Richert, 2012). The lipids present in fish provide health benefits. Omega-3 polyunsaturated fatty acids (PUFA) provide health benefits, such as prevention of cardiovascular disease and strokes (Domingo, 2007). Triacylglycerides in the blood can be reduced by 25 to 40% by consuming 4g of omega-3 a day (Usydus & Szlinder-Richert, 2012). The American Heart Association recommend eating fish at least twice a week (He, 2009). Eating fish

has health and nutritional benefits and by farming more fish species, fish will become more readily available to consumers.

Aquaculture is considered a more sustainable alternative to fishing as it consumes less fossil fuel, which in turn lowers costs and reduces the carbon footprint (Stimpson and Co, 2007). By supplying the increasing seafood demand, aquaculture lightens the pressure on overfished wild fish stocks by decreasing the amount that needs to be caught. In 2013, only 68.6% of fisheries were considered sustainable (FAO, 2016a). Thus, 31.4% of the world's fisheries are being overfished. This is despite a large amount of research and effort going in to restoring fish stocks. In 2015, 17% of the commercial fish stocks in New Zealand were over fished (Ministry for the Environment & Statistics New Zealand, 2016). Over-fishing affects the ecosystem as well as fish production (FAO, 2016a).

Aquaculture still poses its own problems, such as water pollution. Farming a marine-based organism can be achieved both in land-based and sea-based (near, or off shore) facilities (Cao et al., 2007). Land based facilities have the potential to clean water that is returned to the ocean through filtration and purification systems. Water, which is brought into the facility, is also filtrated before entering a containment area, tank or pond (Mussely & Goodwin, 2012). However, off shore facilities are directly in an open, marine environment. Waste products sink until they reach the seabed and/or enter the water column and are dispersed (Australian Government, 2001). Wastes that are dispersed result in a change in the nutrient composition of the water, which in turn can alter the native species found in the area. The entry of nutrients into the water around aquaculture farms can increase the occurrence of toxic algal blooms, especially in areas with more than one farm in close proximity. Increased nitrates in the water contribute to an increased likelihood of algal blooms (Cao et al., 2007). Despite these potential problems there are ways to prevent them. Accumulation of wastes and the development of algal blooms can be prevented by picking locations where a high flow rate will disperse waste products of the farm (Australian Government, 2001). A high flow rate prevents material settling on the seabed and changing the sediment composition, which can cause the flora and fauna in the area to alter. However, effects to the seabed occur in an area around the nets (Brown et al., 1987).

Fish feed can have a large impact on the environment, the extent of which is dependent on management practices (Australian Government, 2001), feed type, and formulation (FAO, 2016a). A primary source of wastes from aquaculture farms comes from feed, either due to uneaten feed or excretory products. Different diet forms behave differently in the water. Pellets tend to result in the smallest amount of pollution as they are eaten whole and hold together

better in the water. Wet diets and raw fish mixes result in increased pollution as they readily disperse in the water. Excreted nitrogenous compounds are some of the worse pollutants from aquaculture (Cao et al., 2007). Ammonia is one of the main excretory products of fish, while urine and faeces also contain other nitrogen-based products. Increasing the level of protein in the feed will not only increase the cost of feed, but will increase nitrogenous waste excretion. In addition, when faeces and uneaten food decompose they can result in decreased oxygen saturation of the water and increased ammonia (Cao et al., 2007). Some species, however, can be farmed without the need for the addition of feed to the water and will, therefore, have no waste from uneaten feed. These "non-fed species", such as carp and bivalve molluscs, do not require the addition of feed as they are filter feeders. Farming non-fed species contributed to 30.8% of the production of all farmed fish in 2014 (FAO, 2016a).

1.1. Culturing a new species

Culturing a new species requires a large amount of research, especially if it, or a similar species, is not being cultured elsewhere in the world. Silver trevally are one of these such species, as no significant commercial-scale culture of this species is currently occurring worldwide. Up to $20 million is invested into seafood research including understanding the physiology of individual fish species (New Zealand Trade and Enterprise, 2015). Plant & Food Research is developing a breeding programme to domesticate New Zealand's endemic fish, including silver trevally and snapper (Seafood New Zealand, 2013). This will allow for the New Zealand aquaculture industry to expand using species not currently farmed. To commercially grow a species, it is important for research to first characterise the species' habitat, nutritional requirements, and reproductive traits (NIWA, 2006).

To farm any fish, in this case silver trevally, their nutrient requirements need to be understood. Species-specific diets are required to meet the differing nutrient requirements of different developmental stages: larval, fingerling, juvenile, adult, and broodstock (FAO, 2016a). This is important as diets are the most expensive long-term component of fish farming.

The purpose of this thesis is to determine the protein requirements of juvenile silver trevally. Providing a well-balanced feed that is cost effective is important for profitable production (FAO, 2016a). By determining the optimum protein requirements, feed efficiency and growth rate can be optimised.

Chapter Two

2. Literature Review

To determine the protein requirement of silver trevally experimental diets first need to be formulated. To formulate diets the ingredients need to be determined and then the diets need to be manufactured. Ideal measurements need to be determined to understand the optimal protein level of silver trevally. This literature review will cover the New Zealand aquaculture industry, previous research on silver trevally as well as methods used in previous studies for fish feed formulation, experimental procedures in fish nutrition trials, physiological measurements, and chemical analysis. This information will then be used to construct the methods for this thesis.

2.1. Introduction to New Zealand's Aquaculture Industry

2.1.1. New Zealand Seafood Exports

Seafood is one of the top 10 largest export items in New Zealand (New Zealand Trade and Enterprise, 2015), as well as the most traded food item in the world (Manan, 2013). Exporting seafood contributes more than $1.5 billion to New Zealand (Ministry of Primary Industries). Seafood is exported to over 70 countries worldwide (New Zealand Trade and Enterprise, 2015) with New Zealand's three main seafood export partners being China, the United States and Australia (Figure 2.5.) (Seafood New Zealand, 2016). New Zealand fish exports into China increased by 202% over 5 years, 2007 to 2012 (Statistics New Zealand, 2013). Both farmed and fished species are exported. Hoki makes up the largest proportion (by tonnage) of fish caught in New Zealand (Ministry for the Environment, 2010). In the first nine months of 2016, New Zealand exported $1,383 million worth of seafood (Figure 2.1.), a large proportion of this being finfish (Seafood New Zealand, 2016). Freezing fish allows for an increased proportion of fish to be exported. This was seen in jack mackerel, which had an increase of 98% (NZD) from 2007 to 2012 despite a lack of increase in terms of catch rate (Statistics New Zealand, 2013). Freezing the jack mackerel allowed for a higher proportion of the fish caught to be exported.

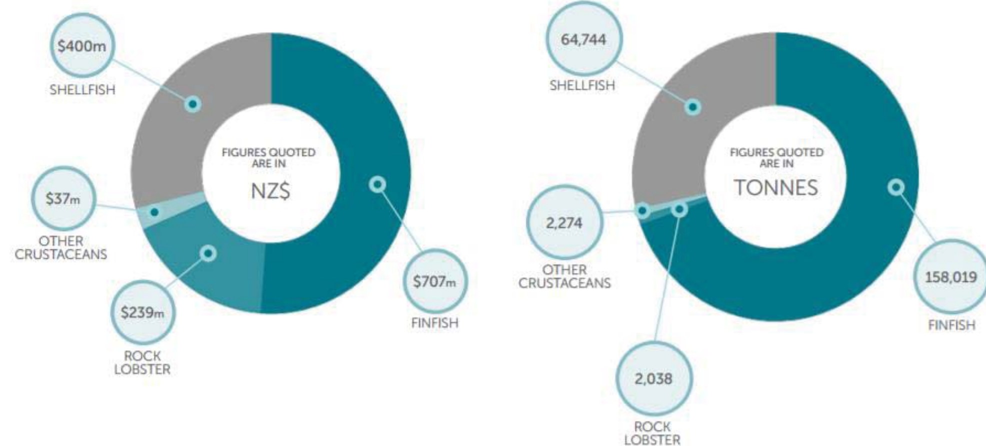

*Figure 2-1 New Zealand's export statistics (Seafood New Zealand, 2016). *FOB=Free on board. The value of the exported product, including the raw material, processing, storage and transport prior to being loaded onto the ship*

2.1.2. Aquaculture Production

New Zealand's primary industries are important for the economy to evolve (New Zealand Goverment, 2012). Of the primary industries, aquaculture is one of the fastest growing worldwide. The last 30 years have seen New Zealand's aquaculture industry grow significantly (Figure 2.2.) resulting in aquaculture being the largest growing rural industry (FAO, 2016c). An increase in the aquaculture industry has caused New Zealand to focus on ways to further increase the productivity, sustainability and quality of New Zealand seafood. In 2016, the New Zealand Aquaculture industry earned around $500 million of revenue (Aquaculture New Zealand, 2015a). However, the aim of the New Zealand government is to grow aquaculture to a $1 billion industry by 2025 (New Zealand Goverment, 2012). To increase revenue, current and new species need to be optimised.

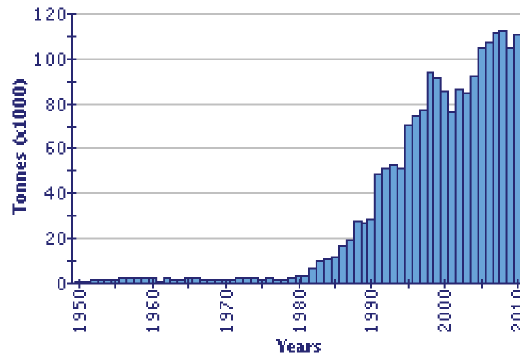

Figure 2-2 Aquaculture production in NZ (FAO, 2016c)

Today the New Zealand aquaculture industry mainly consists of Greenshell mussels, Pacific oysters and King salmon. In 2011, exports of King or Chinook salmon (*Oncorhynchus tshawytscha*), Greenshell mussels (*Perna canaliculus*) and Pacific oysters (*Crassostrea gigas*) earned $63.4 million, $218.1 million, and $16.6 million respectively (Aquaculture New Zealand, 2015a). Aquaculture began in New Zealand in the mid-1960s with oysters (Stimpson and Co, 2007). The majority of oyster farms are found in Northland, Auckland and Coromandel regions (Aquaculture New Zealand, 2015c). Greenshell mussels are farmed all over the country including Northland, the top of the South Island and Stewart Island. The majority of sea-based salmon farms in New Zealand are found in the Marlborough Sounds, Stewart Island and Akaroa harbour (Aquaculture New Zealand, 2015b). King salmon is the only significant finfish farmed in New Zealand and is restricted to the cooler waters found in the South Island (Figure 2.3.).

For New Zealand aquaculture to reach the 2025 government target, expansion is required. Developments in current and new spaces, species, markets, and products needs to occur (Stimpson and Co, 2007). New Zealand's aquaculture industry is large for the size of the land, and available surrounding waters, but has the potential for further growth. The current aquaculture industry takes up 13,000 hectares of New Zealand's marine coastal area (Stimpson and Co, 2007). This equates to only 0.02% of New Zealand's coastline (Aquaculture New Zealand, 2015d). It is estimated that, in terms of finfish, one hectare can result in $4 million in production, while mussels are able to result in a return of only $30,000/ha (NIWA, 2006). Expansion into New Zealand's surrounding waters (Figure 2.3.) that are currently unutilised provides a potential expansion to the industry. It is also possible to expand land-based aquaculture facilities for both marine and freshwater species. Currently salmon (hatchery and early growth stages), oysters, mussels, paua, freshwater prawns, crayfish, brine shrimp, whitebait, eels, kingfish, hapuku, and other species are farmed in land-based facilities at research, pilot scale or full production stages of development (Land Based Aquaculture Assesment Framework; Mussely & Goodwin, 2012).

New Zealand has little land-based aquaculture. However, overseas it contributes to a large proportion of finfish, shellfish and seaweed production (Mussely & Goodwin, 2012). Expansion into both land and off-shore locations will allow for the growth of aquaculture in New Zealand.

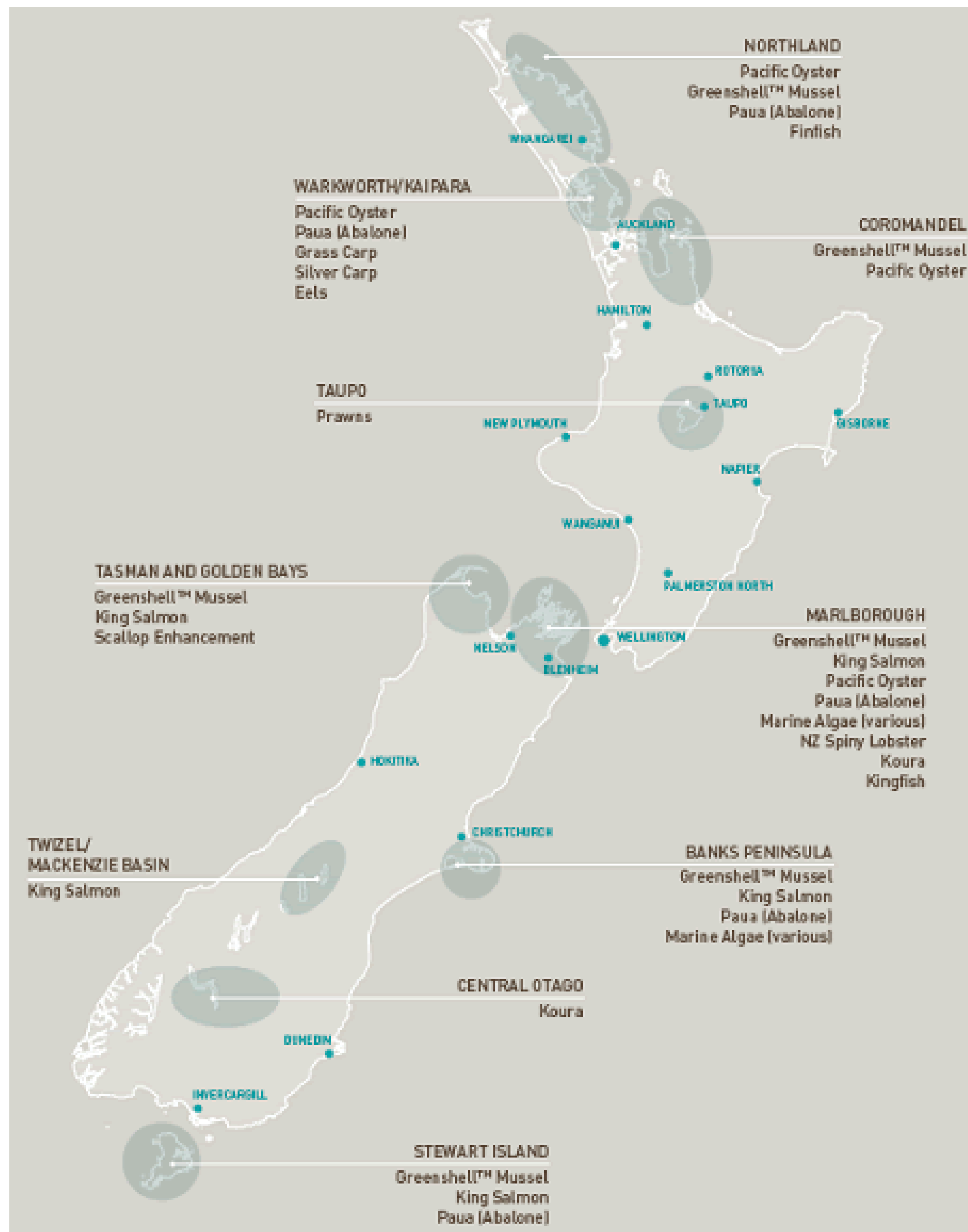

Figure 2-3 Species currently and historically farmed in New Zealand and their geographical location (Stimpson and Co, 2007)

Finding new areas for aquaculture, or related research sites, can be difficult as not all sites are suitable and the public is not always welcoming. Most recently, when the fish feed company Skretting was applying to set up a new aquaculture research and development site in Okiwi Bay public disapproval became apparent (Moore, 2016). Trouble with finding and gaining approval in the Marlborough Sounds is resulting in the need for research and development to be carried

out for new land-based and offshore farms. An example of this is the new area for offshore fish farming in the Firth of Thames in the Waikato. The Waikato regional council has opened 240ha of available space for fish farming in the Coromandel Farming Zone. The space would allow for sea cages and could potentially support the production of 8000 tonnes of fish (Waikato Regional Council, 2017). These waters are too warm for salmon production and, therefore, allows for development in a new high value species that would benefit from being grown in a warmer climate.

