

**Quantitative and qualitative aspects of  
the protein nutrition of barramundi  
(*Lates calcarifer*) larvae fed formulated  
foods.**

Thesis submitted by  
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Leo Nankervis

## **Abstract**

Formulated ‘artificial’ diets have the potential to overcome inherent nutritional and financial drawbacks associated with live foods used for larvae fish culture. Artificial formulations also provide a vehicle for accurate manipulation of nutritional constituents, enabling investigations into the nutrient requirements of fish larvae. In order to develop species-specific larval food formulations, optimal macronutrient requirements and appropriate nutrient sources must be established. The nutritional value of a food ingredient is determined by its nutritional profile and nutrient availability, and therefore nutrient digestibility is an essential part of the evaluation of novel food ingredients. Since the proteolytic capacity of barramundi larvae is limited, optimal sources of dietary protein must be established to optimise both their amino acid profile, and protein digestibility. An integrated approach was therefore adopted in this study to evaluate protein sources for barramundi larvae in terms of their amino acid profile and digestibility and in their capacity to support optimal growth and survival when incorporated into food formulations. Furthermore, the endocrinal mediation of the nutritional control of growth is investigated through thyroid hormone analysis, and nutritional effects on digestive physiology are examined through pepsin development.

The thyrotropic hormone system is a major regulatory mechanism for the control of growth in teleosts. Thyroid hormones (triiodothyronine, T3 and L-thyroxine, T4) mediate extrinsic processes, such as nutrition, to regulate growth in juvenile and adult fish, and are regulated by nutritional quality and quantity. While thyroid hormones regulate growth, survival, development and metamorphosis in fish larvae, data are

lacking on an endocrine-nutrition link at the larval stage. By applying an endocrinal approach to the nutritional control of growth, we may achieve a better understanding of the underlying processes governing the physiological status of fish.

The research described in this thesis was therefore designed to clarify the quantitative and qualitative protein requirement of barramundi, *Lates calcarifer*, larvae, and to investigate possible nutritional links to thyroid hormone concentration.

Dietary protein and energy contents were initially manipulated in larval food formulations to determine baseline macronutrient inclusion levels. Barramundi larvae (14 days after hatch, DAH) were fed microbound diets (MBD), varying in gross dietary protein (45, 50 and 55%) and energy (18 and 21 MJ.kg<sup>-1</sup>) for a period of 14 days. All fish were then sacrificed, measured for total length and a sub-sample taken for dry weight analysis. Carcass T3 and T4 were measured by radioimmunoassay, following chloroform/NH<sub>3</sub>OH extraction.

In following experiments, marine animal meals (fish meal, squid powder, *Artemia* meal, mussel meal, prawn meal and krill meal) were evaluated for their suitability for inclusion into MBD for barramundi larvae. Each of these meals was included in dietary formulations to a total of 50% gross protein. These formulations were evaluated in terms of growth and survival of barramundi larvae, amino acid composition of protein sources, protein digestibility and resulting carcass thyroid hormone levels. To improve the limited digestibility of fish meal, subsequent experiments incorporated fish meal hydrolysates and acid-denatured fish meal into MBD for evaluation in growth trials with barramundi larvae.

An optimal diet was found to contain at least 21 MJ.kg<sup>-1</sup> dietary energy and derived its protein from a combination of fish meal and squid powder (9:1 ratio). The limited digestibility of fish meal was improved approximately two-fold by acid denaturation, and the moderate inclusion of denatured fish meal into food formulations improved larval growth significantly, while the entire replacement of untreated fish meal with denatured fish meal did not improve growth above that of diets containing intact fish meal. The reasons for this are unclear, though carcass pepsin level was depressed for larvae fed the formulation containing no intact fish meal, indicating that larvae may adapt to less digestible protein sources. The high leaching rates typically attributed to MBD are assumed to be responsible for the poor growth and/or survival of larvae fed diets containing autolysates and hydrolysates in this study.

Thyroid hormone levels had no direct correlation to dietary energy level, protein source or protein inclusion level, though T4 correlated with growth independently of these nutritional manipulations. This finding indicates that T4 is important in the growth process of barramundi larvae, but is not directly mediated by specific nutritional inputs.

This study developed a microbound diet which supported up to 58% survival and significant growth in barramundi larvae from 14-28 DAH. The diet that supported the best rates of growth and survival contained 21 MJ.kg<sup>-1</sup> utilisable dietary energy and at least 50% dietary protein, comprised of a 9:1 ratio of fish meal to squid powder.

This study utilised integrated methodology to improve diet composition to increase growth and survival in barramundi larvae fed MBD, and to investigate the underlying mechanisms behind growth promotion. Growth trials remain the most conclusive way to determine optimal nutrient requirements for formulated foods, however, biochemical composition and physical properties can be used to narrow-down the wide range of nutrient sources available. Digestibility is of critical importance to the study of protein sources in food formulations for fish larvae, and a major area for the improvement of native animal meal protein sources.

The diet developed in this study is a critical step in the development of species-specific weaning and larval diets for barramundi. Amino acid profiles for optimal growth have been refined, and the digestibility of fish meal has been increased to improve larval growth, thus potentiating early weaning protocols and diminishing *Artemia* requirements. The development of co-feeding and weaning protocols with this diet is expected to increase growth through optimised amino acid profile and increased energy and fatty acid availability, while reducing costs in barramundi hatcheries through reduced *Artemia* requirements.

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## **Statement on Sources**

### **Declaration**

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education.

Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

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Leo Nankervis

# Chapter 1

## General Introduction

### 1.1. Barramundi

Barramundi (*Lates calcarifer*), or Asian seabass as it is alternatively known, is a euryhaline Perciform fish in the family Centropomidae (e.g. Grey, 1987). It is a popular food and sport fish throughout the Indo-Pacific region, supporting extensive commercial and recreational wild-caught industries, as well as an increasingly important aquaculture industry (Grey, 1987; Tucker *et al.*, 2002).

### 1.2. Hatchery culture

Culture practices for barramundi culture in southeast Asia traditionally revolved around the growout of wild caught juveniles (Tucker *et al.*, 2002). The economic and ecological instability of this practice necessitated the development of captive spawning and larval rearing technologies for this species. The initial larval rearing techniques utilised extensive pond-based systems, relying on natural productivity to provide food for larvae (Geiger, 1983; Rimmer and Russel, 1998). However, inconsistent survival lead to the development of intensive tank-based rearing technologies (Rimmer and Russel, 1998; Tucker *et al.*, 2002), which now provide consistent results under controlled conditions.

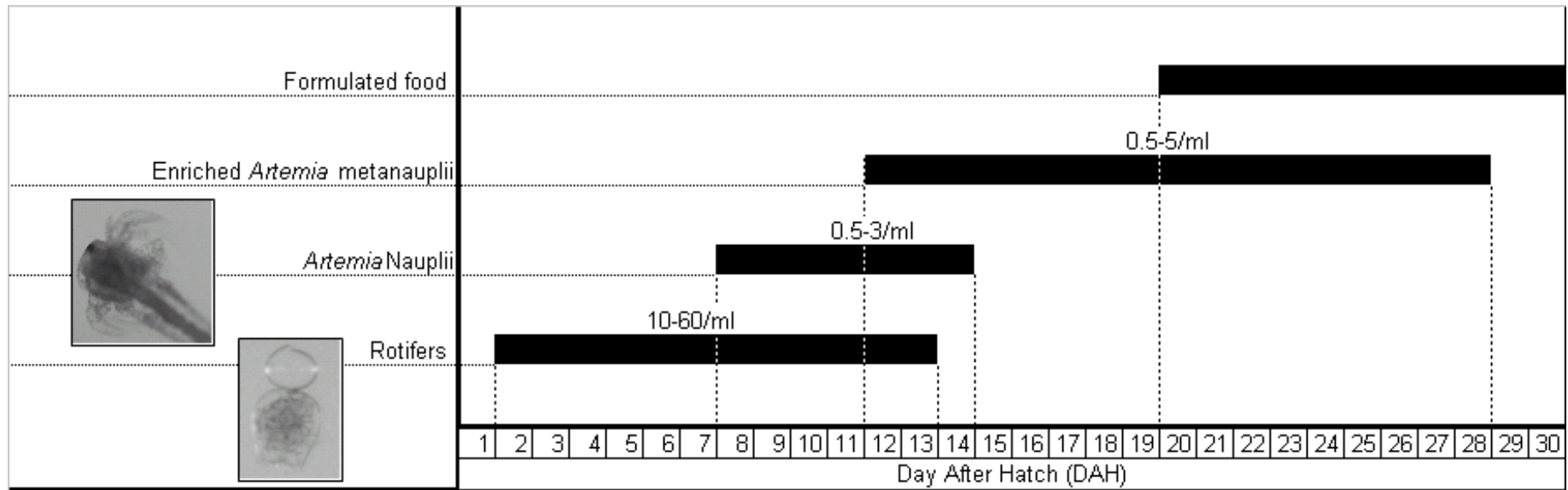
#### 1.2.1. Live foods

The intensive rearing of fish larvae depends heavily upon live foods, predominantly rotifers (*Brachionus* spp.) and brine shrimp (*Artemia* spp.; Fig. 1.1). While rotifers can be cultured at high densities, they are subject to large variations in productivity (Person Le Ruyet *et al.*, 1993; Dhert *et al.*, 2001). Furthermore, the world supply of

*Artemia* cysts is inconsistent and the subsequently inflated prices have increased the requirement for alternatives (Kolkovski, 2001; Sorgeloos *et al.*, 2001; Chong *et al.*, 2002). Culture of live foods requires extensive infrastructure and is labour intensive, accounting for up to 79% of hatchery operating costs (Person Le Ruyet *et al.*, 1993). Further drawbacks with current live food technology are due to their poor nutritional properties. Both *Artemia* and rotifers are naturally deficient in essential nutrients, most notably the highly unsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)(Watanabe *et al.*, 1980; Lubzens *et al.*, 1989; Watanabe, 1993). The nutritional profile of live foods can be enhanced to some extent by enrichment formulae (Southgate and Lou, 1995; Harel *et al.*, 2002; Rolando, 2003), thereby increasing larval performance (Rodgers and Barlow, 1987; Lemm and Lemarie, 1991; Southgate and Lou, 1995; Cho *et al.*, 2001; MacNiven and Little, 2001); however, this process adds to production costs and requires additional infrastructure and time. Furthermore, live food production provides an ideal environment for the proliferation of disease-causing organisms (Dhert *et al.*, 2001; Sorgeloos *et al.*, 2001).

### *1.2.2. Microdiets For Fish Larvae*

The ultimate solution to the above problems is development of inexpensive formulated foods, which are readily accepted and digested by fish larvae and have optimal nutritional profiles. Microparticles can potentially be formulated to fulfil the exact nutritional requirements of fish larvae of different species and developmental stages. Furthermore, there is potential to provide dietary particles of an optimal size for each stage of larval development, ensuring maximal ingestion.



**Fig. 1.1. Standard feeding protocol for *L. calcarifer* larvae (adapted from Mackinnon, 1987; Russell *et al.*, 1987; Rimmer *et al.*, 1994).**



Despite the potential advantages of formulated foods, research investigating total replacement of live foods with formulated microparticles for marine fish larvae has met with limited success. Trials to date have generally reported decreased growth and survival of fish fed microparticulate diets alone when compared to treatments co-fed with live foods and controls fed live foods (Koven *et al.*, 2001; Blair *et al.*, 2003). This has often been attributed to the low level of development and differentiation in larval digestive systems, and their consequent inability to digest complex nutrients (Dabrowski, 1984; Kolkovski, 2001).

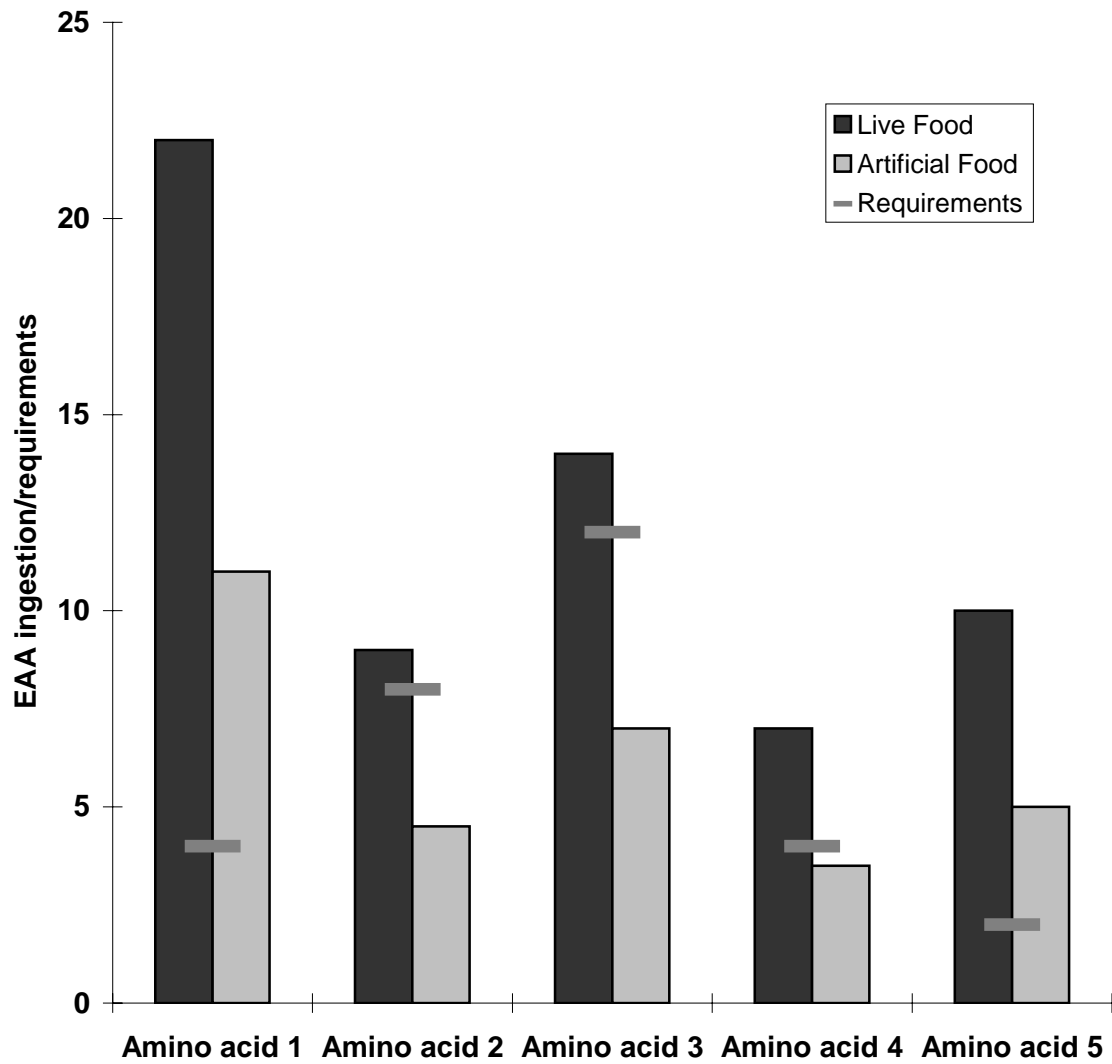
Marine fish larvae are generally altricial and are far less developed at first feeding than their freshwater counterparts (Jones *et al.*, 1993). They have small yolk reserves and generally lack a functional stomach at first feeding (Dabrowski, 1984). The digestive functions in the pancreas and intestine (but not the stomach) of altricial fish larvae are generally efficient before the onset of exogenous feeding (Walford and Lam, 1993; Cahu and Zambonino-Infante, 2001). Barramundi, for example, have high levels of trypsin activity before first feeding, but do not have a fully functional stomach until approximately 17 DAH (days after hatch), at 12.32 mm total length (Walford and Lam, 1993). The absence of a functional stomach, and the associated acid-pepsin-mediated digestion, could be a limiting influence on the digestibility of dietary proteins (Rønnestad *et al.*, 1999). Indeed the combined acid and alkaline protease activity of juvenile seabream (*Sparus aurata*) hydrolyses a range of proteins *in vitro* to a greater extent than either acid or alkaline protease alone (Alacorn *et al.*, 2001). Limited success has subsequently been achieved with weaning first feeding larvae directly onto artificial foods (Rønnestad *et al.*, 2003).

The exception to this trend has come recently when feeding European seabass, *Dicentrarchus labrax*, larvae from first feeding with highly utilisable food ingredients such as protein hydrolysates and phospholipids (Cahu *et al.*, 1998; Cahu *et al.*, 2003). Barramundi, *Lates calcarifer*, have likewise been reported to ingest microdiets from first feeding (Walford *et al.*, 1991); however, no survival past the 10<sup>th</sup> day after hatch was recorded for larvae fed microcapsules alone. Those co-fed with rotifers, however, survived longer and showed signs of microparticle absorption in the rectal epithelia (Walford *et al.*, 1991). The first record of significant pepsin-like activity in larval barramundi started at 17 DAH, coinciding with the acidification of the stomach (Walford and Lam, 1993). Furthermore, fish that lack a stomach as adults are generally herbivorous (e.g. minnows, carp [Cyprinidae], sauries [Scomberesocidae] and parrotfishes [Scaridae]) and therefore have a naturally low dietary protein level, or parasitic (hagfishes and lampreys), feeding on pre-digested food in the host circulatory system (Ringø *et al.*, 2003). The stomach is therefore thought to be integral to digestion of large amounts of complex protein.

One of the assumptions traditionally attributed to the increased growth and survival of larvae fed with live foods, when compared to those fed formulated foods, is that live prey provide a significant contribution to the enzyme activity of the fish larvae (Lauff and Hofer, 1984; Appelbaum, 1985). The mechanism of this enzyme contribution is unclear, whether by autolysis (self-digestion) of the live foods, or the provision of enzyme precursors, or zymogens (Khan and James, 1998). Recent evidence suggests that the contribution of digestive enzymes from live foods to the proteolytic activity in fish larvae may be relatively small. For example Cahu and Zambonino Infante (1995) found the trypsin contribution of *Artemia* to be a maximum of 5% of the enzyme

activity of 20 DAH European seabass, while Kurokawa *et al.* (1998) found that protease activity of *Brachionus* sp. ingested by 2 DAH Japanese sardine (*Sardinops melanoticus*) accounted for less than 1% of the total protease activity of the larvae. This does not, however, negate the theory of live food autolysis, and it is possible that small contributions of enzymes from live foods may be integral to their digestion.

It is also probable that the decreased ingestion rates, often reported for formulated microparticulate foods (Fernandez-Diaz *et al.*, 1994; Kolkovski *et al.*, 1997a), potentiate a lower window of error for nutritional requirements. For example, the quantitative essential amino acid (EAA) profile of *Artemia* may be far from optimal, though relatively high rates of ingestion may mask such deficiency (Fig. 1.2). In this hypothetical example, the EAA profiles of live and artificial foods are identical, though the ingestion of *Artemia* is double that of the formulated food. Larvae fed formulated foods in this example therefore lack sufficient quantities of amino acids 2, 3 and 4 while those fed *Artemia* are able to obtain sufficient quantities of all amino acids. On this basis, formulated foods with an identical amino acid profile to the traditionally used live foods may not satisfy EAA requirements due to lower ingestion levels.



**Fig. 1.2. Hypothetical ingestion rates of 5 essential amino acids (EAA) from live and formulated foods compared to EAA requirements for fish larvae. Amino acid profiles are identical for live and formulated foods, however in this example live food ingestion is double formulated food ingestion.**

### **1.3. Protein Nutrition of Fish Larvae**

#### *1.3.1. Quantitative Protein Requirements*

The quantitative protein requirement in formulated foods for altricial marine fish larvae is very poorly documented, though it is assumed to be higher than for juvenile fish (Cahu and Zambonino-Infante, 2001). The relatively high rates of growth evident in larval ontogeny would logically prescribe a high amino acid requirement (Rønnestad *et al.*, 2003), however, very few studies have sought to quantitatively determine the gross protein requirement of fish larvae (Péres *et al.*, 1996).

The only previous study assessing quantitative protein requirements of barramundi indicated that optimal growth occurred when fed diets containing approximately 47% protein (Partridge, 1996). This study, however, was conducted with weaning-age post-metamorphosis fish and may therefore be of limited relevance to true larval fish. As indicated by Dabrowski (1984), differences in digestion and absorption of nutrients between larval and juvenile fish prevent the interpolation of juvenile nutritional requirements to those of larvae. Further research is required to determine the species-specific protein requirements for marine fish larvae fed formulated foods.

#### *1.3.2. Qualitative Protein Requirements*

The biological importance of a dietary protein source relates to its amino acid composition, and to the availability of amino acids for digestion and intestinal absorption. Amino acids can be divided into essential (EAA) and non-essential amino acids (NEAA). EAA are not able to be synthesised by the animal, and therefore must be included in the diet (DeSilva and Anderson, 1995). All fish are believed to have the same 10 EAA (Table 1.1), while dietary cystine and tyrosine are thought to spare

methionine and phenylalanine, respectively, so are considered to be ‘semi-essential’ (Ketola, 1982). NEAA can be synthesised from other amino acids by the transfer of amino groups to the corresponding  $\alpha$ -keto acid, by transamination (Mambrini and Guillaume, 1999).

NEAA have higher catabolism and lower retention rates in fish larvae than EAA, indicating that they are preferentially catabolised for energy, sparing EAA for protein anabolism (Conceicao *et al.*, 2003). Thus fish larvae have a requirement for each individual EAA, and a cumulative requirement for NEAA.

To identify the optimal EAA levels in juvenile fish, graded levels of a particular crystalline amino acid are used to supplement a base diet which is intentionally deficient in the amino acid in question (Ketola, 1982; Cowey, 1994). Growth responses or signs of pathogenicity then indicate requirements. Due to the small size of larvae and their foods, these amino acid manipulation experiments are not practical with fish larvae. Therefore, representative optimal EAA profiles have been identified, assuming that the optimal EAA profile for larval foods corresponds to the profile of larvae tissue (Conceicao *et al.*, 1998), their eggs (Ketola, 1982), or their natural food (Dabrowski and Rusiecki, 1983).

The amino acid profile of dietary protein sources is likely to act synergistically with its digestibility, in order to determine its value in microparticulate diet formulations. Therefore, both factors must be considered to evaluate the optimal source of dietary protein for microparticulate formulations. In order to increase the dietary value of the protein content of microdiets for larval fish, researchers have recently investigated

methods of increasing the dietary availability of amino acids. Methods have included incorporation of digestive enzymes (Dabrowski and Glogowski, 1977; Kolkovski *et al.*, 1993) into formulated foods or pre-digestion (hydrolysis) of protein components (Zambonino Infante *et al.*, 1997; Cahu *et al.*, 1999; Kolkovski and Tandler, 2000; Kvale *et al.*, 2002).

**Table 1.1. The essential, semi-essential and non-essential amino acids for fish (Ketola, 1982; DeSilva and Anderson, 1995)**

Essential		Semi-essential		Non-essential
Arginine				Alanine
Histidine				Asparagine
Isoleucine				Aspartate
Leucine				Glutamate
Lysine				Glutamine
Methionine	↔	Cystine		Glycine
Phenylalanine	↔	Tyrosine		Proline
Threonine				Serine
Tryptophan				Taurine
Valine				

#### 1.3.2.1. Digestive Enzymes

The presumed reliance of altricial fish larvae on exogenous digestive enzymes has prompted researchers to include proteolytic enzymes or pancreatic extracts (pancreatin) in larval microdiet formulations, with mixed results. The supplementation of trypsin into foods for common carp fry increased proteolytic activity linearly with inclusion level, and increased food utilisation (Dabrowski and Glogowski, 1977). Dietary inclusion of pancreatin increased microdiet assimilation by 30% and dry weight gain by 48% in 20 day-old gilthead seabream, *Sparus aurata*, larvae (Kolkovski *et al.*, 1993); however, the same treatment had no effect on growth or food assimilation in larval seabass, *Dicentrarchus labrax* (Kolkovski *et al.*, 1997b) or juvenile yellow perch, *Perca flavescens* (Kolkovski *et al.*, 2000). These differences between species may represent species-specific digestive processes, or may be due to ontogenetically variable levels in particular digestive enzymes. The pancreas and the associated proteolytic enzymes (trypsin and chymotrypsin) are generally active from first feeding in altricial fish larvae (e.g. Walford and Lam, 1993; Douglas *et al.*, 1999), and therefore the limitation in early digestive processes may be due to other enzymes such as pepsin. The low pH specificity of pepsin, however, has limited its usefulness as a supplement to artificial diet formulations, while the internal compartmentalisation present in live foods may allow for the intact delivery and suitable functional conditions for such enzymes.

#### 1.3.2.2. Protein Hydrolysates

Free amino acids or small-chain peptides are generally considered to be more efficiently absorbed over the gut epithelium of fish larvae than intact protein (Tengjaroenkul *et al.*,



2002). However, microdiets for fish larvae rarely contain free amino acids, reflecting the inherent difficulties that are involved in forming a small water-stable particle, which includes highly soluble, low molecular weight components (Yufera *et al.*, 2003). Artificial formulations instead contain intact proteins and binding or coating agents, that may be difficult for larvae to digest (Partridge and Southgate, 1999; Baskerville-Bridges and Kling, 2000; Kolkovski, 2001). Artificial food formulations also contain large amounts of dry matter when compared with live foods, which may reduce their digestibility (Lee *et al.*, 1996; Partridge and Southgate, 1999; Kolkovski, 2001; Lee, 2003).

In order to make microdiets more utilisable for fish larvae, enzymatic pre-digestion has been investigated as a means of increasing protein assimilation over the larval gut wall (Cahu *et al.*, 1999; Kolkovski and Tandler, 2000; Kvale *et al.*, 2002). Inclusion of pre-digested protein meals in microparticulate foods for larval fish reduces the molecular size of the dietary protein fraction. A percentage of the amino acid supply to larval fish can be met by short peptides, which are readily absorbed over the intestinal wall without prior digestion (Zambonino Infante *et al.*, 1997).

Despite the possible advantages of hydrolysates, use of pre-digested dietary proteins has yielded mixed results. Hydrolysis of 20-25% of the protein fraction of microdiets for European seabass larvae increased growth and survival, while also decreasing the incidence of spinal malformation and increasing intestinal development (Zambonino Infante *et al.*, 1997; Cahu *et al.*, 1999). However, large amounts of hydrolysate inclusion

in microdiet formulations impaired growth of gilthead sea bream, *Sparus aurata* (Kolkovski and Tandler, 2000) and European seabass larvae (Cahu *et al.*, 1999). Poor performance of hydrolysates has been attributed to an excess of amino acids and short peptide proteins that flood over the gut wall at a rate greater than is utilisable by the larva (Kolkovski and Tandler, 2000). Bleeding of the gut of rainbow trout alevins has also been reported when fed diets containing high levels of fishmeal hydrolysate (Dabrowski, 1984), a phenomenon attributed to the hygroscopic nature of this food formulation. Likewise, hydrolysate inclusion rates above 25% reduced growth of European seabass larvae (Cahu *et al.*, 1999). While growth rates were almost identical to a fishmeal-based control, maximum survival was shown by larvae fed diets containing 25% hydrolysate inclusion and significant decreases in skeletal malformation were found with increasing levels of hydrolysate.

Similar growth inhibition resulting from excess levels of individual amino acids has also been described for juvenile barramundi (Murillo-Gurrea *et al.*, 2001), and implies decreased assimilation of dietary protein when internal FAA pools are saturated. In comparison, digestion of intact proteins within the fish gut are likely to result in a controlled release of amino acids for intestinal absorption, resulting in a more balanced amino acid composition of FAA pools. While Applebaum and Rønnestad (2004) determined FAA's to be effectively utilised by Atlantic halibut (*Hippoglossus hippoglossus*) larvae by a tube feeding method, it is likely that this treatment constituted a small percentage of the total amino acid pool of the fish larvae and therefore did not saturate it with individual free amino acids.

The common finding of the majority of studies utilising dietary protein hydrolysates is that a moderate dietary inclusion ( $\leq 25\%$  of total protein) increases larval performance, while higher inclusion levels depress growth and/or survival. Interestingly, Rønnestad *et al* (2003) calculated that a similar proportion (12-26%) of amino acids from rotifers that were retained by turbot larvae came from rotifer free amino acid pools with the remainder (74-88%) coming from rotifer proteins. The commonality of these findings may indicate that there is a physiological limit to the ability of fish larvae to assimilate free amino acids from their food, and that further amino acid requirements beyond this limit must be met by intact proteins.

The only formulated diets that have supported significant growth and survival from first feeding in altricial fish larvae have also included significant proportions of hydrolysed protein (Appelbaum, 1985; Cahu *et al.*, 1998; Fontagne *et al.*, 2000; Cahu *et al.*, 2003). The hydrolysis of dietary proteins may therefore be an integral step in the replacement of significant amounts of live foods with formulated foods. It is therefore important to further investigate the effects of low to medium inclusion levels of protein hydrolysate, or the partial hydrolysis of dietary protein for formulated larval fish diets, to supply dietary protein in a highly utilisable form.

#### 1.3.2.3. Degree of Hydrolysis

Hydrolysis of dietary proteins cleaves peptide linkages, reducing the molecular size of protein delivery. This process may act differentially on different protein chains and

amino acid linkages, depending on the enzymes used and their specificity for particular peptide bonds. Indiscriminate hydrolysis of dietary proteins may therefore free specific amino acids for fast adsorption over the gut wall while those that remain linked as proteins and long peptide chains are stalled in the gut as they are digested. The net result is the formation of an excess of particular amino acids in circulation, without the presence of complementary amino acids required for protein anabolism. Since FAA pools are small relative to total body amino acid pools (DeSilva and Anderson, 1995), the consequent inability to assimilate these amino acids into proteins is likely to cause their catabolism or excretion. Dietary free amino acids are also more susceptible to leaching (López-Alvarado *et al.*, 1994; Kvale *et al.*, 2002), and if essential, their subsequent absence may limit the value of the hydrolysate as a source of protein accretion. For these reasons it has been suggested that short-chain peptides are more useful as a readily digestible source of amino acids than FAA in microdiet formulations for shrimp (Cordova-Murueta and Garcia-Carreno, 2002) and fish larvae (Zambonino Infante *et al.*, 1997; Dabrowski *et al.*, 2003). This has been demonstrated experimentally, at least with the freshwater rainbow trout (*Oncorhynchus mykiss*), with dietary di-peptides being assimilated into body proteins, while free amino acids were catabolised to a greater extent (Dabrowski *et al.*, 2003), resulting in inferior growth and survival. Whether this remains true for marine species is yet to be elucidated.

FAA are regarded as an important energy source for developing fish embryos and larvae (Fyhn and Serigstad, 1987; Sivaloganathan *et al.* 1998), with high levels (25.3 - 200 nmol/egg) of FAA found in the egg and yolk-sac larvae stages. The importance of FAA

as an energy source was supported by depletion of the FAA supply in embryonic and larval development through to yolk adsorption, accompanied in the embryonic form by an increase in stored ammonia. When this finding is compared to the high FAA levels found naturally in marine zooplankton (Dabrowski and Rusiecki, 1983), it may be surmised that comparable levels may be beneficial in microdiet formulations for early marine fish larvae as an energy substrate. Furthermore, free amino acids may play a very important role in the feeding of marine fish larvae through their chemo-attractant properties (Kolkovski *et al.*, 1997a).

Formulated microdiets rarely contain free amino acids or low molecular weight peptides, instead containing intact proteins and other ingredients that may be difficult for larvae to digest, such as binding or coating agents (Partridge and Southgate, 1999; Baskerville-Bridges and Kling, 2000). This reflects the difficulty encountered when attempting to bind low molecular weight materials, such as free amino acids, in a microparticle in an aqueous solution (López-Alvarado *et al.*, 1994). The partial hydrolysis of dietary proteins to form short-chain peptides rather than FAA's may decrease or eliminate the leaching problems inherent in FAA incorporation into formulated foods.