2.1.3. Silver Trevally

Silver trevally (*Pseudocaranx georgianus*) has the potential to be farmed in the warmer waters of the North and South Island. They are from the carangidae family and reach sexual maturation at around 2 – 4 years old and 18 – 24cm long (New South Wales Government, 2009). Silver trevally are able to reach sizes up to 1.2m and 18kg; however, in the wild they are often caught at 35 – 60cm and 0.4 – 2.5kg (Australian Government, 2016). They are a carnivorous species and feed on benthic invertebrates as well as benthic and planktonic crustaceans (New South Wales Government, 2009). Silver trevally are found in warmer oceans, in particular south-eastern regions of Australia and the northern waters in New Zealand, as well as around the top of the South Island (Figure 2.4). In New Zealand, around 3500 tonnes of trevally are harvested a year with 60% coming from the TRE7 region (Figure 2.4), 30% from TRE1 and 10% from TRE2 (McKenzie, Parsons, Bian, & Doonan, 2016). Farming silver trevally would provide a white-fleshed finfish available for exporting as well as local consumers.

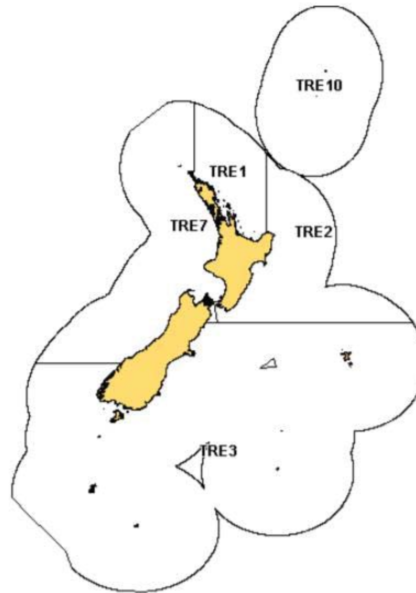

Figure 2-4 New Zealand's main quota management areas (QMAs) with TRE1, TRE2, and TRE7 the highest producing QMAs (McKenzie et al., 2016)

New Zealand currently exports silver trevally to nearby markets in Asia, United States, and Australia (New Zealand Goverment, 2012). Japan is New Zealand's fourth largest seafood export trading partner (Figure 2.5), of which 81% of the exports were finfish (Seafood New Zealand, 2016). Silver trevally is a popular fish for sashimi and sushi in Japan. Striped jack, from the same family as the silver trevally, is considered a premium fish for sashimi, making it a delicacy (Watanabe & Vassallo-Agius, 2003). Fish that make it to this market are sold for a considerable premium. Striped jack are the most expensive fish from the carangidae family (Watanabe & Vassallo-Agius, 2003). By farming silver trevally fish fillet quality can be improved, increasing the likelihood of it entering the sashimi market. Sashimi quality silver trevally could result in Japan importing a larger amount of finfish which, in turn, could increase New Zealand's aquaculture revenue.

TOP 10 EXPORT VALUES (NZ$)	2015	2016	% Change
1 China	$376m	$438m	▲ 14
2 United States	$155m	$189m	▲ 18
3 Australia	$161m	$184m	▲ 13
4 Japan	$85m	$92m	▲ 8
5 Spain	$31m	$46m	▲ 33
6 Hong Kong	$50m	$43m	▼ 16
7 South Korea	$35m	$38m	▲ 8
8 Poland	$15m	$29m	▲ 48
9 Germany	$22m	$29m	▲ 24
10 Thailand	$22m	$28m	▲ 21

Figure 2-5 New Zealand's main seafood exporting partners (Seafood New Zealand, 2016)

2.2. Current Knowledge on Silver Trevally Nutrition

Current knowledge on silver trevally is relatively limited. Striped jack (*Pseudocaranx dentrex*) is from the same family as the silver trevally. The striped jack is a Japanese species that is farmed to some extent in Japan, where wild caught juveniles are on-reared to commercial size, and has been researched more than the silver trevally so will be included in this analysis of silver trevally research. Research includes stimulation of feeding, lipid requirements, inclusion of starch in the diet, and broodstock nutrition.

When exposed to an optical stimulus, transparent polyethylene bag containing krill, and a chemical stimulus, an opaque, perforated polyethylene bag containing krill, equal numbers of silver trevally approached both bags indicating that silver trevally respond to both visual and olfactory stimuli when feeding (Masuda et al., 1995). Understanding how fish sense food and what triggers feeding behaviours can be used to determine the best feeding procedure.

A diet without essential fatty acids results in high mortality rates and poor feed efficiency ratios (Watanabe et al., 1989a; Takeuchi et al., 1992a). Essential fatty acids, in the form of n-3 highly unsaturated fatty acids (n-3 HUFA), are required in the diet of juvenile striped jack at around 1.7% (Watanabe et al., 1989b). The main essential fatty acids are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). DHA is a more efficient essential fatty acid than EPA. The addition of only EPA was unable to prevent poor mortality rates and FCR (Watanabe et al., 1989a). DHA is required at 1.7% of the juvenile striped jack's diet, while EPA is required at less than 0.8% of the diet (Takeuchi et al., 1992a). Sufficient DHA levels are able to almost completely prevent mortalities caused by a lack of essential fatty acids in the diet (Watanabe et al., 1989a).

Marine fish are known for being unable to utilize high levels of carbohydrates (Wilson, 1994). This can cause problems when producing synthetic diets. It is important to determine what types of carbohydrates are able to be used preferentially and at what inclusion level. Feeding gelatinised starch in the striped jack's diet improved growth, feed efficiency, and protein efficiency ratio compared with the inclusion of raw starch in the diet (Takeuchi et al., 1992b). Starch should not be included in the diet of striped jack above 15% and at least 50% of that included should be gelatinised (Arakawa et al., 1993). This indicates that gelatinised starch is the preferred starch source in striped jack.

Optimising broodstock fertility is important as the offspring are required for profit on aquaculture farms. Striped jack fed steam-dry pellets results in poor fecundity compared with fish fed a raw fish mix (mackerel, squid and shrimp fed at a ratio of 2:2:1 respectively). The addition of astaxanthin, 10 mg/kg, to the steam-dry pellets improves total egg production

(Vassallo-Agius et al., 2001a). However, the astaxanthin is not incorporated in the egg (Watanabe & Vassallo-Agius, 2003). Lipids found in striped jack eggs are derived from dietary lipids. However, high levels of dietary lipids can cause excessive abdominal lipid deposition, which can interfere with spawning (Watanabe & Vassallo-Agius, 2003). The replacement of half the fish meal in steam dry pellets with squid meal improves egg quality, increased number of buoyant eggs, and higher fertilisation and hatching rates (Vassallo-Agius et al., 2001b). The combination of both astaxanthin and squid meal improves the overall spawning performance of broodstock striped jack (Vassallo-Agius et al., 2001c).

2.3. Dietary Protein

Determining the optimum protein requirement of individual species can optimise animal production, such as body weight gain, and feed efficiency. Feed is considered the largest expense of fish rearing facilities. Of the feed ingredients protein sources are the most expensive component (Luo et al., 2004). Therefore, it is important to determine the protein requirements to prevent wasting money. Protein requirements vary among species. Fish typically require a diet containing 30–50% crude protein (CP) (Medale & Kaushik, 2009). Protein requirements (as a percentage of the overall diet) have shown to decrease with an increase in size (Watanabe et al., 2000). This is likely due to a decrease in growth in older fish. By optimising the diet to individual species feed costs and wastage can be minimised while feed efficiency and growth can be optimised.

Protein ingested is first broken down to its individual amino acids. The amino acids are then either absorbed or travel down the gastrointestinal tract unaffected. The amino acids that are absorbed can be utilised in different ways depending on the animal's needs (for the synthesis of proteins and other amino acid based compounds) and the amino acids that are available to be used. When a protein that has amino acids in the proportions required by the animal the protein source is considered a "balanced protein". Amino acids in roughly the correct proportions required by the animal are considered to be balanced (Harper, 1959). The body does not have a large capacity for storing free amino acids so unbalanced amino acids are catabolised for energy (Figure 2.6). A lower availability of these amino acids can end up affecting the weight gain and performance of the fish. Fishmeal is considered to be a balanced source of amino acids and is often used when determining protein requirement in fish for this reason.

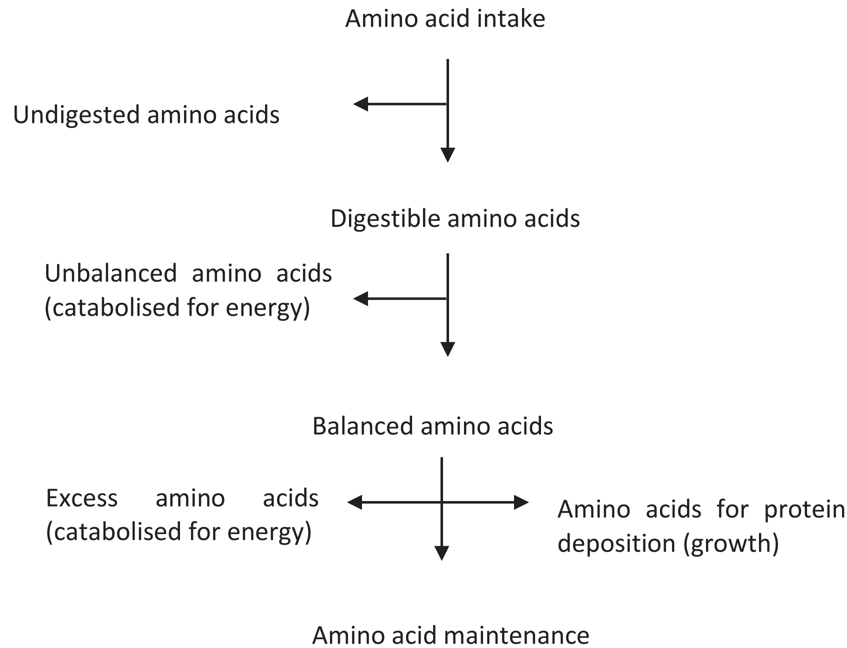

Figure 2-4 Protein and amino acid partitioning

2.4. Diet Formulation

2.4.1. Protein Level

The protein requirements for individual species need to be determined if a species is going to be farmed or reared in a domestic setting. Fish have a large and highly specific need for protein and, therefore, the level in the diet needs to be consideration (Sanz et al., 2000). Protein is one of the most influential factors on growth performance in fish (Luo et al., 2004). This is likely due to its effect on growth and feed utilisation (Chatzifotis et al., 2012). As individual species have different protein requirements the experiments need to be performed *in vivo* as it is not possible to determine this in vitro. To determine protein requirements for fish, synthetic diets need to be formulated to contain a range of protein concentrations, e.g. 30–65%, and each protein level should be fed to triplicate groups of fish. Fish meal is used as the main protein source in fish diets due to its high protein content and ideal amino acid balance (Medale & Kaushik, 2009). Determining the protein requirement is a start in developing a specialised diet. Other factors that need to be considered within the diet include an energy level and source, lipid source, vitamins and minerals (added as premixes), a binder, and, if needed, preservatives.

2.4.2. Energy Level

To determine the protein requirements of a fish species, the diets need to be kept iso-energetic. This means the energy level needs to be kept constant across all experimental diets. Iso-energetic diets often range between 16 and 20 kJ/g (de Silva & Gunasekera, 1991). For example,

Ai et al. (2006) created diets with 20 kJ/g for large yellow croaker, while Eliason, Higgs, and Farrell (2007) developed 16.7 MJ/kg diets for rainbow trout. The energy content of the diets will depend on the ingredients used and their individual energy content.

To create an iso-energetic diet the addition of an ingredient that provides energy without adding protein is required. Ingredients often used in iso-energetic diets include corn starch (Jiang et al., 2015; Schuchardt et al., 2008; Wang et al., 2016; Yigit et al., 2006), dextrin (Lee, Cho, Lee, & Yang, 2001; Lee & Kim, 2001), wheat meal (Ai et al., 2006; Eliason et al., 2007), cellulose (Luo et al., 2004) and starch (Chatzifotis et al., 2012). Most of these ingredients are carbohydrates high in energy and contain little to no protein. This allows for the decrease in energy that occurs with a decrease in fishmeal to be compensated for without affecting the protein content of the experimental diet. The energy level of fishmeal varies; 21.3, 21.5, 20.9MJ/kg (Allan et al., 2000), or 21.3MJ/kg (Allan et al., 1999). Alternative energy sources have a range of energy content. The energy content of wheat ranges from 17.8MJ/kg (Allan et al., 1999) to 22.6MJ/kg (Tibbetts, Milley, & Lall, 2006) while corn gluten meal ranges from 15.0MJ/kg (NZ Starch, 2015), 20.9MJ/kg (Tibbetts et al., 2006), 24.1MJ/kg (Allan et al., 2000), to 23.2MJ/kg (Allan et al., 1999). It can be difficult to make iso-energetic diets as alternative energy sources are not always as high as fishmeal.

As well as feeding diets with the same energy level it is possible to test both protein and energy at the same time. This is usually done by feeding different protein levels under two energy levels. As well as determining the ideal protein level it will also give the ideal protein to energy ratio. This was seen in experiments performed by Suprayudi et al., (2014) and Lee and Kim (2001) for bluefin trevally and masu salmon, respectively. Experiments comparing protein and energy levels can also be used to determine if energy has an effect on growth when protein is low, and vice versa.

2.4.3. Other Ingredients

Lipids are required to supply essential fatty acids as well as serve as an energy source. The lipid source can be marine-based, such as fish oil, krill oil or squid liver oil, or plant-based, such as soybean oil and canola oil. However, plant-based oils can reduce palatability and decrease growth performance especially if 100% of the fish oil is substituted by a plant-based oil (Trushenski et al., 2011). It is important to determine what level of substitution can occur without negatively impacting the fish’s health and performance. Often multiple lipid sources are added to the diet, especially if using a plant-based oil. For protein requirement experiments a

marine source is used to ensure that the essential fatty acids are present in the diet and minimise any palatability problems that could occur from using a plant-based lipid.

Figure 2-5 The molecular structure of CMC with some substitution (Su et al., 2010)

Binders are required to be added to the diet to allow all the ingredients to bind together. Binders used to make diets include carboxy methyl cellulose (CMC) and alginate. The inclusion level of these binders varies. CMC is derived from cellulose and is often used in the form of its sodium salt (Figure 2.7). Experiments using CMC included it at different rates. Jiang et al. (2015) and Lee and Kim (2001) added 3% CMC, Lee et al. (2001) added 4%, Schuchardt et al. (2008) added 5%, while Khosravi et al. (2015) added 10%. Alginate levels appeared to be less variable with both Luo et al. (2004) and Chatzifotis et al. (2012) adding 20g alginate/kg diet. Alginate is a seaweed-based binder and requires the addition of a calcium source to form alginic acid, which in turn results in formation of gel structures that assist in binding.

Preservatives are also often added to pelleted diets to prevent the growth of microbes, which can cause the feed to go rancid when it is kept in a warm, moist location or if stored for long periods of time. A mould inhibitor used in a study on large yellow croaker was 50% calcium propionic acid and 50% fumaric acid (0.1% DM) (Ai et al., 2006). Potassium sorbate can also be used as an antimicrobial in pelleted feed to prevent mould and yeast growth. It is recommended that no more than 2500 mg/kg be added to the diet. However, 18% of the preservative was lost from the feed after 3 months of storage (EFSA, 2014). Mach et al., (2010) used 2.2g/kg diet to prevent the growth of fungi. Potassium sorbate is quickly metabolised to carbon dioxide and expelled (EFSA, 2014). This makes it safe to be used in diets of food producing animals as there are minimal residues found in the products. Potassium sorbate has also been shown to have no effect on the environment (EFSA, 2014).

2.4.4. Diet Production

Diets can be manufactured in several ways depending on the equipment available. While commercial diets are made in a feed mill, scientists often use alternative methods. Firstly, the dry ingredients were mixed thoroughly in a mixer. However, some ingredients required grinding first to reduce the particle size. Next the wet ingredients were added. This included oils and water. The types of oils used included fish oil (Ai et al., 2006; Chatzifotis et al., 2012), squid liver oil (Lee et al., 2001; Khosravi et al., 2015), soybean oil (Ai et al., 2006; Khosravi et al., 2015), and anchovy oil (Eliason et al., 2007). The proportion of water added varied. Chatzifotis et al. (2012) added 50% water (w/v), Jiang et al. (2015) added 30–50ml water/100g DM, and Rahimnejad et al. (2015) added 40% water. Once the wet ingredients were mixed a pellet was formed using a meat grinder (Chatzifotis et al., 2012; Khosravi et al., 2015), or extruder (Jiang et al., 2015). Once the diet was pelletized it was dried by one of three methods: freeze drying at -40°C for 24 hours (Khosravi et al., 2015); using the heat in an oven (60°C for 12 hours (Ai et al., 2006) or at 45°C until moisture is less than 100g/kg (Luo et al., 2004)); under a stream of air (25°C (Jiang et al., 2015) or 35°C (Chatzifotis et al., 2012) for 24 hours); or by leaving out overnight (Rahimnejad et al., 2015). Next, the diets were crushed to get the desired size or left unchanged. Once the ideal particle size was reached the diets were then stored in a freezer, between -15°C and -30°C, until feeding.

2.5. Experimental Procedure

2.5.1. Feeding and Trial Length

The fish selected for the trial were of similar weights. The number of fish per tank varied depending on the fish species and the size of the tank. The amount of food offered to fish throughout the day is determined by one of two methods: to apparent satiation/satisfaction (when fish appear to lack interest in feed and stop eating) or as a percentage of the body weight. In yellowtail, feeding to apparent satisfaction resulted in the growth rate being the highest (Watanabe et al., 2000). Other studies have fed the fish based on their body mass. Jiang et al. (2015) fed 3–9% of the live weight of hybrid grouper per day. This method required measurements throughout the trial to accurately alter the amount of feed offered with an increase in weight. By feeding a ration that is proportional to the fish's body weight, over feeding was prevented (Jiang et al., 2015). Feeding frequency and length of the experiment was highly variable. Both Ai et al. (2006) and Khosravi et al. (2015) fed twice daily to apparent satisfaction for 8 weeks and juvenile meagre were fed twice a day to visual satiety 5 days a week (Chatzifotis et al., 2012). In another trial, rainbow trout were fed twice a day to apparent satisfaction for

eight weeks (Eliason et al., 2007). Feeding experiments were mostly carried out for at least 8 weeks (Luo et al., 2004; Jiang et al., 2015; Rahimnejad et al., 2015). However, Lee et al. (2001) only carried out their trial for 6 weeks. The feeding trial should be sufficient to allow enough time for a significant increase in body weight. Increasing the length of the trial also helped eliminate any variability of the environment. This thesis was carried out for 12 weeks.

2.5.2. Stocking Rate

The number of fish per treatment group can be limited by the size of the cage and the size of the fish. Smaller fish allowed for a larger stocking rate; however, it was important to allow enough space for growth. Cages used in trials were floating sea cages, land-based flow tanks, or multiple small tanks kept within a larger tank. Ai et al. (2006) used floating sea cages (1x1x1.5m) stocked with 180 large yellow croaker that had an initial weight of 1.88g fish while Luo et al. (2004) stocked a slightly larger floating net (1.5x1x1.5m) with only 20 grouper, initially at 10.7g, and Jiang et al. (2015) stocked 22 hybrid grouper, initially at 4.68g, per floating cage (120x70x50cm). As well as floating sea cages it was also possible to do experiments in flow tanks kept on land based facilities, this is the system that was used in this thesis. Lee et al. (2001) kept 30 giant croaker, initial weight of 1.86g, in a 150 L flow tank while Chatzifotis et al. (2012) had 30 meagre, initially at a weight of 23.4g, in a 500 L tank. Despite the method used, triplicates of each group were carried out (Lee et al., 2001; Luo et al., 2004; Ai et al., 2006; Chatzifotis et al., 2012). The floating cages were exposed to natural environments, which made standardising and controlling environmental factors impossible. However, they were more representative of a farming situation. Indoor cages allowed for more control, which allowed many environmental variables to be removed from the trial. This means the protein requirements of fish could be determined without other factors influencing the results. In this thesis the fish were stocked 15 per tank and the tanks were kept on a land-based facility.

2.5.3. Anaesthetic

Anaesthetics are used to reduce the stress experienced by fish when they are removed from water for measurements or moving (from one tank to another). There are multiple marine anaesthetics that can be used on fish. These include AQUI-S® (iso-eugenol), clove oil (eugenol), metomidate (hypnotic agent with no analgesia), and Benzoak® (benzocaine). AQUI-S® is the anaesthetic that was used when the fish needed to be moved or measured in this thesis. Its active component is iso-eugenol, which is a plant based substance (AQUI-S, 2013a). It is an anaesthetic designed for fin fish that is diluted directly by the saltwater in the tank and can be used for a range of uses: light sedation, deep sedation, and partial anaesthetic. Fish have been

shown to have a fast recovery after use and is efficient at low quantities (AQUI-S, 2013a). This is seen in Atlantic salmon. Fish that were untreated with an anaesthetic during movement were shown to take over a week to recover while those administered with AQUI-S® recovered within 12 hours (Iversen & Eliassen, 2009). AQUI-S® has been shown to decrease the production of cortisol and lactate in Atlantic salmon compared to non-treated fish during transport (Iversen & Eliassen, 2009). AQUI-S® was also shown to reduce cortisol levels while Benzoak® did not (Iversen et al., 2003). AQUI-S® has also shown to reduce cortisol levels better than metomidate (Iversen et al., 2013). The metabolic rate of yellow perch is lowered through the use of AQUI-S® (Cupp et al., 2016). The use of AQUI-S® has shown to reduce mortality compared with the use of no anaesthetic when moving Atlantic salmon (Iversen & Eliassen, 2009). The use of AQUI-S® reduces the stress behaviours seen in non-treated fish which made handling easier and, in turn, reduced the handling time, the possibility of injury, and the chance of scale loss.

2.5.4. Digestibility Trials

Digestibility is determined by comparing the indicator concentration in the feed and in the faeces to a nutrient, such as protein or energy, as well as dry matter in the samples. The proportion of the nutrient to indicator remaining in the faeces compared with that of the diet is considered to be digested by the animal (Mach et al., 2010). This gives the digestibility of the nutrient. This method can be used to determine the digestibility of an overall diet or individual ingredients. The main digestibility method used in fish is the apparent faecal digestibility. Digestibility trials are commonly carried out in facilities based on the land as faecal collection is difficult in sea cages unless using a manual stripping technique or post-mortem collection to sample faeces. It is possible to measure ileal digestibility in other species, such as mammals and birds. However, in fish the intestine is not greatly differentiated so while a digesta sample can be taken from the intestine, it cannot be classified as an ileal sample. To determine the true digestibility of a diet the endogenous losses (enzymes, skin, etc. from the animal itself) need to be accounted for. Hossain et al., (1997) determined the endogenous losses by feeding a protein free diet to rohu. However, it does not allow for the full contribution of endogenous losses as enzymes involved in protein digestion are not produced without the presence of protein. True digestibility is not a method commonly used in fish. It is possible to do the apparent intestinal digestibility but the main method used is the apparent faecal digestibility.

2.5.4.1. Indicators

Indicators are used to determine the digestibility of feeds or ingredients. A known amount of indicator needs to be added to the diet. An indicator is an inert marker that is not digested by

the animal and is retrieved unaffected at the end of the digestion process. This can then be used to determine the apparent digestibility coefficient (ADC). One study used 100mg Y_2O_3 (yittrium oxide)/kg diet as an inert indicator to determine the apparent digestibility coefficients (ADC) (Mach et al., 2010). Another indicator, and the most commonly used in fish digestibility trials, is chromic oxide (Cr_2O_3). Khosravi et al. (2015) added 10g Cr_2O_3/kg diet while Eliason et al. (2007) added 5g/kg. Another indicator that can be used is titanium oxide. However, titanium oxide is not a free-flowing powder and can aggregate if not mixed into the diet thoroughly and can result in a lack of uniform distribution (Vandenberg & De la Noue, 2001). Once the indicator has been added to the diet in a known concentration it must be measured in the faecal or digesta sample.

To determine the digestibility coefficients the level of indicator needs to be measured in the faecal samples. Chromic oxide was measured via titration (Vandenberg & De la Noue, 2001). Another method to measure chromic oxide content is microwave acid digestion followed by flame atomic absorption spectrophotometry (Tibbetts et al., 2006). DPC (diphenylcarbazide) colorimetry can be used to measure chromic oxide using a 0.25% 1,5-DPC solution and 3N sulfuric acid in a spectrophotometer at 540nm (Divakaran et al., 2002). Titanium oxide is measured via a spectrophotometer after being boiled in 7.4M H_2SO_4 for 1 hour (Short et al., 1996). However, another method boiled the sample in 7.4M H_2SO_4 for 3 hours (Vandenberg & De la Noue, 2001). Hydrogen peroxide is then added to the samples. The hydrogen peroxide reacts with the titanium and causes the solution to turn orange which can then be measured by a spectrophotometer at 410nm (Short et al., 1996). Once the level of indicator has been determined it can be compared with the concentration of a certain nutrient to determine the digestibility using one of the calculations below.

ADC (%) for dry matter (DM) = 100 – (100 x % ind in diets x % ind in faeces^{-1}) (Mach et al., 2010)

ADC (%) for CP, CL, & GE = 100 – (100 x (% ind in diets x % nutrient in faeces) x (% nutrient in diets x % ind in faeces)$^{-1}$) (Mach et al., 2010)

(CP – crude protein, CL – crude lipid, GE – gross energy)

Protein Digestibility (%) = [1-((% CP in faeces / % CP in diet) x (% ind in diet / % ind in faeces))] x 100 (Eliason et al., 2007)

ADC = 1 – ((ind in diet / ind in faeces) x (nutrient content in faeces / nutrient content of diet)) (Khosravi et al., 2015)

As different indicators can be used it is important to determine if they all have the same efficiency when used in fish. Titanium oxide (5g/kg) resulted in higher ADC values than chromic oxide (5g/kg) and acid insoluble ash (10g/kg) in rainbow trout which had relatively similar results (Vandenberg & De la Noue, 2001). Another study, by Weatherup and McCracken (1998), found that titanium oxide (1g/kg) resulted in lower values compared with chromic oxide (3g/kg). However, results using chromic oxide were shown to be more repeatable than those using titanium oxide or acid insoluble ash (Vandenberg & De la Noue, 2001). Adding a mixture of indicators was shown to give a higher ADC when compared with that of the individual indicators (Vandenberg & De la Noue, 2001). However, this may not be practicable due to unknown interactions between the markers or the detection methods. These results indicate that chromic oxide is a better indicator than titanium oxide in fish. Chromic oxide will be used to determine the digestibility in this thesis.

2.5.4.2. Faecal Collection Methods

There are multiple methods to collect a faecal sample. An example of faecal collection methods include: the Guelph system, where faecal material passes from the tank (assisted by an angled tank bottom) into a vertical settling column (Figure 2.8); the St-Pee system, which uses an automatic faeces collection device; or manual stripping, where gentle pressure is applied to the ventral abdominal area of the fish to remove faeces via the anus (Black, 2000). It is also possible to collect digesta directly from the intestine post-mortem. Vandenberg and De la Noue (2001) tested three of the methods (Guelph, St-Pee, and manual stripping) by setting up three replicates per method and allowing a 5-day adjustment period before a 5-day collection period. The strip method was carried out twice a day, 60 minutes following feeding (Vandenberg & De la Noue, 2001). Samples from each treatment were pooled to measure digestibility (Allan et al., 1999; Eliason et al., 2007). Faeces collected via the Guelph system were centrifuged and the supernatant was discarded. All the samples were frozen for storage, freeze dried, and analysed for dry matter, ash, CP, lipid and energy content.

After feeding a diet containing an indicator it is important to allow a few days before faecal collection. This time allows the animal to adapt to the new diet containing the indicator and for the digesta, which does not contain any indicator, to be excreted. The acclimatisation period varies between studies. Mach et al. (2010) carried out the acclimatisation period of the digestibility trial for a week before sampling while Ai et al. (2006) collected samples after a 5-week trial to determine the apparent digestibility. Another study fed the indicator diet during the last week of the growth trial and for a further 2 weeks (digestibility trial) after the growth

trial had ended (Eliason et al., 2007). The faeces were stripped from anaesthetised fish on day 4 and 14 of the digestibility trial and was frozen until analysis.

Figure 2-6 Modified version of a Guelph faecal collection system (Velazquez & Martinez., 2005)

Short adjustments periods are also possible. One study carried out sample collections over three periods of 10 days with a 3 day acclimatization period and faecal collection for the next 7 days (Khosravi et al., 2015). Each tank of fish was exposed to three different diets (one every 10 days) providing three samples per diet, which could be pooled and tested. By feeding different diets to the same tanks, individual variance was minimised. Another study fed the test diets for at least 4 days before collection was started (Weatherup & McCracken, 1998). The adjustment period differs between studies but should be for at least 3 days to allow the indicator to pass through the gastrointestinal tract.

The faecal collection method used can result in a slight variation of the ADC. The stripping method resulted in the lowest ADC while the Guelph system resulted in the highest value (Vandenberg & De la Noue, 2001). The reason for this decreased value when stripping is thought to be due to several reasons including injury to the abdomen from repeated sampling, incomplete digestive processes resulting in wasted nutrients that would otherwise be utilised by the fish, and contamination of the samples from blood, urine, and gametes. However, stripping reduced the contact of the sample with water, therefore avoiding leaching of nutrients from the faeces (Ambardekar et al., 2009). Stripping was not an ideal method for small fish such

as silver perch that are less than 10g as it was difficult to get sufficient sample sizes (Allan et al., 1999). Faecal collection units can be useful in receiving larger sample sizes. However, unlike the manual stripping technique, it requires a special tank set up. ADC values for most nutrients, excluding lipids, when using the Guelph system were higher than that of a collection method (Vandenberg & De la Noue, 2001). This may have been due to leaching occurring from the faeces sitting in water for long periods of time. However, faeces collected through a settlement method in another trial showed similar digestibility values regardless of the time (2, 6, 12, or 18 hours) between sample collections (Espe et al., 1999).

As well as using faecal samples it is possible to determine digestibility values when using digesta removed from the intestine via dissection. Getting digesta from the posterior end of the intestine gave a higher digestibility than a sample taken from the anterior end (Allan et al., 1999). This is due to incomplete digestion of the feed at the beginning of the intestine. However, it can be difficult to remove digesta from the intestine and, depending on the size of the individual, can result in a small amount of sample. This method required the fish to be euthanized so only one sample per individual was possible. It is less commonly used than faecal collection methods.

2.5.5. Palatability

Palatability is influenced by both the gustatory system and the olfactory system. The feeding process is considered to start when the fish become aware of the feed and ends when the feed is either consumed or rejected (Kasumyan & Døving, 2003). Fish have been shown to learn from other fish. If more than one fish rejects an item of food, other fish are also likely to reject it (Schulte & Bakus, 1992). The amount of feed that is eaten and then rejected is often used as a determination of palatability as the feed comes in contact with taste buds before being rejected. Feed rejected before this is likely due to a lack of attractant in terms of smell. Amino acids, nucleotides, amines, sugars, etc. influence the palatability of a diet. A study by Schulte and Bakus (1992) showed that fish did not reject a piece of bread soaked in the aqueous extract of sponges, which have a natural deterrent, until it had first been placed in the mouth. As taste buds are found in the oral cavity, the pharynx and the oesophagus, this indicates the importance of feed intake to determine palatability (Kasumyan & Døving, 2003). Taste and texture were both shown to influence the ingestion of feed in Atlantic salmon (Stradmeyer, 1989). The time when feed was kept in the mouth before swallowing or rejection is considered the retention time. During this period, which is often quite short, the feed is analysed by recognising the taste substances to analyse palatability and make the decision to swallow or reject the food (Kasumyan & Døving, 2003). Some species, such as channel catfish, have been shown to also possess taste buds on their external surface. Stimulation of these external taste buds has been shown to assist with

grasping, biting and snapping behaviours, especially in fish with a low visual capacity (Kasumyan & Døving, 2003).

To determine the palatability of a diet, a fish's reaction needs to be observed and analysed. Juvenile Senegalese sole were observed for 10 minutes following the addition of feed to determine their reactions based on three definitions: (1) distant orientation, immediate reaction to feed entering the water and whether they will approach the feed (2) near orientation, whether the fish will eat or ignore the food once near the food particle, and (3) continuation of intake, does the fish go on to continue eating the new feed items (positive response) or expel the feed initially ingested (negative response) (Barroso, Rodiles, Vizcaino, Martínez, & Alarcón, 2013). Orientation is classified as the fish's reaction to the feed but without movement towards it. Similarly, Atlantic salmon were observed for one of five behavioural aspects: (1) orientation, (2) approach, (3) capture, (4) capture-rejection, and (5) capture-ingestion (Stradmeyer et al., 1988; Stradmeyer, 1989). The time taken for an animal to approach the feed can be used to determine palatability. Feed intake is shown to be highest in the first minute and decrease substantially after the second minute. Rainbow trout will increase feed intake in the first minute after a few days of being fed the diet (Oikawa & March, 1997). Feeding a diet with high palatability will increase feed intake and decrease feed wastage.

The experimental diets fed in the growth trial of this thesis have differing levels of protein. It is possible that because of this, the palatability will differ. A fish's taste buds are sensitive to free amino acids in the diet; this indicates that free amino acids are a strong attractant to fish (Kasumyan & Døving, 2003). It has been shown, in a study comparing 32 fish species, that unessential amino acids have a higher attractiveness in terms of taste than essential amino acids (Kasumyan, 2016). A lower level of protein in the diet will likely have a lower amount of free amino acids, which in turn could result in a less palatable diet and decreased feed intake.