While microparticles are ingested from first-feeding, limited success has been achieved with complete early weaning of barramundi larvae (Walford *et al.*, 1991; Southgate and Lee, 1993). As high levels of pinocytosis have been documented across the rectal epithelium in barramundi larvae prior to the onset of acidified and pepsin-mediated digestive processes (Walford and Lam, 1993), the delivery of small peptides may be

important in the utilisation of microparticulate formulations in this species. Therefore, the delivery of amino acids in microdiet formulations for barramundi larvae may hinge on the development of a hydrolysis technique that yields low molecular weight peptides, which may be effectively incorporated into formulated microparticles with a minimum of leaching.

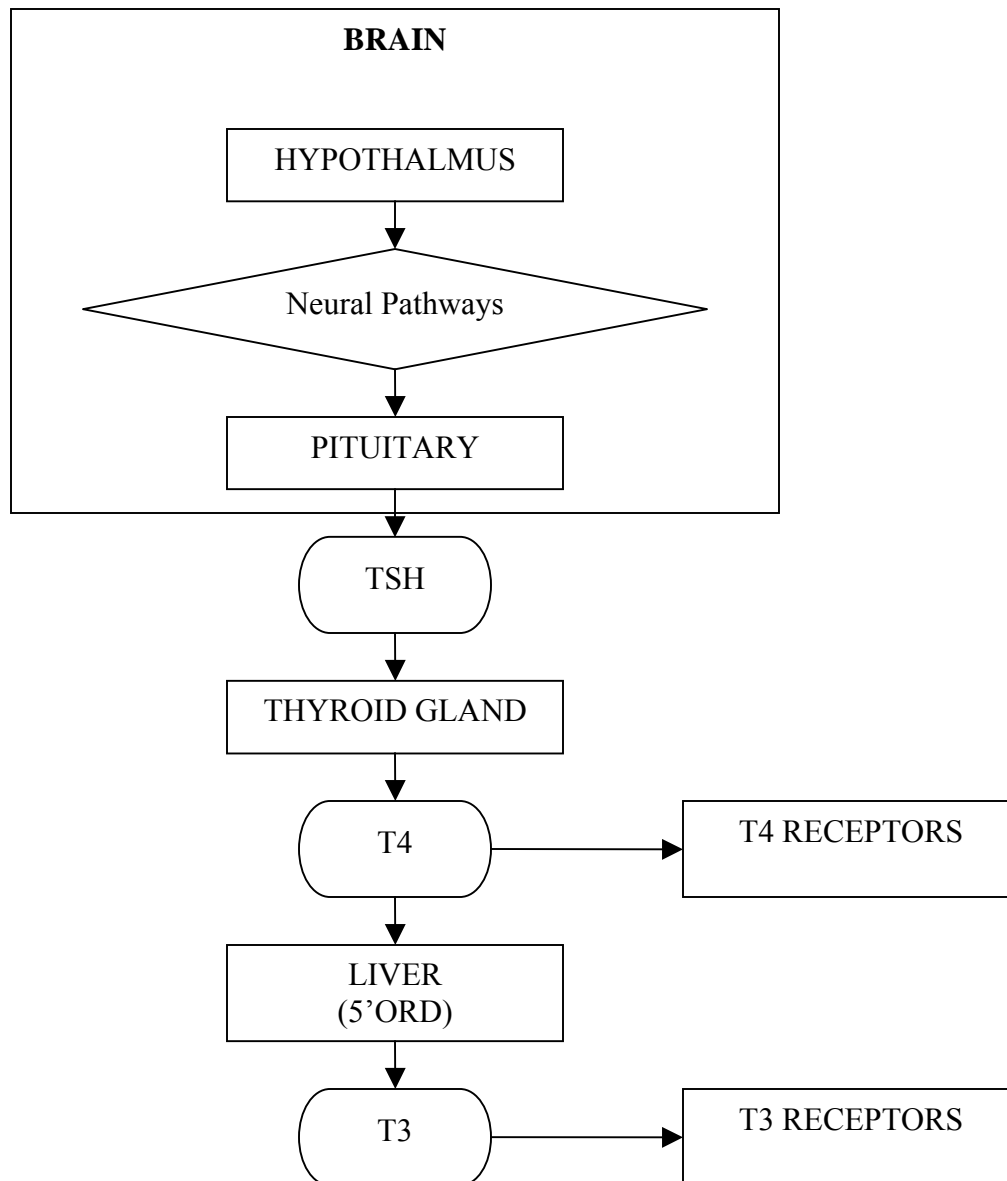
#### **1.4. Thyroid Hormones in Fish Larvae**

The thyroid hormones L-Thyroxine (T4) and Triiodothyronine (T3) are iodinated tyrosine molecules, which have been demonstrated to play an important role in various aspects of growth and development of the larvae of several fish species. Thyroid hormone administration promotes various responses in the larvae of different fish species, including improved egg hatchability and viability (Lam and Sharma, 1985), accelerated yolk-sac absorption (Nacario, 1983), increased swimbladder inflation rates (Brown *et al* 1988), accelerated stomach formation (Huang *et al* 1998 a, b; Soffientino and Specker 2001) improved larval survival (El-Zibdeh *et al.*, 1996; Tachihara *et al.*, 1997) and improved growth and development (Lam, 1980; Lam and Sharma, 1985; Lam, 1991; Nayak *et al.*, 2000). Thyroid hormones have also been closely linked to metamorphosis (Lam and Sharma, 1985; Inui *et al.*, 1995; Boeuf *et al.*, 1999) and subsequent cannibalistic behavioural patterns (Hey *et al.*, 1996; Hutchinson and Iwata, 1998), however more recent evidence suggests that thyroid hormones likely exert control over all processes of rapid development and body reorganisation in larval ontogeny (Liu and Chan 2002). Both TH and TH receptors are developed within the first two days of larval development (Nugegoda *et al* 1994; Tanaka *et al* 1995; Liu and Chan 2002), indeed the

importance of thyroid hormones to the development of zebrafish can be traced as far back as maternal contributions to the egg (Brown *et al* 1988). Since *L. calcarifer* continuously undergo rapid body development and reorganisation throughout the larval phase, it is likely that thyroid hormone levels are indicative of growth and development potential in the larval stages of this species.

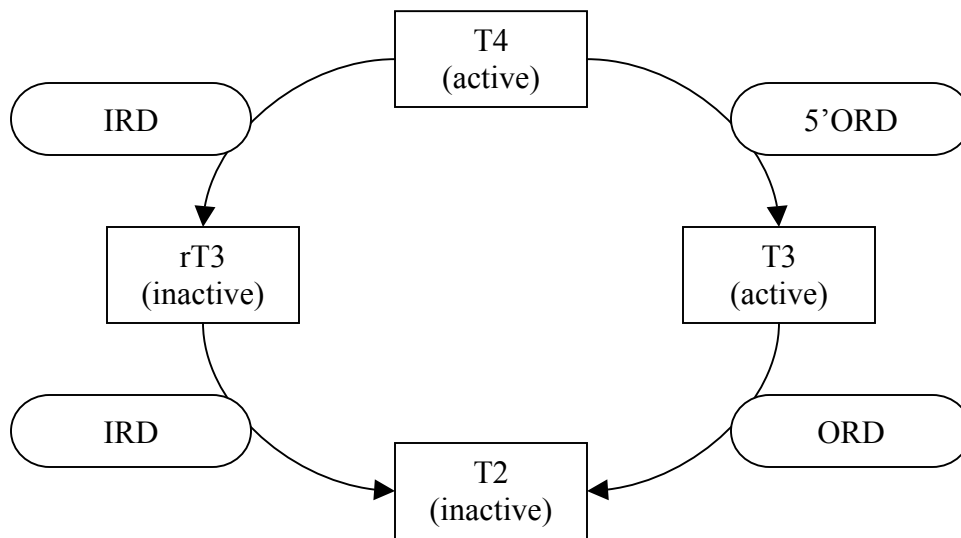
The natural stimulation of thyroid hormones is mediated through the thyrotropic hormone axis (Fig. 1.3). External stimuli are received by the hypothalamus, which stimulates the release of thyroid-stimulating hormone (TSH) from the pituitary, by specific neural pathways (Eales, 1995; MacKenzie *et al.*, 1998). Circulating TSH stimulates T4 secretion from the thyroid gland (Higgs *et al.*, 1982). T4 is converted to T3 in a variety of tissues, predominantly the liver, catalysed by the 5'outer-ring monodeiodinase (5'ORD) enzyme (Figure 1.4, Higgs *et al.*, 1982).

L-thyroxine (T4) is generally regarded to be the relatively inactive precursor of triiodothyronine (T3) in juvenile fish (Higgs *et al.*, 1982; Eales *et al.*, 1992). However, T4 may be of greater importance in larval development (Nacario, 1983; Lam and Sharma, 1985; Yamano *et al.*, 1994; Wan *et al.*, 1997). T4 also has a closer affinity with binding proteins in fish plasma, and therefore has a higher retention time (Higgs *et al.*, 1982), and consequently T4 levels may be less affected by short term stimulation such as overnight starvation.



**Fig. 1.3. The thyrotropic hormone axis (adapted from Eales, 1985).**





**Fig. 1.4. Thyroid hormone outer ring (ORD) and inner ring (IRD) deiodination (adapted from Eales, 1985).**

#### 1.4.1. Thyroid Hormones and Growth

Of all hormone systems studied in fish, the somatotropic, thyrotropic and gonadotropic axes are the most important in the control of growth (Boeuf *et al.*, 1999). Thyroid hormones (TH) such as L-thyroxine (T4), triiodothyronine (T3) and thyroid-stimulating hormone (TSH) have been shown to be very effective in stimulating growth in larvae of flounder, *Paralichthys olivaceus* (Inui *et al.*, 1995), rabbitfish, *Siganus guttatus* (Ayson and Lam, 1993b), Atlantic halibut, *Hippoglossus hippoglossus* (Solbakken *et al.*, 1999), two species of tilapia, *Oreochromis mossambicus*, barramundi, *Lates calcarifer* (Lam, 1980; Lam, 1991; Wan *et al.*, 1997), and catfish, *Heteropneustes fossilis* (Nayak *et al.*, 2000). Thyroid hormones are also thought to have a regulatory action on other endocrine processes in fish, facilitating the action of other anabolic hormones, such as growth

hormone (Higgs *et al.*, 1982). Nugegoda *et al.* (1994) found no effect of T4 and T3 administration on the growth of barramundi larvae. While Nugegoda *et al.* (1994) treated fish by immersion with 0.25-0.5 mg.L<sup>-1</sup> T4 and 0.01 mg.L<sup>-1</sup> T3 from the time just after hatching to day 14, Wan *et al.* (1997) used microspheres to deliver T4 through the oral-gastric route to larvae, from day 16 to day 42 after hatching. As results with thyroid hormone immersion have proven to be effective in regulating growth for the larvae of other fish species (Lam, 1991; Nayak *et al.*, 2000), the difference in these results has been attributed to the age of the larvae; seabass larvae not responding to thyroid hormone treatment until later in their larval development. One possible explanation for the lack of response of early seabass larvae to TH treatment is that high rates of thyroxinogenesis in early larvae satisfy the potential for thyroid-mediated growth stimulation in this species (Nugegoda *et al.*, 1994). This is supported by experimental evidence, with Nugegoda *et al.* (1994) finding a rapid rise in whole body T4 concentration from 24 hours after hatch to a peak on day 12 of 8-12 ng/g wet weight. Likewise, Wan *et al.* (1997) found no significant difference in the measured growth parameters in the first 15 days of their experiment (30 day-old larvae), while there was a significant difference in length and weight by day 27 (42 day-old larvae). This may, however, be a factor of the extended experimental period rather than an ontogenetic effect.

This paradox between studies in thyroid hormone treatment continues when studies with flounder are considered. While thyroid hormone treatment of *Paralichthys olivaceus* resulted in increased growth and development (Inui *et al.*, 1995), thyroid hormone treatment did not increase survival in *Paralichthys dentatus* (Bengston *et al.*, 2000), nor

did it positively affect the ability of these fish to wean to formulated foods, as determined by survival. This indicates that the thyroid hormone influence on fish larvae may be mediated by other factors, which may include complimentary hormones such as those in the growth hormone-insulin like growth factor-I hormone axis, the antagonism of inhibiting hormones such as dopamine, or the availability of hormone pre-cursors such as tyrosine or iodine (Higgs *et al.*, 1992). This may also be a factor of a species-specific and/or dose-dependent response to TH treatment in fish larvae. Alternatively the latter study may have used larvae that contained sufficient endogenous thyroid hormones for optimal survival and weaning ability, and exogenous hormone treatment therefore had no net benefit.

One such factor, which has been studied in conjunction with thyroid hormones in larval fish, is cortisol. Studies on Pacific threadfin, *Polydactylus sexfilis*, larvae have shown that combined treatment with cortisol and T3 accelerated gut development, increased survival and resulted in larger fish of a more uniform size than untreated controls and fish treated with the individual hormones alone (Brown and Kim, 1995; Kim and Brown, 1997).

A strong correlation has been reported between specific growth rate and plasma T3 levels in juvenile Arctic char, *Salvelinus alpinus* (Eales and Shostak, 1985) when studying food ration influences. A modest correlation between body weight and 5'ORD activity was found in rainbow trout (Eales *et al.*, 1992), while other studies have found no specific

relationship between growth parameters and thyroid hormone levels (Farbridge *et al.*, 1992; Nankervis *et al.*, 2000).

#### *1.4.2. Thyroid Hormones and Metamorphosis*

TH have been shown to be important in fish metamorphosis and have been linked to subsequent changes in body protein constituents (Lam and Sharma, 1985; Inui *et al.*, 1995; Boeuf *et al.*, 1999). Administration of TH influences the morphometry and physiology of fish larvae with effects such as increasing the rate of yolk-sac resorption, increasing fin development, head size and body width (Lam, 1980; Lam, 1991; Wan *et al.*, 1997), while in other studies accelerating yolk sac resorption and gut formation (Huang *et al.*, 1998a; Nayak *et al.*, 2000; Soffientino and Specker, 2001). TH have also been shown to increase larval survival in some species of fish and have been implicated in the stimulation of digestive processes (Solbakken *et al.*, 1999; Nayak *et al.*, 2000). Exogenous T4 treatment accelerated metamorphosis by 12 days in summer flounder, *Paralichthys dentatus* (Huang *et al.*, 1998b), while T4 synthesis inhibition by thiourea treatment suppressed metamorphosis. Further applications of the thyroidal regulation of metamorphosis is the synchronisation of metamorphic events, as occurs with summer flounder (Gavlik *et al.*, 2002), where TH-inhibition by thiourea, followed by thyroid-mediated initiation of metamorphosis reduced the within-tank size variation which gives rise to cannibalistic behaviour.

#### *1.4.3. Nutritional Influences on Thyroid Hormones*

By applying an endocrinal approach to the nutritional control of performance indices such as growth and survival, we can achieve a better understanding of the underlying processes governing the physiological status of fish. Such an approach has been taken by several authors seeking to further understand such growth processes, predominantly working with post-metamorphosis juvenile animals. The majority of this research has been performed on salmonids (e.g. Leatherland *et al.*, 1977; Himick *et al.*, 1991; Eales *et al.*, 1992; Farbridge *et al.*, 1992; Gelineau *et al.*, 1996; Dosanjh *et al.*, 1998), with very few studies on tropical species (e.g. Nankervis *et al.*, 2000). Furthermore this research has been applied very little if at all to larval stages of fish.

Numerous studies have highlighted the relationship between nutrition and circulating TH levels (Himick *et al.*, 1991; Eales *et al.*, 1992; Farbridge *et al.*, 1992; Dosanjh *et al.*, 1998), and have implicated TH's in the nutritional control of growth (Plohman *et al.*, 2002). Dietary protein inclusion levels regulate circulating T3 levels and 5'ORD activity in juvenile rainbow trout (Eales *et al.*, 1990; Eales *et al.*, 1992). While carbohydrate influences 5'ORD activity, this influence was thought to be a reflection of protein metabolism reacting to a higher dietary caloric content. Similar results have been found with juvenile barramundi, with dietary carbohydrate inclusion levels influencing both protein metabolism and serum T3 levels (Nankervis *et al.*, 2000).

Thyroid hormones have been shown to stimulate digestive processes in fish, and may lead to an enhanced absorption of ingested amino acids and peptides (Solbakken *et al.*, 1999). Furthermore, the inclusion of a moderate amount of fish protein hydrolysate in

compound microdiets for European sea bass larvae facilitated the onset of the adult mode of digestion (Cahu *et al.*, 1999). Given the importance of TH in the transition from the larvae to juveniles (i.e. metamorphosis), it is reasonable to suspect an influence of dietary protein sources on TH levels. The potential for predictable nutritional stimulation of increased TH levels may have implications toward the manipulation of growth, increasing survival and synchronisation of metamorphosis.

### **1.5. Aims of This Study**

The major objective of this study was to further develop our understanding of the processes involved in feeding formulated foods to altricial fish larvae. Dietary macronutrients (energy level, protein level and protein source) were investigated for their affect on growth and survival of barramundi larvae when fed formulated foods. Digestibility analysis and amino acid composition of various marine-derived protein sources was used to determine the underlying reasons behind the observed growth and survival differences. The potential of thyroid hormones as markers of nutritional status in barramundi larvae was also investigated.

The specific aims of this study were:

1. To examine the pepsin development of barramundi larvae on a daily basis, and to characterise pepsin development in relation to external morphological and size characteristics.
2. To investigate the protein and energy requirements of barramundi larvae, and the interactions between dietary energy and protein levels.
3. To determine the effectiveness of marine animal meals as protein sources in promoting growth and survival of barramundi larvae.
4. To investigate the utilization of partially hydrolysed fish meal in formulated microbound diets for barramundi larvae.
5. To evaluate the digestibility of acid denatured fish meal *in vitro* and the affect of denatured fish meal inclusion in formulated foods on the growth and survival of barramundi larvae.

6. To assess dietary effects on thyroid hormones to achieve a greater understanding of endocrinal mediation of growth promotion in barramundi larvae.
7. To assess dietary effects on whole homogenate pepsin level in barramundi larvae.

This research will aid our knowledge of specific nutritional requirements of barramundi larvae and provide valuable insights into the effects of nutrition on growth and larval physiology. By applying a digestive physiology, biochemical and endocrinal approach to nutritional research, this study aims to elucidate the reasons for the variable nutritional quality of dietary protein sources in microbound diets for barramundi larvae. The outcome of this research will therefore be an integrated data set, outlining dietary protein and energy affects on growth and survival in barramundi larvae fed microbound diets, and relating this data to the nutritional and physiological mechanisms behind such performance indices.



## Chapter 2

### General Materials and Methods

#### 2.1. Diet Formulations

Microbound diets were prepared by an adaptation of the methods of Kolkovski *et al.* (1993). Microbound diets (MBD) use a binder, in this case 3% gelatin (Partridge, 1996), to form a bound matrix of nutrients without an outer coating agent (Southgate and Partridge, 1998). Dietary protein sources incorporated into MBD were not defatted to prevent the loss of protein associated with the defatting process (Anderson *et al.*, 1993). Squid oil was used as a source of essential fatty acids and dietary energy, and has previously been demonstrated to be a rich source of highly unsaturated fatty acids (Ritar *et al.* 2004). All dietary ingredients, with the exception of gelatin, vitamins and minerals, were weighed to the nearest milligram before being added together and mixed by hand. Remaining ingredients were pre-dissolved in warm water before being added to the feed mixture. Sufficient distilled water was added to give the formulation a liquid consistency before mixing well with a Braun Multiquick barmix. The feed mixture was then spread thinly onto plastic trays (36 cm<sup>2</sup> per gram dry weight ingredients) and oven-dried at 50°C to constant weight. Dry diets were ground using a mortar and pestle before being sieved to achieve a particle size range between 100 and 300 µm. These diets were stored at <6°C until used.

#### 2.2. Biochemical Analysis

All major feed ingredients were analysed for dietary protein and energy contents.

### *2.2.1. Protein Analysis*

The protein levels of feed ingredients were determined using a Kjeldahl Nitrogen method, adapted from Baethgen and Alley (1989). Samples to be analysed were digested in a solution of sulphuric acid and hydrogen peroxide, with a selenium catalyst. Total nitrogen levels of digested samples were determined colorimetrically, using a salicylate and hypochlorite-based colour reagents. Total protein was calculated using a conversion factor of 6.25 g protein per gram nitrogen. All samples were analysed in triplicate.

#### 2.2.1.1. Protein digestion

Before being analysed for total protein content, samples were ‘digested’ by complete acid hydrolysis, at high temperatures. Samples (0.02 g) were weighed into clean, dry 75 mL volumetric digestion tubes. A 4.4 mL aliquot of digestion mixture (420 mL concentrated sulphuric acid, 350 mL hydrogen peroxide, 0.42 g selenium, 14 g lithium sulphate) was then added to each sample. All samples were then gradually heated in a block digester to 365°C over a one-hour period, and maintained at that temperature for approximately two hours. Digestion was determined to be complete when all samples became colourless. The samples were allowed to return to room temperature before being made up to 75 mL with distilled water.

#### 2.2.1.2. Colorimetric Analysis

After the digestion and dilution of proteins within each sample, total nitrogen levels were analysed colourimetrically by reaction with salicylate and hypochlorite (Baethgen and

Alley, 1989). The colourimetric reaction involved two reagents; N1 (34 g sodium salicylate, 25 g sodium citrate, 25 g sodium tartate and 0.12 g sodium nitroprusside, made to 1 L with distilled water) and N2 (30 g sodium hydroxide and 10 mL sodium hypochlorite, made to 1 L with distilled water). Nitrogen standards (ammonium sulfate equivalent to 0, 10, 20, 30, 40, 50 and 60  $\mu\text{g N}\cdot\text{mL}^{-1}$ ) and samples (100  $\mu\text{l}$ ) were added to clean, dry, 20 mL glass vials. A 5 mL aliquot of reagent N1 was added to each vial and allowed to stand at room temperature for 15 minutes. A 5mL aliquot of reagent N2 was added to each vial and allowed to stand for a further 1 hour. The absorbance of standards and samples was read on a spectrophotometer at 655 nm. Nitrogen concentrations of samples were determined against a standard curve, developed from the nitrogen standards outlined above.

### *2.2.2. Energy analysis*

Energy contents of feed ingredients were determined by bomb calorimetry. Samples of dry ingredients were compressed into pellets (0.05-0.15 g), and lipid samples (0.04-0.06 g) were combusted in the liquid form. All samples were combusted in a Parr 1421 Semimicro Calorimeter (Parr Instrument Company, Moline, Illinois, USA). Energy output was graphed using an Activon plotter (Houston Instrument, Austin, Texas, USA). All samples were assayed in triplicate. Benzoic acid was used as a standard of known energy level (26.455  $\text{MJ}\cdot\text{kg}^{-1}$ ). Peak height of samples and standards were used to calculate their energy content.

### 2.3. Fish Larvae

Barramundi larvae used throughout this project were spawned at the Queensland Department of Primary Industries (DPI) Northern Fisheries Centre, Cairns and at Bluewater Barramundi, Mourylian, Queensland, Australia. Larvae for each experiment were spawned from one or two female broodstock and were fertilised by one or two male broodstock correspondingly. Regardless of the number of broodstock used, eggs were incubated and transported together, effectively mixing the larval population and preventing any genetic bias on experimental treatments. Larvae were obtained one day after hatching (DAH), at the yolk-sac stage, were stocked at a density of 20-50.L<sup>-1</sup> in a cylindrico-conical tank (2,000 L), and grown to the stage required for experiments according to the protocol outlined in Fig. 1.1 (Chapter 1).

Cultured microalgae, *Nannochloropsis oculata*, was added to larvae rearing tanks to a density between 400,000 and 600,000 cells.mL<sup>-1</sup>. Rotifers, *Brachionus rotundiformis* were added to larval rearing tanks at a density of 20.mL<sup>-1</sup>. Rotifer populations were maintained in the larval rearing tanks for approximately eight days by addition of 500,000 cells.mL<sup>-1</sup> *N. oculata* as a food source. Rotifer density was monitored daily by counting the total number of rotifers in triplicate 1 mL samples of culture water, using a Sedgewick-Rafter counting cell. When the rotifer density dropped below 20.mL<sup>-1</sup>, rotifers were added to give a total density of 20 rotifers.mL<sup>-1</sup>.

Newly hatched *Artemia* (GSL, INVE, Ghent, Belgium) nauplii were first introduced to larvae rearing tanks when larvae were 8 DAH, and added at a density of 0.5.mL<sup>-1</sup> from 8-

10 DAH. When larvae were 11 DAH *Artemia* nauplii were added to larvae rearing tanks at a density of  $1.\text{mL}^{-1}$ , and at 12 DAH and 13 DAH *Artemia* metanauplii were added twice and three times daily respectively, at a density of  $1.\text{mL}^{-1}$ , after enrichment with Super Selco (INVE, Belgium). All feeding trials began 14 DAH.

### 2.3.1. Microalgae culture

The microalga *Nannochloropsis oculata* was cultured using a rotating batch culture method. A 1500 L capacity translucent tank was filled with 1100 L saltwater (30-35 ppt), which was then sterilised with 100 mL liquid chlorine (12.5% hypochlorite). The chlorine was removed with vigorous aeration overnight, prior to the inoculation of algae culture from another batch culture tank to a density of  $6 \times 10^6 \text{ cells.mL}^{-1}$  (250-350 L). Inoculation was performed through a  $10 \mu\text{m}$  filter bag to remove contaminants. Nutrients (Aquasol, Hortico (Aust.) Ltd) were added at  $40 \text{ mg.L}^{-1}$ . This culture was aerated heavily and maintained under ambient conditions for 5 days, reaching  $25 - 32 \times 10^6 \text{ cells.mL}^{-1}$ , and was used in the following three days for rotifer culture, “greenwater” additions to larvae rearing tanks and inoculation of other algae tanks.

### 2.3.2. Rotifer culture

*Brachionus rotundiformis* were cultured using semi-continuous culture methods. Densities of  $600-1000.\text{mL}^{-1}$  were maintained with up to 25% harvest per day. Rotifers were maintained in saltwater, at 35 ppt salinity and  $29 \pm 1^\circ\text{C}$ . Half of the rotifer culture water was drained daily and rotifers were trapped on a  $63 \mu\text{m}$  submerged screen. Harvested rotifers were then added to fish larvae rearing tanks or returned to rotifer

culture tanks. Rotifer culture tanks were then filled with *N. occulata* culture at  $10\text{-}20 \times 10^6 \text{ cells.mL}^{-1} \cdot \text{day}^{-1}$  and saltwater.

#### **2.4. Feeding Trials**

To facilitate transfer of larvae from feeding on live foods to formulated foods, formulated foods were added to larvae rearing tanks from 8 DAH to 12 DAH, at a rate of  $0.5 \text{ g.day}^{-1}$ . To prevent any bias toward any particular artificial diet, equal proportions of each of the experimental diets to be used in the subsequent feeding trial were mixed before addition to the larvae tank.

Feeding trials were conducted in 50 L parabolic-based tanks, with flow-through ( $1.2 \text{ L.min}^{-1}$ ) seawater ( $30\text{-}32 \text{ g.L}^{-1}$  salinity) and gentle aeration, maintained at  $28 \pm 1^\circ\text{C}$ . Photoperiod was maintained at 12 h light: 12 h dark. Barramundi larvae were counted individually into each experimental tank at an age of 13 DAH, to a density of  $6 \cdot \text{L}^{-1}$ . The feeding experiment began on the following day (14 DAH). Larvae were fed *ad libitum*, four times daily.

Any individuals showing obvious cannibalistic behaviour were removed from their tanks by siphon. The removal of such fish was recorded, and they were included in calculations of survival rates. Each growth trial was conducted for 14 days, after which all fish were removed from their tanks and sacrificed in a refrigerator at  $<5^\circ\text{C}$ . Survival was calculated as the final number of surviving larvae at the end of the experiment, plus the number of removed cannibalistic larvae, as a percentage of the initial number of larvae stocked.

## **2.5. Analyses**

### *2.5.1. Weight and Length measurements*

All fish were counted and photographed using a Leica DC300 camera, through a Lieca MZ12<sub>5</sub> stereo dissecting microscope (Leica Microsystems AG). Total length of each fish was subsequently determined using Leica IM50 Image Manager (V1.2) software. A random sub-sample of fish (0.2 g wet weight) was taken from each tank for thyroid hormone extraction and analysis, while the remainder were oven-dried at 50°C to constant weight. Dry larvae were weighed to the nearest 0.001 mg using a CAHN C-33 Microbalance.

### *2.5.2. Thyroid Hormone Analysis*

Larvae samples were prepared according to (Hey *et al.*, 1996) and thyroid hormones extracted according to (Tagawa and Hirano, 1987).

#### 2.5.2.1. Sample Preparation

A random sub-sample of fish from each treatment was blotted briefly on absorbent paper to remove surface moisture before weighing a sample of 0.1 g ( $\pm$  0.02 g) into microcentrifuge tubes. Each of these samples was homogenised on ice with a hand-held pestle. Extraction fluid (1 mL; 1%10 N NaOH, in 1 mM 6-N-propylthiouracil made up in methanol) was added to each sample at -5°C and further homogenised . Samples were then sonicated for 2 minutes and vortexed for ten minutes, before being centrifuged for ten minutes, at 3,700 rpm and 5°C.

The supernatant was removed to a 16 mm diameter glass test tube. A further 0.5 mL extraction fluid was added to the sample before being sonicated, vortexed and centrifuged as above. The supernatants were then combined and 5 mL chloroform and 0.5 mL 2 N NH<sub>4</sub>OH added. This solution was vortexed for 15 minutes before being centrifuged for 10 minutes at 3,700 rpm and 5°C. The aqueous phase was carefully removed from the chloroform phase and added to a clean 5 mL vial. A 0.5 mL aliquot of 2 N NH<sub>4</sub>OH was added to the chloroform phase and vortexed and centrifuged before the aqueous phase was removed as above. A further 0.5 mL 2 N NH<sub>4</sub>OH and 1 mL extraction fluid was added to the chloroform phase and vortexed, centrifuged, and aqueous phase removed as above. All aqueous phases were combined.

The aqueous phases were heated to 30°C using a block heater and dried under nitrogen. These samples were stored at -80°C until required. When required for analysis, samples were re-suspended in 200 µL 0.1N NaOH (Hey *et al.*, 1996).

#### 2.5.2.2. Radioimmunoassay Procedure

Radioimmunoassays were performed using coated tube antibody radioimmunoassay kits (ICN Diagnostics, New York). Aliquots (100 µL) of each sample and standard (50 µL, 100 µL, 200 µL, 400 µL and 800 µL) were added to tubes, coated on the inner surface with a T3 or T4 antibody. A 1 mL aliquot of T3 or T4 tracer solution (I-125 labelled hormone in a phosphate buffer with sodium salicylate and 0.1% sodium azide) was added to each tube before vortexing. Samples were incubated at 37 ± 1°C for 1 hour before

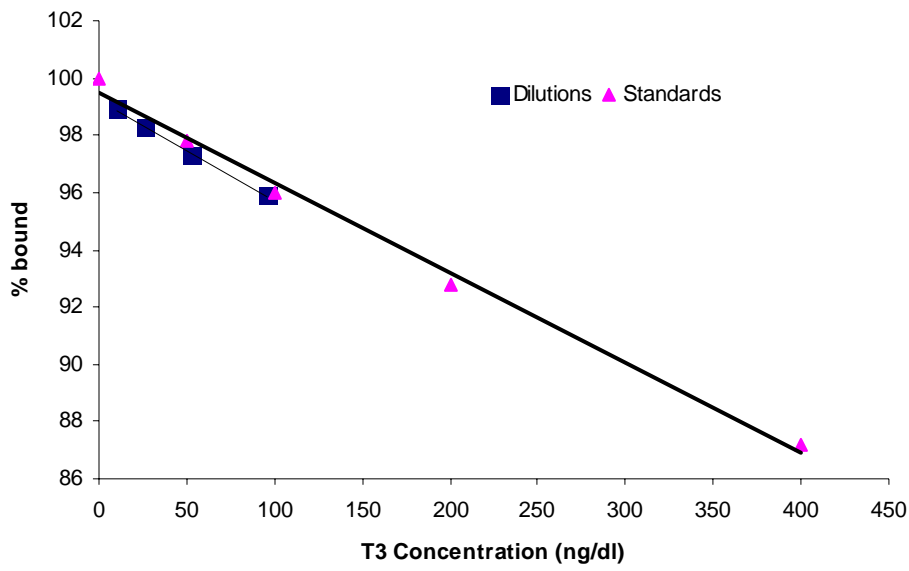


aspirating all liquid from the tubes. All tubes were rinsed once with distilled water to remove any non-bound tracer and aspirated again, before reading radiation on a gamma counter (Packard Instrument Company) for three minutes per sample.

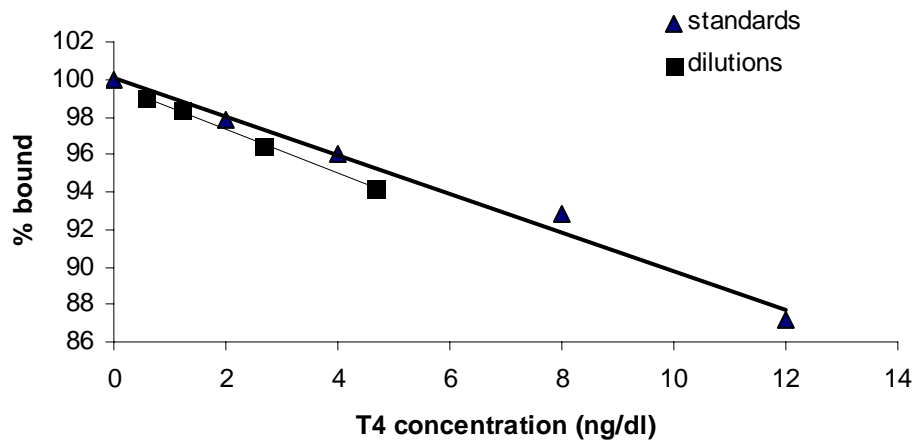
A quality control sample was prepared to determine intra-assay coefficient of variation, by extracting 5 g blotted wet weight of 14 DAH larvae as described for samples above. This extract was re-suspended with 5 mL 0.1 N NaOH. Serial dilutions of this solution were analysed and gamma counts compared to those of standards to determine parallelism, thus validating the method. Three quality control samples were also run in each analysis to determine intra-specific coefficient of variation. The intra-assay coefficient of variation was 8.18% and 5.94% for T3 and T4, respectively, the inter-assay coefficient of variation was 11.66% and 9.68% for T3 and T4, respectively, and the extraction efficiency 82.2% and 86.0% for T3 and T4, respectively. Hormone concentrations were not corrected for extraction efficiency.

#### 2.5.2.3. Radioimmunoassay Validation

The RIA methods used for T3 and T4 were validated for the barramundi larvae extracts by comparison of the concentration of the hormone detected in serial dilutions of the quality control sample and standards with the percentage of tracer bound to analysis tubes. This procedure validated the assay by confirming that expected values from the standards relate to assayed values from the dilutions, and is displayed by parallel regression lines (Figs. 2.1 and 2.2).



**Fig. 2.1. Triiodothyronine assay validation. A comparison of the percentage radioactive T3 bound to the T3 antibody and concentrations of T3 standards and dilutions of larvae hormone extract.**



**Fig. 2.2. L-Thyroxine assay validation. A comparison of % radioactive T4 bound to the T4 antibody and concentrations of T4 standards and dilutions of larvae hormone extract.**

### 2.5.3. *Pepsin analysis*

#### 2.5.3.1. Enzyme extraction

When analysed, larvae were defrosted and homogenized with a hand-held pestle. Samples were sonicated before vortexing for 10 min and centrifugation (10,000 g; Eppendorf 5415C microfuge, Germany). The supernatant was then removed for analysis.

#### 2.5.3.2. Assay procedure

Enzyme samples were analysed in triplicate for pepsin by the methods of Anson (1938), modified for use with 96-well microtitre plates. Hemoglobin was used as the substrate. Ten samples of hemoglobin (0.125 g) were each added to 5 mL purified water before being vortexed for 10 min, and centrifuged for 10 min at 2,000 g in a Beckman GS-6R bench centrifuge. Supernatants were removed and combined to form the hemoglobin (Hb) stock. For each assay an acidified Hb substrate was made by adding 4 mL Hb stock to 1 mL 0.3 N HCl.

A 10  $\mu$ L aliquot of each enzyme extract was added to 50  $\mu$ L Hb substrate in a microcentrifuge tube, before being vortexed and incubated for 10 min at 28°C. The reaction was terminated and proteins precipitated by the addition of 100  $\mu$ L 0.3 N trichloroacetic acid (TCA). Blanks were prepared for each extract sample by the same method, though delaying the addition of extract until after the addition of TCA. L-tyrosine (Sigma) standards (100 mM, 50 mM, 25 mM, 12.5 mM and 6.25 mM) were

made and included by the same method as extract blanks. All samples, blanks and standards were vortexed before centrifugation (10,000 g, 5 min).

A 50  $\mu\text{L}$  aliquot of each supernatant was transferred to 96-well microtitre plates, before adding 100  $\mu\text{L}$  NaOH and 30  $\mu\text{L}$  Folin - Ciocalteu's phenol reagent (Sigma product number F9252). This was incubated for 5 min before reading the absorbance at 620 nm. Pepsin activity was expressed in terms of pepsin units, equivalent to the amount of pepsin required to liberate 1  $\mu\text{M}$  tyrosine per hour.

## Chapter 3

### The external morphological development and pepsin ontogeny in barramundi, *Lates calcarifer*, larvae

#### 3.1. Introduction

The failure of artificial foods to promote high levels of growth and survival in the larvae of many species of fish larvae has been widely attributed to their inability to digest complex molecules, most notably complex protein sources (reviewed by Kolkovski, 2001). The proteolytic digestive enzymes of the pancreas (chymotrypsin and trypsin), but not the stomach (pepsin), are active before first feeding activity in many altricial marine fish larvae (reviewed by Dabrowski, 1986). As chymotrypsin and trypsin have specific sites of action (Blow, 1971; Kiel, 1971), whereas pepsin has a broad range of active sites (Fruton, 1971), the digestive processes combining pepsin with pancreatic enzymes is likely to hydrolyse proteins to a greater extent than when pepsin is missing. Furthermore, the acidic nature of peptic digestion (optimal pH 2-3) is thought to increase protein digestibility by denaturation (Lauff and Hofer, 1984). When subjected to acidic environments (pH 2-3) many proteins will progressively 'unravel', losing their quaternary, tertiary and then secondary structure (Fink *et al.*, 1994). As denaturation proceeds, unraveling of polypeptide chains reveals more binding sites for the action of proteolytic enzymes, thus increasing the digestibility of dietary proteins (Jany, 1976).

It has been hypothesized that live foods make a significant contribution to the digestive capacity of altricial fish larvae, through autolysis or contributing enzymes and zymogens

to the natural enzyme profile of the larvae (Lauff and Hofer, 1984; Walford and Lam, 1993; Kolkovski, 2001). Co-feeding live foods with formulated foods has increased the assimilation of microdiets, which may relate to the contribution of digestive enzymes from live foods (Kolkovski, 2001), or may represent increased ingestion by live food stimulation, as demonstrated by Kolkovski *et al.* (1997a).

While juvenile barramundi can efficiently utilize formulated foods, little success has been previously evident with live food replacement for barramundi larvae (Walford *et al.*, 1991; Southgate and Lee, 1993). This has been generally attributed to a low digestive capacity, as microcapsules and microbound diets have been effectively ingested (Walford *et al.*, 1991; Southgate and Lee, 1993). Therefore the onset of pepsin activity may be integral to the effective capacity of barramundi larvae to wean effectively onto formulated foods. The digestive tract development has previously been described for barramundi larvae (Walford and Lam, 1993), along with the trypsin and pepsin ontogeny. However, this previous study had limited sampling points and described pepsin development in terms of larvae age, with little reference to the stage of morphological development.

This study therefore determines pepsin development of barramundi larvae on a daily basis, and characterizes pepsin development in relation to external morphological and size characteristics.

## 3.2. Material and Methods

### 3.2.1. Source of Larvae

Experimental animals were reared as described in Chapter 2 (section 2.2), with the following exceptions: Larvae were reared in four 270 L cylindrico-conical tanks, which were static during the day, and had 1 L.min<sup>-1</sup> water-flow overnight. Rotifers (*Brachionus rotundiformis*; 20.ml<sup>-1</sup>) and algae (*Nannochloropsis*; 500 000 cells.ml<sup>-1</sup>) were added daily to the rearing tanks, and flushed from the system overnight with seawater (1 L.min<sup>-1</sup>).

### 3.2.2. Larvae sampling

A sample of larvae was removed from all tanks daily (100 µg-1 g wet weight), by means of a siphon running into a submerged nylon screen (200 µm mesh). The timing of sampling was immediately after the lights were turned on, before larvae were fed. Larvae were euthanased by chilling to <5° C before being weighed, photographed (a sub-sample of 30 larvae; see Chapter 2, section 2.4.1), and frozen in 500 µL purified water to -80° C.

### 3.2.3. Pepsin analysis

Carcass pepsin levels were extracted and analysed as described in Chapter 2, section 2.4.3. Larvae samples were homogenised, sonicated and vortexed before being centrifuged and supernatants removed. Supernatants were then analysed for pepsin activity according to the method of Anson (1938), modified for use with 96-well microtitre plates, using Folin - Ciocalteu's phenol reagent as the amino acid binding dye and haemoglobin as the protein standard.

### **3.3. Results**

#### *3.3.1. Morphological development*

At 1 DAH (day(s) after hatch), barramundi larvae ( $2.3 \pm 0.06$  mm) did not have an open mouth, and the gut was an undifferentiated and apparently blind-ended tube (Fig 3.1). There was a small yolk-sac present, immediately posterior to an oil globule. The eyes were lightly pigmented, with a transparent pupil. Otoliths were obvious as two small circles, 14  $\mu$ m diameter, immediately posterior to the eye. The notochord was straight, running from the eye to the posterior end of the body, where it tapered to a point. A transparent envelope of tissue surrounded the entire body, with the exception of the anterior and ventral sides of the head.

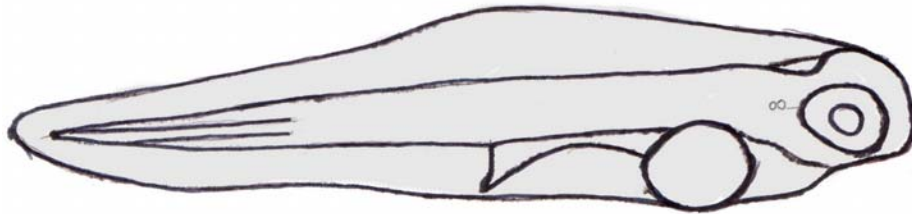
By 2 DAH ( $2.4 \pm 0.02$  mm) the mouth was open and rotifers were present in the digestive tract. The oil globule had reduced to 290-302  $\mu$ m at its anterior-posterior axis to 307-310  $\mu$ m at its dorso-ventral axis. The gut had expanded to 60  $\mu$ m in width, and the intestinal-rectal valve developed. The iris of the eye was fully pigmented. There were small areas of pigmentation, around the persistent oil globule and along the notochord and flanks of the larvae.

The first appearance of the swim bladder occurred at 3 DAH ( $2.5 \pm 0.06$  mm). At this stage 66% of larvae had partially inflated swim bladders, measuring 80-100  $\mu$ m at its dorso-ventral axis and 108-122  $\mu$ m at its antero-posterior axis. At 3 DAH the oil globule was persistent, though had reduced in size to 138-154  $\mu$ m at its dorso-ventral axis and

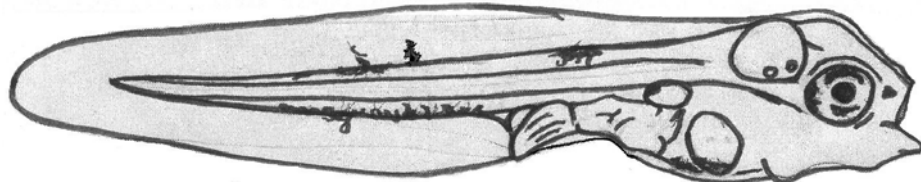


148 – 163  $\mu\text{m}$  at its antero-posterior axis. The major feature of the ventral side of the body at this stage was the gut, which has become folded at its anterior end.

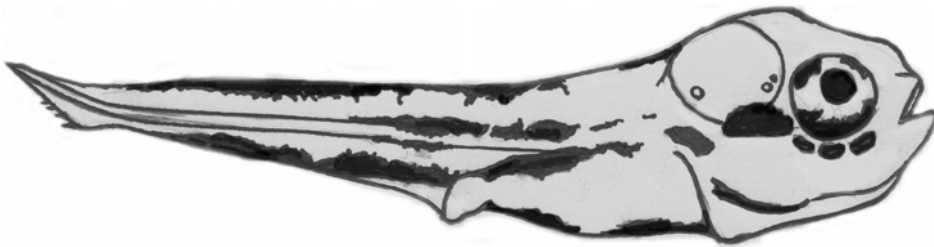
a)



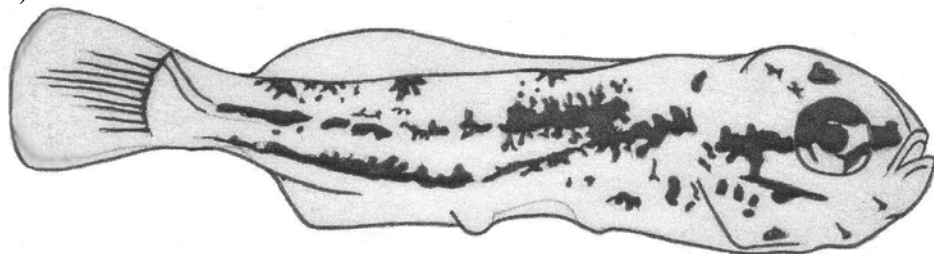
b)



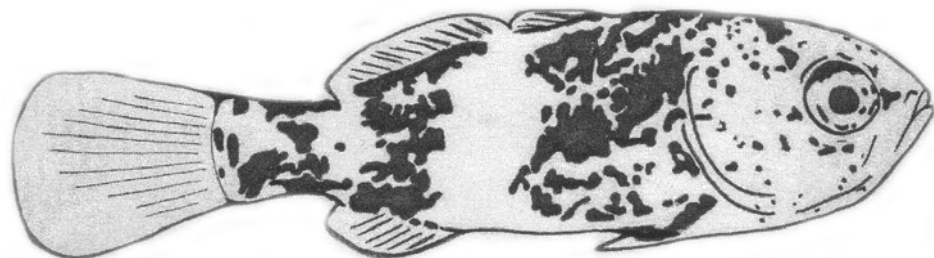
c)



d)



e)



**Fig 3.1. Morphological development of barramundi larvae; a) 1 DAH, yolk-sac larvae (2.3 mm), b) 3 DAH, swim bladder inflation (2.5 mm), c) 9 DAH, flexion (3.5 mm), d) 15 DAH, post-flexion (4.8 mm), e) 20 DAH, metamorphosed (7.2 mm)**

At 4 DAH ( $2.5 \pm 0.03$  mm) 100% of fish sampled had inflated gas-filled swim bladders, with a characteristic shiny appearance from the presence of gas. At this stage swim bladders range from 108-128  $\mu\text{m}$  at its dorso-ventral axis and 197-216  $\mu\text{m}$  at its anterior-posterior axis. There were no larvae sampled which had oil globules at this stage.

The development from 4 to 6 DAH consisted of intestinal convolution and development of mouth structures. During this period there was also a progressive increase in pigmentation along the notochord and along the dorsal and ventral flanks of the larvae.

At 7 DAH ( $2.8 \pm 0.04$  mm) the first indication of notochord flexion was present, with a slight upturn near the posterior end of the notochord, and a budding development on the ventral side of the curved section of the notochord (Fig 3.1). From 7-9 DAH an increasing number of larvae began notochord flexion, and at 10 DAH ( $3.7 \pm 0.07$  mm) the first post-flexion larvae were identified, with an upturned notochord and developed caudal fin (Fig 3.1). At 12 DAH ( $4.2 \pm 0.01$  mm ) all larvae had completed flexion. From 7-15 DAH there was a progressive increase in the body pigmentation, until the majority of the body surface became pigmented.

At 15 DAH ( $4.8 \pm 0.09$ ), the first appearance of dorsal and ventral fins was identified, and there was a pink gill pigmentation under the developing operculum. At 16 DAH ( $5 \pm 0.05$  mm) the peritoneal cavity of some individuals was pigmented and all individuals had pigmented gills.

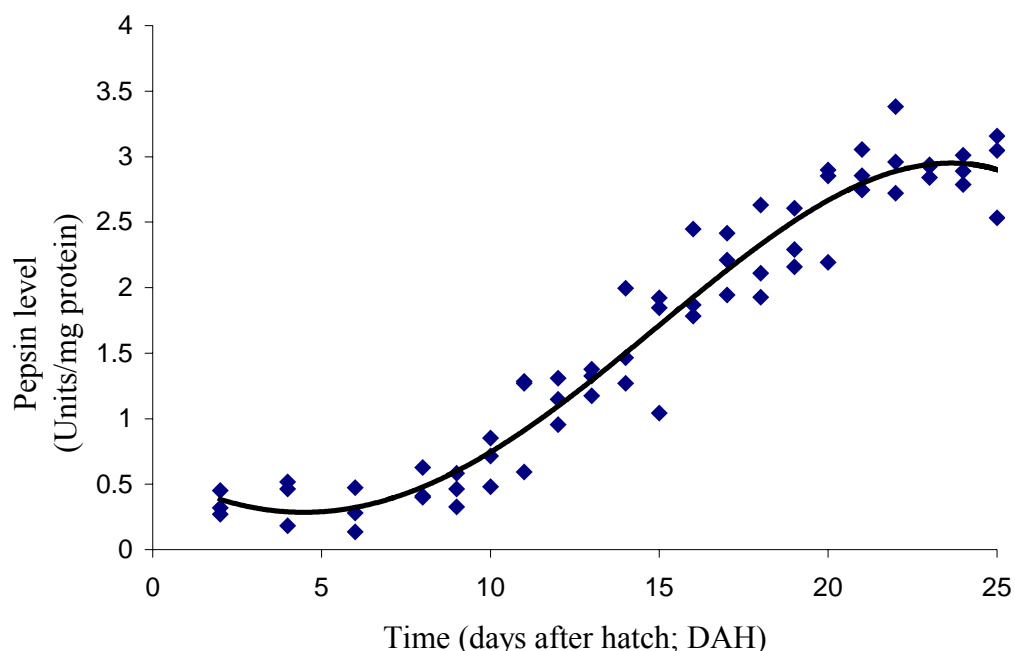
**Table 3.1. Numbers of barramundi larvae sampled at each developmental stage, sorted according to developmental age (days after hatch, DAH).**

DAH	Developmental stage				
	Yolk-sac	Pre-flexion	Flexion	Post-flexion	Metamorphosed
1	30	-	-	-	-
2	-	30	-	-	-
3	-	30	-	-	-
4	-	30	-	-	-
5	-	30	-	-	-
6	-	30	-	-	-
7	-	17	13	-	-
8	-	8	22	-	-
9	-	1	29	-	-
10	-	-	23	7	-
11	-	-	12	18	-
12	-	-	-	30	-
13	-	-	-	30	-
14	-	-	-	30	-
15	-	-	-	30	-
16	-	-	-	30	-
17	-	-	-	21	9
18	-	-	-	20	10
19	-	-	-	11	19
20	-	-	-	4	26
21	-	-	-	-	30
22	-	-	-	-	30
23	-	-	-	-	30
24	-	-	-	-	30
25	-	-	-	-	30

At 17 DAH ( $5.4 \pm 0.12$  mm) some individuals had fully pigmented operculum and peritoneal cavity, preventing any internal details from being observed. Pigmentation at this stage was concentrated in two broad bands, giving the fish a striped appearance which is indicative of completed metamorphosis (Moore 1982). Metamorphosed larvae were further defined by the presence of all fin structures (dorsal, caudal, pectoral, pelvic and anal), the presence of scales and a well defined caudal peduncle. From 18 to 21 DAH, progressively more individuals had this appearance, and from this point until the last sampling point there were no further developmental changes.

### 3.3.2. Pepsin Development

Pepsin levels in larvae were generally less than  $0.5$  units.mg protein<sup>-1</sup> until 9 DAH (Fig. 3.2). From this point there was a consistent increase until 21 DAH. Between 21 and 25 DAH there was no further increase in larvae pepsin level.



**Fig. 3.2. Pepsin levels in barramundi larvae from 2 days after hatch (DAH) until 25 DAH.**

### 3.4. Discussion

Nutritional input is entirely endogenous at 1 DAH, classifying them as yolk-sac larvae. Initial development is concentrated around the formation of the mouth and the gut in order to ingest and digest prey. This is typical of planktonic marine fish larvae, which have small endogenous reserves and rapid larvae development (Mihelakakis *et al.*, 2001).

Early barramundi larvae possess an oil globule, which is resorbed coinciding with the inflation of the swim bladder at 3-4 DAH. This is common to a variety of marine fish larvae (Mihelakakis *et al.*, 2001), with the swim bladder assuming the oil globule's role in buoyancy maintenance. The timing of swim bladder inflation is critical for hatchery management of physostomous larvae. Physostomous fish larvae appear to preferentially inflate swim bladders during dark or low light periods (Trotter *et al.*, 2003) and oily surface films can inhibit the surface gulping behavior that facilitates swim bladder inflation (Chatain and Ounais-Guschemann, 1990). Lack of swim bladder inflation potentially leads to a lack of buoyancy control, decreased growth and survival, and increasing spinal deformation (Mihelakakis *et al.*, 2001; Trotter *et al.*, 2001; Trotter *et al.*, 2003; Zilberg *et al.*, 2004).

The appearance of post-flexion larvae at 10 DAH coincides with the initial development of pepsin in larvae homogenates. Walford and Lam (1993) reported initial stomach formation for this species at 11 DAH and stomach acidification achieved at 22 DAH in fed larvae. The timing of stomach formation in this previous study coincides with the plateau found in pepsin development in the present study, from 21-25 DAH. This corresponds to the timing of full metamorphosis as determined by morphological indices. Interestingly, Walford and Lam (1993) found pepsin levels to increase until at least 30

DAH. This discrepancy may be due to digestive enzyme secretion as a response to feeding. It is assumed that Walford and Lam (1993) used fed larvae for their pepsin analysis, while the present experiment used larvae that were starved overnight prior to sampling. Since Walford and Lam (1993) found an influence of feeding on stomach pH, it is also likely that pepsin levels are affected by food ingestion, and this should be evaluated in further studies.

Pepsin-mediated digestion has been indicated as a limiting factor for the capacity of larvae to digest the complex proteins present in weaning diets. The presence of pepsin in the stomach may therefore be a cue to weaning readiness in species that readily ingest formulated foods from a young age, but are limited by digestive capability, such as barramundi. The timing of initial pepsin development in the stomach of barramundi larvae is coincident with the first appearance of post-flexion larvae, while the maximum pepsin level for unfed larvae occurs at the time of metamorphosis. This study is the most comprehensive and detailed description of *L. calcarifer* morphological development to date, and represents the first record of morphological predictors of gastric formation. Applications of this work to *L. calcarifer* larviculture therefore exist as a references guide to larval development and in the visual assessment of weaning readiness.

## Chapter 4

### **The influence of dietary protein and energy levels on the growth, survival and thyroid hormone (T3 and T4) composition of barramundi (*Lates calcarifer*) larvae**

#### **4.1. Introduction**

The relatively high growth rate of marine fish larvae would logically prescribe a high amino acid requirement (Rønnestad *et al.*, 2003). However, few studies have quantitatively manipulated the protein component of diets for larval fish (Péres *et al.*, 1996). Furthermore, although the interaction of dietary energy and protein has been very well documented in juvenile fish (Nankervis *et al.*, 2000; Lee *et al.*, 2002; Meyer and Fracalossi, 2004) no previous studies have accounted for the influence of dietary energy on the protein requirement of fish larvae. As amino acids and lipids are the major energy substrates in larval fish nutrition (Fyhn, 1989; Watanabe and Kiron, 1994), lipid-derived energy may influence their quantitative protein requirement.

Thyroid hormones are integral to the control of growth and development in fish larvae (Ayson and Lam, 1993a; Nugegoda *et al.*, 1994; Kim and Brown, 1997; Gavlik *et al.*, 2002), and are related to nutritional inputs in juvenile fish (Eales *et al.*, 1992; Farbridge *et al.*, 1992; Nankervis *et al.*, 2000), particularly protein and caloric inputs. Nutritional inputs have been recently shown to regulate thyroid hormones in larval Atlantic halibut (Solbakken *et al.*, 2002), however, no qualitative or quantitative data are available to elucidate the nutritional components responsible.



This chapter investigated the protein and energy requirements of barramundi larvae, and the interactions between dietary energy and protein levels. The interrelationship between whole body T3 and T4 concentration and these nutritional variables was also examined.

## **4.2. Materials and Methods**

### *4.2.1. Larval rearing*

Larvae were reared as described in Chapter 2 (section 2.2).

### *4.2.2. Feeding Trials*

The feeding trial was conducted as described in Chapter 2 (section 2.3). Four replicate 50 L parabolic tanks were used per dietary treatment in Experiment 1, and larvae were stocked at a density of 6.L<sup>-1</sup>. Four replicate 18 L conical tanks were used per dietary treatment in Experiment 2, and larvae were stocked at a density of 16.6.L<sup>-1</sup>.

### *4.2.3. Experimental diets*

Experimental diets were prepared as described in Chapter 2 (section 2.1). Fishmeal was included as the main protein source, while squid oil was used as the primary lipid source and non-protein energy derivative. Other lipids were derived from soy lecithin and fishmeal. Cellulose was used as an inert filler to balance dietary formulations, and gelatin was used as a binder.

#### 4.2.3.1. Experiment 1 - The effect of varying dietary protein and energy on growth, survival and thyroid hormone levels in barramundi larvae.

A factorial experimental design was used with six diets, formulated to two dietary utilisable (excluding cellulose) energy levels (18 and 21 MJ.kg<sup>-1</sup>) and three dietary protein levels (45, 50 and 55%; Table 4.1).

#### 4.2.3.2. Experiment 2 - The effect of varying dietary energy on growth, survival and thyroid hormone levels in barramundi larvae.

To clarify the results from Experiment 1, an experiment was devised to examine the effect of dietary energy level, as adjusted by dietary lipid content, on growth, survival, T3 and T4 level. Microbound diets were formulated to 18, 19, 20, 21 and 22 MJ.kg<sup>-1</sup> utilisable energy levels (total energy – cellulose energy), according to Table 4.2.

#### *4.2.4 Analyses*

At the end of each 14-day experiment, all fish were counted before being photographed using a stereo dissecting microscope as described in Chapter 2 (Section 2.4.1). A subsample was removed for hormone analysis and the remainder dried to constant weight and weighed to the nearest 0.001 mg as described in Chapter 2 (Section 2.4.1).

Triiodothyronine (T3) L-thyroxine (T4) were analysed as described in Chapter 2 (Section 2.4.2). A quality control sample was prepared to determine intra-assay coefficient of variation, as described in Chapter 2 (Section 2.4.2). The intra-specific coefficient of variation was 8.88% and 6.63% for T3 and T4, respectively. Hormone concentrations were not corrected for extraction efficiency.

**Table 4.1. Formulation and analysis of the six microparticulate diets used in this study containing three different protein levels (45, 50 and 55%) and two different energy levels (18 and 20 MJ.kg<sup>-1</sup>)**

Ingredient (%)	Diet (Protein/Energy content)					
	45/18	45/21	50/18	50/21	55/18	55/21
Fish Meal <sup>1*</sup>	65.74	65.74	73.41	73.41	81.09	81.09
Gelatin <sup>*</sup>	3	3	3	3	3	3
Squid Oil	12.52	20.19	8.78	16.45	5.03	12.7
Soybean Lecithin	1	1	1	1	1	1
Choline Chloride <sup>*</sup>	0.5	0.5	0.5	0.5	0.5	0.5
Mineral Mix <sup>2*</sup>	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin Mix <sup>3*</sup>	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin C <sup>4*</sup>	0.6	0.6	0.6	0.6	0.6	0.6
Cellulose <sup>*</sup>	15.64	7.97	11.71	4.04	7.78	0.11
<b>Analysis</b>						
Protein <sup>a*</sup>	45.34	45.13	50.31	49.38	55.51	56.2
Energy <sup>b*</sup>	18.61	21.78	18.59	21.66	18.69	21.22

\* % dry matter by mass

<sup>1</sup>Skretting Australia, 65% protein, 18 MJ/kg<sup>-1</sup> gross energy

<sup>2</sup>Magnesium sulphate, zinc sulphate, iron sulphate, manganese sulphate, copper sulphate, cobalt sulphate, sodium selenate

<sup>3</sup>Citric acid, ascorbic acid, alpha tocepherol,, myo-inositol, ethoxyquin, nitotinic acid, pantothenate acid, menadione, riboflavin, pyridoxine, retinylacetate, thiamine, biotin, folic acid, cyanocobalamin, cholecalciferol,

<sup>4</sup> Ascorbic acid

<sup>a</sup>% Kjeldhal nitrogen (N X 6.25) <sup>b</sup> MJ.kg<sup>-1</sup> bomb calorimetry, corrected for cellulose inclusion

Significant differences between treatments were determined by two-way analysis of variance (ANOVA), while Tukey's HSD was used post-hoc. Pearson's correlation was used to determine relationships between thyroid hormones and growth parameters.

### **4.3. Results**

Larvae from all treatments grew throughout both experiments. Feeding and gut fullness observations indicated that all diets were ingested throughout both experiments and there were no obvious visual differences in feeding rates between treatments.

#### *4.3.1. Experiment 1 - The effect of varying dietary protein and energy on growth, survival and thyroid hormone levels in barramundi larvae*

Increasing dietary energy levels from 18 to 21 MJ.kg<sup>-1</sup> led to increased growth performance in terms of final dry weight and total length of larvae ( $p < 0.05$ , Figs. 4.1 and 4.2, Tables 4.3 and 4.4). Final dry weight of larvae ranged from 0.44-1.16 mg.larvae<sup>-1</sup>, with the highest mean dry weight ( $1.03 \pm 0.09$  mg) recorded for larvae fed the 50% protein, 21 MJ.kg<sup>-1</sup> energy diet (Fig. 4.1). Final total length of barramundi larvae ranged from 7.97 to 10.09 mm, with the highest mean total length ( $9.85 \pm 0.25$  mm) recorded for larvae fed the 50% protein, 21 MJ.kg<sup>-1</sup> energy diet (Fig. 4.2).

Survival ranged from 13% to 33.67% (Fig. 4.3). There was no significant influence of dietary protein level on survival, though larvae fed the 50% protein, 21 MJ.kg<sup>-1</sup> diet had a slightly higher mean survival ( $28 \pm 1.67\%$ ) than larvae from all other treatments.