2.5.6. Environmental Measurements

2.5.6.1. Water Temperature

Water temperature can be reliant on the natural temperature of the seawater or be controlled by heating and maintained at a constant temperature. Facilities that control water temperature should keep the temperature close to the natural environmental temperature for the species. The temperature of sea cages will be dependent on the location and the season. Water temperature can affect the feed intake of fish. This is seen in Baltic salmon, where a drop of 4°C resulted in a decrease in energy intake by over half (Koskela et al., 1997). Therefore, it ideal to maintain a constant temperature across tanks and throughout the experiment, if possible, to

minimise the effects on feed intake. In this thesis the water temperature will be manually controlled.

2.5.6.2. Oxygen

Dissolved oxygen (DO) concentration (% or mg/L) in water is an important measurement as it can be used to determine water quality. Feeding when the oxygen level is low should be restricted as feeding will further decrease water quality through increased carbon dioxide and ammonia production as well as pollution from the feed. A moist diet can increase water pollution more so than feeding a pellet diet as it dissociates in the water. When oxygen levels are high (>80%) feed can be administered to apparent satisfaction. In land-based facilities, the use of an aeration device (e.g. airstone or low pressure blower) is often used to increase the DO (Lee et al., 2001; Yigit et al., 2006). The AQUI-S® airstone diffusers are made of silica glass and produce medium air bubbles, which prevent irritation of fish gills (AQUI-S, 2013b).

2.5.6.3. Photoperiod

Depending on the facility, the photoperiod the fish are exposed to will be natural or artificially determined. Those facilities able to control the light period tend to use fluorescent lighting and maintain a 12:12 hour light:dark photoperiod (Lee et al., 2001; Jiang et al., 2015; Rahimnejad et al., 2015). Facilities reliant on natural photoperiod are influenced by geographical location and season.

2.6. Physiological Measurements

2.6.1. Weight Gain

Weight gain is measured by the weight difference at the beginning and at the end of an experiment (Calculation One). Fish are often starved for 24 hours before weighing (Watanabe et al., 2000) to allow the digestive tract to empty, which reduces handling stress (Chatzifotis et al., 2012). It also allows for the body weight independent of feed intake and gut fill to be determined. Weight gain can also be expressed as a total weight gain (Calculation One) or as a percentage of the starting body weight (Calculation Two). If feeding according to the body weight, weighing fish multiple times allows the feed allotment to be adjusted accurately. Jiang et al. (2015) weighed grouper weekly during an 8 week experiment while Lee et al. (2001) weighed their fish every 2 weeks. Fish can be weighed at various points throughout the experiment to determine if fish growth is linear and if there is any variation due to water temperature, season, size, etc. Multiple weigh-ins allow for a more accurate average daily growth to be calculated. However, it could also affect growth due to the stress of handling. Fish also tend to have lowered feed intake the day before and the day of the moving.

Calculation One

Weight gain (g) = final mean wet mass (g) – initial mean wet weight (g) (Eliason et al., 2007)

Calculation Two:

Weight gain (%) = (final weight – initial weight) x 100/initial weight (Ai et al., 2006)

Weight gain is one of the main factors used to determine the protein requirement of different fish species. This is due to weight gain being highly influenced by the protein level of the diet. Growth rates were highest at 45% dietary protein in juvenile giant croaker (Lee et al., 2001), hybrid grouper juveniles (Jiang et al., 2015; Luo et al., 2004), and Indian and Chinese major carp (de Silva & Gunasekera, 1991). Hybrid grouper had higher levels of weight gain when fed a diet containing 50% CP (Rahimnejad et al., 2015) as did juvenile red porgy (Schuchardt et al., 2008). The decreased weight gain occurring at lower levels of protein are likely due to a decrease in available amino acids (Jiang et al., 2015). Weight gain may not improve above a certain level of dietary protein due to the genetic restrictions of the fish. Once the maximum protein deposition is reached, the amino acids will be used for energy, lipid synthesis or excreted unused. Feeding at a protein level that results in the highest level of weight gain, has no additional benefit. Protein sources, such as fishmeal are expensive, so adding additional protein will only increase costs and will not be beneficial. However, in some species feeding above the optimum protein level resulted in a decrease in weight gain. This was seen in hybrid grouper (Rahimnejad et al., 2015). The differences in protein requirements indicate how important it is to determine the protein requirements of individual species.

The combination of protein and energy also influences the weight gain. Weight gain of juvenile masu salmon increased with increasing levels of dietary protein (40–50%) and energy (Lee & Kim, 2001). However, when comparing protein level at the same energy level, the protein concentration had no effect on weight gain. Increasing the dietary protein level of juvenile bluefin trevally resulted in increased growth (Suprayudi et al., 2014). Based on this the optimum protein level for bluefin trevally was found to be 45% protein when fed with an energy:protein ratio of 9kcal GE/g protein. However, this study did not feed a diet higher than 45% so the true protein requirement may be higher than this value. However, if feeding low levels of protein, growth can be improved by increasing the energy content of the diet (Suprayudi et al., 2014). This is likely due to a change in the composition of the weight gain. An increase in lipid deposition is likely occurring due to the maximal protein synthesis having been reached due to the limited

amino acids. As well as understanding the amount of protein required the level of energy may also be required.

2.6.2. Specific Growth Rate

Specific growth rate (SGR) is a log measurement of growth. It is calculated using one of the equations in calculation Three. SGR is influenced by the level of protein in the diet. SGR was highest in meagre fed a diet containing 50 and 54% protein compared with diets containing lower levels of protein (Chatzifotis et al., 2012). Juvenile groper (Luo et al., 2004) and red porgy (Schuchardt et al., 2008) were shown to require 45% protein to optimise SGR. While 50% protein was required to optimise SGR in hybrid grouper Rahimnejad et al. (2015), brown-marbled grouper (Shapawi, Ebi, Yong, & Ng, 2014), and Mediterranean yellowtail (Jover, García-Gómez, Tomás, De la Gándara, & Pérez, 1999). However, SGR in rainbow trout was largely unaffected by the CP (Eliason et al., 2007). Overall, SGR is influenced by the protein levels in the diet making it a good determinant of the optimum protein requirement.

Calculation Three:

SGR = ln(final body weight – initial body weight)/days of experiment x 100 (Chatzifotis et al., 2012)

OR

SGR = (ln final weight – ln initial weight) x 100/days of experiment (Ai et al., 2006)

OR

SGR (%/day) = ((ln final weight (g) – ln initial weight (g))/days of experiment) x 100 (Eliason et al., 2007; Schuchardt et al., 2008)

2.6.3. Feed Efficiency

The ability of an animal to use feed can be represented by the feed efficiency ratio (Lee et al., 2001) or feed conversion ratio (FCR) (Chatzifotis et al., 2012). Determining the feed efficiency of a diet indicates how effectively the diet is used by that particular species. The FCR is the amount of feed required for one gram of weight gain (Calculation Four). Meagre were found to have improved FCR when fed a diet containing 50% protein (Chatzifotis et al., 2012), as were red porgy (Schuchardt et al., 2008). Hybrid gropers were shown to have higher FCR at 40% (Jiang et al., 2015). Above this level of protein, the FCR did not differ significantly among the diets so other factors may need to be considered to determine the overall protein requirement. The FCR of

grouper was found to be lowest in fish fed the 45%, 50% and 60% diets (Luo et al., 2004). Feed efficiency is the weight gain per one gram of feed intake (Calculation Five). Feed efficiency was shown to be improved in the giant croaker when fed a diet of at least 45% protein (Lee et al., 2001). Rahimnejad et al. (2015) found that feed efficiency significantly improved with an increase in dietary protein in juvenile hybrid grouper. A poor feed conversion ratio in fish fed a low protein diet is likely due to an unbalanced amino acid profile. This is seen in juvenile groper and resulted in poor feed conversion (Jiang et al., 2015). Optimising feed efficiency for individual species will reduce the amount of feed required to be fed out and the cost spent on feed.

Calculation Four:

FCR = feed consumed (g) / weight gain of the tank (g) (Chatzifotis et al., 2012; Schuchardt et al., 2008)

Calculation Five:

Feed efficiency ratio = weight gain (g)/feed intake (g) (Eliason et al., 2007; Glencross, Booth, & Allan, 2007)

Some experiments measured the effect of both protein and energy level, which can influence the feed efficiency ratio. The feed efficiency ratio of masu salmon increases with increasing levels of dietary crude protein and energy (Lee & Kim, 2001). However, as seen with weight gain in the masu salmon, this increase was not seen with changes to protein only (i.e., different levels of protein in iso-energetic diets). The bluefin trevally was shown to have decreasing feed efficiency with decreasing protein (Suprayudi et al., 2014). The effect of energy to protein ratio varied and had no consistent effect.

2.6.4. Protein Efficiency Ratio

Protein efficiency ratio (PER) is used to determine how well the dietary protein is used by the fish for growth (Calculation Six). PER is influenced by the protein level in the diet. PER did not improve in giant croaker fed a diet containing more than 45% CP (Lee et al., 2001). PER in red porgy was found to be maximised when fed a diet containing 45% protein (Schuchardt et al., 2008), while in brown-marbled grouper PER was highest when fed 50% protein (Shapawi et al., 2014). PER was unaffected by protein level at the same energy level in masu salmon. However, feeding the same dietary protein at a higher energy level resulted in a higher PER (Lee & Kim, 2001). Below the optimum protein level PER is reduced, which indicates that the protein level is not sufficient to meet the requirements for protein synthesis.

PER was found to be lowest in groper fed 55 and 60% CP (Luo et al., 2004). In the hybrid groper the PER decreased as the crude protein level increased also (Jiang et al., 2015). PER is shown to be decrease with an increase in dietary protein in rainbow trout (Eliason et al., 2007). This indicates that feeding a high level of protein does not increase the utilisation of the dietary protein. It may be more cost effective to add a lower amount of protein to the diet. A decreased PER when fed high levels of protein indicates that dietary protein is being wasted. The protein supplied in the diet is above that required for protein synthesis and the excess is not being utilised for protein synthesis. When paired with a high growth rate, it is possible that the increased weight gain is due to lipid deposition over protein deposition. Optimising PER allows for growth to be optimised without wasting protein in the feed.

Calculation Six:

PER = weight gain (g) / protein consumption (g) (Eliason et al., 2007; Schuchardt et al., 2008)

Protein retention is determined by comparing the protein content of the fish before and after being fed the experimental diets in a feeding trial (Calculation Seven). Protein retention increased with protein content in the diet of the bluefin trevally (Suprayudi et al., 2014). The amount of protein deposition (Calculation Eight) can be used to determine how much of growth is due to protein and not lipid. Protein deposition has been shown to be inversely related to protein content in rainbow trout, as dietary CP increases protein deposition decreases (Eliason et al., 2007). By determining the protein retention of the animal it is possible to determine how much of the protein in the diet is going towards muscle growth and not being stored as energy in adipose.

Calculation Seven:

Protein retention = (final weight x protein content in final fish body – initial body weight x initial protein content) / (feed intake as DM x protein content of diet) (Ai et al., 2006)

OR

Protein retention = (final protein content – initial protein content) (protein offered)$^{-1}$ (Espe et al., 1999)

Calculation Eight:

Protein deposited (%) = (protein gained (g) / total protein consumed (g)) x 100 (Eliason et al., 2007)

2.6.5. Body Condition Indices

2.6.5.1. Condition Factor

Condition factor (CF, Calculation Nine) can be measured as a way to determine the effect of the diet on the condition of the fish (Jiang et al., 2015). It is a ratio between the weight and the length of the fish (similar to that of the body mass index, BMI, in humans). In Mediterranean yellowtail, feeding 50% CP increased the CF compared with a diet containing 45% protein (Jover et al., 1999). Feeding 35% CP diets in juvenile groper significantly decreased the CF (Luo et al., 2004). This is likely due to insufficient protein for growth let alone to be converted into energy stores. CP level had no effect on CF in juvenile red spotted grouper (Wang et al., 2016), Rainbow trout (Eliason et al., 2007), and juvenile grouper (Jiang et al., 2015). The effect of the dietary protein level on CF is highly variable.

Calculation Nine:

CF = (body weight (g) x 100)/total length^3 (cm) (Jiang et al., 2015; Lee et al., 2001)

CF = ((body weight (g)/length (cm)^3) x 100) (Eliason et al., 2007)

2.6.5.2. Hepatosomatic Index

The hepatosomatic index (HSI) is used as a way of determining the body reserves a fish possesses in the liver, in particular glycogen and lipid. This is achieved by determining the contribution of the liver weight to the live weight of the fish (Calculation Ten). As this requires euthanasia and a dissection, a representative sample of fish in a tank is used to calculate the HSI. Chatzifotis et al. (2012) weighed the livers of five fish per tank while Jiang et al. (2015) sampled three fish per tank, and Shapawi et al. (2014) 10 fish per tank. A sample of the liver can also be taken at this time, after weighing, to determine its proximate composition (Jiang et al., 2015).

Calculation Ten:

HSI = (Liver weight (g)/Live weight (g)) x 100 (Chatzifotis et al., 2012; Eliason et al., 2007; Jiang et al., 2015)

Feeding a diet with 50% CP resulted in higher HSI values in juvenile giant croaker (Lee et al., 2001), and Mediterranean yellowtail (Jover et al., 1999). HSI was unaffected by the protein level in the diet fed to juvenile meagre (Chatzifotis et al., 2012) as well as brown-marbled grouper (Shapawi et al., 2014), and juvenile groper (Luo et al., 2004). A lower level of protein (40 and 45%) has shown to result in a higher HSI than diets higher in protein (50 and 55%) in hybrid groper (Jiang et al., 2015). Bluefin trevally (Suprayudi et al., 2014) and red spotted grouper (Wang et al., 2016) also have decreased HSI with increased levels of protein. HSI is likely high

when fed low levels of protein due to the increase in dietary carbohydrates, which results in increased levels of glucose and, therefore, glycogen.

2.6.5.3. Intraperitoneal Fat

Intraperitoneal fat (IPF) is measured by dissecting and measuring all fat found in the abdominal cavity, including that attached to the intestines (Calculation Eleven). Lee et al. (2001) sampled five fish per tank to determine the IPF while Jiang et al. (2015) sampled three.

Calculation Eleven:

IPF ratio = (IPF weight/body weight) x 100 (Jiang et al., 2015)

OR

IPF ratio = (IPF weight x100) / body weight (Lee et al., 2001)

The effect of dietary protein on IPF is variable in different fish species. Grouper fed a diet containing 40% protein resulted in significantly higher levels of intraperitoneal fat than those fed 55% (Jiang et al., 2015). This is likely due to the increased carbohydrate content of the diet (corn starch) being hydrolysed to glucose, which in turn was used to synthesise lipid (Jiang et al., 2015). IPF was not significantly affected by the CP content of the diet in juvenile giant croaker (Lee et al., 2001) and juvenile red spotted grouper (Wang et al., 2016). Protein likely has little influence on IPF as dietary lipid is the main influencer.

2.6.5.4. Viscerosomatic Index

Viscerosomatic index (VSI) can be used to determine what percentage of the body weight is contributed to by the weight of the visceral organs (calculation Twelve). The viscera needs to be dissected from the abdominal cavity and weighed (Luo et al., 2004). Shapawi et al. (2014) sampled 10 fish per tank while Mach et al. (2010) sampled five to determine the VSI of each diet.

Calculation Twelve:

VSI (%) = 100 x viscera weight (g) x whole body weight^{-1} (Mach et al., 2010)

VSI = 100 x (Viscera weight/body weight) (Luo et al., 2004; Shapawi et al., 2014)

Dietary protein has varying effects on the VSI in different species. Juvenile grouper fed 35% CP had a significantly reduced VSI compared with those fish fed a higher level of protein (Luo et al., 2004). VSI was highest in brown-marbled grouper fed 45% protein and above (Shapawi et al., 2014). This is likely due to sufficient levels of protein in the diet to contribute to organ development. However, in juvenile red spotted grouper VSI decreased with an increase in

protein content of the diet (Wang et al., 2016) and VSI was unaffected by the protein content of the diet in Mediterranean yellowtail (Jover et al., 1999).

2.7. Chemical Analysis

After the experiment whole body analysis: ash, moisture, protein, and lipid content (Watanabe et al., 2000). Whole body analysis is often determined by grinding down the fish to a homogenized mixture. Initial body compositions should be performed as a comparison.

2.7.1. Body Composition Analysis

A sample of fish, from the same population the experimental fish are coming from, should be taken to analyse the initial body composition. Wang et al. (2016) sampled 30 fish for initial analysis, Khosravi et al. (2015) 20 fish, and Rahimnejad et al. (2015) and Lee et al. (2001) both sampled 10 fish. The body composition of a selected number of fish per tank should also be analysed at the end of the experiment. The body composition includes the overall protein, lipid, moisture, and ash content of the body. Lee et al. (2001) and Chatzifotis et al. (2012) sampled 5 fish per tank, Jiang et al. (2015) sampled two fish per tank, and Khosravi et al. (2015) three fish. To determine the overall body composition, the fish needs to be homogenised. This can be achieved using a blender or a stick blender. However, this can be difficult for fish that have a large amount of bone and cartilage structures, like the trevally. Autoclaving prior to homogenisation minimised the time required to prepare samples by softening bony structures and produced results similar to drying before homogenisation and homogenisation before drying (Glover, DeVries, Wright, & Davis, 2010). Once the sample has been homogenised it can then be analysed.

2.7.1.1. Moisture and Ash

Moisture and ash are two components of the overall body composition that are analysed. Moisture content of the fish is determined by drying a sample at 105°C (Ai et al., 2006) or 90°C (Chatzifotis et al., 2012) until the weight becomes constant, i.e., no further water is being lost. Ash is measured by burning a sample at 600°C for 7 hours (Chatzifotis et al., 2012). Ash is made up of inorganic matter, such as minerals. The protein level had little to no impact on the moisture and ash in grouper (Jiang et al., 2015; Luo et al., 2004) and the red spotted grouper (Wang et al., 2016).

2.7.1.2. Lipid

The overall lipid content of the body is influenced by the level of protein in the diet. Feeding a diet containing 50% protein to meagre resulted in the highest level of lipid in the overall body

composition of the fish; this was considered to be the protein requirement (Chatzifotis et al., 2012). The increase in lipid content seen in meagre indicates that the faster growing fish tend to have higher levels of lipid deposition. The increase of body lipid levels coincides with a decrease in overall water content (Chatzifotis et al., 2012). This inverse relationship between moisture and lipid deposition was also seen in hybrid grouper (Rahimnejad et al., 2015), and juvenile giant croaker (Lee et al., 2001). Malabar grouper were shown to have the highest level of lipid deposition when fed the highest CP diet (Tuan & Williams, 2007). Primarily amino acids are used for protein synthesis and growth. However, if the amino acids present in the diet are in excess they can be catabolised for energy. This energy can then be used for other bodily processes. Once the energy requirements of the animal have been met the left-over amino acids are converted into lipids to be used as body stores. However, increasing protein content in juvenile groper decreased the lipid content of the body (Luo et al., 2004). Carbohydrates can be stored as lipids like proteins but the conversion process from carbohydrates is less energetically expensive. An increased carbohydrate content of a diet may result in more lipids to be formed at the same dietary energy level than in diets higher in protein. In most fish, the lipid content of the body increases with increasing dietary protein.

2.7.1.3. Protein

The amount of protein present in the body depends on the dietary protein level. Grouper fed 60% protein had the highest overall protein and water content, g/kg DM (Luo et al., 2004), while the overall protein content of red spotted grouper was highest when fed 56 and 62% CP (Wang et al., 2016). These species showed a linear relationship between body protein content and dietary protein. However, this was not seen in all species. In juvenile malabar grouper, protein was highest when fed the lowest level of protein (Tuan & Williams, 2007). Furthermore, the body's protein content of other species, such as the giant croaker (Lee et al., 2001), hybrid grouper (Jiang et al., 2015), and the brown-marbled grouper (Shapawi et al., 2014) were unaffected by the level of protein in the diet.

2.8. Plasma Biochemical Analysis

To determine the biochemistry of the plasma, blood samples are required. Three fish per tank were bled from the caudal vasculature (Jiang et al., 2015). In another experiment six fish per tank were used and a blood sample was taken from the caudal vein (Wang et al., 2016). Blood samples then need to be centrifuged to remove the plasma for analysis. Protein, lipid and glucose are able to be measured in the plasma. Plasma protein levels were shown to be highest

in fish fed the middle levels of protein (Wang et al., 2016). However, in hybrid grouper protein levels in the plasma were unaffected by the amount of dietary protein (Jiang et al., 2015).

Chapter Three

3. Materials and Methods

3.1. Fish

The silver trevally used in this experiment were hatched on site from Plant & Food Research's (PFR) broodstock eggs. Once the fish reached 150mm in length (152.57±6.30 mm, mean±SD), 180 were selected and placed in 12 tanks, 15 fish per tank (Image One). In each tank eight fish were tagged while the other seven remain untagged. The tags used were AVID DNAChip (disposable needle assembly). By leaving some untagged it was possible to determine if the tag/tagging process affected the fish. AQUI-S® was used to move and measure fish. AQUI-S® antifoaming solution is also added (a few drops). The addition of AQUI-S® decreases stress and makes handling easier. The acclimatisation period allows the fish to become adjusted to the experimental system and was carried out for 4 1/2 weeks. During this time, they were fed 25g/day of a commercially available diet (3mm Ridley). Throughout the experiment any dead fish were removed from the tank before feeding.

Figure 3-1 12 800L tank set up for the experiment. Tank lay out: B8 is the top right hand tank, B9 the top left hand tank through to B19 the bottom left-hand tank

3.2. Tanks

The experimental system was comprised of 12 800 L tanks (Image 3.1) filled with filtered, sterilised seawater. Seawater is collected in a sea water well, which fills with the tide, before being pumped into the facility. The water is first filtered by passing through a set of 5µm bag filters, a set of 5 µm cartridge filters, and a set of 1 µm cartridge filter. Once the seawater has been filtered, it is sterilised using a UV system before being collected in a 7000L silo where the water temperature was controlled at around 21°C using heat exchangers. However, as the ambient water temperature decreased the silo was unable to maintain the temperature, this resulted in a drop of temperature in the experimental tanks (Figure 3.1). Three temperature monitoring devices were used to determine the temperature every 30 minutes. They were placed in with 30a, 40b, and 50c on the airstone hose, the 30a monitoring device stopped measuring in April. Temperature of the seawater averaged 20.5±0.92°C. Each tank had an

airstone to provide aeration and keep oxygen levels at a satisfactory level. The oxygen concentration of each tank was measured prior to each feed and averaged 101±2.6% and 9.2±0.34 mg L^{-1}. The tanks were arranged in two lines of seven (two extra tanks used when moving fish during weighing) in a tunnel house. The tunnel house allows the tanks to be exposed to the ambient photoperiod while protecting them from wind, rain, and predators.

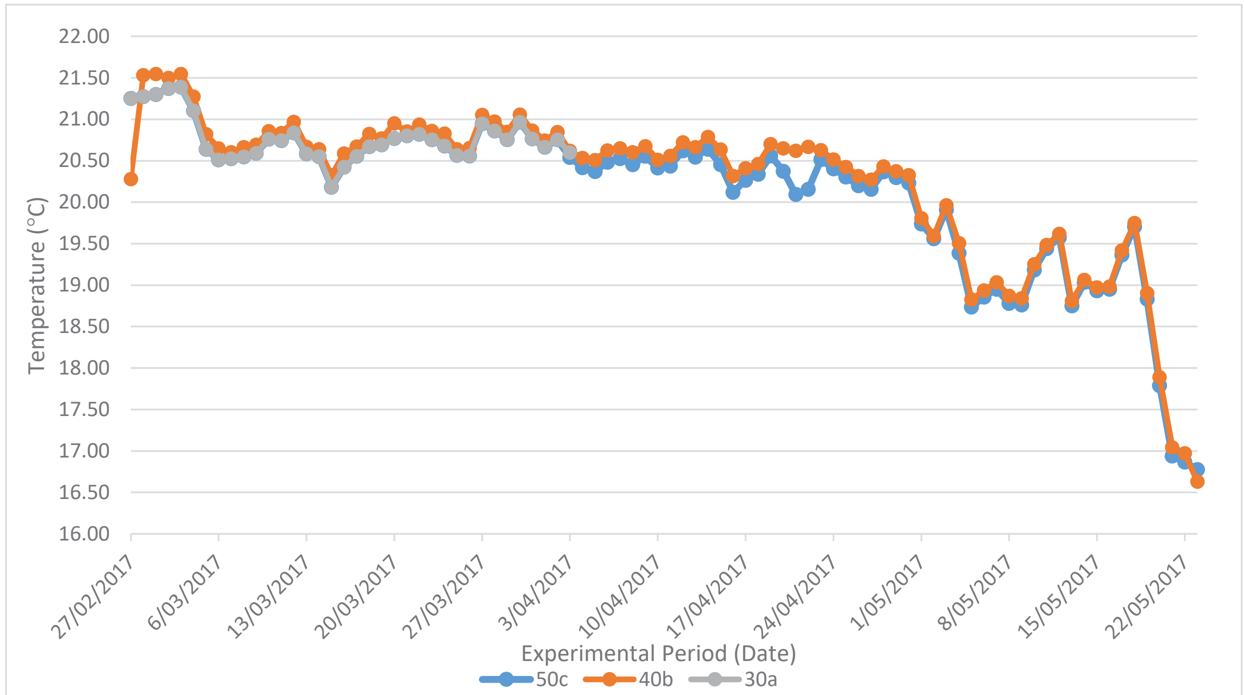

Figure 3-2 Water temperature in the experimental tanks throughout the experimental period

Prior to use the tanks were chlorinated to sterilise them. Chlorine was added at around 160–200 g to each tank filled with fresh water and left over night. In the morning the tanks were neutralised using sodium thiosulfate at a dose double that of the chlorine added. The tanks were then drained and filled with seawater. Once the tank was filled with seawater the flow of fresh seawater into the tank was determined by filling a 2 L jug in 15 seconds from the spout (Image 3.2). The flow was set to 8 L min^{-1} at low tide. This ensures the minimum flow rate. However, the tanks can lose flow for an hour or two after low tide due to the drop in pressure in the silo. The air stones help oxygen content stay normal until flow returns. Each tank was covered by mesh (held down by bungee cord) as a precaution to prevent fish from jumping out of the tank (Image 3.3). Tanks are cleaned every 2-3 days, as needed. Tanks were scrubbed, carefully as not to frighten the fish, and the air stone hose (Image 3.3) was wiped with a cloth. Tanks are also purged daily, the drain is opened for 5 minutes to allow water to flow through and rinse the

pipes. Purging the tanks removed less than a quarter of the tank's water. However, the tanks had a failsafe on them so the tanks could not be drained completely.

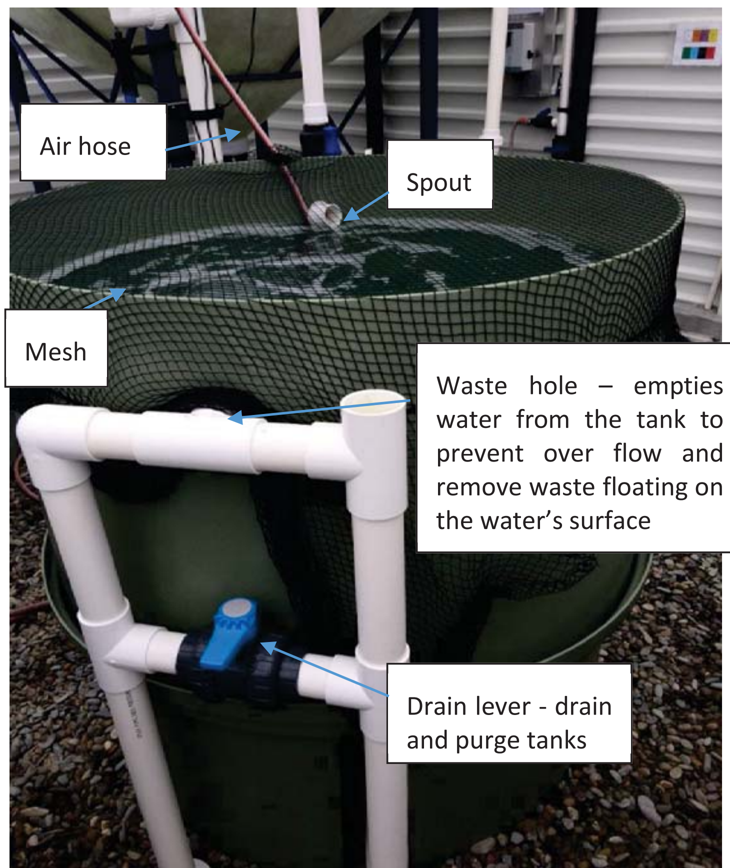

Figure 3-3 Set up of single 800L tank

3.3. Experimental Diets

3.3.1. Ingredients

3.3.1.1. Fish Meal

Fish meal was sourced from Sanford. Five 30kg bags were ordered but all came from different ships or were made on different days. Therefore, to get a consistent composition all the bags were mixed to form a homogenous mixture. Composition was calculated at the Cawthron Institute in Nelson who were contracted to carry out the measurements. Ash, moisture, total fat, and crude protein (Table 3.2.) was measured using AOAC methods and energy and carbohydrates via calculations.

3.3.1.2. Fish Oil

Fish oil was supplied by Talley's. The Cawthron Institute calculated the energy, lipid content, crude protein, moisture and ash (Table 3.2) using AOAC methods and calculations. The lipid

composition was measured using gas chromatography and mass spectrophotometry (Table 3.1) by the Marine Products Group at PFR.

Table 3-1 Fatty acid profile of fish oil used in the experimental diets (% area is the area each substance is represented on the graph output).

Fatty Acid	**Amount (% area)**	**Fatty Acid**	**Amount (% area)**
Pristane	0.4	22:6n-3c (DHA)	11.2
14:0	2.9	22:5n-3c	1.3
15:0	0.4	22:1-A	1.7
16-1-OH	1.1	22:1-B	0.5
16:1-A	0.3	24:1	0.4
16:1n-7c	5.8		
16:0	16.6	Saturated	24.3
16:0-1,1-dimethoxy	0.2	Monosaturated	47.1
16:1n-10-7-methyl	0.5	Polyunsaturated	25.7
16:0-14-methyl	0.2	Omega-3	13.9
17:0	0.4	Omega-6	5.0
Unknown	0.5		
18:4n-3c	0.5	Triacylglycerides	86±0.2
18:2-A	0.5	Wax and sterol esters	13±0.2
18:1n-9c	1.1	Sterols	1±0
18:1n-7c	30.3	Monoacylglycerides	1±0
18:0	4.0	Polar Lipids	1±0.1
20:4n-6c (AA)	4.6	Free fatty acids	0
20:5n-3c (EPA)	1.0		
20:3	6.8		

20:1n-9c	7.0	

3.3.2. Trial to Determine Diet Form

PFR does not possess feed processing equipment so alternative methods were required. The first method used to manufacture the diets was freeze drying. First the diet needed to be made into pellets. To determine the best method to make the diet in to a pellet two methods were used. The first was a meat mincer (Everest TCE 32 2000) and the second a sausage maker (Image 3.4a). A sample of the 30% CP diet was made up to compare the different methods. Enough water was added to the mix to reach a consistency that would go through the mincer and hold its shape. The mincer set up required 2 blades, 3 dies (pierced disks) and an auger (screw conveyor). The dies were arranged so feed was forced through the die with the largest diameter first and continued until the desired size was reached, (8 mm; Image 3.4c). Samples of the strands of feed exiting the dies were collected whole or were roughly broken up or chopped (Image 3.4b). The sausage maker diameter is around 10mm and was used without any casing. The diet required an addition 60mL of water to go through the sausage maker than through the mincer. Like before some were left whole while others were broken up. These were placed in the second freeze dryer tray (Image 3.4b). The trays were then placed in a plastic zip lock bag and placed in the -40°C freezer until freeze dried.

Figure 3-4 a) Sausage maker b) Sausage pellets left tray and mincer pellets right tray c) Mincer with 8mm pierced disk on

To freeze dry they were placed in the freeze drier (VirTis AdVantage Freeze Dryer) at -40°C for two hours, -20°C for 4 hours, 20°C for 3 hours, and 15°C until removed from the freeze drier. The freeze dried (FD) samples were placed in zip locked bags (Image 3.5.). The level of water added to feeds before pelleting had no effect on the freeze drying process. Then a small handful of the diet was place in the tanks to determine how it would react in the water and the fish's reaction to it. The diet floated for a couple of seconds before sinking slowly. The fish were interested in the feed. The mincer pellets were less favoured likely due to the pellets being too long. The sausage pellets diameter was too large for the fish to eat at this stage. However, crushing it into smaller pieces increased the consumption by the fish.

Figure 3-5 FD 30% CP pellets a) Mincer pellets b) Sausage maker pellet

A sample of 60% CP diet was passed through the mincer using a 10mm diameter die. The pellets were then placed in the -40°C freezer until freeze drying. The product was freeze dried using the same programme as before (-40°C for 2 hours, -20°C for 4 hours, 20°C for 3 hours and 15°C until the end). The diet made with the largest diameter die resulted in a FD strand that could be crumbled into the water for feeding. It appeared to float more than the 30% CP trial pellet but the fish swam to the top of the water to eat it.

As a comparison some of the mixture that was not freeze dried was fed to the fish. The wet mix resulted in increased feeding behaviour of the tank, which indicated that the feed may have been more palatable before it was FD. By not freeze drying the diet the time required to make the diets was decreased. While a freeze dried diet would have had a longer shelf-life the combination of increased palatability and decreased time to manufacture the diets resulted in the decision to feed the diet as a wet mix.