**Table 4.2. Formulation and analysis of the five microparticulate diets used in this study containing different utilisable energy levels (18-22 MJ/kg).**

<b>Ingredient:</b>	<b>Formulated Dietary Energy Level (MJ/kg)</b>				
	<b>18</b>	<b>19</b>	<b>20</b>	<b>21</b>	<b>22</b>
Fish Meal <sup>1*</sup>	73.41	73.41	73.41	73.41	73.41
Gelatin <sup>*</sup>	3	3	3	3	3
Squid Oil	8.78	11.33	13.89	16.45	19.01
Soybean Lecithin	1	1	1	1	1
Choline Chloride <sup>*</sup>	0.5	0.5	0.5	0.5	0.5
Mineral Mix <sup>2*</sup>	0.5	0.5	0.5	0.5	0.5
Vitamin Mix <sup>3*</sup>	0.5	0.5	0.5	0.5	0.5
Vitamin C <sup>4*</sup>	0.6	0.6	0.6	0.6	0.6
Cellulose <sup>*</sup>	11.71	9.15	6.60	4.04	1.48
<b>Analysis</b>					
Protein <sup>a*</sup>	49.97	50.16	50.03	50.06	50.11
Energy <sup>b*</sup>	17.96	19.08	20.12	20.92	22.14

\* % dry matter by mass

<sup>1</sup>Skretting Australia, 65% protein, 18 MJ/kg<sup>-1</sup> gross energy

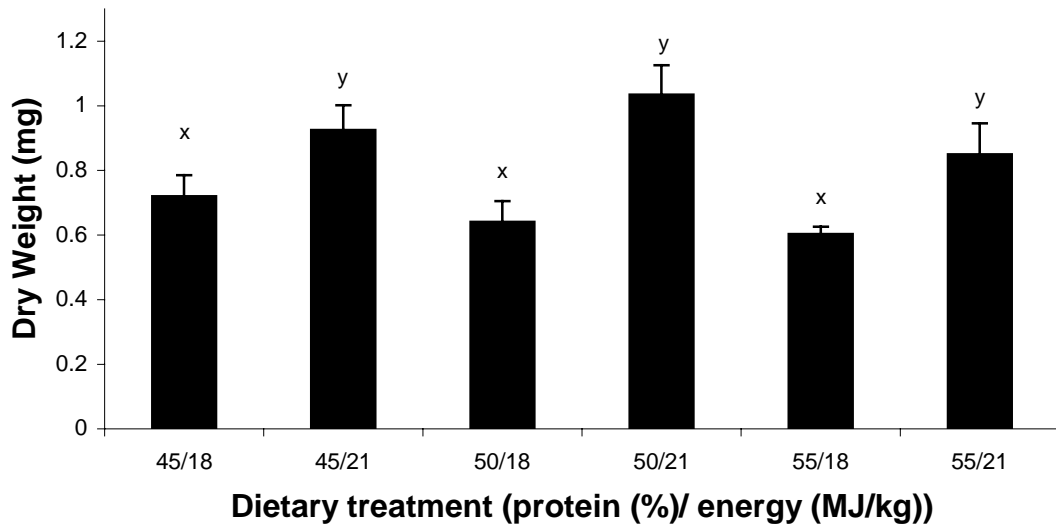
<sup>2</sup>Magnesium sulphate, zinc sulphate, iron sulphate, manganese sulphate, copper sulphate, cobalt sulphate, sodium selenate

<sup>3</sup>Citric acid, ascorbic acid, alpha tocepherol, myo-inositol, ethoxyquin, nitotinic acid, pantothenate acid, menadione, riboflavin, pyridoxine, retinylacetate, thiamine, biotin, folic acid, cyanocobalamin, cholecalciferol,

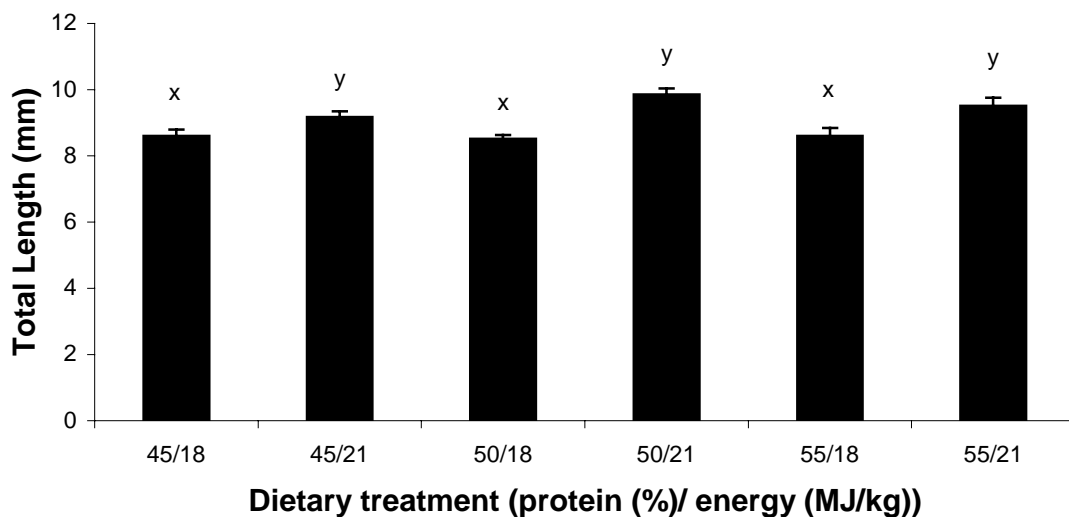
<sup>4</sup> Ascorbic acid

<sup>a</sup>% Kjeldhal nitrogen (N X 6.25)

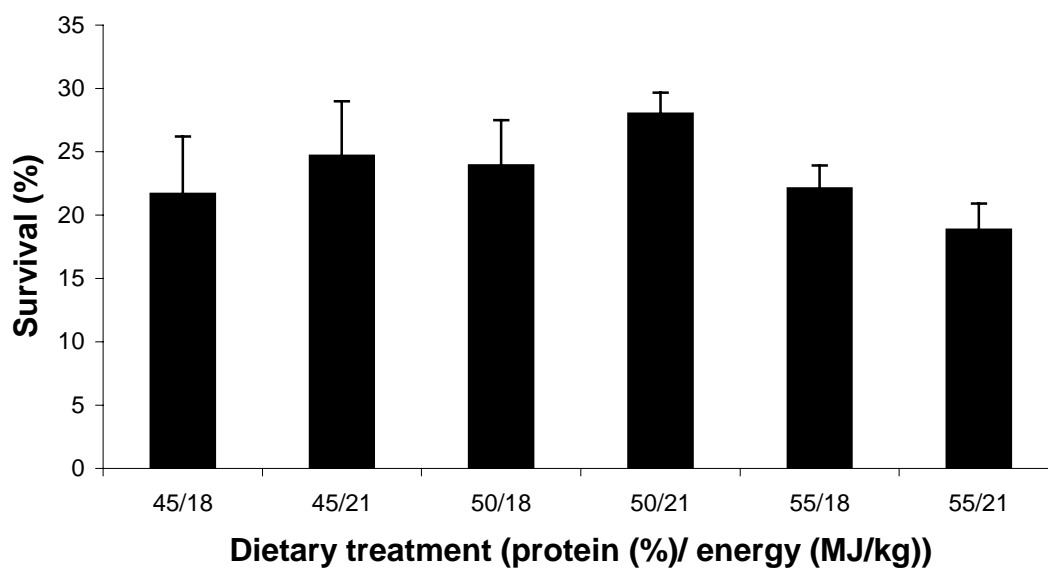
<sup>b</sup> MJ.kg<sup>-1</sup> bomb calorimetry, corrected for cellulose inclusion



**Fig. 4.1.** Final mean ( $\pm$ SE) dry weight (mg) of barramundi larvae fed microparticulate diets containing three different protein levels (45, 50 and 55%) and two different energy levels (18 and 20 MJ.kg<sup>-1</sup>). Means with different superscripts (x-y, two-way ANOVA, Table 4.3) are significantly different.



**Fig. 4.2.** Final mean ( $\pm$ SE) total length of barramundi larvae fed microparticulate diets containing three different protein levels (45, 50 and 55%) and two different energy levels (18 and 20 MJ.kg<sup>-1</sup>). Means with a different superscript (x-y, two-way ANOVA, Table 3) are significantly different.



**Fig. 4.3.** Mean ( $\pm$ SE) survival of barramundi larvae fed microparticulate diets containing three different protein levels (45, 50 and 55%) and two different energy levels (18 and 20 MJ.kg<sup>-1</sup>). There were no significant differences between means (one-way ANOVA;  $p>0.05$ ).

**Table 4.3.** Two-way ANOVA calculated on final dry weight of barramundi larvae fed microparticulate diets containing three different protein levels (45, 50 and 55%) and two different energy levels (18 and 20 MJ.kg<sup>-1</sup>).

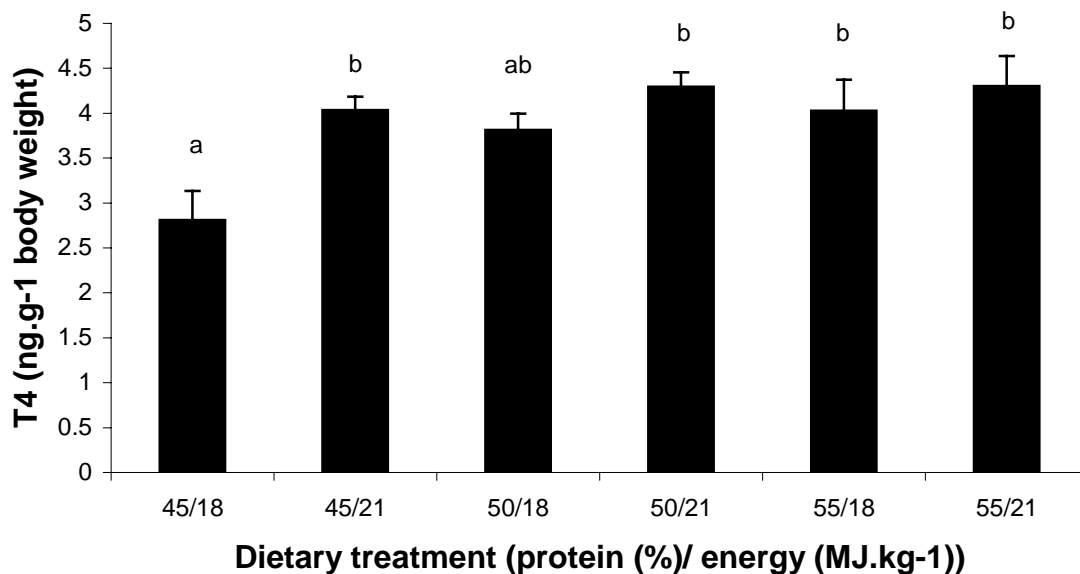
Source	SS	DF	MS	F	Sig.
Protein	0.039	2	0.02	0.986	0.395
Energy	0.514	1	0.514	25.933	0
Interaction	0.069	2	0.035	1.748	0.206
Error	0.317	16	0.0198		

**Table 4.4. Two-way ANOVA calculated on final total length of barramundi larvae fed artificial diets containing three different protein levels (45, 50 and 55%) and two different energy levels (18 and 20 MJ.kg<sup>-1</sup>).**

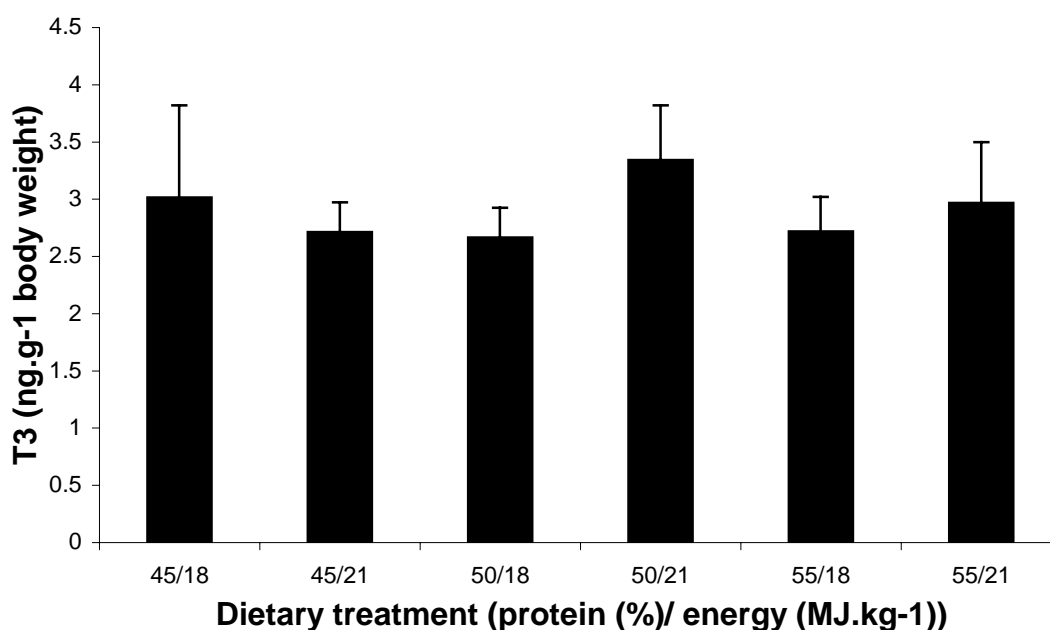
Source	SS	DF	MS	F	Sig.
Protein	0.32	2	0.16	0.989	0.394
Energy	4.76	1	4.76	29.38	0
Interaction	0.51	2	0.255	1.574	0.238
Error	2.592	16	0.162		

The whole carcass T4 level of larvae fed the diet containing 45% protein and 18 MJ.kg<sup>-1</sup> ( $2.81 \pm 0.33$  ng.g<sup>-1</sup> body weight), was significantly lower than in those fed the 45% protein, 21 MJ.kg<sup>-1</sup> ( $4.04 \pm 0.15$  ng.g<sup>-1</sup>), 50% protein, 21 MJ.kg<sup>-1</sup> ( $4.3 \pm 0.16$  ng.g<sup>-1</sup>), 55% protein, 18 MJ.kg<sup>-1</sup> ( $4.02 \pm 0.34$  ng.g<sup>-1</sup>) and 55% protein, 21 MJ.kg<sup>-1</sup> diets ( $4.3 \pm 0.33$  ng.g<sup>-1</sup>; Fig. 4.4). Larvae fed the 50% protein, 18 MJ.kg<sup>-1</sup> diet had an intermediate value for carcass T4 concentration ( $3.81 \pm 0.18$  ng.g<sup>-1</sup>). Mean whole carcass T3 levels ranged from 2.67 to 3.34 ng.g<sup>-1</sup> fish body weight (Fig. 4.5). There were no significant differences between dietary treatments.





**Fig. 4.4.** Whole carcass L-thyroxine levels of barramundi larvae fed microparticulate diets containing three different protein levels (45, 50 and 55%) and two different energy levels (18 and 20 MJ.kg<sup>-1</sup>).

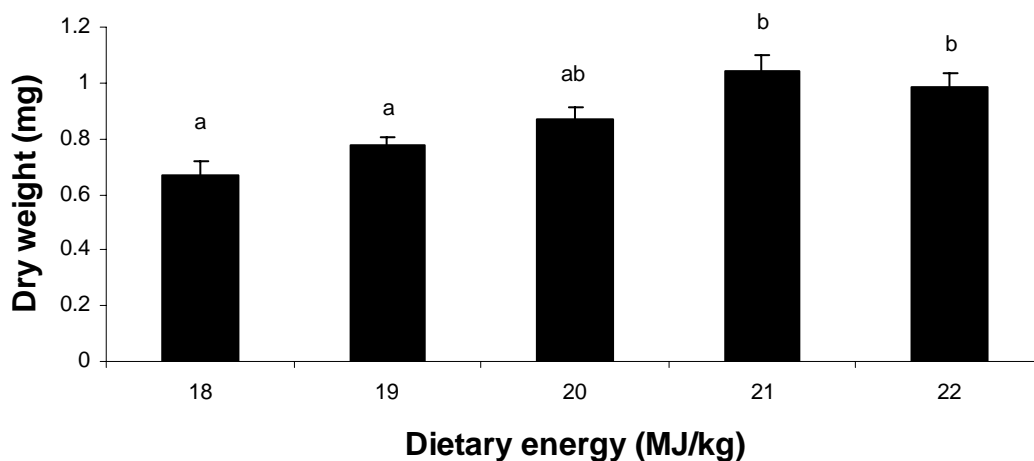


**Fig. 4.5.** Whole carcass triiodothyronine concentration of barramundi larvae fed microparticulate diets containing three different protein levels (45, 50 and 55%) and two different energy levels (18 and 20 MJ.kg<sup>-1</sup>).

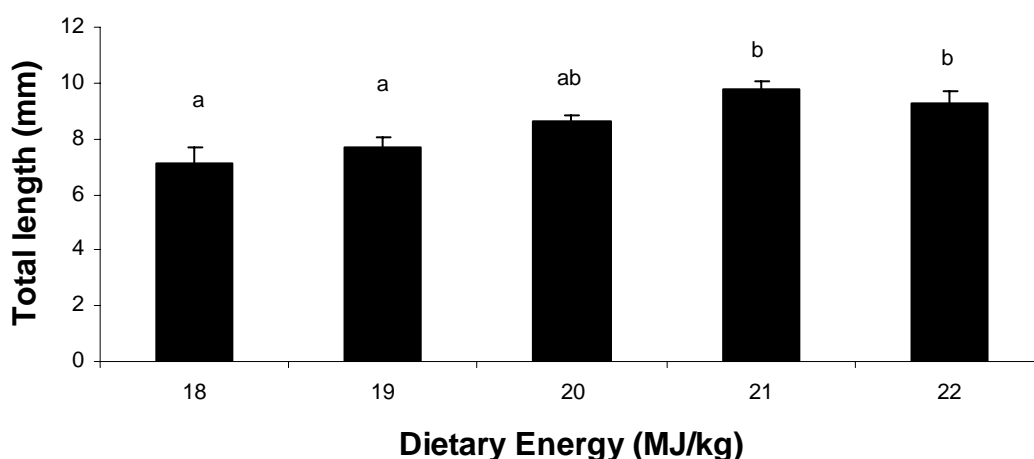
*4.3.2. Experiment 2 - The effect of varying dietary energy on growth, survival and thyroid hormone levels in barramundi larvae.*

The final dry weight and total length for larvae fed diets containing 21 and 22 MJ.kg<sup>-1</sup> dietary utilisable energy was significantly higher than those fed the 18 and 19 MJ.kg<sup>-1</sup>, while those fed the diet containing 20 MJ.kg<sup>-1</sup> had intermediate values for both parameters (Figs. 4.6 and 4.7). The total length at the end of the experiment ranged from 6.03 to 10.23 mm. The dry mass of larvae at the end of the experiment ranged from 0.601 to 1.15 mm. Survival of fish larvae throughout this experiment ranged from 24.6 – 55.2%, with no significant differences between dietary treatments (Fig 4.8).

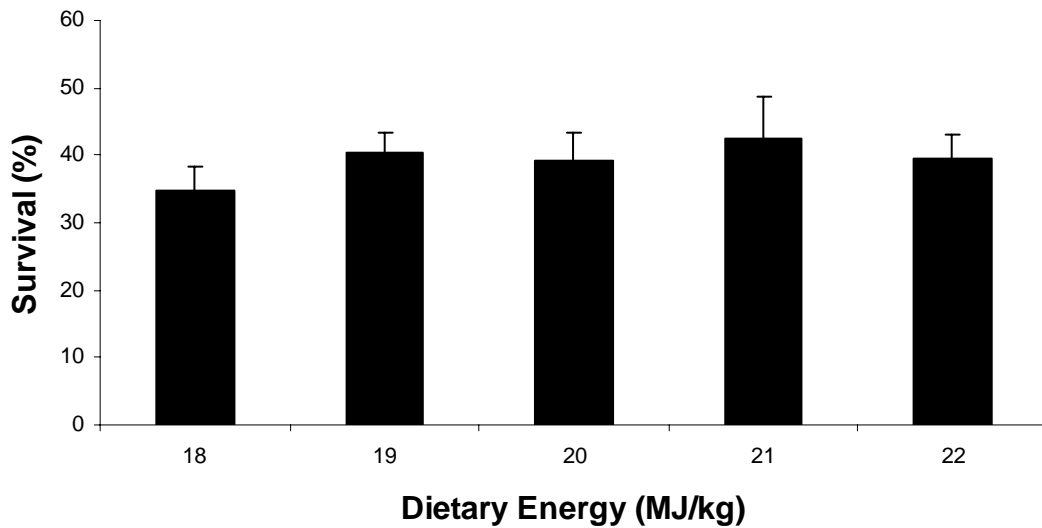
Carcass T4 and T3 levels ranged from 2.65 to 4.55 ng.g<sup>-1</sup> and 2.53 to 4.85 ng.g<sup>-1</sup> body weight respectively, with no significant differences between treatments (Figs. 4.9 and 4.10). There was, however, a significant regression, relating carcass T4 level to dietary energy inclusion ( $r^2 = 0.203$ ,  $P < 0.05$ ). Carcass T4 level was also correlated to the total length of larvae at the end of the experiment ( $r^2 = 0.288$ ,  $p < 0.05$ ), but not to dry weight ( $p > 0.05$ ).



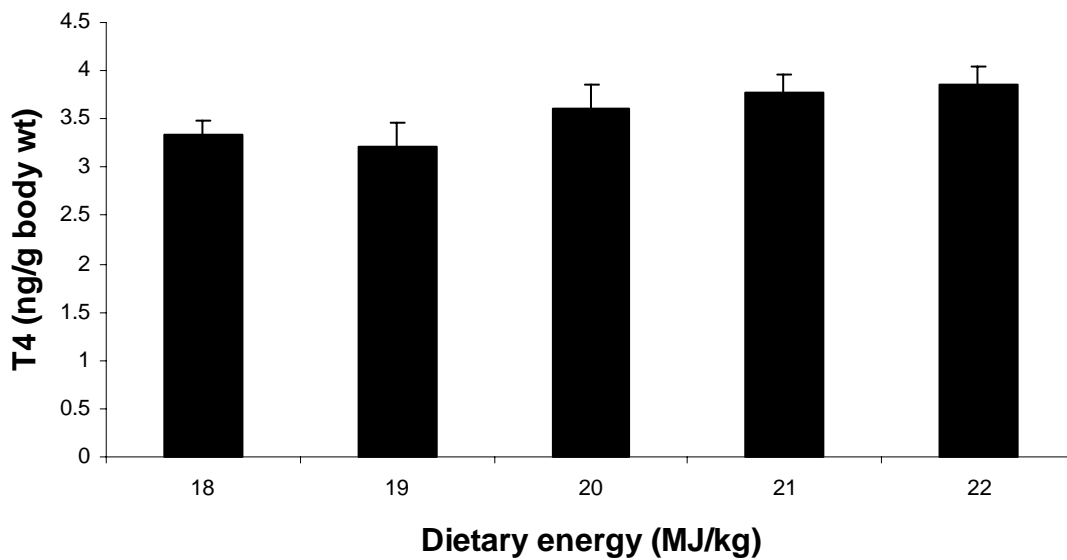
**Fig. 4.6.** Final mean ( $\pm$ SE) dry weight (mg) of barramundi larvae fed microparticulate diets containing five different utilisable energy levels (18–22 MJ.kg<sup>-1</sup>). Means with different superscripts (a-b) are significantly different (1-way ANOVA).



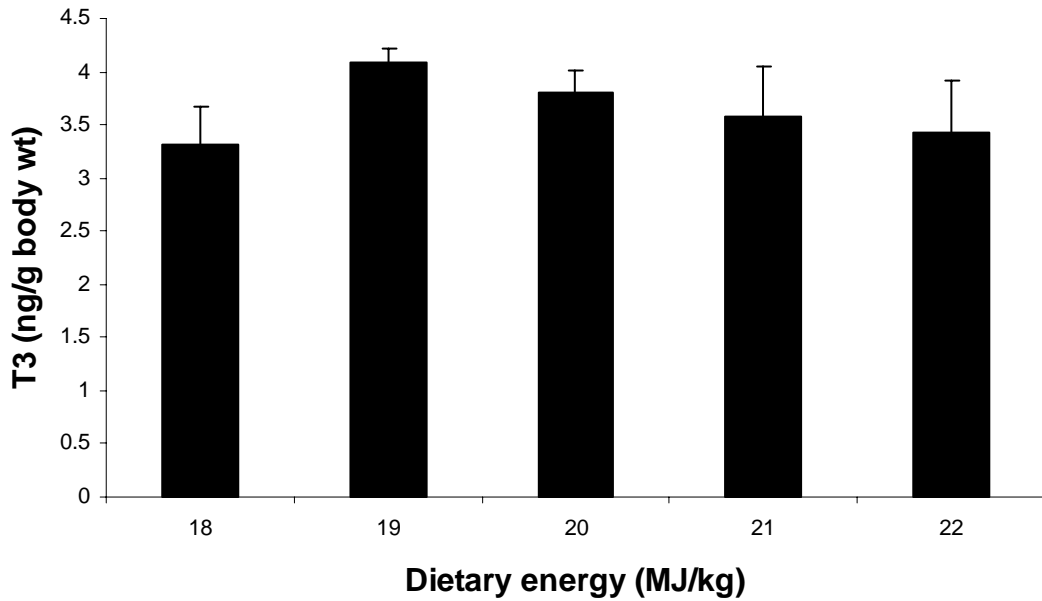
**Fig. 4.7.** Final mean ( $\pm$ SE) total length (mm) of barramundi larvae fed microparticulate diets containing five different utilisable energy levels (18–22 MJ.kg<sup>-1</sup>). Means with different superscripts (a-b) are significantly different (1-way ANOVA).



**Fig. 4.8.** Mean ( $\pm$ SE) survival of barramundi larvae fed microparticulate diets containing five different utilisable energy levels (18–22 MJ.kg<sup>-1</sup>). There are no significant differences in mean survival levels between treatments ( $p>0.05$ ; 1-way ANOVA).



**Fig. 4.9.** Mean ( $\pm$ SE) carcass L-Thyroxine (T4) level of barramundi larvae fed microparticulate diets containing five different utilisable energy levels (18–22 MJ.kg<sup>-1</sup>). There are no significant differences in mean T4 levels between treatments ( $p>0.05$ ; 1-way ANOVA).



**Fig. 4.10.** Mean ( $\pm$ SE) carcass triiodothyronine (T3) level of barramundi larvae fed microparticulate diets containing five different utilisable energy levels (18–22 MJ.kg<sup>-1</sup>). There are no significant differences in mean T3 levels between treatments ( $p>0.05$ ; 1-way ANOVA).

#### 4.4. Discussion

This is the first record of barramundi larvae effectively ingesting formulated microparticulate diets at this stage, while supporting significant growth and survival. While ingestion of microencapsulated diets has previously been reported for barramundi at 3 DAH and 8 DAH (Walford *et al.*, 1991), low survival was recorded (0% and 2.4%) over the short experiment (10 days and 1 week, respectively). Other studies have begun the weaning process for barramundi at 18-23 DAH (Lee *et al.*, 1996; Partridge and Southgate, 1999), while commonly accepted commercial practice is to begin weaning onto microparticulate diets from 20-25 DAH (Mackinnon, 1987; Fuchs and Nedelec, 1991; Moullac *et al.*, 2003). Earlier weaning of fish larvae has the potential to impart significant savings in commercial hatchery operations (Person Le Ruyet *et al.*, 1993).

The total length and dry weight of fish larvae at the end of both experiments was directly related to dietary energy level and no affect of dietary protein content was evident. Dietary energy was manipulated by squid oil inclusion, and as such the results may also reflect the availability of particular fatty acids. However, the consistent increase in growth of larvae fed diets containing higher utilisable dietary energy levels, irrespective of squid oil content, indicates that dietary energy is relevant to this study and has a significant effect on growth of barramundi larvae. High dietary lipid levels have previously been shown to increase growth, survival and development in European seabream (Zambonino Infante and Cahu, 1999) and red drum, *Sciaenops ocellatus*, (Buchet *et al.*, 2000), and the importance of utilisable dietary energy to such lipid requirements has previously been highlighted (Buchet *et al.*, 2000).

All dietary protein inclusion levels supported similar rates of growth of barramundi larvae during this study. This suggests that the minimum protein requirement for optimal growth of barramundi larvae may not have been assessed in this study and would therefore be  $\leq 45\%$  of the total dry mass of the diet. Interestingly, this is far less than the protein content (dry weight basis) of copepods in the genera *Acartia*, *Calanus*, *Temora* and *Eurytemora* (52.4-57.6%; Evjemo *et al.*, 2003), which are widely recognized as important natural food sources for marine fish larvae in the wild and are often used as indicators of nutritional requirements (Evjemo *et al.*, 2003). Previous assumptions of a generically high protein requirement for fish larvae (eg. Rønnestad *et al* 2003) may therefore be over-stated.

While the protein requirement for freshwater fish larvae is generally thought to be high (Eguia *et al.*, 2000; Cahu and Zambonino-Infante, 2001), few previous studies have manipulatively studied the quantitative protein requirements of marine fish larvae (Péres *et al.*, 1996). Given the fact that barramundi larvae develop and grow rapidly, and that amino acids are regarded as an important energy substrate during early larval development (Fyhn, 1989; Sivaloganathan *et al.* 1998), barramundi larvae are likely to have a high requirement for dietary protein (Rønnestad *et al.*, 2003). Indeed, this has been shown for the larvae of European sea bass, *Dicentrarchus labrax*, (Péres *et al.*, 1996) with diets containing 50% protein producing a far greater larval weight gain than those containing 40% protein. However, the recent use of diets containing wide ranging protein levels (46-62%) in experiments with *D. labrax* and gilthead seabream, *Sparus aurata*, larvae (Cahu *et al.*, 1999; Fontagne *et al.*, 2000; Yúfera *et al.*, 2000), indicates the need for further research to quantify the protein requirements of marine fish larvae.

There was no discernable interaction between dietary protein and dietary energy inclusion levels on any of the physical performance indices used in this study. This suggests that, under the conditions of this study, dietary protein did not become limiting to the extent that a protein sparing effect became evident. However, it is difficult to exclude the possibility of such an interaction without an accurate measure of protein utilisation.

Mean T4 levels were consistently (though non-significantly) lower for larvae fed diets with lower dietary energy levels. This relationship is supported by the significant correlation between T4 level and total length of larvae in both experiments, and the regression relating carcass T4 level to dietary energy inclusion in Experiment 2. The

growth stimulatory effects of T4 have been previously established for barramundi larvae (Wan *et al.*, 1997), among other species (Nacario, 1983; Lam and Sharma, 1985). Whole carcass T4 levels were found to be depressed in the lowest dietary protein and energy combination in Experiment 1, and while there was no significant physical manifestation of this depressed T4 level, it may represent decreased potential for growth.

Dietary protein deficiency has previously been found to depress circulating T3 levels and 5'ORD activity in juvenile rainbow trout (Eales *et al.*, 1990; Eales *et al.*, 1992). Non-protein energy derivatives also influence thyroid status in juvenile fish, with the effect attributed to the caloric manipulation of protein metabolism (Eales *et al.*, 1990; Eales *et al.*, 1992; Nankervis *et al.*, 2000).

T4 is the main hormone released from the thyroid as a result of TSH stimulation (Eales, 1985). Circulating T4 levels may therefore have a permissive action, representing the potential for 5'outer-ring monodeiodinase (5'ORD) activity, converting T4 to T3. The subsequent influence on T3 levels may not be readily apparent since circulating T3 is dependent on both conversion from T4 and physiological clearance. Since T4 is predominantly present non-covalently bound to carrier proteins (Eales, 1985), it is also likely to have a much longer circulating retention than T3 and, therefore, more likely to be representative of a longer term thyroid stimulation. Since T3 has a shorter circulating retention time, the lack of any relationship between T3 and growth parameters in the present study may also be a product of the early morning termination of both experiments, after an over-night period of starvation. Consequently, it is postulated that the results of this study indicate a depressed thyroidal status for fish fed the diet containing 45% protein and 18 MJ.kg<sup>-1</sup> energy, as represented by decreased T4 levels.