3.3.3. Final Diets

The experimental diets were made to have differing levels of protein while remaining iso-energetic. The diets were made iso-energetic through the addition of corn starch. The experimental diets were made up in batches of the dry ingredients. The dry ingredients were

mixed in a mixer and kept in plastic bags. When making feed the bags of dry ingredients were shaken to redistribute the contents. The appropriate amount was mixed with the corresponding fish oil and water in a Kenwood food processor. The water content varied between diets (Table 3.2) in order to reach an ideal consistency. These diets were sent to the Massey University Nutrition Lab for analysis.

Table 3-2 Composition and proximate analysis of the diets

	Diets			
Nominal Protein Fraction	**30**	**40**	**50**	**60**
Ingredients (g/kg)				
Fishmeal[1]	489.9	644.6	763.6	890.9
Fish oil[2]	121.8	109.3	100.4	89.1
Corn starch[3]	377.8	235.6	125.5	9.5
Vitamin mix[4]	4	4	4	4
Mineral mix[5]	1.5	1.5	1.5	1.5
CMC[6]	5	5	5	5
Water[7]	50%	60%	70%	70%
Proximate Composition (% of wet diet)				
Moisture	39.9	42.8	45.5	45.1
Ash	6.5	7.8	8.7	10.1
Crude lipid	10.5	10.4	9.6	9.4
Crude protein	22.6	27.7	30.9	36.0
Crude fibre	0.3	0.1	0.2	0.1
GE (kJ/g)	13.1	12.4	11.9	11.9
Proximate Composition (% of dry weight)				
Ash	10.8	13.6	16.0	18.4
Crude lipid	17.5	18.2	17.6	17.1

Crude protein	37.6	48.4	56.7	65.6
Crude fibre	0.5	0.2	0.4	0.2
GE (kJ/g)	21.8	21.7	21.8	21.7

[1]fish meal sourced from Sanford. Proximate composition (/100g): GE 1440kJ, crude protein 67.8g, lipid 7.9g, ash 19.1g, moisture 5.9g.

[2]fish oil sourced from Talley's. Proximate composition (/100g): GE 3680kJ, crude protein 0.2g, lipid 99.1g, ash <0.1g, moisture 0.3g.

[3]corn starch sourced from NZStarch.

[4]vitamin premix, sourced from Hawkins Watts, contains (mg/kg or IU/kg of premix): vitamin A 4332IU, vitamin D3 2000IU, vitamin D3 220mg, vitamin B1 27.5mg, vitamin B2 33.2mg, vitamin B3 199mg, vitamin B5 58.8mg, vitamin B6 27.78mg, vitamin B12 0.135mg, vitamin B9 9.9mg, biotin 1mg, & inositol 250mg.

[5]mineral premix, sourced from Hawkins Watts, contains (mg/kg or g/kg of premix): zinc 75mg, iodine 5mg, copper 10mg, manganese 20mg, selenium 1.6mg, & calcium 1.448g.

[6]carboxy methyl cellulose, sourced from Hawkins Watts.

[7]water is added as a percentage based on the weight of the dry ingredients (including fish oil)

3.4. Experimental Procedure

The fish used in the experiment were randomly distributed into 12 experimental tanks. This allowed for triplicates of each diet, triplicates were labelled a, b, and c within each protein level. The fish were placed in the experimental tanks 36 days prior to the experiment starting, this was the acclimatisation period. The 12 tanks were filled over a 5-day period and, the acclimatisation period was defined as the day the last fish were moved into the tanks. During the moving period 8 out of 15 fish were PIT tagged. The tag was placed in the abdomen on the left side of the fish, just before the pectoral fin (Image 3.6) using a tagging gun (Image 3.7a). The needle comes in a sterilised container with a sticker of the tag's ID number (Image 3.7b). A new needle was used for each fish.

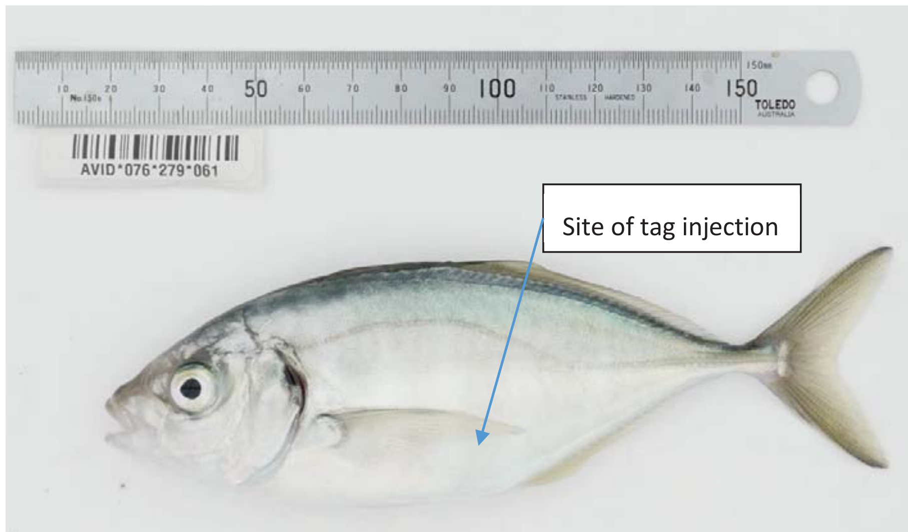

Figure 3-6 Screenshot of the video of a to be tagged fish being moved

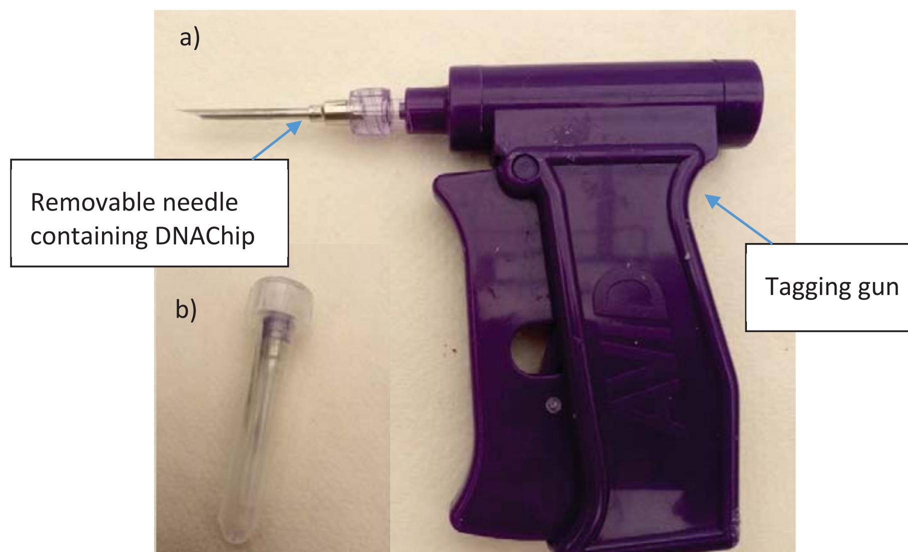

Figure 3-7 a) AVID tagging gun with needle attached b) Packaged sterilised needle with ID sticker

During the move the fish were videoed to record the weights. This allows images of individual fish to be captured from the video. The images were then input into NIS Elements D to determine the fork length of individual fish, which was measured from the mouth to the fork in their tails. This was more accurate than measuring the fish on a ruler during the move and minimised handling time as well as time spent out of the water. A 15 cm ruler was placed in the photos for scale (Image 3.6). This ruler was used to calibrate the programme. Fish were placed in front of the camera in the same order that their weights were taken so the lengths could be paired with the corresponding weight. Tagged fish had the tag number in the photo with them for identification (Image 3.6). The average length of the fish at the beginning of the acclimatisation period was 152.57±6.30 mm with an average weight of 56.0±8.7 g.

After the acclimatisation period the fish were again weighed and moved. The average fish weighed 67.5±12.0 g and had a fork length of 158.09±7.23 mm. They were then fed the experimental diets three times a day, 6 days a week. The amount of feed offered to the fish was recorded daily. The fish were fed to apparent satisfaction. This was determined to be when the feeding response became less frenzied and feed began to settle on the bottom of the tank; however, the trevally will still eat food that has settled on the bottom of the tank. From day 30 of the trial the 60% protein diet was fed frozen to prevent it from dissipating in the water before it could be consumed. Before it was fed frozen, much of the feed was wasted.

3.4.1. Sampling

The fish were weighed and photographed (for lengths) on days 0 (week 0), 22–23 (week 4), 52–54 (week 8) into the trial and at the end of the trial (85–86 days, week 12). At the end of the experiment the fish were scanned with a PIT tag reader. The fish were then weighed and videoed. The seven untagged fish from each tank were separated and euthanized for sampling. Euthanasia was carried out by overdosing the fish on AQUI-S®. Three of the fish from each tank had blood samples taken from the base of the tail in a BD 5 mL Vacutainer tube spray-coated with EDTA (an anti-coagulant). The weights of the liver and viscera were taken from five fish, including the fish that had blood samples taken, and the final two fish were frozen for whole body composition. These fish were broken down by a Kenwood food processor and then homogenised using a Kenwood Triblade 700W hand blender. The samples were then frozen in a -80°C freezer. Ten fish were euthanized and frozen at the beginning of the experiment from the original population. The fish selected were all around 150mm. These fish were also homogenised and stored at -80°C until testing. Whole body composition was measured by the Massey University Nutrition Lab.

The blood samples were used to measure the haematocrit and haemoglobin concentrations. A sample of the blood was placed in a 75mm capillary tube, plugged with wax, and centrifuged at 10,000rpm for 5 minutes in a capillary rotor. The scale on the lid of the capillary rotor was used to measure the haematocrit concentration. To measure haemoglobin 10 µL of blood was mixed with 1 mL of Drabkin’s reagent and left to sit for 10 minutes at room temperature. The sample was then read in a spectrophotometer at 540nm with the absorbance reading placed into the equation in Section 3.4.2.5 to determine haemoglobin concentration. The remaining sample was centrifuged at 5,600rpm for 5 minutes and the plasma stored at -80°C until used to measure ammonia and protein content. Ammonia was determined using Megazyme (Rapid) Ammonia Assay Procedure (Megazyme International, Ireland) and protein content was determined using

a ThermoScientific Pierce™ Protein Assay Kit (ThermoScientific, Rockford Illinois, USA). The microplate methods of the kits were used.

All the fish in tanks 60a, 30b, 60b and 30c were euthanized. Seven of the tagged fish from these tanks were blood sampled, and liver and viscera weights recorded. Seven of the tagged fish were used as liver samples were also taken for RNA profiling to be done for another project being carried out at Plant & Food Research. Two untagged fish from each tank were used for whole body composition.

3.4.2. Diet Palatability

Three of the tagged fish from each tank were kept for a palatability trial, except for tanks 60a, 30b, 60b and 30c (remaining tagged fish were returned to the original population in a bigger tank). A GoPro Hero3 was placed in 2 tanks (B8 and B9), each with 12 silver trevally from the growth trial. The palatability trial started 3 days after the growth experiment and was carried out for three days. The GoPro recorded for 3 minutes while the fish were fed the four experimental diets, a commercially available 3mm pellet (Ridley's, 50% protein, 18% fat, and 22.8MJ/kg) and an alternative gel-based diet (20.4% crude protein, and 2.1% fat, 63.6% moisture) typically fed by PFR to their fish. The gel diet was made up of fishmeal, vitamins, minerals, and raw fish and used alginate as a binder. Fish were fed three times a day with a different diet offered at each feeding until all the diets were tested.

The video was then used to analyse the feeding behaviour of the fish. The feeding behaviour categories used were based on the feeding behaviour observed during the growth experiment. Silver trevally a) ignored the food in the water, b) approached the food and then swam away without consumption, c) ingested the food but spit some out (assumed to be reducing the particle size to assist feeding), or d) eaten the food in its entirety. The amount of food offered varied so for comparison a percentage (of the overall observed behaviours) was used. For each time fish were fed, the number of each specific responses was divided by the total of all responses observed. Each item of food was able to have more than one response acted on it due to fish taking the item of food into their mouths and then spitting it out and allowing another fish to eat the same piece of food. The different behaviours were counted on each item of food until it was consumed or reached the bottom of the tank.

3.4.3. Digestibility

Chromic oxide was used as an inert marker. It was added to the diets at 10g/kg for the last 10 days of the growth experiment. The diet was fed the same as the experimental diets in the protein requirement growth trial. The chromic oxide was mixed with the dry ingredients in a

Kenwood food processor before adding the fish oil and water. The diets were made three times throughout this experiment and the food was stored in a chiller (5°C). The last feed was given to the fish less than 24 hours prior to sampling. Faecal samples were manually stripped from the fish when they were under anaesthetic and collected in a plastic pottle.

3.4.4. Calculations

At the end of the experiment the total number of remaining fish was determined and the survival rate of the fish was calculated.

Survival = 100 x (final no. fish/initial no. fish)

3.4.4.1. Growth

Using the lengths and weights measured throughout the experiment the average daily gain (ADG), specific growth rate (SGR), and length growth rate (LGR) were determined for the three intervals as well as for the overall experiment.

ADG = (final weight – initial weight)/days of experiment

SGR = (ln final weight – ln initial weight)/days x 100

LGR = (final length – initial length)/days of experiment

3.4.4.2. Condition Indices

The fish weights, lengths, and liver and viscera weights were used to determine the body condition of individual fish. Condition factor was determined for all fish at the beginning of each interval as well as at the end of the experiment. HSI and VSI were determined at the end of the experiment for three fish (or seven in tanks used for the RNA experiment).

HSI = liver weight/body weight x 100

VSI = viscera weight/body weight x 100

CF = body weight x 100 / total length^3

3.4.4.3. Protein

PER = weight gain (g)/protein offered (g)

Protein retention (PR) = (final weight x protein content in final fish body – initial body weight x initial protein content)/(feed intake as DM x protein content of diet)

3.4.4.4. Feed Efficiency

Feed conversion ratio (FCR) = total feed offered/total weight gain of fish per tank

The FCR of each diet was determined using the wet weight (as fed to the fish) of the diets as well as the dry weight of the diets (based on the analysis, seen in Table 3.2). Due to the loss of six fish in 60c throughout the experiment, adjustments to the initial weight and feed intake was required to calculate FE and FCR for the last interval (8–12) and the overall experiment as follows. The total fish weight at 8 weeks in tanks 60c was adjusted by subtracting the post-mortem weights of fish that died in that period. The FE and the FCR for the overall experiment were adjusted by multiplying the average fish weight at the start of the experiment in tank 60c by 9 (the number of fish at the end of the experiment). Feed intake for each period was divided by the number of fish in the tank (0–4 weeks = 15 fish, 4–8 weeks = 14 fish, 8–12 weeks = 9 fish). The average of each interval was then multiplied by 9 and added together. This represented the feed intake used for nine fish throughout the entire experimental period.

3.4.4.5. Blood

Blood parameters were calculated as follows:

Haemoglobin concentration [Hb] (mg/dL) = (mean absorbance at 540nm x 64458 x DF)/(44 x d x 1000)/10

d = path length (cm) = 1

44 = millimolar extinction coefficient for cyanmetHb

DF = dilution factor = 101

Mean corpuscular haemoglobin concentration = ([Hb]/mean haematocrit) x 100

The mean corpuscular haemoglobin concentration gives a good indication on whether the fish have anaemia and hyperemia.

$$\text{Ammonia g/L} = \frac{\Delta A\text{sample}}{\Delta A\text{standard}} \times \text{g/L standard} \times F$$

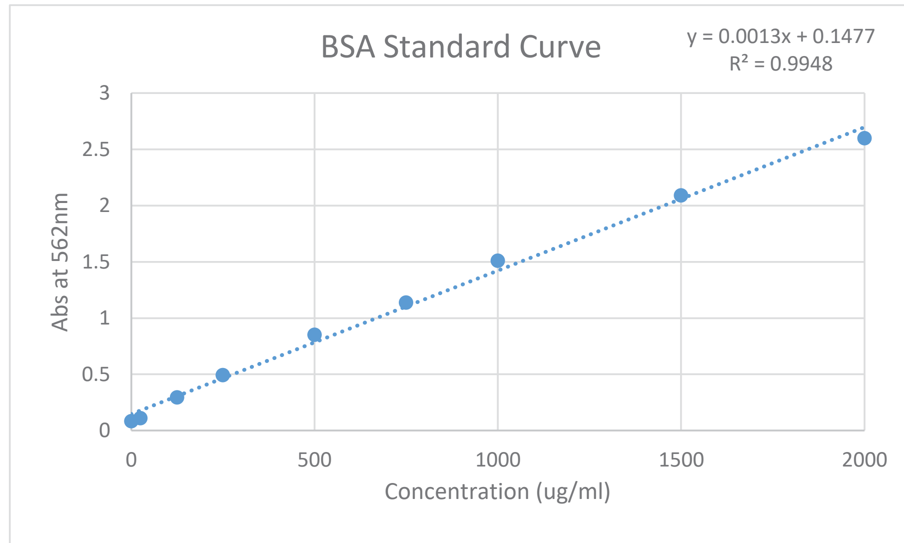

Figure 3-6 BSA protein assay standard curve

The calculation from the BSA standard curve is then rearranged and used to determine the concentration of the samples by substituting the absorbance into the equation.