High variability in circulating T3 levels may represent a combination of variable T4 to T3 conversion (5'ORD activity) and T3 clearance. Interestingly, circulating T3 level was found to be nutritionally stimulated in juvenile barramundi (Nankervis *et al.*, 2000), while T4 was not, indicating that the thyroid hormone axis may be nutritionally stimulated at the 5'ORD level (T4 to T3 conversion) in juvenile barramundi, but at the T4 genesis level in larvae.

The only previous records of nutritionally mediated thyroid hormone levels in larval fish relate to thyroid stimulation by wild zooplankton (Nugegoda *et al.*, 1994; Solbakken *et al.*, 2002) attributed to iodine availability. While the direct mechanism is still unclear, it is certain that diet quality influences thyroid status in marine fish larvae, which in turn has implications for survival, growth, development and metamorphic success.

While growth data would suggest that the minimum dietary protein inclusion level for optimal growth in 14-28 DAH barramundi larvae is 45% or less, thyroid hormone analysis indicates that at 18 MJ.kg<sup>-1</sup> dietary energy level this is an underestimate and that optimal dietary protein inclusion level is approximately 50%. While there was no statistically significant difference between dietary protein levels in terms of final larval dry weight or length data, depressed T4 levels in larvae fed the diet containing 45% protein, 18 MJ.kg<sup>-1</sup> indicates that 45% protein is less than optimal at this dietary energy level. The lack of a corresponding T4 decrease in larvae fed the diet containing 45% protein at the 21 MJ.kg<sup>-1</sup> dietary energy level indicates that there may well be a protein sparing effect of dietary energy, though this is difficult to clarify without accurate protein utilisation data. There is a strong influence of dietary energy on growth in barramundi larvae, with 21 MJ.kg<sup>-1</sup> being preferable to 18 MJ.kg<sup>-1</sup>. This estimate was refined in the

second experiment, indicating that at least 20 MJ.kg<sup>-1</sup> dietary energy is necessary for optimal growth for barramundi larvae from 14-28 DAH.

The present data is presented as a baseline study into the nutrient requirements of barramundi larvae, upon which further research may determine optimal formulations for this species, to maximise growth and survival. This study is a significant step in our understanding of macronutrient requirements of altricial marine fish larvae when fed formulated foods. The application of thyroid hormone levels to evaluate the physiological state of such larvae seems to be an effective tool in evaluating microparticulate foods and warrants further development.

## Chapter 5

### **An assessment of gross marine protein sources in formulated microdiets for barramundi (*Lates calcarifer*) larvae**

#### **5.1. Introduction**

Animals have no biological requirement for dietary protein; rather, they require a readily available source of amino acids. The small diet particle sizes used to feed fish larvae, and the solubility of free amino acids (FAA, Baskerville-Bridges and Kling, 2000), confound the use of FAA manipulations to discern the exact amino acid requirements of fish larvae. Therefore, representative essential amino acid (EAA) profiles have often been used to approximate the ideal protein component of formulated foods for fish larvae (Baskerville-Bridges and Kling, 2000; Eguia *et al.*, 2000; Gisbert *et al.*, 2002). These can be used as a reference for perceived optima against which protein sources can be evaluated; however, the relevance of these representative amino acid profiles is rarely tested with growth studies. By comparing each essential amino acid in food ingredients with the perceived optima (aa/AA) food ingredients can be analysed for their limiting amino acids, while the essential amino acid index (EAAI) can be used to compare the whole amino acid profile of novel protein sources with their perceived optima (Penaflorida, 1989).

Since altricial marine fish larvae are considered to have a poorly developed digestive capability, especially in their proteolytic capacity, digestibility is likely to be a major contributor to the dietary value of protein sources in microdiet formulations. The ideal protein source in such foods must therefore be evaluated by both their EAA profile, and their digestibility (Lidner *et al.*, 1995).

Since the small size of fish larvae limits the use of traditional *in vivo* inert marker techniques used to evaluate nutrient digestibility for larger fish (Alacorn *et al.*, 1999), *in vitro* digestibility techniques have been developed to analyse the capacity of fish larvae to liberate amino acids or peptides from proteins.

It is generally recognised that growth studies are the best method of evaluating food ingredients for fish (Eid and Matty, 1989). However by applying growth and survival data to the digestibility and amino acid profile we can hope to determine limiting factors associated with particular protein sources and achieve a greater understanding of growth promotion and inhibition in fish larvae.

This experiments reported in this chapter therefore aims to determine the effectiveness of various sources of dietary protein in promoting growth and survival of barramundi larvae. Such growth performance will be analysed with respect to the amino acid profile of the protein sources and their relative digestibility by *in vitro* analysis. Any influences of the dietary inclusion of such protein sources on the thyroid hormones triiodothyronine (T3) and L-thyroxine (T4) will be determined in order to further elucidate any nutritional mediation of these growth-promoting hormones.

## **5.2. Materials and Methods**

This chapter reports four experiments with the overall aim of determining the optimum marine animal protein sources (squid powder, fish meal, mussel meal, *Artemia* meal, prawn meal and krill meal) for inclusion in microbound diets for barramundi larvae. These protein sources are evaluated exclusively and in unison to determine the combination that supports the greatest growth and survival when fed to barramundi

larvae. Subsequently, amino acid composition, digestibility analysis and solubility analysis were used to determine the reasons for varying growth and survival. Thyroid hormones are analysed in relation to growth to evaluate endocrine control of nutritional growth promotion, and as a physiological indicator for barramundi larvae.

#### *5.2.1. Larval rearing*

Larvae were reared as described in Chapter 2 (section 2.2).

#### *5.2.2. Experimental diets*

Experimental diets were prepared as described in Chapter 2 (section 2.1).

*5.2.3. Experiment 1- The effect of varying dietary protein source (fish meal, squid powder, prawn meal, mussel meal and decapsulated Artemia cyst meal) on growth, survival and thyroid hormone levels in barramundi larvae.*

Six isocaloric (20 MJ.kg<sup>-1</sup> body weight), isonitrogenous (48% protein) diets were formulated, incorporating five different protein sources (fish meal, squid powder, prawn meal, mussel meal and decapsulated *Artemia* cyst meal) and one colour control diet (fish meal plus orange food dye) according to Table 5.1.

*5.2.4. Experiment 2 - The effect of varying dietary protein source (fish meal, squid powder and mussel meal), on growth, survival and thyroid hormone levels in barramundi larvae.*

Six isocaloric ( $20 \text{ MJ.kg}^{-1}$ ), isonitrogenous (48% protein) diets were formulated, incorporating varying proportions of three different protein sources (fish meal, squid powder and mussel meal), according to Table 5.2.

*5.2.5. Experiment 3 - The effect of varying dietary protein source (fish meal and squid powder) on growth, survival and thyroid hormone levels in barramundi larvae.*

Ten isocaloric ( $21 \text{ MJ.kg}^{-1}$ ), isonitrogenous (50% protein) diets were formulated, incorporating varying proportions of two different protein sources (fish meal and squid powder), according to Table 5.3.

*5.2.6. Experiment 4 - The effect of varying dietary protein source (fish meal, squid powder and krill meal) on survival and thyroid hormone levels in barramundi larvae.*

Ten isocaloric ( $21 \text{ MJ.kg}^{-1}$ ), isonitrogenous (50% protein) diets were formulated, incorporating varying proportions of three different protein sources (fish meal, squid powder and krill meal), according to Table 5.4.

#### *5.2.7. Feeding Trials*

Feeding trials were conducted as described in Chapter 2 (section 2.3). Four replicate 50 L parabolic tanks were used per dietary treatment and larvae were stocked at a density of  $6.L^{-1}$ . Experiments were conducted for 14 days, when larvae were sacrificed by refrigeration at  $<5^{\circ}\text{C}$ .

**Table 5.1. Formulation and analysis of experimental diets used in Experiment 1.**

Ingredient	Diet					
	Fish Meal	Colour Control	Artemia Meal	Squid Powder	Prawn Meal	Mussel Meal
Fishmeal <sup>1</sup>	70.34	70.34	0	0	0	0
Artemia Meal <sup>2</sup>	0	0	81.84	0	0	0
Squid Powder <sup>3</sup>	0	0	0	53.88	0	0
Prawn Meal <sup>2</sup>	0	0	0	0	55.55	0
Mussel Meal <sup>2</sup>	0	0	0	0	0	86
Gelatin	3	3	3	3	3	3
Squid oil	14.88	14.88	1.40	17.15	18.87	7.77
Soybean Lecithin	1	1	1	1	1	1
Choline Chloride	0.5	0.5	0.5	0.5	0.5	0.5
Mineral Mix <sup>4</sup>	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin Mix <sup>5</sup>	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin C <sup>6</sup>	0.6	0.6	0.6	0.6	0.6	0.6
Cellulose	8.68	8.28	10.66	22.87	19.48	0.14
Food Dye	0	0.5	0	0	0	0
<b>Analysis</b>						
Protein <sup>a*</sup>	48.21	48.14	48.28	48.14	48.18	48.04
Energy <sup>b*</sup>	19.89	19.94	19.72	19.86	19.95	19.77

\* % dry matter by mass

<sup>1</sup>Skretting Australia, 65% protein, 18 MJ/kg<sup>-1</sup> gross energy

<sup>2</sup> Homogenised/freeze-dried

<sup>3</sup> Rieber & Son, Bergen, Norway

<sup>4</sup> Magnesium sulphate, zinc sulphate, iron sulphate, manganese sulphate, copper sulphate, cobalt sulphate sodium selenate

<sup>5</sup> Citric acid, ascorbic acid, alpha tocoferol, myo-inositol, ethoxyquin, nitotinic acid, pantothenate acid, menadione, riboflavin, pyridoxine, retinylacetate, thiamine, biotin, folic acid, cyanocobalamin, cholecalciferol

<sup>6</sup> Ascorbic acid

<sup>a</sup> % Kjeldhal nitrogen (N X 6.25)

<sup>b</sup> MJ.kg<sup>-1</sup> bomb calorimetry, corrected for cellulose inclusion

**Table 5.2. Formulation and analysis of experimental diets used in Experiment 2, containing varying proportions of squid powder (S), fish meal (F) and mussel meal (M).**

	Diet Composition									
	100S	66S 33F	66S 33M	100M	66M 33F	66M 33S	100F	66F 33M	66F 33S	33F 33M 33S
<b>Formulated to contain (% dietary protein):</b>										
Fishmeal	0	33.33	0	0	33.33	0	100	66.67	66.67	33.33
Squid Powder	100	66.67	66.67	0	0	33.33	0	0	33.33	33.33
Mussel Meal	0	0	33.33	100	66.67	66.67	0	33.33	0	33.33
<b>Ingredient *:</b>										
Fishmeal <sup>1</sup>	0	23.45	0	0	23.45	0	70.34	46.89	46.89	23.45
Squid Powder <sup>2</sup>	53.88	35.92	35.92	0	0	17.96	0	0	17.96	17.96
Mussel Meal <sup>3</sup>	0	0	28.67	86.00	57.33	57.33	0	28.665	0	28.67
Gelatin	3	3	3	3	3	3	3	3	3	3
Squid oil	17.15	16.40	14.02	7.77	10.14	10.90	14.88	12.51	15.64	13.27
Lecithin	1	1	1	1	1	1	1	1	1	1
Choline Chloride	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Mineral Mix <sup>4</sup>	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin Mix <sup>5</sup>	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin C <sup>6</sup>	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
Cellulose	22.87	18.14	15.29	0.14	2.99	7.72	8.68	5.83	13.41	10.56
<b>Analysis:</b>										
Protein <sup>a</sup>	47.9	48.31	48.43	48.11	48.46	48.28	48.06	48.35	48.22	47.81
Energy <sup>b</sup>	19.77	19.8	19.82	19.75	19.84	19.74	19.82	19.88	19.74	19.92

\* % dry matter by mass

<sup>1</sup>Skretting Australia, 65% protein, 18 MJ/kg<sup>-1</sup> gross energy

<sup>2</sup>Rieber & Son, Bergen, Norway

<sup>3</sup>Homogenised/freeze-dried

<sup>4</sup>Magnesium sulphate, zinc sulphate, iron sulphate, manganese sulphate, copper sulphate, cobalt sulphate, sodium selenate

<sup>5</sup>Citric acid, ascorbic acid, alpha tocepherol, myo-inositol, ethoxyquin, nitotinic acid, pantothenate acid, menadione, riboflavin, pyridoxine, retinylacetate, thiamine, biotin, folic acid, cyanocobalamin, cholecalciferol,

<sup>6</sup>Ascorbic acid

<sup>a</sup>% Kjeldhal nitrogen (N X 6.25)

<sup>b</sup>MJ.kg<sup>-1</sup> bomb calorimetry, corrected for cellulose inclusion



**Table 5.3. Formulation and analysis of experimental diets used in Experiment 3, containing varying proportions of fish meal (F) and squid powder (S).**

	Diet Composition <sup>#</sup>									
	100F	90F/10S	80F/20S	70F/30S	60F/40S	50F/50S	40F/60S	30F/70S	20F/80S	10F/90S
<b>Formulated to contain (% dietary protein):</b>										
Fishmeal	100	90	80	70	60	50	40	30	20	10
Squid Powder	0	10	20	30	40	50	60	70	80	90
<b>Ingredient*:</b>										
Fishmeal <sup>1</sup>	73.41	66.07	58.73	51.39	44.05	36.71	29.37	22.02	14.68	7.34
Squid Powder <sup>2</sup>	0.00	5.62	11.25	16.87	22.49	28.11	33.74	39.36	44.98	50.60
Gelatin	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Squid oil	16.45	16.69	16.92	17.16	17.40	17.64	17.87	18.11	18.35	18.59
Lecithin	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Choline Chloride	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Mineral Mix <sup>3</sup>	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin Mix <sup>4</sup>	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin C <sup>5</sup>	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60
Cellulose	4.04	5.52	7.00	8.48	9.96	11.44	12.93	14.41	15.89	17.37
<b>Analysis:</b>										
Protein <sup>a</sup>	50.13	50.07	50.11	50.06	50.08	50.16	50.02	50.09	50.12	49.96
Energy <sup>b</sup>	21.31	21.06	21.26	21.17	21.07	21.14	21.07	21.18	21.13	21.21

<sup>#</sup> Proportion (%) of squid powder (S) and fish meal (F)

\* % dry matter by mass

<sup>1</sup>Skretting Australia, 65% protein, 18 MJ/kg<sup>-1</sup> gross energy

<sup>3</sup>Magnesium sulphate, zinc sulphate, iron sulphate, manganese sulphate, copper sulphate, cobalt sulphate sodium selenate

<sup>4</sup>Citric acid, ascorbic acid, alpha tocepherol, myo-inositol, ethoxyquin, nitotinic acid, pantothenate acid, menadione, riboflavin, pyridoxine, retinylacetate, thiamine, biotin, folic acid, cyanocobalamin, cholecalciferol,

<sup>5</sup>Ascorbic acid

<sup>a</sup>% Kjeldhal nitrogen (N X 6.25)

<sup>b</sup> MJ.kg<sup>-1</sup> bomb calorimetry, corrected for cellulose inclusion

Table 5.4. **Formulation and analysis** of experimental diets for Experiment 4, containing varying proportions of fish meal (F), squid powder (S) and krill meal (K).

	Diet Composition <sup>#</sup>									
	90F/10S	80F/10S/10K	70F/10S/20K	60F/10S/30K	50F/10S/40K	40F/10S/50K	30F/10S/60K	20F/10S/70K	10F/10S/80K	10S/90K
<b>Formulated to contain (% dietary protein):</b>										
Fishmeal	90	80	70	60	50	40	30	20	10	0
Squid Powder	10	10	10	10	10	10	10	10	10	10
Mussel Meal	0	10	20	30	40	50	60	70	80	90
<b>Ingredient*:</b>										
Fishmeal <sup>1</sup>	70.22	62.42	54.61	46.81	39.01	31.21	23.41	15.60	7.80	0.00
Squid Powder <sup>2</sup>	5.98	5.98	5.98	5.98	5.98	5.98	5.98	5.98	5.98	5.98
Krill Meal <sup>1</sup>	0.00	7.03	14.05	21.08	28.11	35.13	42.16	49.19	56.21	63.24
Gelatin	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Squid oil	17.01	17.42	17.83	18.25	18.66	19.07	19.48	19.90	20.31	20.72
Lecithin	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Choline Chloride	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Mineral Mix <sup>3</sup>	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin Mix <sup>4</sup>	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin C <sup>5</sup>	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60
Cellulose	0.70	1.06	1.42	1.79	2.15	2.51	2.87	3.24	3.60	3.96
<b>Analysis:</b>										
Protein <sup>a</sup>	53.92	53.33	53.68	53.30	53.23	53.30	53.22	53.51	53.10	53.87
Energy <sup>b</sup>	22.31	22.17	22.42	22.40	22.62	22.06	22.55	22.13	22.37	22.20

<sup>#</sup> Proportion (%) of squid powder (S), fish meal (F) and krill meal (K)

\* % dry matter by mass

<sup>1</sup>Skretting Australia, 65% protein, 18 MJ/kg<sup>-1</sup> gross energy

<sup>2</sup>Rieber & Son, Bergen, Norway

<sup>3</sup>Magnesium sulphate, zinc sulphate, iron sulphate, manganese sulphate, copper sulphate, cobalt sulphate sodium selenate

<sup>4</sup>Citric acid, ascorbic acid, alpha tocepherol, myo-inositol, ethoxyquin, nitotinic acid, pantothenate acid, menadione, riboflavin, pyridoxine, retinylacetate, thiamine, biotin, folic acid, cyanocobalamin, cholecalciferol,

<sup>5</sup>Ascorbic acid

<sup>a</sup>% Kjeldhal nitrogen (N X 6.25)

<sup>b</sup>MJ.kg<sup>-1</sup> bomb calorimetry, corrected for cellulose inclusion

### 5.2.8. Analysis

At the end of each 14-day experiment, all fish were counted before being photographed using a stereo dissecting microscope, as described in Chapter 2 (section 2.4.1). A subsample was removed for hormone analysis and the remainder dried to constant weight and weighed to the nearest 0.001 mg, as described in Chapter 2 (section 2.4.1). Triiodothyronine (T3) L-thyroxine (T4) were analysed as described in Chapter 2 (section 2.4.2).

#### 5.2.8.1. Digestibility - pH-Stat.

The digestibility of the dietary protein sources used in these experiments was evaluated by an adaptation of the pH-stat method of Cordova-Murueta and Garcia-Carreno (2002). A suspension was made from each sample by adding 1 g protein sample to 10 mL purified water (adjusted to pH 8.0, 1 N NaOH) in a 50 mL glass beaker, with a magnetic stirrer. This suspension was adjusted to pH 8.0 by titration with 0.5 N NaOH, and enough purified water (adjusted to pH 8.0, 1 N NaOH) added to make the total mass equal to 15 g (Sample + H<sub>2</sub>O (pH = 8.0) = 15 g). This solution was adjusted to 28 ± 1°C with a heater plate before adding 5 mL enzyme solution (14 DAH *L. calcarifer* larvae extract (20 g larvae homogenised in 100 mL purified water, adjusted to pH 8.0 with 0.5 N NaOH) or porcine trypsin (5 g.L<sup>-1</sup>)/ pancreatin (1 g.L<sup>-1</sup>) solution, pH 8.0). This solution was incubated for 30 min, while maintaining pH by titration with 0.25 N NaOH. After this period, the volume of 0.25 N NaOH used was determined and used to calculate degree of hydrolysis (DH%):

$$DH\% = (B \times N_B \times 1.4 \times [(S/100)/8]) \times 100$$

Where:

$B$  = Volume 0.25 N NaOH (mL) used to maintain mixture at pH 8.0 ± 0.1

$N_B$  = Normality of NaOH solution = 0.25

$S$  = % protein in initial solution (Cordova-Murueta and Garcia-Carreno, 2002)

#### 5.2.8.2. Soluble protein

Water-soluble proteins were extracted in triplicate from the animal meals used in this study. Samples ( $65 \pm 5$  mg) were weighed into microcentrifuge tubes, to which were added 1.25 mL purified water. Samples were vortexed for 10 min before centrifuging at 14,000 rpm for 10 min. The supernatant was removed, diluted 1:100 with purified water and analysed for water-soluble protein with the Bradford (1976) protein assay, using a Bio-rad assay kit; 200  $\mu$ L sample was added to microtitre wells in triplicate, followed by 50  $\mu$ L Bradford reagent. Bovine serum albumin was used as a standard; 25  $\mu$ g.mL<sup>-1</sup>, 18.75  $\mu$ g.mL<sup>-1</sup>, 12.5  $\mu$ g.mL<sup>-1</sup>, and 6.25  $\mu$ g.mL<sup>-1</sup>.

#### 5.2.8.3. Essential Amino Acid Index

Essential amino acid profiles of food ingredients were performed according to standard techniques by the Queensland Department of Primary Industries Natural Resource Sciences Laboratories, Indooroopilly. Essential amino acid (A/E) ratio and Essential Amino Acid Index (EAAI) was determined according to Penafiora (1989);

The A/E ratio was determined as the percentage composition of total essential amino acids (including cystine and tyrosine) that a specific essential amino acid comprises.

$$EAAI = \sqrt[n]{[(aa_1/AA_1) \times (aa_2/AA_2) \times \dots \times (aa_n/AA_n)]}$$

Where  $aa_n$  = the A/E ratio in the feed;  $AA_n$  is the A/E ratio in 21 DAH barramundi larvae (Rimmer *et al* 1984).

### 5.2.9. Statistics

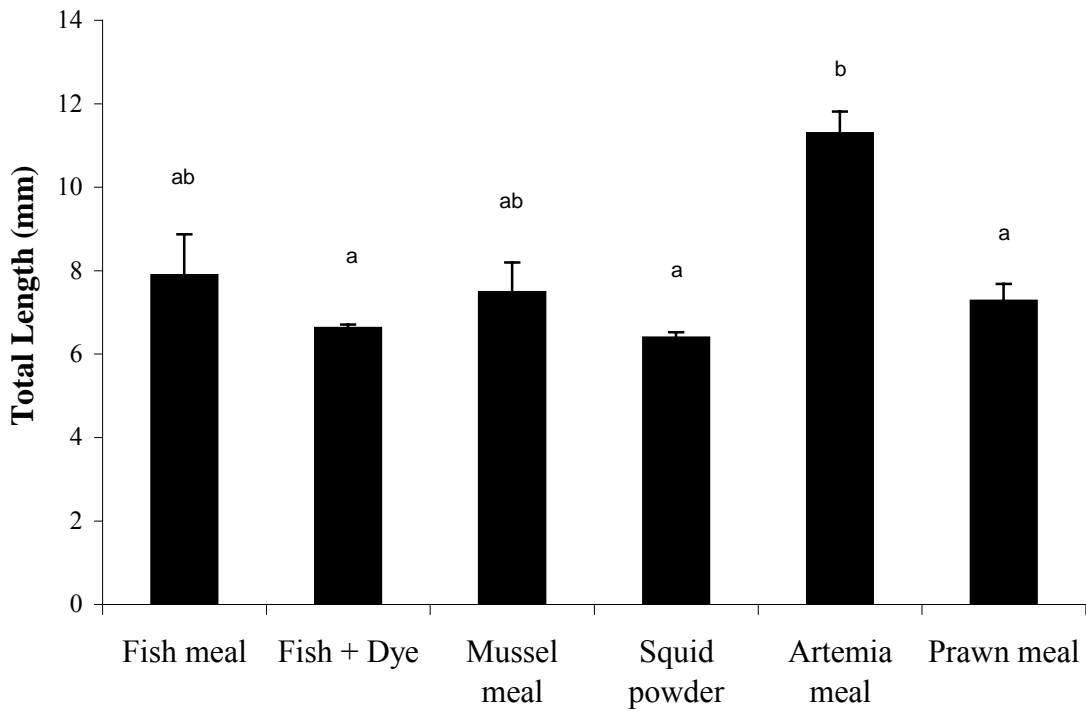
Significant differences were determined by one-way ANOVA. All analysis was performed with SPSS 10.0.

## 5.3. Results

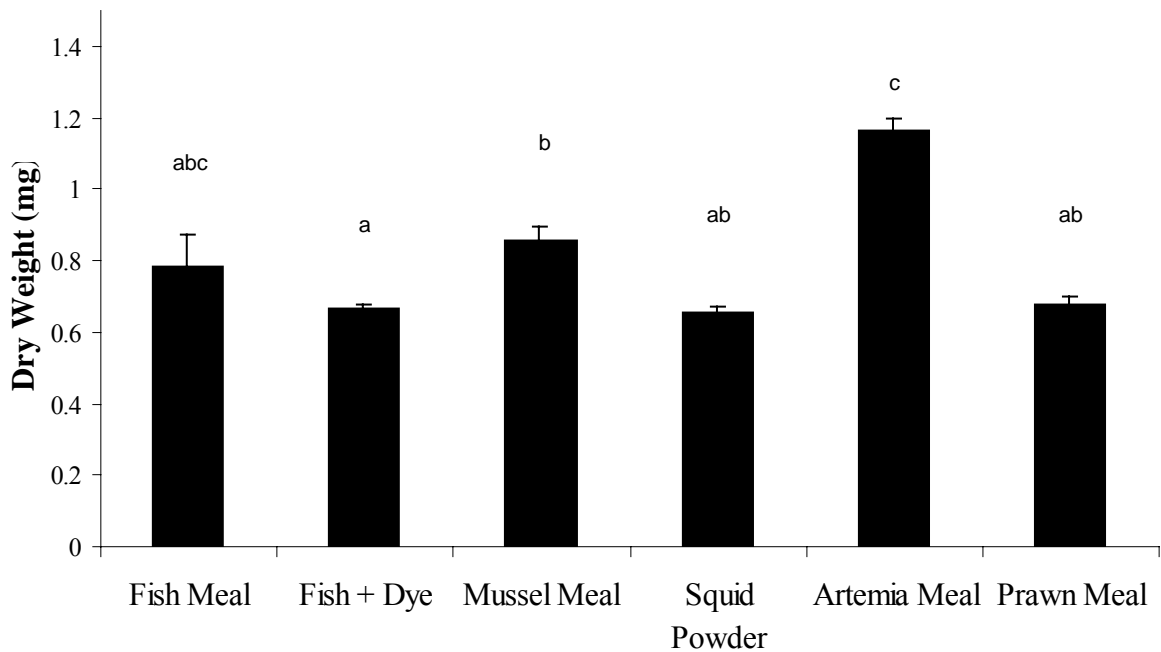
5.3.1. *Experiment 1 - The effect of varying dietary protein source (fish meal, squid powder, prawn meal, mussel meal and decapsulated Artemia cyst meal) on growth, survival and thyroid hormone levels in barramundi larvae.*

Fish fed diets containing *Artemia* cyst meal as the main protein source had the greatest total length (Fig. 5.1) and dry weight (Fig. 5.2). However, the total length of those larvae fed diets based on *Artemia* cyst meal was not significantly different to the total length of larvae fed fish meal or mussel meal based diets ( $p>0.05$ ).

Likewise the dry weight of fish fed *Artemia* based diets was not significantly different to those fed fishmeal based diets (Fig. 5.2). The dry weight of fish fed the mussel meal based diet was significantly higher than the dyed fish meal diet.

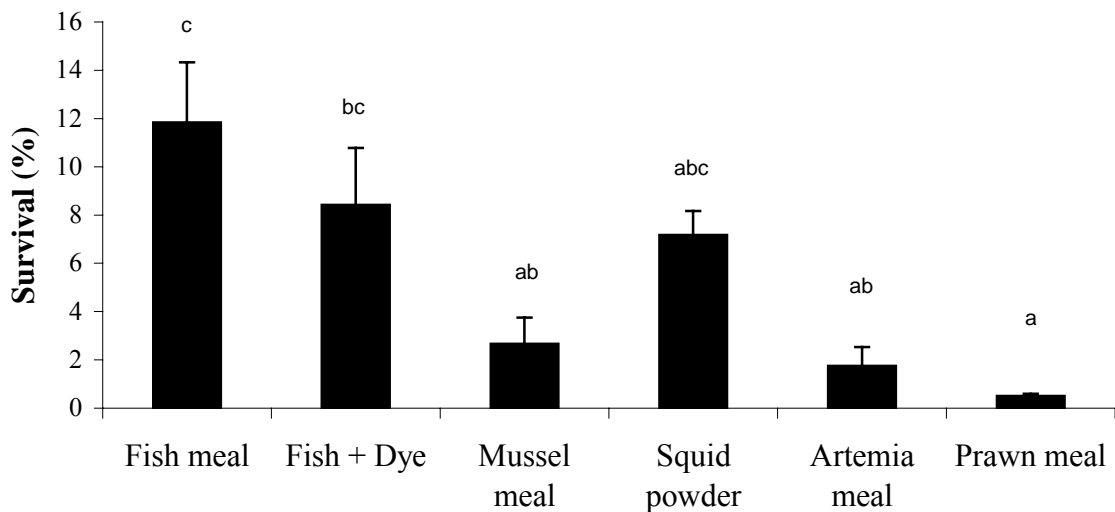


**Fig. 5.1.** Mean ( $\pm$  SE) total length of barramundi (*Lates calcarifer*) larvae, fed for 14 days on six isocaloric ( $20 \text{ MJ.kg}^{-1}$ ), isonitrogenous (48% protein) formulated microbound diets, varying in protein source. Means with different superscripts<sup>(a-c)</sup> are statistically different (1-way ANOVA).



**Fig. 5.2.** Mean ( $\pm$  SE) dry weight of barramundi (*Lates calcarifer*) larvae, fed for 14 days on six isocaloric ( $20 \text{ MJ.kg}^{-1}$ ), isonitrogenous (48% protein) formulated microbound diets, varying in protein source. Means with different superscripts<sup>(a-c)</sup> are statistically different (1-way ANOVA).

Survival was significantly lower ( $p < 0.05$ ) for fish fed the prawn meal based diet (0.5%) to those fed the fish meal (11.8%) and dyed fish meal (8.4%) diets (Fig. 5.3). All other treatments had intermediate values, though the mean survival of larvae fed the squid powder based diet (7.2%) was more than two-fold higher than that of those fed the mussel meal based diet (2.7%) and four times that of larvae fed the *Artemia* based diet (1.75%).



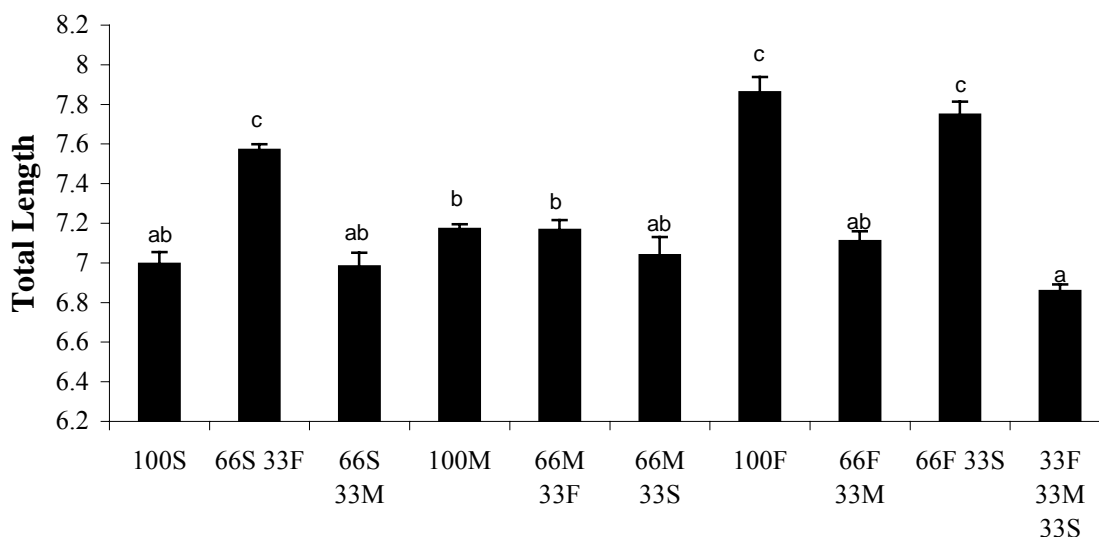
**Fig. 5.3.** Mean ( $\pm$  SE) survival of barramundi (*Lates calcarifer*) larvae, fed for 14 days on isocaloric ( $20 \text{ MJ.kg}^{-1}$ ), isonitrogenous (48% protein) formulated microbound diets, containing varying in protein source. Means with different superscripts<sup>(a-c)</sup> are statistically different (1-way ANOVA).

5.3.2. *Experiment 2 – The effect of varying dietary protein source (fish meal, squid powder and mussel meal), on growth, survival and thyroid hormone levels in barramundi larvae.*

Fish fed the 66S/33F, 100F and 33F/66S diets had a significantly greater total length at the end of the experiment than all other treatments (Fig. 5.4). Larvae fed the 100M and 66M/33F diets had a greater total length than those fish fed the 33F/33M/33S diet.

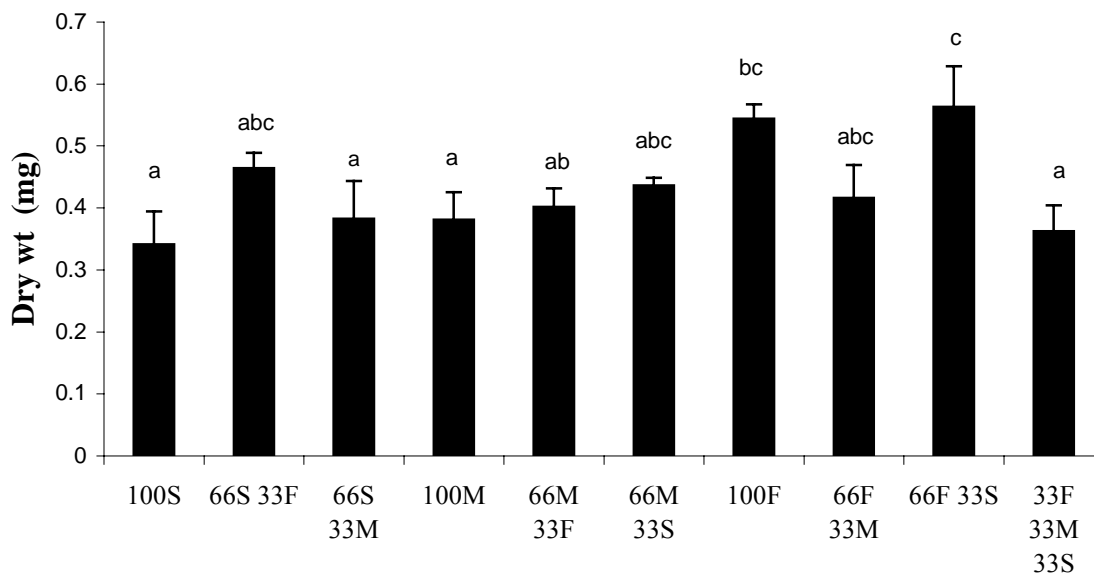
There was a significant difference in final mean dry weight between treatments, with larvae fed the 100F and 66F/33S diets having a significantly higher final dry weight than those fed the 100S, 66S/33M, 100M and 33F/33M/33S diets (Fig. 5.5). Larvae fed the 66F/33S diet *also* had a significantly higher mean dry weight than those fed the 66M/33F diet. All other values were intermediate.

Survival was variable, ranging from 2% to 50%, with no significant differences between treatments (Fig. 5.6). However the 33F/33M/33S diet had particularly low and variable survival.

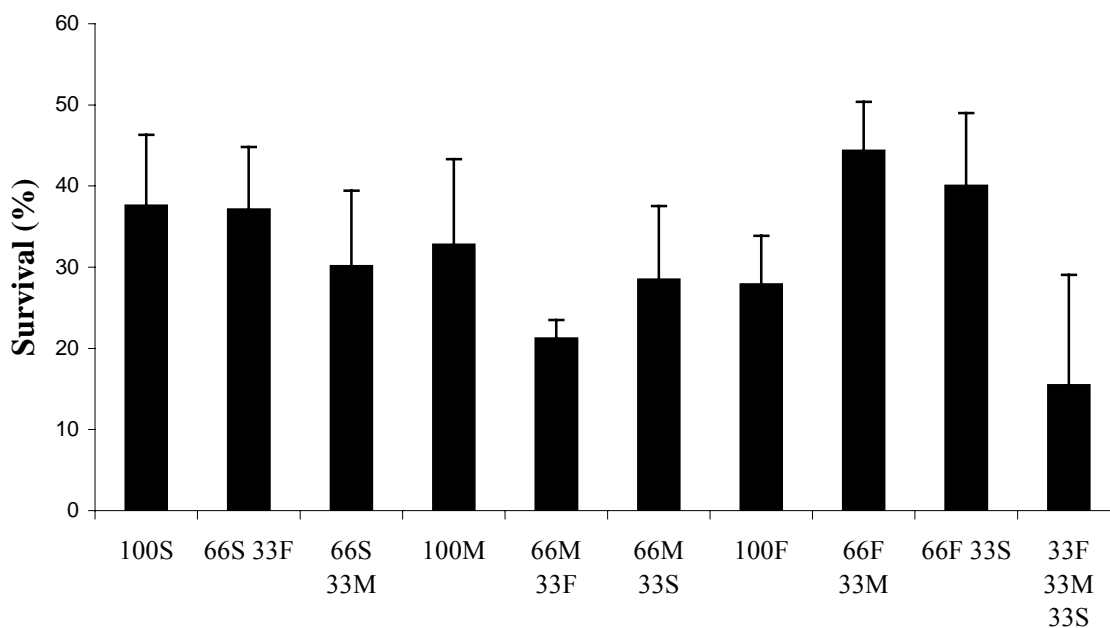


**Fig. 5.4.** Mean ( $\pm$  SE) total length of barramundi (*Lates calcarifer*) larvae, fed for 14 days on isocaloric ( $20 \text{ MJ.kg}^{-1}$ ), isonitrogenous (48% protein) formulated microbound diets, containing varying proportions of squid powder (S), fish meal (F) and mussel meal (M) in protein source. Means with different superscripts <sup>(a-c)</sup> are statistically different (1-way ANOVA).





**Fig. 5.5.** Mean ( $\pm$  SE) dry weight of barramundi (*Lates calcarifer*) larvae, fed for 14 days on isocaloric ( $20 \text{ MJ.kg}^{-1}$ ), isonitrogenous (48% protein) formulated microbound diets, containing varying proportions of squid powder (S), fish meal (F) and mussel meal (M) in protein source. Means with different superscripts <sup>(a-c)</sup> are statistically different (1-way ANOVA).



**Fig. 5.6.** Mean ( $\pm$  SE) survival of barramundi (*Lates calcarifer*) larvae, fed for 14 days on isocaloric ( $20 \text{ MJ.kg}^{-1}$ ), isonitrogenous (48% protein) formulated microbound diets, containing varying proportions of squid powder (S), fish meal (F) and mussel meal (M). There were no significant differences between treatments (1-way ANOVA;  $p > 0.05$ ).

Larvae fed the 100F diet and the 66S/33F diet had a significantly higher mean T4 level than those fed the 100M, 66M/33S and 33/33/33 diet (Fig. 5.7). Larvae fed the 66F/33S and 100S diets also had significantly higher mean T4 levels than the 100M and 33/33/33 diets, with all other values being intermediate. There was a significant correlation between T4 and total length ( $r^2 = 0.497$ ;  $P < 0.01$ ) and between T4 and dry weight ( $r^2 = 0.148$ ;  $P < 0.05$ ).

*5.3.3. Experiment 3- The effect of varying dietary protein source (fish meal and squid powder) on growth, survival and thyroid hormone levels in barramundi larvae.*

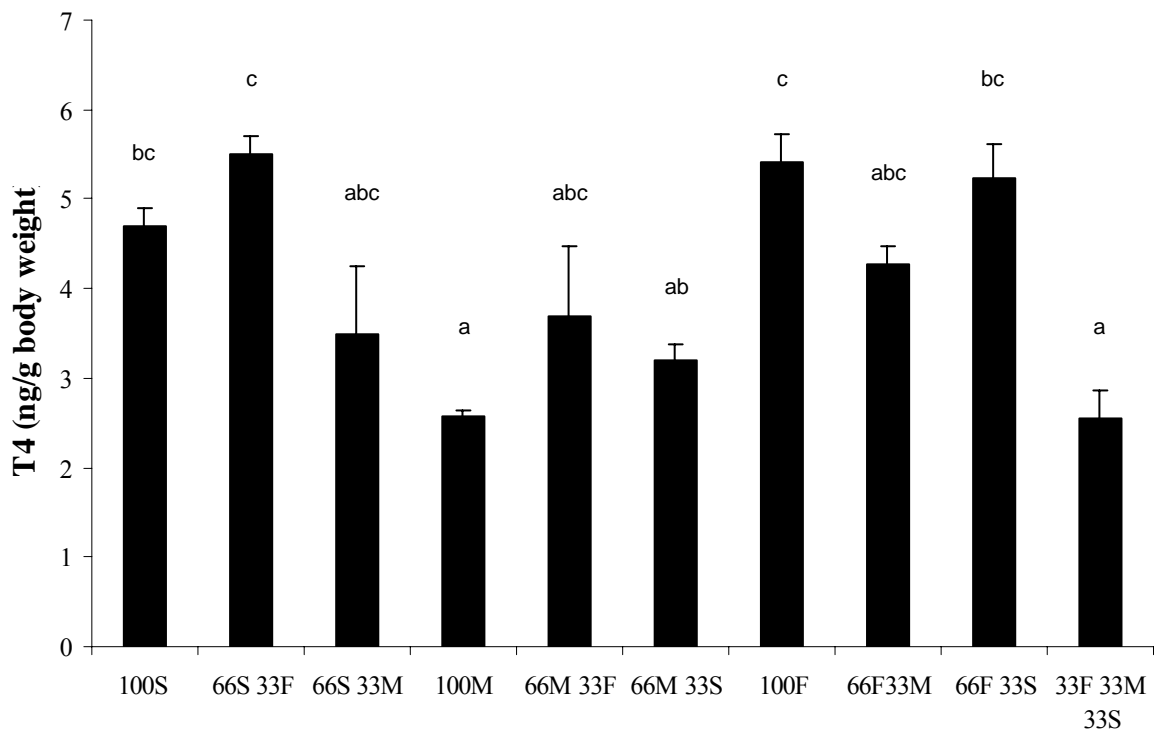
Mean total length of larvae at the end of the experiment ranged from 5.64-6.51 mm. Larvae fed the 90F/10S diet had a significantly higher total length at the end of the experiment than all other diets with the exception of the 80F/20S and 30F/70S diets (Fig. 5.8).

Final mean dry weight of larvae ranged from 0.48-0.65 mg per fish (Fig. 5.9). The dry weight of fish fed the 90F/10S diet was significantly higher than that of larvae in all other treatments with the exception of those fed the 80F/20S diet. The 100F/0S, 60F/40S, 50F/50S and 10F/90S treatments resulted in significantly lower mean dry weight of larvae than the 80F/20S diet. All other values were intermediate.

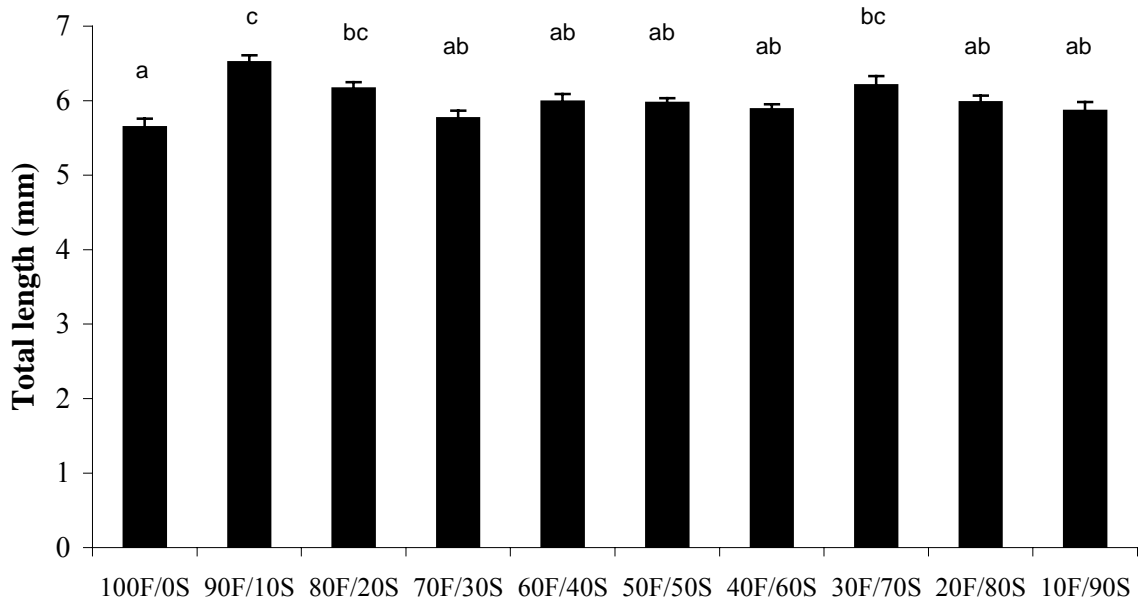
No significant differences occurred between treatments for survival (Fig. 5.10) or carcass T4 levels of larvae in this experiment. Survival ranged from 33.6%, in the 60F/40S and 40F/60S diets, to 50%, in the 80F/20S diet.

5.3.4. Experiment 4. - The effect of varying dietary protein source (fish meal, squid powder and krill meal) on survival and thyroid hormone levels in barramundi larvae.

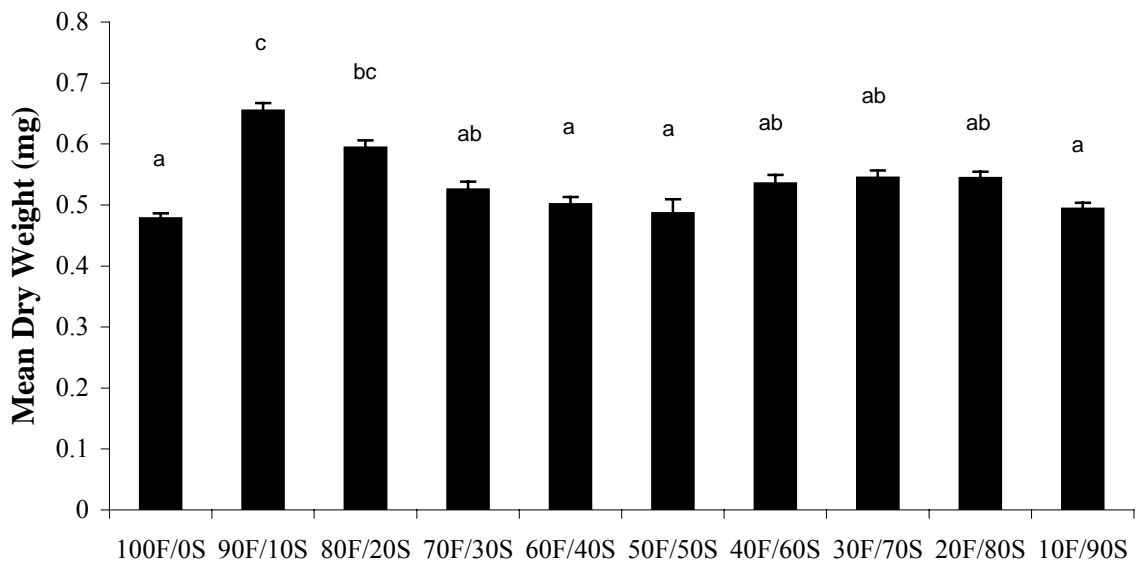
Both dry weight and total length were significantly lower for larvae fed the diet with the highest krill meal inclusion level (Figs. 5.11 and 5.12). Significant linear regressions exist, relating decreased total length ( $r^2 = 0.611$ ,  $p < 0.01$ ), dry mass ( $r^2 = 0.524$ ,  $P < 0.01$ ) and carcass T4 level ( $r^2 = 0.536$ ,  $P < 0.01$ ) to increasing krill meal inclusion. Larvae fed diets containing the highest krill meal inclusion level (10S/90K) had a significantly lower T4 level than those fed diets containing 0–50% krill meal, with all other treatments having intermediate values (Fig. 5.13).



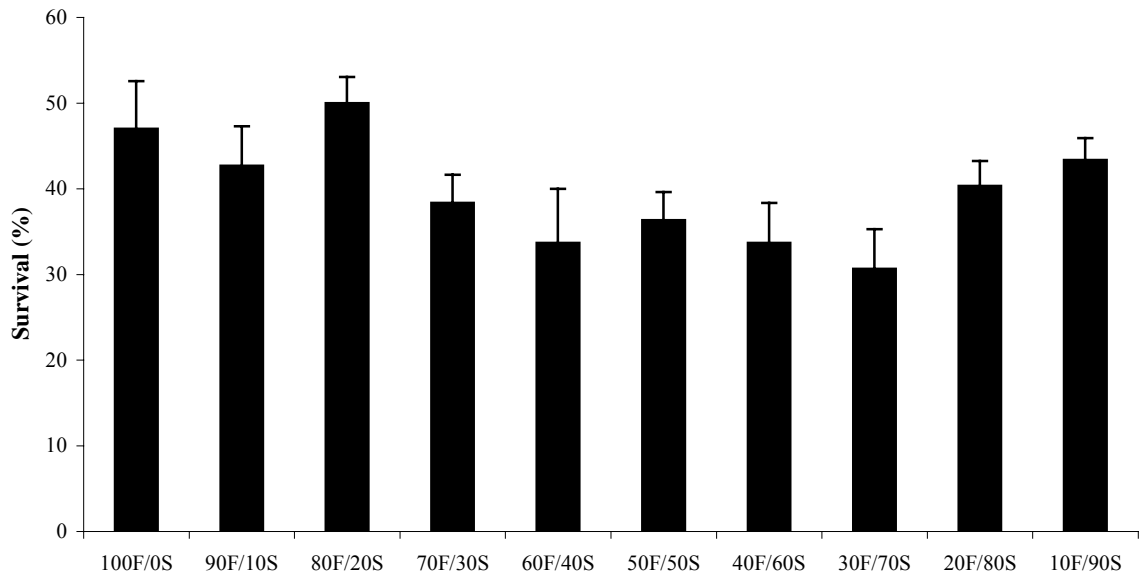
**Fig. 5.7.** Mean ( $\pm$  SE) T4 level of barramundi (*Lates calcarifer*) larvae, fed for 14 days on isocaloric ( $20 \text{ MJ.kg}^{-1}$ ), isonitrogenous (48% protein) formulated microbound diets, containing varying proportions of squid powder (S), fish meal (F) and mussel meal (M). Means with different superscripts <sup>(a-c)</sup> are statistically different (1-way ANOVA).



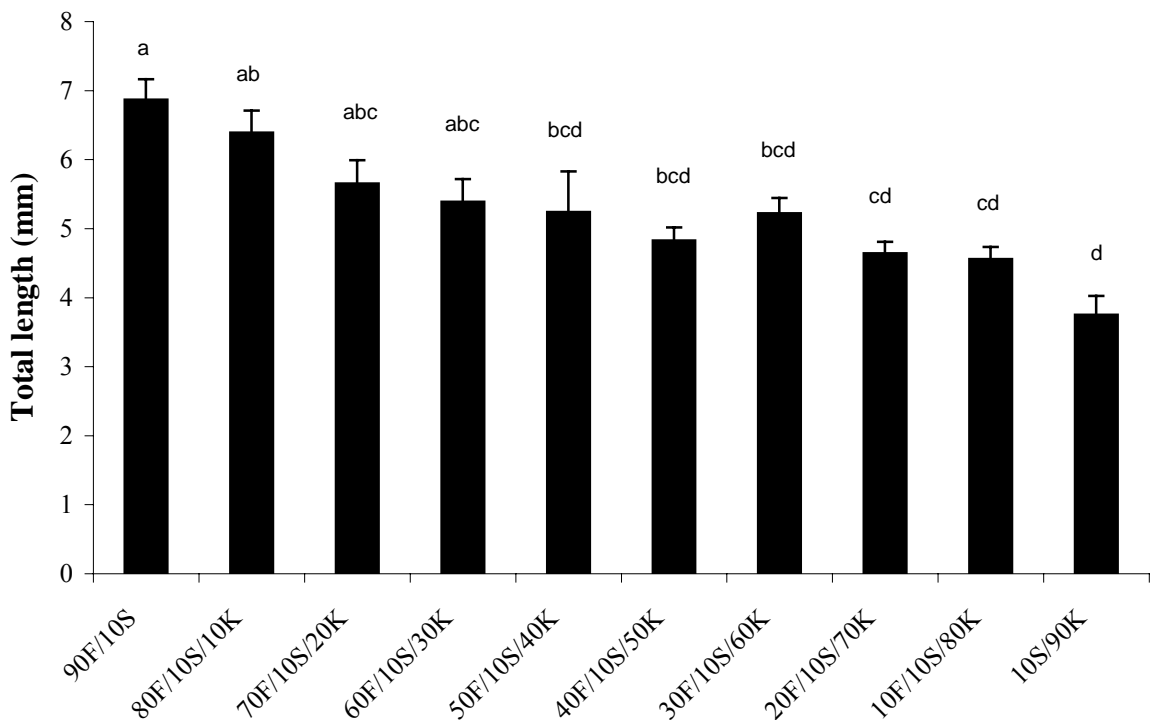
**Fig. 5.8.** Mean ( $\pm$  SE) final total length of *L.calcarifer* larvae fed for 14 days on formulated microbound diets containing ten different combinations of fish meal (F) and squid powder (S). Means with different superscripts <sup>(a-c)</sup> are statistically different (1-way ANOVA).



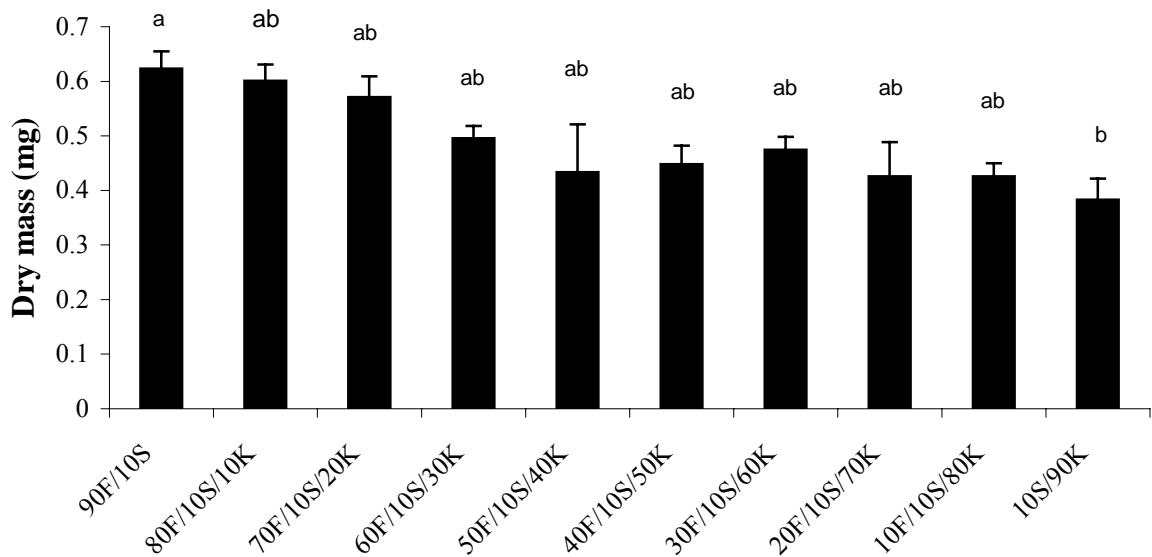
**Fig. 5.9.** Mean ( $\pm$  SE) dry weight of *L.calcarifer* larvae fed for 14 days on formulated microbound diets containing ten different combinations of fish meal (F) and squid powder (S). Means with different superscripts <sup>(a-c)</sup> are statistically different (1-way ANOVA).



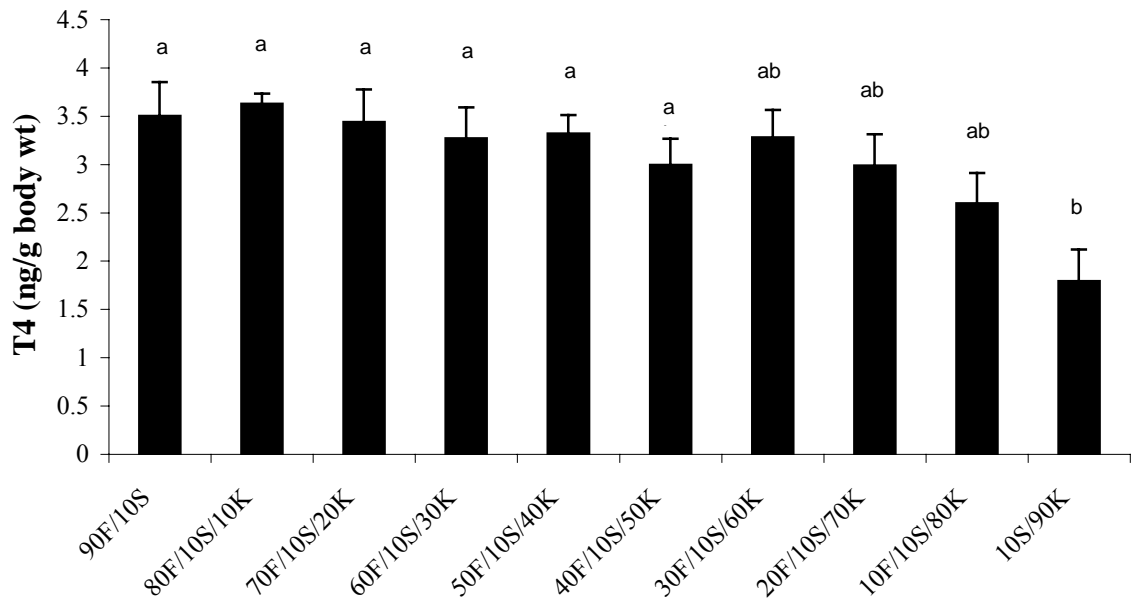
**Fig. 5.10.** Mean ( $\pm$  SE) survival (%) of *L.calcarifer* larvae fed for 14 days on formulated microbound diets containing ten different combinations of fish meal (F) and squid powder (S). There were no significant differences between treatments (1-way ANOVA;  $p>0.05$ ).



**Fig 5.11.** Mean ( $\pm$  SE) final total length of *L.calcarifer* larvae fed for 14 days on formulated microbound diets containing ten different combinations of fish meal (F), squid powder (S), and krill meal (K). Means with different superscripts <sup>(a-d)</sup> are statistically different (1-way ANOVA).



**Fig. 5.12.** Mean ( $\pm$  SE) final dry mass of *L.calcarifer* larvae fed for 14 days on formulated microbound diets containing ten different combinations of fish meal (F), squid powder (S), and krill meal (K). Means with different superscripts <sup>(a-b)</sup> are statistically different (1-way ANOVA).



**Fig. 5.13.** Mean ( $\pm$  SE) carcass T4 level of *L.calcarifer* larvae fed for 14 days on formulated microbound diets containing ten different combinations of fish meal (F), squid powder (S), and krill meal (K). Means with different superscripts <sup>(a-b)</sup> are statistically different (1-way ANOVA).

The EAAI was generally high for all protein sources used, with mussel and prawn meals having the lowest values (Table 5.5). The AA/aa ratio was lowest for leucine in *Artemia* meal, methionine in fishmeal, lysine in krill meal, mussel meal and squid powder, and valine in prawn meal.

Prawn meal and squid powder had the highest degree of hydrolysis (DH) values from pH-stat analysis with both commercial porcine enzymes and homogenous barramundi larvae enzyme extracts (Table 5.6). The DH value of fish meal was significantly lower than that for both squid powder and prawn meal, while the DH of mussel meal and krill meal were significantly lower than all other DH values for both the commercial enzyme and barramundi enzyme reactions. The DH of mussel meal was significantly higher than that for krill meal when using commercial enzymes, though they were not statistically separable when using homogenous barramundi larvae enzymes.

The fish meal, squid powder and prawn meal used in this study all had relatively low levels of water-soluble protein, with mean values ( $\pm$  SE) of  $2.89 \pm 0.82\%$ ,  $3.73 \pm 0.39\%$  and  $2.9 \pm 0.66\%$ , respectively. Mussel meal, krill meal and decapsulated *Artemia* cyst meal all had much higher soluble protein levels, with mean values ( $\pm$  SE) of  $18.66 \pm 3.8\%$ ,  $24.19 \pm 0.93\%$  and  $32.52 \pm 2.11\%$ , respectively.

Table 5.5. Ratio of essential amino acids in protein sources to that of 21 DAH *L.calcarifer*\* (aa/AA ratio<sup>b</sup>) and essential amino acid index (EAAI).

Protein Source	Arg.	His.	Ile.	Leu.	Lys.	Met. (+ Cys.)	Phe. (+ Tyr.)	Thr.	Val.	EAAI
Fish meal	1.00	1.00	0.94	0.94	0.93	0.83	0.98	0.95	0.95	0.95
Squid powder	1.00	0.95	1.00	0.96	0.80	1.00	0.97	0.97	0.81	0.94
Mussel meal	1.00	0.98	0.91	0.82	0.75	0.99	1.00	1.00	0.86	0.93
Krill meal	1.00	0.96	1.00	0.92	0.80	1.00	1.00	0.98	0.96	0.96
Prawn meal	1.00	0.87	0.94	0.94	0.86	1.00	1.00	0.87	0.82	0.93
<i>Artemia</i> meal	1.00	0.98	1.00	0.86	0.91	0.91	1.00	1.00	0.96	0.96

\* EAA profile of 21 DAH *L.calcarifer* from (Rimmer *et al.*, 1994)

<sup>b</sup> AA/aa ratio set at 0.01 minimum and 1.00 maximum (Hyashi 1986 in Penafiorida, 1989)



**Table 5.6. Alkaline digestibility of the protein sources used in experimental diets indicated by the degree of hydrolysis (DH %) by pH-stat, using a mixture of commercially available enzymes (trypsin and pancreatin) and barramundi larvae (14 DAH) enzyme extracts. Different superscripts (a-d) in each column indicate significant differences.**

	DH % (commercial enzymes)	DH % (barramundi enzymes)
Fish meal	2.54 ± 0.027 <sup>c</sup>	1.95 ± 0.057 <sup>b</sup>
Squid powder	3.90 ± 0.045 <sup>d</sup>	2.61 ± 0.053 <sup>c</sup>
Mussel meal	1.30 ± 0.022 <sup>b</sup>	0.92 ± 0.012 <sup>a</sup>
Krill meal	1.07 ± 0.051 <sup>a</sup>	1.02 ± 0.034 <sup>a</sup>
Prawn meal	3.76 ± 0.020 <sup>d</sup>	2.63 ± 0.028 <sup>c</sup>
<i>Artemia</i> meal	-	-

#### 5.4. Discussion

When the native protein sources used in these experiments were included in experimental diets alone, larval growth did not vary greatly, but survival was depressed for those larvae fed diets containing mussel meal, prawn meal and *Artemia* meal. While not significantly different, the mean survival of larvae fed diets containing squid powder was more than two-fold higher than those fed diets containing prawn and mussel meals. The colour control treatment produced similar results to the fish meal diet, indicating that colour of the diet had little influence over these results. The relatively high growth of larvae fed the *Artemia*-based diet is attributed to either unchecked cannibalism, or reduced density due to mortality, allowing faster growth.