3.4.5. Statistical Analysis

SAS 9.4 was used as the statistical programme throughout this experiment. The weights and lengths throughout the different experimental periods were analysed using repeat measures. The remaining factors measured in the growth trial were analysed using ANOVA followed by least significant differences (LSD) for specific comparisons. The palatability results were analysed using ANOVA followed by LSD for specific comparisons. Significance was accepted at $P<0.05$.

Chapter Four

4. Results

4.1. Protein Requirement Growth Trial

4.1.1. Growth Parameters

Live weight and length of silver trevally increased over time ($P<0.001$, Table 4.1), the increase was similar for all the diets as the interaction between week and diets was not significant. However, the week of the experiment did have an effect on growth. The survival rate of the protein requirement growth trial was 94.4%.

ADG, SGR, and LGR of silver trevally was not significantly affected by diet (Table 4.2). Individual growth varied as seen in SGR (Figure 4.1). The temperature of the tanks began to decrease in the last month (Figure 3.1 and 4.2). This decrease in temperature coincided with a decrease in feed intake (Figure 4.2). The FCR, based on the wet weight of the feed, did not differ significantly among diets. However, the FCR, based on the dry weight of the diets, was highest in 40% CP ($P<0.05$) but the other three diets were not significantly different. The PER did not differ among fish fed different experimental diets. The protein retention (PR) was highest in fish fed the 30% diet and lowest in fish fed the 40% and 60% experimental diets. Protein consumption of each individual increased with increasing CP level in the diet (Figure 4.3).

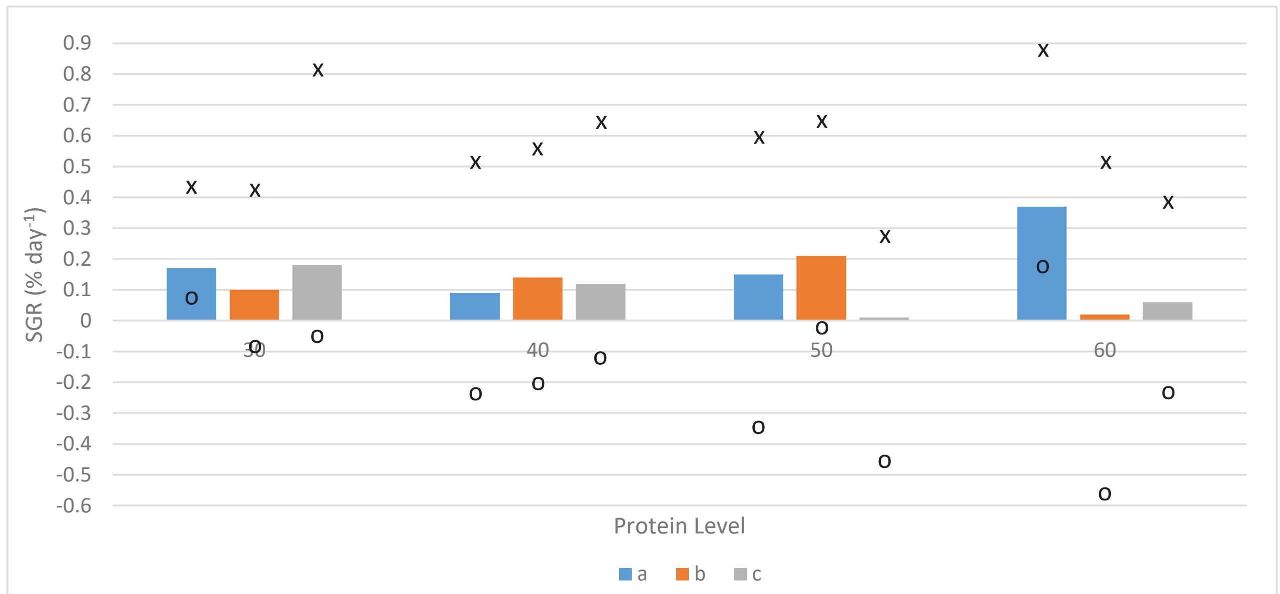

Figure 4-1 The average SGR of the overall experimental period for all four experimental diets and their replicates (a-c). The "x" represents the maximum SGR in each tank and the "o" represents the minimum SGR.

Table 4-1 Live weight and length of fish fed diets containing 30, 40, 50 and 60% of protein at the start of the experiment (week 0) and weeks 4, 8 and 12

Diet	**Live weight (g)**	**Length (mm)**
30	72.0	163.8
40	67.0	161.1
50	77.0	166.8
60	75.1	165.8
SE	2.34	1.76
Week		
0	67.5[a]	158.1[a]
4	69.8[b]	163.0[b]
8	73.7[c]	167.8[c]
12	79.9[d]	168.5[c]
SE	0.50	0.27
Diet x Week		
30 / 0	67.2	157.5
30 / 4	69.5	162.5
30 / 8	74.1	167.6
30 / 12	77.2	167.5
40 / 0	62.3	155.8
40 / 4	64.7	159.9
40 / 8	67.7	164.0
40 / 12	73.2	164.7
50 / 0	70.7	159.7
50 / 4	74.2	165.7
50 / 8	77.6	170.0
50 / 12	85.2	171.6

60 / 0	70.0	159.4
60 / 4	70.8	164.0
60 / 8	75.4	169.6
60 / 12	84.0	170.3
SE	2.50	0.54
P-Value		
Diet	0.07	0.19
Week	<.0001	<.0001
Diet x Week	0.16	0.24

[a,b,c,d] values with different superscripts are significantly different (LSD, P<0.001)

Table 4-2 Growth factors, feed conversion ratio and protein efficiencies of fish fed the experimental diets throughout the overall experimental period

	30	**40**	**50**	**60**	**SE**	**P-value**
SGR[1] (% day^{-1})	0.15	0.12	0.15	0.21	0.053	0.85
ADG[2] (g day^{-1})	0.11	0.10	0.12	0.16	0.051	0.84
LGR[3] (mm day^{-1})	0.11	0.09	0.11	0.14	0.027	0.59
Wet Weight of Diets						
FCR[4]	50.1	57.2	37.1	33.3	9.32	0.29
Dry Weight of Diets						
FCR[4]	21.2[a]	32.2[b]	15.4[a]	21.0[a]	3.06	0.03
PER[5]	0.12	0.11	0.11	0.09	0.01	0.44
PR[6] (%)	0.033[a]	0.013[b]	0.023[c]	0.015[b]	0.002	0.01

[a,b,c] values within the same row with different superscripts are significantly different (LSD, P<0.05).

[1]specific growth rate [2]average daily gain [3]length growth rate

[4]feed conversion ratio [5]protein efficiency ratio [6]protein rentention

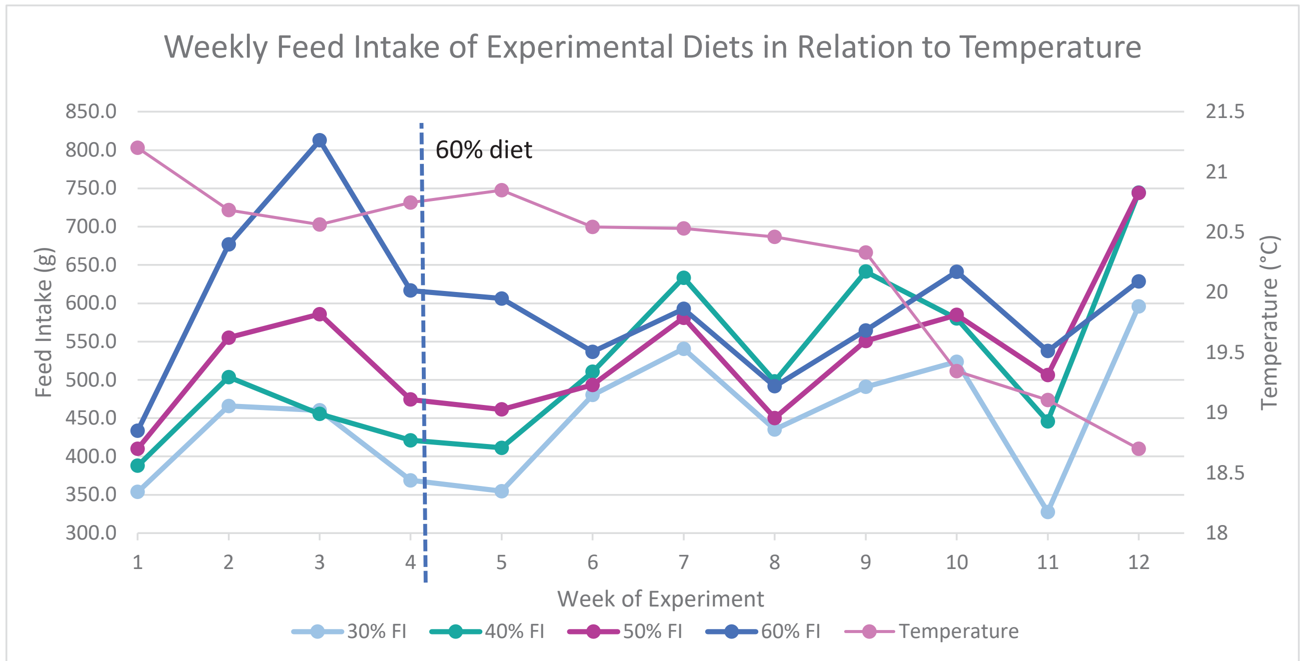

Figure 4-2 Weekly feed intake of each diet (wet weight as fed to the fish), three tanks averaged, in relation to the average temperature. Blue line represents the time in the experiment when the 60% diet was fed frozen due to high pollution properties of this diet

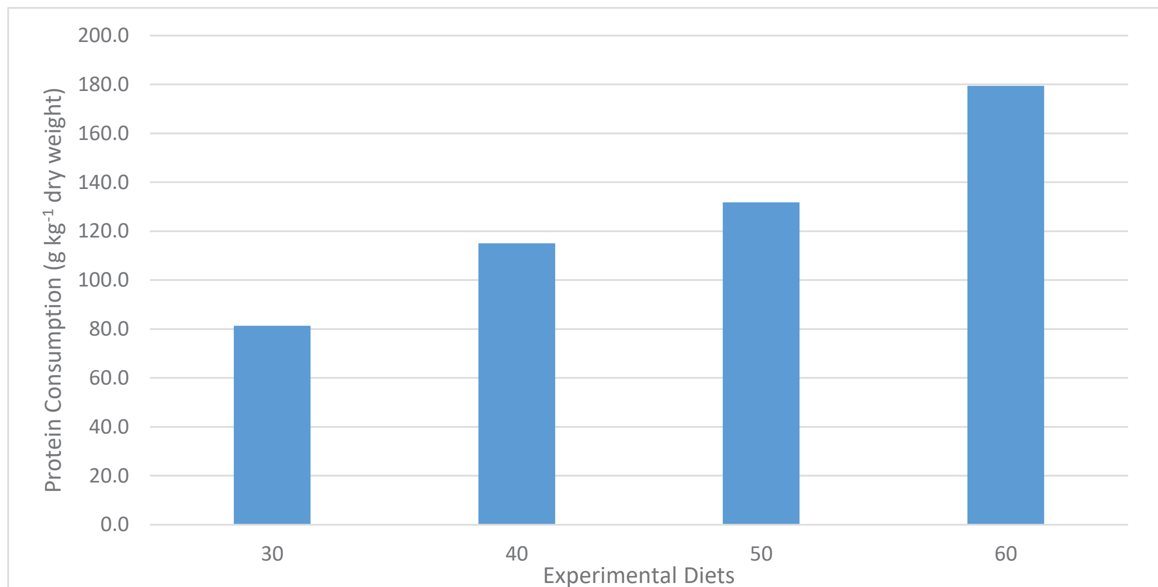

Figure 4-3 Protein offered (g kg-1 diet (DM)) per fish, on average, fed the experimental diets

4.1.2. Body Condition and Composition

The body condition data is shown in Table 4.3. CF, HSI and VSI were not significantly affected by diet. There was no significant difference between the body composition of the initial fish and the final fish after being fed the experimental diets for 12 weeks, shown in Table 4.4.

Table 4-3 The effects of differing dietary protein levels on condition indices of silver trevally

	30	40	50	60	SE	P-Value
CF[1]	0.0016	0.0016	0.0017	0.0017	0.00002	0.12
HSI[2]	0.92	0.87	1.01	0.99	0.115	0.81
VSI[3]	4.94	4.73	5.29	5.12	0.287	0.58

[1]condition factor [2]hepatosomatic index [3]viscerosomatic index

Table 4-4 Body composition of silver trevally before the experiment and at the end of the 12 week period on the experimental diets

(%)	Initial	Initial SE	30	40	50	60	SE	P-value
Protein	17.9	0.83	18.5	18.0	18.3	18.3	0.48	0.95
Ash	3.2	0.35	3.5	3.6	3.5	3.17	0.20	0.55
Lipid	4.9	2.46	4.2	3.8	5.6	8.9	1.42	0.19
Moisture	74.2	2.76	74.5	75.3	73.4	70.2	1.59	0.28

4.1.3. Haematological Parameters

Haematological parameters of fish fed the experimental diets are shown in Table 4.5. The concentration of protein in the plasma did not differ significantly among diets. Fish fed 30% and 60% CP treatments showed significantly higher ammonia content. The corpuscular haemoglobin concentration does not significantly differ among diets. This indicates that the diets did not adversely affect the animal's haematology, nor result in anaemia or hyperemia.

Table 4-5 The effects of differing dietary protein levels on haematological parameters of silver trevally

	30	40	50	60	SE	P-value
Corpuscular Hb Conc (mg dL^{-1})	32.8	33.2	33.1	33.4	1.54	0.99
Protein (µg mL^{-1})	481.3	551.6	499.0	519.4	48.38	0.76
Ammonia (µM L^{-1})	288.6^{a}	172.0^{b}	185.1^{b}	258.0^{a}	22.41	0.03

^{a,b} values within the same row with different superscripts are significantly different (LSD, P<0.05).

4.2. Palatability

Behavioural responses of the palatability trial are shown in Table 4.6. There is not a significant difference between the diets for the number of food particles that are ignored, but the gel and 60% CP diets had the least number of ignored food items. The number of food particles approached and those partially consumed did not greatly differ between diets. The 60% CP experimental diet, pellet diet, and gel diet are more palatable than the other diets based on the percentage of food items that were eaten completely. The 30% CP experimental diet resulted in the lowest percentage being consumed entirely. The survival rate of the palatability trial was 100%.

Table 4-6 Percentage of feed behavioural responses of silver trevally when fed different diets

(%)	30	40	50	60	Pellet	Gel	SE	P-value
Ignore	17.8^{a}	15.3^{ab}	16.7^{a}	9.8^{bc}	17.3^{a}	3.8^{c}	2.20	0.022
Approach	16.4	14.6	19.4	14.8	8.4	10.9	5.40	0.749
Eat Some	26.8	14.0	9.5	7.0	0.5	11.7	4.52	0.069
Eat	39.1^{a}	56.2^{b}	54.5^{b}	68.5^{c}	74.0^{c}	73.8^{c}	3.31	0.002

^{a,b,c} values within the same row with different superscripts are significantly different (LSD, $P<0.05$).

4.3. Digestibility

At sampling it was not possible to get sufficient, or any, faecal samples from the fish. Due to this the digestibility trial was unable to be carried out.

Chapter Five

5. Discussion

5.1. Protein Requirement Growth Experiment

A lack of significant differences in weight and length growth may be due to the energy level of the diet. A low energy level can result in protein being converted to energy and used for general metabolic processes, reducing the amount of protein available for growth. Growth rates are shown to be improved through increased energy levels in the diet, even if the protein level remains constant, seen in masu salmon and Pacific threadfin (Deng et al., 2011). Weight gain of masu salmon was significantly improved feeding the higher level of energy (21 MJ/kg instead of 19 MJ/kg) regardless of whether the diet contained 40% or 50% CP (Lee & Kim, 2001). In Atlantic salmon, increasing the dietary energy level (21.4 MJ/kg to 23.5 MJ/kg) while decreasing the protein content (as low as 35% CP) resulted in improved growth (Hillestad & Johnsen, 1994). This indicates that in some species energy content is more influential on fish growth. However, it may also be the ratio between the protein and the energy content. Increasing the protein to energy ratio in the diet increased the weight gain of hybrid grouper (Jiang et al., 2015). The optimum protein level is influenced by the ideal protein to energy ratio in the diet (Chatzifotis et al., 2012). Based on the wet weight of the diets in this experiment the protein and energy levels were lower than in other literature. A suboptimal protein to energy ratio in the experimental diets likely had a poor effect on silver trevally growth despite the difference in protein intake seen throughout the experiment.