Based on these results mussel meal, squid powder and fish meal were selected to further define the qualitative protein requirements of *L. calcarifer* larvae, and refine our understanding of the optimal protein composition of experimental diets. Survival was generally much higher in the second experiment, and not significantly different between

treatments, allowing growth to give a better indication of larval performance. Total length analysis at the end of the experiment clearly indicated that diets containing fish meal or a combination of fish meal and squid powder were superior to any diet containing mussel meal, even when mixed with both fish meal and squid powder. Dry weight analysis showed similar trends.

The third experiment investigated the optimum inclusion levels of fish meal and squid powder in formulated diets for barramundi larvae and indicated that 10% of the total dietary protein supplied as squid powder is beneficial to growth, in terms of total length and dry weight of larvae. However, greater than 20% squid powder (% protein basis) at the expense of fish meal was generally detrimental to growth. The fourth experiment determined krill meal to be detrimental to growth in a dose-dependent manner.

For any protein source, the amino acid present in the lowest amount in relation to its requirement (AA/aa ratio) is likely to be nutritionally limiting, and is therefore referred to as the first limiting amino acid (Eggum *et al.*, 1985). For the purposes of this study the amino acid profile of 21 DAH barramundi larvae was used as a reference for the ideal amino acid composition of formulated food ingredients (Rimmer *et al.*, 1994). The first limiting amino acid in fish meal for barramundi larvae is methionine (+ cysteine). As squid powder has relatively high amounts of methionine, its combination with fish meal compensates for the primary amino acid deficiency of fish meal. The first limiting amino acid for squid powder in this study is lysine, closely followed by valine. As these two amino acids are relatively high in fish meal, combining fish meal and squid powder likewise compensates for the amino acid limitations of squid powder. Squid powder also has a higher digestibility than fish meal, allowing for the digestion of more protein per

unit mass of food eaten. This is interesting in itself, considering the high substrate specificity of trypsin, cleaving peptide bonds associated with lysine and arginine (Lidner *et al.*, 1995). The relatively low lysine levels present in squid powder possibly did not limit its alkaline digestibility because of the high availability of arginine binding sites for tryptic digestion.

The amino acid profile of krill meal is favourable when compared to 21 DAH barramundi. Krill meal, however, contains abnormally high levels of fluorine (Julshamn *et al.*, 2004), which is toxic to animals which do not have high levels in their natural diets (Camargo, 2003). This limits the applicability of krill meal to food formulations for fish which do not naturally consume krill. Krill does, however, have a favourable amino acid composition for 21 DAH barramundi, and has been demonstrated to enhance food ingestion (Shimizu *et al.*, 1990), possibly due to its high FAA content, which has been demonstrated to stimulate feeding behaviour in fish larvae (Kolkovski *et al.*, 1997a). Furthermore, krill meal contains high levels of carotenoid pigments, and may therefore have a higher applicability to diet formulations for salmonids (Storebakken, 1988), or fish with red skin pigmentation, such as Australian snapper, *Pagrus auratus* (Booth *et al.*, 2004).

The essential amino acid index (EAAI) was generally high for all protein sources used in this study, however, the EAAI showed that the EAA profile of mussel meal and prawn meal were more dissimilar to 21 DAH barramundi larvae than all others. Interestingly, *Artemia* meal had the highest EAAI, though the high solubility of this protein is thought to be a limiting factor in its utilisation as a protein source in the present study, leading to

high leaching rates , as has previously been described in MBD (Baskerville-Bridges and Kling, 2000).

As thyroid hormones are iodinated tyrosine residues (Eales, 1985), the amino acid profile of food sources may directly affect the availability of tyrosine and/or phenylalanine for thyroid hormone synthesis. This is difficult to assess in the present study, as the phenylalanine and tyrosine levels were not limiting for any of the protein sources used. There was, however, a strong relationship between growth (most notably total length) and T4 levels, reaffirming the status of this hormone as a growth performance indicator, as proposed in Chapter 4.

It is clear from digestibility analysis that prawn meal and squid powder are highly digestible protein sources for barramundi larvae, while fish meal was slightly less digestible, and mussel and krill meals were relatively poorly digested. The poor digestibility of mussel and krill meals is attributed to high levels of soluble proteins present in these protein meals, indicative of autoproteolysis. This has been previously demonstrated for krill (Ellingsen and Mohr, 1987), being responsible for the liberation of approximately 30% of krill amino acids to a water-soluble form in less than one day when stored at 0°C. The soluble protein levels found in the present study are consistent with these previous findings, and are thought to be responsible for the decreased growth evident for larvae fed formulations containing large amounts of krill, *Artemia* and mussel meals. The microbound diets used in the present study are susceptible to high levels of leaching of water-soluble nutrients (Baskerville-Bridges and Kling, 2000), which would be expected to decrease the dietary availability of water-soluble peptides and FAA present in formulations containing mussel meal, *Artemia* meal and krill meal. The

protein that remains has a lower digestibility, possibly due to lower amount of protein being present, and fewer enzyme activation sites being available. The poor performance of barramundi fed diets with large amounts of autolysates larvae in this study is supported in the literature, with high levels of protein hydrolysate inclusion in larvae food formulations commonly meeting little success in terms of growth and survival (Dabrowski, 1984; Cahu *et al.*, 1999; Kolkovski and Tandler, 2000).

The naturally occurring zooplanktonic foods of fish larvae contain high levels of free amino acids (FAA) (Helland *et al.*, 2000; Koven *et al.*, 2002). Such FAA are effectively delivered to the larval digestive system with a minimum of leaching, as the differentially permeable membranes within zooplankton prevent their release. The high availability of FAA may be integral to the success of live foods in larval rearing, however, the formulation of a finely ground animal meal in the present study releases these 'biologically encapsulated' FAA, rendering them susceptible to leaching from subsequently formulated diets. Indeed, greater than 80% of the protein found in decapsulated *Artemia* cysts has been previously found to be water soluble (Garcia-Ortega *et al.*, 2000b), and therefore susceptible to leaching from microbound diets. This is high compared to the results of the present study, which may indicate a denaturation of *Artemia* proteins by the oven drying procedure used in the present study (Garcia-Ortega *et al.*, 2000b). Digestibility analysis of decapsulated *Artemia* cyst meal was not performed in the present study, due to its tendency to coagulate when the pH was adjusted to 8.0, though *Artemia* cysts processed in a similar way resulted in protein digestibility levels in excess of 80% (Garcia-Ortega *et al.*, 2000a). This previous result must be treated with caution however, as the method used measured insoluble nitrogen as

an estimate of indigested protein, and their own results indicated that greater than 80% of the protein in *Artemia* cysts is soluble (Garcia-Ortega *et al.*, 2000b).

The ideal source of dietary protein in microdiets for fish larvae is dependent not only on their amino acid profile, but on the dietary availability of such amino acids. The results of the present study indicate that a large part of the dietary availability of amino acids in microbound diets relates to the solubility of dietary proteins, and the potential for leaching of water-soluble proteins. This confirms the requirement to assess ingredients for microdiet manufacture with all available means, rather than relying on individual quality indices such as EAA profile, or digestibility. This study also reaffirms the value of feeding experiments for the evaluation of such food ingredients (Eid and Matty, 1989).

The results of this study indicate that an appropriate protein source for barramundi larvae, 14-28 DAH fed formulated microbound diets consists of fish meal and squid powder at a 9:1 ratio, total protein basis. However, as the fish meal used was less digestible than squid powder, further processing in terms of denaturation or hydrolysis may be beneficial to increase the dietary availability of amino acids from the fish meal component of this food. This aspect was further investigated in Chapter 6.

## Chapter 6

### **Substitution of untreated fish meal with partially hydrolysed fish meal in formulated foods for barramundi larvae**

#### **6.1. Introduction**

The early proteolytic capacity of fish larvae is generally defined by the pancreatic enzymes trypsin and chymotrypsin, (Chapter 3, DeSilva and Anderson, 1995). Since these enzymes are specific in substrate activation sites (Blow, 1971; Kiel, 1971), they act primarily as endopeptidases (Guillaume and Choubert, 1999), cleaving the internal rather than terminal bonds of peptide chains, thus reducing proteins to smaller peptide chains. Since the peptidase enzymes act synergistically to break down proteins to small peptides (Guillaume and Choubert, 1999), the lack of pepsin in fish larvae has been suggested as a limiting factor for protein digestion (Rønnestad *et al.*, 1999). Therefore pre-digestion of dietary protein by pepsin may serve to increase its capacity for intestinal absorption, and thereby increase the total amino acid availability for protein accretion in larval fish.

Free amino acids or small chain peptides are generally considered to be more efficiently absorbed over the gut epithelium of fish larvae than intact protein (Tengjaroenkul *et al.*, 2002). Therefore researchers have investigated the incorporation of predigested proteins (hydrolysates) into formulated microdiets. As well as increasing growth (Carvalho *et al.*, 1997) and survival (Cahu *et al.*, 1999) of fish larvae, the inclusion of moderate levels of hydrolysates decreased malformation rates in European seabass larvae (Zambonino Infante *et al.*, 1997), and is thought to facilitate the transition of the larval gut to the adult mode of digestion (Zambonino Infante *et al.*, 1997; Cahu *et al.*, 1999).

Previous studies have generally included fully hydrolysed proteins and have often met considerable success with low levels of hydrolysate inclusion, though often limited success with higher levels of inclusion (Cahu *et al.*, 1999; Kolkovski and Tandler, 2000). This has been explained by a glut of amino acids, which are not readily assimilated into body tissue (Kolkovski and Tandler, 2000), bleeding of the gut due to the hygroscopic nature of this food formulation (Dabrowski, 1984), and by the leaching of the small water-soluble molecules that are released by the hydrolysis process, thus decreasing the value of the protein source.

To avoid such problems associated with the liberation of large amounts of water-soluble protein subunits, this study investigated the utilization of partially hydrolysed fishmeal in formulated microbound diets. The intended outcome was to find a level of hydrolysis that maintains the benefits of hydrolysates, but does not subject the subsequently made food to large amounts of leaching or amino acid imbalances. Further, pepsin was hypothesized as an appropriate agent for hydrolysis, as the natural pepsin deficiency of barramundi larvae is thought to limit their digestive capability (Walford and Lam, 1993). Since fish meal was found to have a relatively low digestibility for barramundi larvae in Chapter 5 (section 5.3, Table 5.6), the partial hydrolysis of fish meal may increase its dietary value when formulated into microbound diets for barramundi larvae.

## **6.2. Materials and Methods**

### *6.2.1. Hydrolysis*

The hydrolysis method used was based on Kolkovski and Tandler (2000) and Kvale *et al.*(2002) A suspension of fish meal (300 mg.mL<sup>-1</sup>) was hydrolysed under acidic



conditions (pH 3.0), with pepsin (Sigma, porcine – product number P 7000) at a rate of 0.1 mg.g<sup>-1</sup> fish meal. Temperature was maintained at 28 ± 1°C with a hotplate, and the solution was constantly mixed with a magnetic stirrer. The pH of the solution was maintained through titration of 2 M HCl. Samples were removed from the hydrolysate at 7.5 min, 15 min, 30 min and 60 min, at which time the pH of the removed sample was adjusted to 8.0 with 2 M NaOH to stop the hydrolysis and match the intestinal pH of barramundi larvae (Walford and Lam, 1993). To prevent further hydrolysis, pepsin remaining in the samples was denatured by heating to 80°C for 10 min (Kolkovski and Tandler, 2000). Hydrolysate samples were subsequently dried at 50°C to constant weight before being ground and sieved to <100 µm.

#### *6.2.2. Analysis of hydrolysis – digestibility and degree of hydrolysis*

Two triplicate sets of each hydrolysate sample and intact fish meal (<100 µm particle size) were re-suspended in purified water (250 mg.mL<sup>-1</sup>) and vortexed for 10 min. All samples were centrifuged for 10 min at 2,000 g in a Beckman GS-6R bench centrifuge, and supernatants removed. Triplicate supernatant samples were removed and analysed for total soluble protein (Bradford, 1976).

The remaining triplicate supernatant samples were precipitated by 12.5% TCA (1 ml 25% TCA added to 1 ml supernatant) and centrifuged as above. Supernatants were removed and analysed for amino acids with the cadmium-ninhydrin method of Volden *et al.* (2002) and soluble protein (Bradford, 1976). Since TCA-soluble protein includes amino acids and small peptides (4 amino acids or less)(Greenberg and Shipe, 1979), the quantitative difference between TCA soluble protein and amino acids indicates the composition of di-, tri- and tetra-peptides.

#### 6.2.2.1. Soluble protein analysis

The water-soluble protein content of hydrolysates was analysed as described in Chapter 5 (section 5.2.6.2).

#### 6.2.2.2. Amino acid analysis

A cadmium-ninhydrin reagent was formulated by dissolving 0.8 g ninhydrin (Sigma catalogue #N4876) in absolute ethanol, before adding 10 mL acetic acid (-20°C) and 1 mL CdCl<sub>2</sub> solution (1 g.mL<sup>-1</sup>). This reagent was stored in a stoppered opaque bottle at 5°C.

Samples, standards and blanks were analysed by adding 100 µl TCA soluble hydrolysate samples, tyrosine standards (6 mg.L<sup>-1</sup>, 3 mg.L<sup>-1</sup>, 1.5 mg.L<sup>-1</sup>, 750 µg.L<sup>-1</sup> and 375 µg.L<sup>-1</sup>) or purified water, to microcentrifuge tubes and mixed with 200 µl CdCl<sub>2</sub>-ninhydrin reagent. All tubes were heated to 84°C in a water bath for 5 min before being centrifuged at 10,000 g (Eppendorf 5415C microfuge, Germany) for 10 min. Supernatants (200 µl) were added to microtitre plates and absorbance read with a microplate reader at 490 nm.

### 6.2.3. *Growth trial*

#### 6.2.3.1. Microbound diet formulations

Microbound diets were made according to the method outlined in Chapter 2 (section 2.1). Formulations were identical to the 90F/10S diet from Experiment 4 in Chapter 5 (section 5.2.5), with the exception that the fish meal component was derived from either intact fish meal or the hydrolysates described above. Each hydrolysate sample and an

intact fish meal control were incorporated into a microbound diet at 100% of the fishmeal component of the formulation (Table 6.1).

**Table 6.1. The proportion of fish meal, fish meal hydrolysate and squid powder incorporated into experimental diets.**

Diet	Proportion of protein component (%)		
	Fish Meal	Hydrolysate inclusion (hydrolysis period)	Squid Powder
1	90	0	10
2	0	90 (7.5 min)	10
3	0	90 (15 min)	10
4	0	90 (30 min)	10
5	0	90 (60 min)	10

#### 6.2.3.2. Experimental procedure

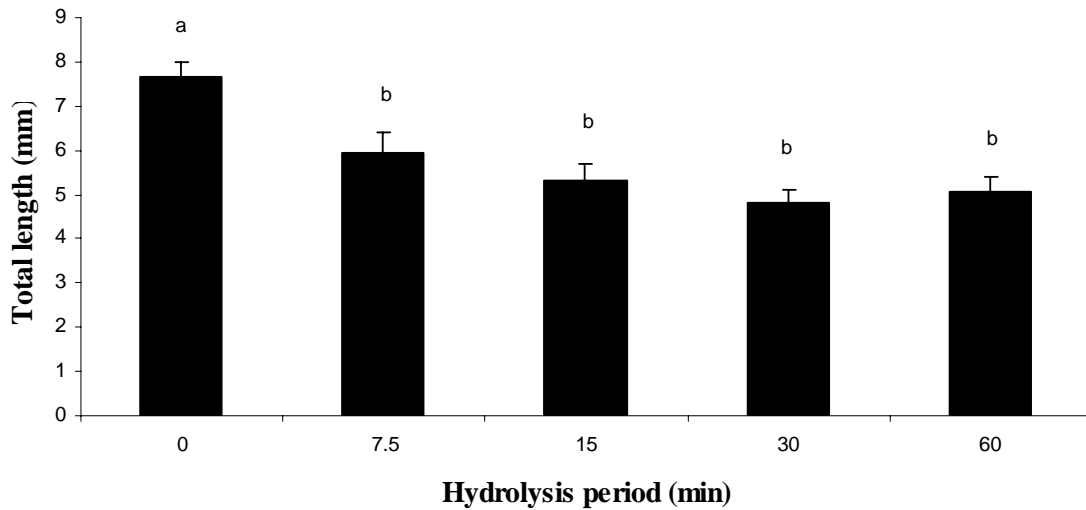
A growth trial was then performed as described in Chapter 2 (section 2.3), with three replicates per treatment. Each treatment was fed a different microbound diet for a total of 14 days.

#### 6.2.4. *Effect of dietary hydrolysate inclusion on pepsin levels*

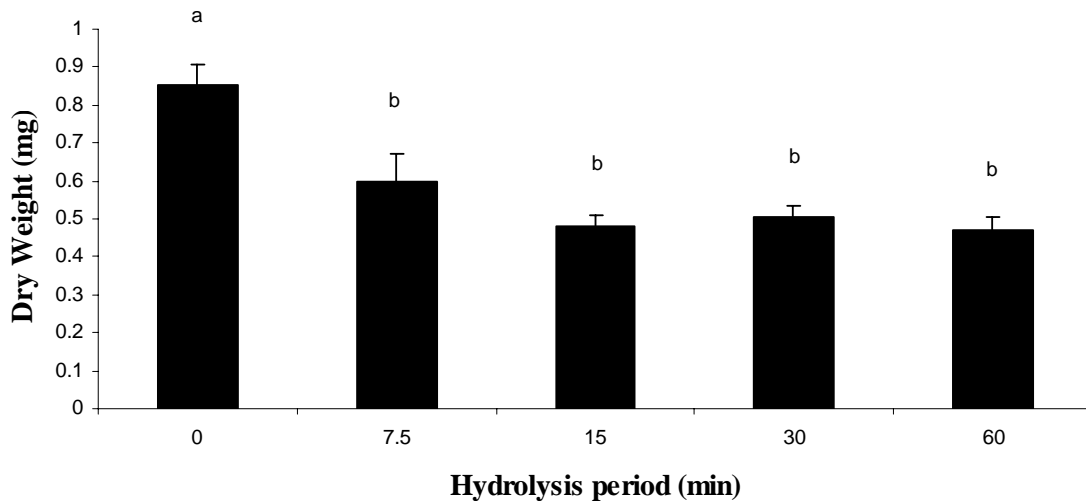
At the end of the experiment, samples of larvae (50 mg) were assayed for pepsin as described in Chapter 3 (section 3.2.3).

### 6.3. Results

Total length and dry weight at the end of the experiment were both higher for larvae fed diets containing untreated fish meal than those fed diets containing any level of hydrolysate (Figs. 6.1 and 6.2).



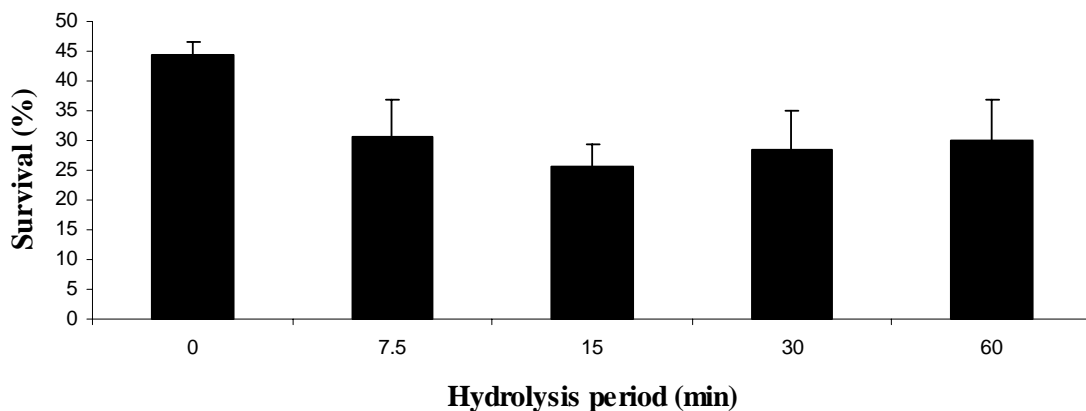
**Fig. 6.1. Mean ( $\pm$  SE) final total length of barramundi larvae fed experimental diets containing fish meal hydrolysed for varying period (7.5 – 60 min), from 14-28 DAH. Means with different superscripts (<sup>a-b</sup>) are significantly different ( $p < 0.05$ ).**



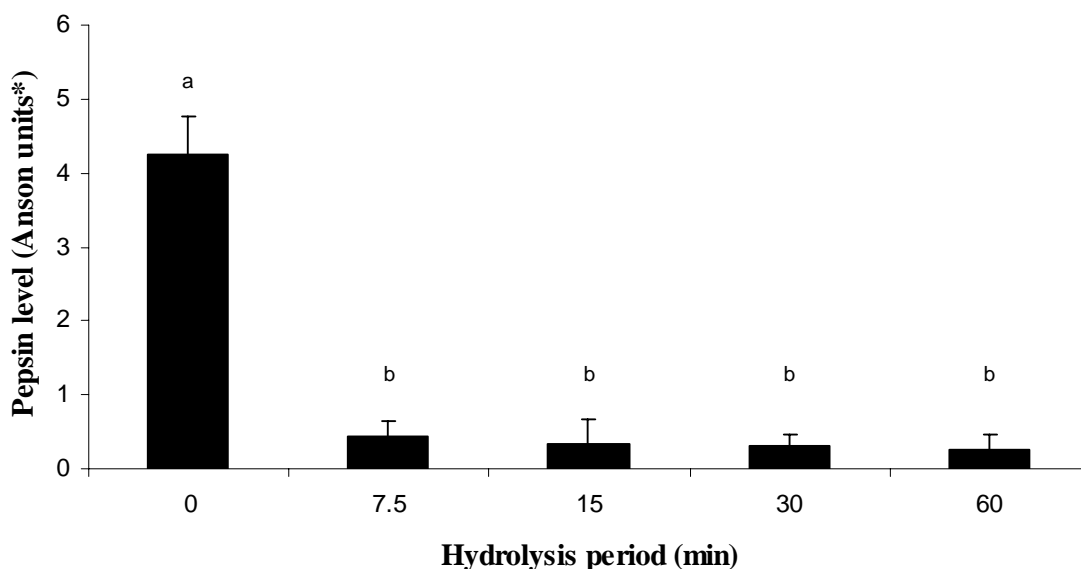
**Fig. 6.2. Mean ( $\pm$  SE) final dry weight of barramundi larvae fed experimental diets containing fish meal hydrolysed for varying period (7.5 – 60 min), from 14-28 DAH. Means with different superscripts (<sup>a-b</sup>) are significantly different ( $p < 0.05$ ).**

Survival of barramundi larvae in this experiment ranged from 15.8% to 47.2%, with no significant differences between treatments (Fig. 6.3;  $p>0.05$ ). Mean survival of the fish larvae fed diets containing untreated fish meal was at least 30% greater than for any of the treatments fed diets containing hydrolysates; however, high variation in survival for those larvae fed hydrolysates prevented these differences from being statistically significant.

Carcass pepsin levels of barramundi larvae fed diets containing untreated fish meal were significantly higher than pepsin levels of larvae fed all other diets ( $p<0.05$ ; Fig. 6.4). Larvae fed diets containing untreated fish meal had almost 10-fold higher pepsin levels ( $4.24 \pm 0.52$  units) than larvae from the next highest treatment, fed diets containing the 7.5 min hydrolysate ( $0.44 \pm 0.20$  units)(Fig. 6.4).



**Fig. 6.3. Mean ( $\pm$  SE) survival of barramundi larvae fed experimental diets containing fish meal hydrolysed for varying period (7.5 – 60 min), from 14-28 DAH. There were no significant differences between treatments ( $p<0.05$ ; 1-way ANOVA).**



**Fig. 6.4. Mean ( $\pm$  SE) carcass pepsin level of barramundi larvae fed experimental diets containing fish meal hydrolysed for varying period (7.5 – 60 min), from 14-28 DAH. Means with different superscripts (<sup>a-b</sup>) are significantly different ( $p < 0.05$ ).**

**\*Anson units are equivalent to 1  $\mu$ g tyrosine liberated in one min per gram of protein in solution.**

Analysis of soluble protein products shows that 2.63% of the total protein component of the untreated fish meal was water-soluble (Table 6.1). This water-soluble protein was entirely precipitated by 12% TCA, and did not react with the Cd-Ninhydrin reagent. After 7.5 min hydrolysis a further 11.63% of the total protein was broken down to a soluble form. At this stage, 3.05% of total protein was not able to be precipitated by 12.5% TCA and 0.64% was in the form of FAA's. After 15 min hydrolysis, a further 4.15% of the total protein was degraded to a water-soluble state, while the 12.5% TCA-soluble protein FAA levels increased by 5.54% and 0.2% of the total protein content, respectively. After 30 min hydrolysis, the total soluble protein content increased by a further 3.74% of total protein, 12.5% TCA-soluble protein increased by 5.44%, and

FAA's increased by 1.53% of the total fish meal protein. After the longest duration of hydrolysis (60 min), water-soluble protein accounted for 35.68% of total protein levels, while 12.5% TCA-soluble protein and FAA's accounted for 22.73% and 3.81% of total protein levels, respectively.

**Table 6.1. Total water soluble protein, 12.5% TCA soluble protein and free amino acid content of hydrolysates incorporated into experimental diets.**

	Total water-soluble protein (%)	12.5% TCA soluble protein (%)	Free Amino acids (%)
Fish meal (untreated)	2.63	-	-
7.5 min hydrolysate	14.26	3.05	0.64
15 min hydrolysate	18.41	8.59	0.84
30 min hydrolysate	22.15	14.03	2.37
60 min hydrolysate	35.68	22.73	3.81

#### 6.4. Discussion

Barramundi larvae fed formulated foods containing untreated fish meal grew faster than those fed diets containing fish meal hydrolysates, regardless of the degree of hydrolysis. Larvae fed formulations containing untreated fish meal also had far higher carcass pepsin levels than larvae fed formulations containing any of the hydrolysates. Since carcass pepsin levels are generally developed by 28 DAH (Chapter 3, Walford and Lam, 1993), the more complex protein source offered by untreated fish meal may stimulate pepsin secretion through provision of increased binding substrate than hydrolysates. A similar trend has been reported for European seabass larvae, with larvae fed formulated foods having higher pepsin activity than those fed live foods (Zambonino Infante and Cahu, 1994a), which are generally more digestible (Garcia-Ortega *et al.*, 2000a) and contain more soluble proteins (Garcia-Ortega *et al.*, 2000b; Helland *et al.* 2000).

Despite the relatively poor growth and survival of barramundi larvae fed formulations containing hydrolysates, inert diets containing protein hydrolysates have been formulated for gilthead seabream (*Sparus aurata*), carp (*Cyprinus carpio*) and European seabass larvae (Cahu *et al.*, 1998; Cahu *et al.*, 1999; Yúfera *et al.*, 2000), which support higher growth and survival than formulations containing fish meal alone. Such formulations generally contain approximately up to 20-25% hydrolysate by mass, with higher inclusion levels resulting in diets which support reduced growth and/or survival of larvae (Zambonino Infante *et al.*, 1997; Cahu *et al.*, 1999; Kolkovski and Tandler, 2000). These successful food formulations use microencapsulated particles to retain the associated water-soluble molecules. The microbound particles used in the present study are far more subject to leaching of water-soluble ingredients (Baskerville-Bridges and Kling,



2000), and therefore upon ingestion the water soluble peptides and FAA in food particles are likely to be lower than the formulation.

Microencapsulated food particles were not investigated in the present study due to poor digestibility of such diets by barramundi larvae in the absence of live foods (Walford *et al.*, 1991). In the absence of a highly digestible particle membrane, the incorporation of hydrolysates into formulated foods for fish larvae may therefore be limited to the feed attractant role described by Kolkovski (1997a) and Wright (2003).

The 7.5 and 15 min hydrolysates had less than 1% of the total dietary protein as FAA's. As formulations containing these hydrolysates did not support any greater growth than those containing hydrolysates with higher levels of FAA's, this would indicate that the growth limitation of hydrolysates was not due to saturation of the larvae FAA pools, as has been postulated by Kolkovski and Tandler (2000). Furthermore, fish larvae have been demonstrated to effectively assimilate FAA's when they are delivered directly into the larval gut (Ronnestad *et al.*, 2000). Therefore, the poor growth performance of larvae fed microdiet formulations containing hydrolysates in the present study is attributed to the large amounts of water-soluble nitrogen (predominantly peptides) in hydrolysates. Kvale *et al.* (2002) likewise found increasing levels of soluble protein in microbound diets decreases survival for Atlantic halibut (*Hippoglossus hippoglossus*) larvae.

As microbound diets typically leach large amounts of water-soluble nutrients (Baskerville-Bridges and Kling, 2000), further development of hydrolysate use in larvae foods may require development of microparticle types which prevent or reduce such leaching, while retaining high digestibility. Alternatively, research into optimizing the

digestibility of fish meal without increasing the soluble protein content may improve its value as a protein source for barramundi larvae.

## Chapter 7

### **Substitution of untreated fish meal with acid denatured fish meal in formulated foods for barramundi larvae**

#### **7.1. Introduction**

The decreased capacity for protein digestion in altricial marine fish larvae has been associated with the lack of a functional stomach and the associated denaturing and hydrolyzing action of low pH and pepsin (DeSilva and Anderson, 1995). While pepsin is responsible for the hydrolysis of proteins into smaller peptide chains (Grabner and Hofer, 1989), reduced pH can electrostatically break the bonds responsible for the three-dimensional folding of proteins (Fink *et al.*, 1994), thus decreasing the structural complexity of the protein and subjecting more active sites to the action of proteolytic enzymes.

The ability of chymotrypsin and trypsin to break down native proteins is thought to be limited in fish and higher vertebrates (Jany, 1976), and therefore the action of pepsin and HCl in the stomach is thought to be integral to the efficient digestion of feed proteins. Since protein hydrolysates have met limited success as a total protein source in larval food formulations for barramundi, European seabass and gilthead seabream (Chapter 6, Cahu *et al.*, 1999; Kolkovski and Tandler, 2000), the presentation of protein in an acid-denatured form, without the liberation of small water soluble molecules (small peptides and free amino acids; FAA's), may be a more effective means of delivering amino acids in a readily utilizable form. It is hypothesized therefore that acid denaturation of proteins may be an effective means of improving protein digestibility, thus providing a more

effective form of amino acids for delivery to altricial marine fish larvae fed microbound diets.

There is evidence that nutritional inputs, more specifically the digestibility of food, influences the enzyme secretions of fish larvae (Zambonino Infante and Cahu, 1994a; Zambonino Infante and Cahu, 1994b). This is of interest in the capacity of larvae to wean to a more complex (less processed) food source and in their ability to adapt their physiology to cope with foods of varying digestibility.

This chapter evaluated the digestibility of acid denatured fish meal *in vitro* and the affect of denatured fish meal inclusion in formulated foods on the growth and survival of barramundi larvae. To assess the capacity of acid denatured fish meal to aid weaning to less processed foods, fish from the growth trial were analysed for pepsin levels and their capacity to digest intact and denatured fish meal *in vitro*.

## **7.2. Materials and Methods**

### *7.2.1. Denaturation*

Fish meal (Skretting Australia, sieved to  $<100\ \mu\text{m}$ ) was suspended in purified water at a rate of  $300\ \text{mg}\cdot\text{mL}^{-1}$ . Temperature was adjusted to  $28^{\circ}\text{C}$  using a hotplate, and the suspension was mixed thoroughly with a magnetic stirrer. Hydrochloric acid (2 M) was used to adjust the pH of the protein suspension to pH 3.0. The solution was incubated at  $28 \pm 1^{\circ}\text{C}$  for 30 min, while the required pH was maintained through 2 M HCl titration.

At the end of the incubation period the pH was adjusted to 8.0 with 2 M NaOH, to end the denaturation process and match the intestinal pH of barramundi larvae (Walford and

Lam, 1993). The protein suspension was then dried to constant weight at 50°C before being ground and sieved to <100 µm.