Growth and feed conversion efficiency in active finfish species is improved through induced swimming and non-stop exercise (Khan et al., 2014). The increase in growth rate depends on the species, water temperature, and water flow (Brown et al., 2011). Improved growth and feed efficiency is seen in yellowtail kingfish (Brown et al., 2011; Palstra et al., 2015), gilthead sea bream (Felip et al., 2013), and Arctic charr (Christiansen et al., 1992). The flow required for yellowtail king fish differed with size. Palstra et al. (2015) found that 4.83 BL (body length) s^{-1}, 3.25BL s^{-1}, and 2.73 BL s^{-1} was required for fish 145 mm, 206 mm, and 311 mm long, respectively. Brown et al. (2011) found that a 10% increase in growth can be achieved in yellowtail kingfish with a flow of 0.75 BL s^{-1}. In Arctic charr, growth improvement is constricted by the stocking density, a high density stocking rate (47kg m^{-3}) prevents exercise from improving growth (Christiansen et al., 1992). In gilthead sea bream exercise increased the use of carbohydrates and lipids as a fuel source, and increased dietary protein retention (Felip et al., 2013).

During the current experiment the flow was 8 L min^{-1}, which resulted in no directional water current in the tanks. This could arguably have resulted in a lack of exercise in the silver trevally, as when not feeding they tended to sit at the bottom of the tank, which may have led to the low growth rates and feed conversion observed in this experiment. Unfortunately, it was not possible to increase the flow of the tank to a level that allowed a current owing to the design of the tanks. Approaching and following low tide often resulted in no flow to most, if not all, of the tanks. By increasing the flow of the tank this lack of flow would have become more apparent. As pelagic fish, silver trevally possess a comparable lifestyle to yellowtail kingfish, and are in fact a closely related species, which indicates that sustained exercise would have likely improved growth.

It is also possible that the use of a wet diet decreased nutrient intake due to the high water content (Table Two). A high water intake limits how much overall nutrients can be ingested at one time. The water content makes the diet bulkier and less concentrated than a pelletised diet. The low moisture content of a pellet allows for increased dry matter to be ingested. The wet diet also resulted in a larger amount of pollution than a pellet would have. However, food that was lost as pollution was unable to be measured, so all food that was placed in the tank was assumed to be consumed, potentially resulting in an overestimation of what was being consumed. If pellets were used the uneaten feed could have been collected and measured as seen in studies by Allan et al. (1999) and Allan et al. (2000). In a study by Eliason et al. (2007) uneaten pellets were collected and multiplied by their air-dry weight (mean weight per pellet). This was then subtracted from the weight fed out to determine the feed intake. However, previous studies done at PFR have been unsuccessful at retrieving uneaten pellets. It was also observed that the fish were eating algae off the bottom and sides of the tanks. Algae growth did not appear to differ between tanks. This intake was unable to be accounted for and the contribution to the nutrients consumed is unknown.

In the current experiment the fatty acid (FA) and amino acid profiles were not measured. The inclusion level of certain fatty acids and amino acids is important for the health and growth of fish. Essential FA deficiencies result in impaired growth, increased mortalities, and fatty liver (Watanabe et al., 1989a; FAO, 2017). The overall FA profile of the fish oil was measured (Table 3.1); however, the water content of the diet and inclusion level of fish oil will influence the FA content of the diets. Fish meal also contributed lipids, of an unknown composition, to the diets. Measuring the FA of the diets will determine if the requirements seen in the Japanese studies by Watanabe et al. (1989b) and Takeuchi et al. (1992a). On average fish required their protein source to contain 4–5% arginine, 1.5–2.1% histidine, 2–2.6% isoleucine, 3.3–4% leucine, and 3–

4% valine (Wilson & Halver, 1986). A deficiency in essential amino acids results in impaired growth rates, increased rates of mortalities, scoliosis (curvature of the spine), and other health problems (FAO, 2017). Amino acid deficiencies have also been shown to decrease feed intake but the effects of this fade after a couple of weeks (Dabrowski et al., 2007). This study would have benefited from the amino acid of the experimental diets being measured to ensure these requirements are met.

Within treatments there was a large amount of variation, as seen in the SGR of individual tanks (Figure 4.1). This indicates that while some species responded well to the experimental diets others did not. Due to this it was difficult to determine statistically significant differences. It is possible that using the overall sample for analysis is not the best method of statistical analysis. Comparing the larger and smaller fish in each replicate within-treatments may result in more accurate results. For this to work all fish would need to be PIT tagged so identification throughout the entire experiment is possible. The within treatment variation suggests that, as a species, silver trevally would benefit from a breeding programme to create a more equal species before beginning to commercially farm silver trevally.

Based on the dry weight of the feed, the 40% CP diet had a poor FCR compared to the other experimental diets. A poor FCR is seen in low protein diets: hybrid grouper had a low FCR at 40% CP (Jiang et al., 2015); malabar grouper at 44% CP (Tuan & Williams, 2007); brown-marbled grouper at 45% CP (Shapawi et al., 2014); and Pacific threadfin at 35% CP (Deng et al., 2011). A poor feed efficiency is likely due to a deficiency in amino acids, in particular essential amino acids, as they are not available for protein synthesis. This may have contributed to the poor FCR in the 40% CP diet. However, it is therefore surprising that this effect was not observed in the 30% CP diet, as no differences in FCR were observed between the 30%, 50%, and 60% CP diets.

The increased level of carbohydrates in the lower protein experimental diets could also contribute to the poor FCR. Dietary carbohydrate content is highly influential on the feed efficiency of a diet (Hemre et al., 2002). This is due to the inability of carnivorous fish to digest high levels of carbohydrates. Coldwater and marine fish tend to lack the ability to digest high levels of dietary carbohydrates that warm water fish possess (Wilson, 1994). In juvenile cobia high levels of starch reduced the apparent digestibility coefficient and above 12.5% starch feed efficiency no longer improved (Ren et al., 2011). The inclusion of carbohydrates, such as wheat, should not exceed 10% of yellowtail kingfish diets as growth becomes impaired. In the experimental diets of the current trial the corn starch inclusion level was 38%, 24%, 13%, and 1% in the 30%, 40%, 50%, and 60% CP diets, respectively. This was required to balance the energetic content of the four, protein varied diets. However, this inclusion rate of the

carbohydrate may have had a detrimental effect on the FCR and growth of the experimental diets.

The protein efficiency ratio is not significantly different among the different experimental diets. Protein efficiency ratio can be improved by increasing the lipid content of the diet (Tibaldi et al., 1996; Chatzifotis et al., 2012). The addition of lipids is more effective in diets with low dietary protein. The increased lipid content allows for the lipid to be broken down for energy leaving the protein for protein synthesis.

Protein retention was highest in fish fed the 30% CP experimental diet and lowest in fish fed 40% and 60% CP in the current trial. Protein retention is often highest in fish with restricted protein intake (Booth et al., 2013). This is due to the effect of protein sparing. Protein sparing is seen in animals fed less than their requirements, when there is no excess protein in the diet. A lower level of dietary protein results in a higher level of carbohydrates that can be used for energy leaving protein for protein synthesis. However, this did not occur with fish fed the 40% CP diet. The reason for this is unknown.

The 60% CP diet had a low protein retention in the current experiment. This is likely due to low level of carbohydrates to use for energy. High levels of dietary lipid improved protein retention in dentex (Company et al., 1999). The inclusion of increased dietary lipids results in a protein sparing effect (Helland & Grisdale-Helland, 1998). The use of lipids as an energy source results in a low fat content in the whole body composition (Company et al., 1999). However, once lipid reaches a certain level, dependent on the species, lipid in the whole body composition increases (Helland & Grisdale-Helland, 1998). This can be undesirable as it can affect the fillet. The protein sparing effect is less significant in high protein diets (Company et al., 1999), and was observed in the current study by the low protein retention of fish fed the 60% CP diet. However, the body composition of silver trevally, in this current study, was unchanging (Table 4.4).

Body condition indices are dependent on the amount of body stores, glycogen and lipid, the animal has. Due to the lack of difference in growth rates observed in the current study it would indicate that the four experimental diets are not high enough in energy to meet the demands of silver trevally. Due to this, there are not enough spare nutrients available for the fish to store so the CF, HSI, and VSI of the fish do not differ between diets. HSI is a way of quantifying the liver's energy or nutritional stores. Energy stores in the liver are glycogen and lipid deposits. An increase in dietary protein has shown to increase HSI in bluefin trevally (Suprayudi et al., 2014). This is likely due to the increase of amino acids available for conversion into glucose and, therefore, glycogen. Liver glycogen content in Atlantic halibut increased with dietary starch

content (Hatlen et al., 2005). Increasing the protein to energy ratio in the diet increases HSI in bluefin trevally (Suprayudi et al., 2014) while increased levels of dietary lipids increase the HSI as well as lipid content of the liver in Atlantic cod (Hansen et al., 2008). The similar HSI values of treatments in the current study emphasise the lack of nutritional difference provided by the four diets. By increasing the energy content and/or the lipid content of the silver trevally's diet a difference in HSI may appear.

HSI would be expected to be higher in low protein diets, as they are high in carbohydrates. This is seen in hybrid grouper (Jiang et al., 2015) and red spotted grouper (Wang et al., 2016) but not in juvenile meagre (Chatzifotis et al., 2012). Like in the juvenile meagre, a lack of variation between diets was also seen in silver trevally. This is likely due to the internal competition between dietary glucose and endogenous glucose synthesised by the animal. Fish are able to produce high levels of endogenous glucose independent of the amount of dietary carbohydrates (Enes, Panserat, Kaushik, & Oliva-Teles, 2009). This is likely a contributor to the poor dietary carbohydrate utilization seen in fish and why the HSI does not differ significantly among protein levels. The lack of difference of the HSI in silver trevally is likely due to internal glucose production being relatively constant across treatments.

In this study, the dietary protein content did not influence the protein content of the plasma. However, in black sea bream, plasma protein increases with dietary protein (Zhang et al., 2010). The protein ranges from 32% to 49% CP. In red spotted grouper protein plasma levels were higher in the fish fed moderate levels of dietary protein, 50% CP (Wang et al., 2016). Corpuscular haemoglobin concentration is not affected by the diet. 30% and 60% CP experimental diet had higher ammonia concentrations than 40% and 50% CP. The higher ammonia level in fish fed the 60% CP diet is likely due to the catabolism of amino acids. Breaking down amino acids to enter the citric acid cycle and be converted into glucose results in the amino groups being converted to ammonia to be excreted, therefore increasing ammonia content of the plasma.

5.2. Palatability

The four experimental diets in the current study were studied to determine if palatability across the four protein levels were comparable. Differences in palatability could in turn affect the feed intake of the different diets. Amino acids have been shown to be a strong attractant to fish (Kasumyan & Døving, 2003; Velez et al., 2007). While the amino acid composition of the diets has not been determined it stands to reason that the higher the protein level of the diet the higher the amino acid content. However, the balance of amino acids cannot be estimated. Non-essential amino acids are more palatable than essential amino acids (Kasumyan, 2016). The 60%

CP experimental diet had significantly higher eat responses than the other experimental diets. This is likely due to the increase in dietary protein. A low concentration of amino acids does not contribute to olfactory potency (Velez et al., 2007). The low concentration of protein in the 30% experimental diet likely contributes to the poor ingestion rates as the attractiveness of the diet is poor. Differences in palatability (palatability increasing with the protein content of the diets) may have an effect on the differences in feed intake seen in Figure 4.2. Feed intake then limits nutrient intake and, thus, has an effect on growth.

For comparison the experimental diet was also compared with alternative diets: a gel diet and a commercial pellet. Palatability is a combination of olfaction and gustation of the diet. The two detection methods can be hard to differentiate in fish as they are both determined by molecules dissolved in the water (Hara, 1986). In terrestrial animals, olfaction is the detection of air-borne particles via the nose and gustation is the detection of molecules via the taste buds in the mouth. Despite the olfaction and gustation being transported through the water the two systems are separated in fish. Olfaction is important in terms of determining food location as well as ingestion (Velez et al., 2007). The gel diet used in the palatability trial contains raw seafood. This likely contributes to an increased olfactory response promoting increased intake. The taste of the diet determines whether the food will be eaten. A low ignore rate minimises feed loss. The gel diet also had a high palatability value despite being low in protein. The inclusion of raw seafood (including fish mince, squid, and mussel) in the gel diet is likely the reason for the increased palatability of this low protein diet. It likely contributes to a higher olfaction response in fish.

Taste and texture have an influence on ingestion (Stradmeyer, 1989). Softer textures increase feed ingestion (Stradmeyer et al., 1988). Texture between experimental diets would be relatively similar so the differences between intakes is likely due to the taste. When comparing hard and soft pellets it was observed that the same proportion were captured but soft pellets had a higher proportion of being completely ingested; hard pellets were spit out after being captured (Stradmeyer et al., 1988). Texture may contribute to the gel diet's high intake despite the low protein content.

5.3. Digestibility

The inability to get faecal samples may have been due to an overestimation of the time taken for food to pass through the gastrointestinal tract. The application of anaesthetic can cause fish to defecate (Spyridakis et al., 1989). Gut motility in fish is controlled by the presence of food, autonomic nerves, and hormones, the same as with mammalian species (Olsson & Holmgren,

2001). The presence of food in the gut results in peristalsis, contractions of the gut wall, which moves food down the gut. Gastric emptying is influenced by stomach fullness, and frequency of feeds (Olsson & Holmgren, 2001). The time for gut emptying to occur differs between species, but appears to be fast for trevally at these temperatures. To determine the digestibility in silver trevally in the future further understanding of the transit time through the gastrointestinal tract or a faecal collection unit on the tank is required.

5.4. Conclusions

Due to the results of the growth experiment it is not possible to conclude the ideal protein level for silver trevally. This experiment should be repeated to determine the optimum protein level. The formulation of the experimental diets meets the protein levels required on a dry matter basis, as shown in Table 3.2. Feeding the experimental diets as a pelletised diet would decrease the water content of the diets thus increasing the nutrient density of the feed. The wet diet likely limited the feed intake resulting in feed intake being controlled by the gut space rather than the nutrient requirements of the animal. It was shown in the palatability trial that the use of a pelletised diet would not affect feed intake. When repeating this experiment the addition of a current to the tanks for this species would be beneficial. If it is not possible to achieve a sufficient current with the tank's water flow the addition of a water propulsion system could be used to create a current. A reduced period of time between the last feed and sampling would increase the chance of getting sufficient faecal samples and allow the digestibility of the diets to be determined. These adjustments can be used to improve the experimental design and determine the protein requirements of silver trevally.

5.5. Opportunities for Future Research

An adjustment to this experiment is to firstly determine the ideal energy level of silver trevally diets. This can be done by feeding different levels of energy and observing the growth. The diets should be iso-nitrogenous, at around 50% CP. The lipid level can be adjusted to alter the energy level of the diet, as seen in the experiment done by Deng et al. (2011). Reducing the water content of the diets can also be used to improve the energy content of the diets (Table 3.2.). A higher quality fish meal and fish oil can be used to improve the quality of the experimental diets. The protein to energy ratio required by silver trevally should also be determined.

Determining the lipid requirements of silver trevally can be used to improve the energy content and FCR of the diet. Fish oil used in this experiment was not ideal in terms of quality. The fish oil contains higher levels of oleic acid than expected in fish oil. There is also a large number of wax esters that can impair digestion. In Atlantic salmon high levels of wax esters decrease lipid

digestion and growth (Bogevik, 2011). However, salmon have adaptations to digest wax esters and are likely able to process higher levels of these lipids than silver trevally. It is possible that some of the essential fatty acids were not present at sufficient levels in the lipid source used. By obtaining a higher quality fish oil growth may be improved.

Another study could be done to determine the effect of moisture on feed intake, nutrient intake, growth, and feed efficiency. It is likely that by feeding the diet in a pellet, and reducing the moisture content of the feed, the fish is able to increase nutrient intake and improve fish performance. Feeding a low moisture diet will increase the concentration of the energy in the diet, which could improve fish performance. The moisture content of the diet may also have an effect on the palatability. A longer term behavioural experiment could also be conducted to determine if food preference changes over different seasons and times of the day.

To determine the digestibility of different diets and/or ingredients in silver trevally their gut motility needs to be further understood. A trial should be run to determine how long it takes for feed to pass through the gastrointestinal tract. There is also the need to determine the effect of anaesthetic on gastric evacuation as it is possible that a faecal collection unit may be more ideal than manual stripping.

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