#### *7.2.2. Analysis of denaturation – digestibility*

Digestibility of acid denatured protein was determined by the pH-stat method described in Chapter 5 (section 5.2.6), and compared to the digestibility of intact fish meal.

#### *7.2.3. Food formulation*

Microbound diets were made according to the method described in Chapter 2 (Section 2.1). All formulations were isocaloric and contained 50% protein. Formulations were identical to the diet composed of 9:1 fish meal: squid powder used in Experiment 4 in Chapter 5 (Section 5.2.7), with graded levels of fish meal and denatured fish meal (Table 7.1).

#### *7.2.4. Growth trial*

A 14-day growth trial was conducted to determine the effects of acid-denatured protein inclusion in microbound diet formulations on growth, survival and pepsin development for barramundi larvae. The growth trial was conducted using the methods described in Chapter 2 (section 2.4). Analysis of growth and survival were conducted as described in Chapter 2 (section 2.5).

#### *7.2.5. Effect of acid treatment on soluble protein levels*

Soluble protein levels of intact fish meal and denatured fish meal were analysed as described in Chapter 5, section 5.2.8.2.

**Table 7.1. The proportion of total protein derived from fish meal, denatured fish meal and squid powder in experimental diets in Chapter 7.**

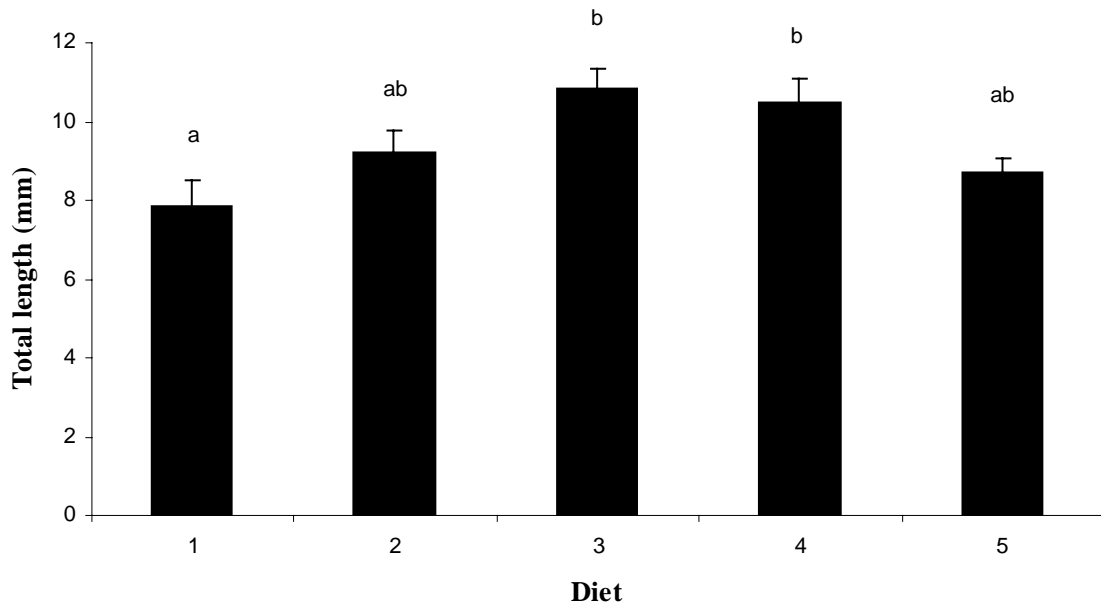
Diet	Proportion of protein component (%)		
	Fish Meal	Denatured Fish Meal	Squid Powder
1	90	0	10
2	78.75	11.25	10
3	67.5	22.5	10
4	45	45	10
5	0	90	10

#### *7.2.6. Effect on digestive capacity of larvae*

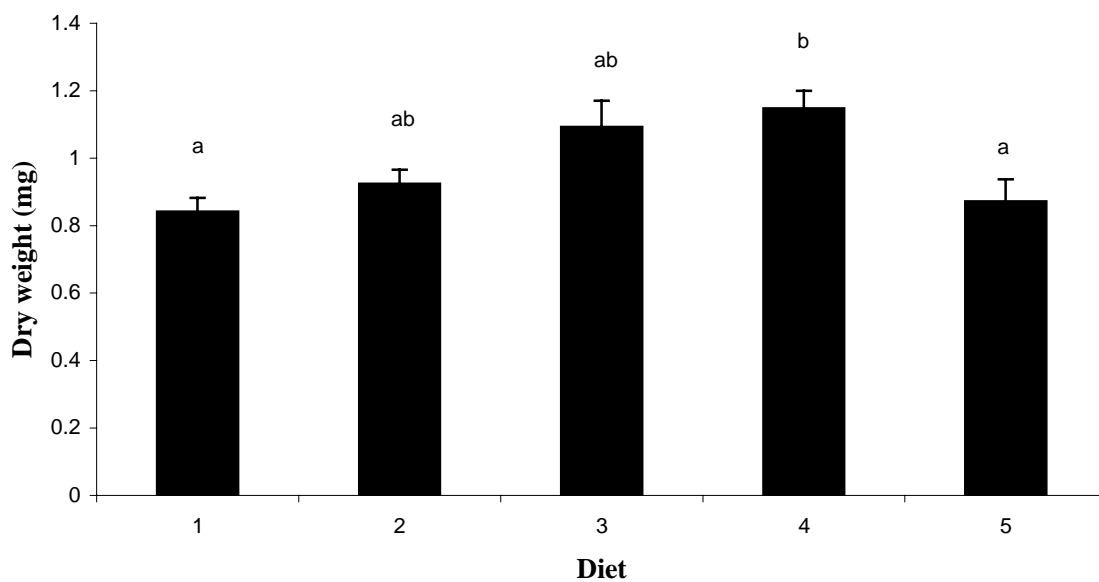
At the end of the experiment samples of larvae (50 mg) were assayed for pepsin as described in Chapter 3 (section 3.2.3).

### **7.3. Results**

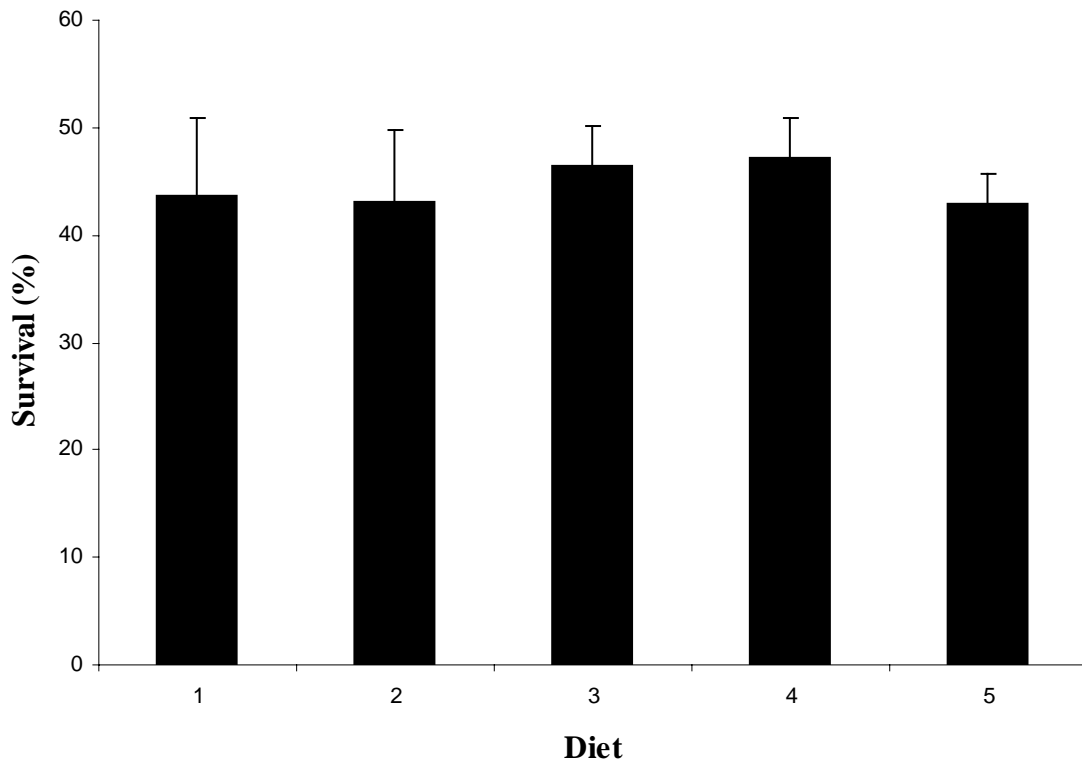
Barramundi larvae fed diets 3 and 4 had significantly higher total length at the end of the growth trial than those fed diet 1 (Fig. 7.1). Larvae fed diets 2 and 5 had intermediate values. Larvae fed diet 4 had significantly higher dry weight at the end of the experiment than those fed diets 1 and 5 (Fig. 7.2), while larvae fed diets 2 and 3 had intermediate dry weight values. Survival values ranged from 30.2% to 58.2%, with no significant differences between treatments (Fig. 7.3).



**Fig. 7.1. Mean ( $\pm$  SE) final total length of barramundi fed diets containing different levels of acid denatured fish meal (% protein basis) from 14 - 28 DAH. Means with different superscripts are significantly different ( $p < 0.05$ ).**



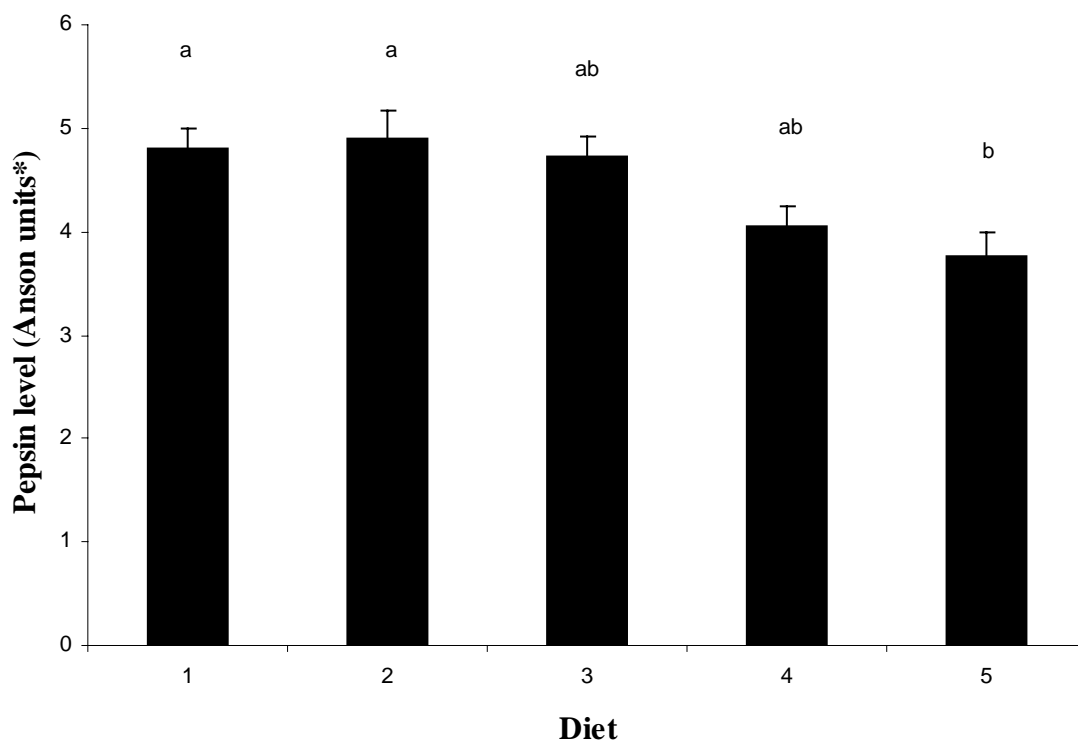
**Fig. 7.2. Mean ( $\pm$  SE) final dry weight of barramundi fed diets containing different levels of acid denatured fish meal (% protein basis) from 14 - 28 DAH. Means with different superscripts are significantly different ( $p < 0.05$ ).**



**Fig. 7.3. Mean ( $\pm$  SE) survival of barramundi fed diets containing different levels of acid denatured fish meal (% protein basis) from 14 - 28 DAH. There were no significant differences between treatments ( $p>0.05$ ).**

Whole homogenate pepsin levels were significantly affected by the level of dietary denatured fish meal inclusion. Larvae fed diets 1 and 2 contain higher pepsin levels than those fed diet 5, containing 100% denatured fishmeal (Fig. 7.4). Larvae fed diets 3 and 4 had intermediate pepsin levels. There was a significant regression relating dietary denatured fish meal inclusion level to decreasing pepsin level ( $r^2 = 0.569$ ,  $p<0.01$ ). Larval pepsin level did not correlate to either dry weight or total length (Pearson correlation,  $p>0.05$ ).





**Fig. 7.4. Mean ( $\pm$  SE) carcass pepsin level\* of barramundi fed diets containing different levels of acid denatured fish meal (% protein basis) from 14 - 28 DAH. Means with different superscripts are significantly different ( $p < 0.05$ ).**

**\*Anson units are equivalent to 1  $\mu$ g tyrosine released in 1 min per mg protein in the homogenate supernatant.**

The DH % of the acid-denatured fish meal used in this study was significantly higher than untreated fish meal, when assessed by both commercial enzymes (trypsin and pancreatin) and barramundi enzyme extracts (Table 7.1). Intact fish meal had a significantly lower percentage of soluble protein than denatured fish meal, with values of  $2.89 \pm 0.22\%$  and  $5.24 \pm 0.71\%$ , respectively.

**Table 7.1. Degree of hydrolysis (DH%) of fishmeal and acid denatured fishmeal by the pH-stat method, incubated for 30 minutes with a commercial enzyme mixture and homogenous barramundi larvae extracts. Means within columns with different superscripts (<sup>a-b</sup>) are significantly different (paired t-test).**

	DH % (commercial enzymes)	DH % (barramundi enzymes)
Fishmeal	2.54 ± 0.027 <sup>a</sup>	1.95 ± 0.057 <sup>a</sup>
Denatured fishmeal	4.47 ± 0.143 <sup>b</sup>	4.28 ± 0.125 <sup>b</sup>

#### **7.4. Discussion**

While the inclusion of pre-hydrolysed food ingredients in microbound diets for barramundi larvae met with little success (Chapter 6), the inclusion denatured fish meal increases growth significantly. The acidification of fish meal to pH 3 mimics the denaturing processes of a fully functional stomach (Walford and Lam, 1993), with a small increase in soluble protein. Acid denaturation of fish meal prior to incorporation into formulated microbound diets increased the digestibility of the food while not increasing soluble protein levels to the extent of the hydrolysates in Chapter 6. The inclusion of 25-50% of the fishmeal derived protein in the experimental microbound diet in denatured form resulted in an increase in larvae growth of 38.7% on the basis of total length and 36.4% on the basis of dry weight.

The pepsin activity of larvae decreased with increasing denatured fish meal level, indicating that there is a response of the larvae pepsin development to the digestibility of the food presented. As there was no correlation between growth indices and whole carcass pepsin level, it is thought that this trend represents a diet-induced adaptation rather than a developmental acceleration in the natural increase in pepsin activity through

ontogeny (Walford and Lam, 1993, Chapter 3). A similar trend has been reported for European seabass larvae, with larvae fed formulated foods having higher pepsin activity than those fed live foods (Zambonino Infante and Cahu, 1994a), which are generally more digestible (Garcia-Ortega *et al.*, 2000a).

There are few data on the effects of protein denaturation on digestibility and nutritional value for fish. Urea denaturation of bovine serum albumin (BSA), has been shown to increase the digestion rate of this protein eight-fold by the stomachless bonefish (*Carassius auratus gibellio*) (Jany, 1976). While this does not relate directly to larval fish nutrition, the common lack of functional stomach presents a similar digestion problem, and combined with the results of the present study, indicate that acidification of foods in the stomach may account for a significant amount of gastric digestion. This is supported by studies into digestion in higher vertebrates, with HCl, heat and pressure treatments all changing the secondary and tertiary structure of various proteins, thereby increasing subsequent protein digestibility *in vitro* (Venkatachalam and Sathe, 2003; Van der Plancken *et al.*, 2004). Therefore while the lack of a functional stomach in barramundi larvae (Walford and Lam, 1993) may not limit the digestion of live prey (Day *et al.*, 1997), the lack of an acid denaturing stage may limit digestion of processed protein sources.

HCl incubation was also found to partially hydrolyse plant protein (phaseolin) (Venkatachalam and Sathe, 2003), which may be responsible for the slight increase in soluble protein found in the acid denatured fish meal in the present study. This may be responsible for the decreased growth of larvae fed the diet containing 100% denatured fish meal in a similar way to that found in protein hydrolysates. There may also be

bioactive proteins or peptides in the untreated fish meal which are denatured in the acidification process, limiting its usefulness as a total fish meal substitute. Fish meal has also been found to be an effective feed stimulator (Kubitza and Lovshin, 1997), and the increased digestibility of denatured fish meal may be negated at high levels by decreased food ingestion if such attractants are affected by the denaturation process.

Reducing the pH of proteins changes their conformational state, either increasing or decreasing structural complexity, depending on the pH and the nature of the protein itself (Fink *et al.*, 1994). Therefore, the pH used for protein treatment is likely to influence the consequent effects on digestibility. Indeed, different patterns of protein denaturation have been shown to increase or decrease digestibility of protein in higher vertebrates, depending on the effect of the treatment on protein structure (e.g. Liu and Xiong, 2000). In the present study, the pH used to denature fish meal was selected on the basis of the stomach pH of juvenile barramundi (Walford and Lam, 1993), and was therefore thought to be representative of optimal digestive processes. These conditions are thought to ‘unravel’ the three-dimensional structure of the fish meal used, thus subjecting more activation sites to proteolytic enzyme activity. Further study into the conformational changes of such proteins under different pH treatments may further refine the optimal pH and time required to promote maximum protein digestibility for fish larvae.

The results of this experiment are consistent with the function of the developing digestive system. As live prey typically contains large amounts of free amino acids (Dabrowski and Rusiecki, 1983), and are thought to undergo significant autolysis (Kolkovski, 2001), the digestive functions of the stomach are not necessary for efficient digestion of live prey in early stages of marine fish larvae. However, incorporation of processed protein

sources into formulated microdiets removes the autolytic nature of natural feeds and high leaching rates in microbound diets negate the incorporation of low molecular weight water-soluble molecules such as free amino acids (Chapter 6). It has long been believed that the pepsin digestion and acid denaturing activity within the stomach is integral to the digestion of fish (Rønnestad *et al.*, 1999) however, recent evidence indicates that gastric pepsin levels have a limited effect on digestibility (Venkatachalam and Sathe, 2003) and larval growth (Zambonino Infante and Cahu, 1994a). The acid denaturing process used in this study is therefore thought to mimic the functional digestive processes of the stomach, preparing fish meal for effective hydrolysis by endogenous pancreatic proteases, followed by intracellular digestion.

## Chapter 8

### General Discussion

Barramundi larvae begin to develop significant levels of pepsin at approximately 10 DAH, but levels do not peak until approximately 21 DAH. This developmental trend corresponds to previously reported trends in stomach formation for this species (Walford and Lam (1993), beginning 11 DAH and full acidification achieved at 22 DAH in fed larvae. However, Walford and Lam (1993) reported an increasing pepsin level until at least 30 DAH, and did not report the plateau in pepsin level that was found in the present experiment. As the present experiment sampled larvae before food was administered each day, the data represent the basal pepsin level for barramundi larvae, in the absence of any live food contribution or stimulation. Since Walford and Lam (1993) found an influence of feeding on stomach pH, it is also likely that pepsin levels are affected by food ingestion, and this should be evaluated in further studies. In view of these findings, the nutritional studies reported in this study began during the stomach formation stage of larval development, and continued until after the stomach was fully functional. It is therefore considered that the alkaline digestibility of dietary protein is critical at least in the early stages of the experimental period. Alkaline digestibility was therefore used as a measure of the quality of the protein source.

Prior to the evaluation of various protein sources for formulated diets for barramundi larvae, however, a basal diet had to be formulated for this species, with macronutrient (protein and energy) levels which support significant growth and survival. The relatively high growth rate of marine fish larvae would logically prescribe a high amino acid requirement (Rønnestad *et al.*, 2003), and concomitant high dietary protein inclusion

levels. Contrary to this assumption, growth and survival indices used in the present study to evaluate the performance of barramundi larvae fed isocaloric food formulations with variable protein levels (45-55%) did not indicate any statistically significant differences. However, at a dietary energy level of 18 MJ.kg<sup>-1</sup>, there was a suppressed T4 level for those larvae fed diets containing 45% protein, when compared to higher dietary protein levels. Thyroid hormones (T3 and T4) have a strong association with growth and development in fish larvae (Lam *et al.*, 1985; Ayson and Lam, 1993a; Soffientino and Specker, 2001) and juveniles (Nacario, 1983; Gelineau *et al.*, 1996; Boeuf *et al.*, 1999), and their levels have been linked to endocrinal mediation of nutritional growth control (Higgs *et al.*, 1992; Nankervis *et al.*, 2000; Gaylord *et al.*, 2001). It is therefore likely that T4 suppression indicates a decreased potential for growth, which may become evident in measured growth indices in longer-term studies. On this basis, it is considered that the minimum protein level for optimal growth in barramundi larvae fed formulated microbound diets is 50%.

There was a strong association between dietary energy level and growth, with 21 MJ.kg<sup>-1</sup> dietary utilisable energy being the minimum level for optimal growth in barramundi larvae fed microbound diets. The effect of dietary energy on growth may be indicative of fatty acid requirements, indeed highly unsaturated fatty acid levels have been consistently shown to influence growth and survival in fish larvae (Rodriguez *et al.*, 1994; Salhi *et al.*, 1994; Buchet *et al.*, 2000). However there was consistently higher growth found at higher dietary energy levels irrespective of lipid inclusion level, and dietary energy was therefore considered to have nutritional significance.

The optimal source of dietary protein had a far greater affect on growth and survival than protein inclusion level. From the untreated proteins studied (fish meal, squid powder, mussel meal, prawn meal, decapsulated *Artemia* cyst meal and krill meal), formulated foods containing fish meal and squid powder, at a 9:1 ratio, stimulated the best growth and survival in barramundi larvae. The essential amino acid index (EAAI) of the protein sources used in this experiment indicates that fishmeal and squid powder are complementary sources of essential amino acids, each supplementing the first limiting amino acid of the other.

Digestibility analyses indicated that prawn meal and squid powder are highly digestible protein sources for barramundi larvae, while fish meal is slightly less digestible, and mussel and krill meals are relatively poorly digestible. High levels of soluble proteins found in mussel and krill meals indicate autoproteolysis at some stage in their preparation, as has previously been reported for krill meal (Ellingsen and Mohr, 1987). Leaching of these soluble proteins from the microbound diets formulated in this study is thought to reduce the nutritional value of these animal meals. The proteins that remain were also found to be less digestible, further reducing the nutritional value of krill and mussel meals.

The inclusion of pre-digested (pepsin-hydrolysed) fish meal in formulated microbound diets also supported decreased growth and lower pepsin levels in barramundi larvae, compared to fish fed diets containing intact fishmeal. Hydrolysis of fish meal liberated significant amounts of soluble proteins, which have a low water-stability in microbound diets, limiting the nutritional value of this protein source in a similar way to the autoproteolysis of krill and mussel meals. This is consistent with growth data for



barramundi larvae fed microbound diets containing graded levels of hydrolysates. The reduced pepsin level found in fish fed hydrolysed fish meal is thought to be due to reduced pepsin substrate availability of pre-digested proteins and thus reduced pepsin secretion by the larvae.

The inclusion of 25-50% of the total fish meal component of microbound diets in an acid-denatured form increased growth of barramundi larvae, which is attributed to an approximately 2-fold higher alkaline digestibility than intact fish meal. Denatured fish meal did have a higher level of soluble protein (5.24%) than untreated fish meal (2.89%), though this was far less than hydrolysed fish meal (14.26 – 35.68%). There was a decreased dry weight gain in larvae fed formulations containing 100% of the fish meal component in a denatured state, the reason for which is difficult to elucidate through this study.

### **8.1. Conclusion**

The present study developed a microbound diet which supported up to 58% survival and significant growth in barramundi larvae from, 14-28 DAH. The diet which supported the best growth and survival contained 21 MJ.kg<sup>-1</sup> utilisable dietary energy, and at least 50% dietary protein, comprised of a 9:1 ratio of fish meal to squid powder.

It is anticipated that development of co-feeding and weaning regimes with the diet developed in this study will further improve the growth and survival values presently recorded. It is anticipated that this will directly translate to significant savings in live food costs and time for barramundi culture, as has been found for European seabass (Person Le Ruyet *et al.*, 1993).

Digestibility with both homologous barramundi proteolytic enzymes and commercial preparations were found to be a useful tool in the determination of useful sources of dietary protein for formulated diets. The application of digestibility analysis has led to the discovery that fish meal digestibility is improved by acid treatment, and formulated foods for barramundi larvae containing 11-22% denatured fish meal significantly increased growth compared to larvae fed formulations without denatured fish meal.

While highly digestible food ingredients increased growth significantly, larvae fed diets containing protein hydrolysates or autolysates had decreased growth, due to the leaching of water-soluble peptides from the food particle prior to ingestion. Future studies into hydrolysis of dietary protein for inclusion into larval foods should consider leaching as a major influence on the utilisation of hydrolysates, and the development of a highly digestible microencapsulated diet may allow a better understanding of the dietary effects of hydrolysates on barramundi larvae.

Thyroxine levels were generally found to correlate well with growth in barramundi larvae rather than any particular nutritional component. It is clear that carcass T4 level is increased by improved nutrition, however, the lack of a clear link to nutritional manipulations indicates that it is indirectly stimulated by nutrition rather than directly mediating nutritional control of growth.

## References

- Alacorn, F. J., Moyano, F. J. and Diaz, M. (2001). Use of SDS-page in the assessment of protein hydrolysis by fish digestive enzymes. *Aquacult. Int.* 9(255–267,).
- Alacorn, F. J., Moyano, F. J., Diaz, M., Fernandez-Diaz, C. and Yufera, M. (1999). Optimization of the protein fraction of microcapsules used in feeding of marine fish larvae using in vitro digestibility techniques. *Aquacult. Nutr.* 5(2): 107-113.
- Anderson, J. S., Lall, S. P., Anderson, D. M. and McNiven, M. A. (1993). Evaluation of protein quality in fish meals by chemical and biological assays. *Aquaculture* 115: 305-325.
- Anson, M. L. (1938). The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. *J. Gen. Physiol.* 22: 79-89.
- Appelbaum, S. (1985). Rearing of the dover sole, *Solea solea* (l), through its larval stages using artificial diets. *Aquaculture* 49( 3-4): 209-221.
- Applebaum, S. L. and Rønnestad, I. (2004). Absorption, assimilation and catabolism of individual free amino acids by larval Atlantic halibut (*Hippoglossus hippoglossus*). *Aquaculture* 230: 313–322.
- Ayson, F. and Lam, T. (1993a). Thyroxine injection of female rabbitfish (*Siganus guttatus*) broodstock: Changes in thyroid hormone levels in plasma, eggs, and yolk-sac larvae, and its effect on larval growth and survival. *Aquaculture* 109(1): 83-93.
- Ayson, F. and Lam, T. J. (1993b). Thyroxine injection of female rabbitfish (*Siganus guttatus*) broodstock: changes in thyroid hormone levels in plasma, eggs, and yolk-sac larvae, and its effect on larval growth and survival. *Aquaculture* 109: 83-93.
- Baethgen, W. E. and Alley, M. M. (1989). A manual for the colorimetric procedure for measuring ammonium nitrogen in soil and plant Kjeldahl digests. *Commun. Soil Sci. Plan.* 20: 961-969.
- Baskerville-Bridges, B. and Kling, L. (2000). Development and evaluation of microparticulate diets for early weaning of Atlantic cod *Gadus morhua* larvae. *Aquacult. Nutr.* 6(3): 171-182.
- Bengston, D. A., Slimlick, T. L., Binette, E. W., Lovett IV, R. R., Alves, D., Schreiber, A. M. and Specker, J. L. (2000). Survival of larval summer founder *Paralichthys dentatus* on formulated diets and failure of thyroid hormone treatment to improve performance. *Aquacult. Nutr.* 6: 193-198.
- Blair, T., Castell, J., Neil, S., D'Abramo, L., Cahu, C., Harmon, P. and Ogunmoye, K. (2003). Evaluation of microdiets versus live feeds on growth, survival and fatty

- acid composition of larval haddock (*Melanogrammus aeglefinus*). *Aquaculture* 225: 451-461.
- Blow, D. M. (1971). The structure of chymotrypsin. *The Enzymes*. Boyer, P. D. LA, Academic Press. **III** pp. 185-212.
- Boeuf, G., Boujard, D. and Person-Le ruyet, J. (1999). Control of the somatic growth in turbot. *J. Fish Biol.* 55(A): 128-147.
- Booth, M. A., Warner-Smith, R. J., Allan, G. L. and Glencross, B. D. (2004). Effects of dietary astaxanthin source and light manipulation on the skin colour of Australia snapper *Pagrus auratus* (Bloch & Schneider, 1801). *Aquaculture Res.* 35(5): 458-464.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.
- Brown, C. L. and Kim, B. G. (1995). Combined application of cortisol and triiodothyronine in the culture of larval marine finfish. *Aquaculture* 135(1-3): 79-86.
- Brown, C. L., S. I. Doroshov, J. M. Nunez, C. Hadley, J. Vaneennaam, R. S. Nishioka and H. A. Bern (1998) Maternal triiodothyronine injections cause increases in swimbladder inflation and survival rates in larval striped bass, *Morone saxatilis*. *J. Exp.Zool.*248(2):168-176
- Buchet, V., Zambonino Infante, J. L. and Cahu, C. (2000). Effect of lipid level in a compound diet on the development of red drum (*Sciaenops acellatus*) larvae. *Aquaculture* 184: 339-347.
- Cahu, C. and Zambonino Infante, J. L. (1995). Maturation of the pancreatic and intestinal functions in sea bass (*Dicentrarchus labrax*): effect of weaning with different protein sources. *Fish Physiol. Biochem.* 14: 431-437.
- Cahu, C. and Zambonino-Infante, J. (2001). Substitution of live food by formulated diets in marine fish larvae. *Aquaculture* 200: 161–180.
- Cahu, C., Zambonino-Infante, J., Quazuguel, P. and Le Gall, M. (1999). Protein hydrolysate vs. fish meal in compound diets for 10-day old sea bass *Dicentrarchus labrax* larvae. *Aquaculture* 171(1-2): 109-119.
- Cahu, C. L., Zambonino Infante, J. L. and Barbosa, V. (2003). Effect of dietary phospholipid level and phospholipid:neutral lipid value on the development of seabass (*Dicentrarchus labrax*) larvae fed a compound diet. *Br. J. Nutr.* 90(21-28).
- Cahu, C. L., Zambonino Infante, J. L., Escaffre, A. M., Bergot, P. and Kaushik, S. (1998). Preliminary results on sea bass (*Dicentrarchus labrax*) larvae rearing with compound diet from first feeding. Comparison with carp (*Cyprinus carpio*) larvae. *Aquaculture* 169: 1-7.

- Camargo, J. A. (2003). Fluoride toxicity to aquatic organisms: a review. *Chemosphere* 50(3): 251-264.
- Carvalho, A. P., Escaffre, A.-M., Teles, A. O. and Bergot, P. (1997). First feeding of common carp larvae on diets with high levels of protein hydrolysates. *Aquacult. Int.* 5: 361–367.
- Chatain, B. and Ounais-Guschemann, N. (1990). Improved rate of initial swim bladder inflation in intensively reared *Sparus auratus*. *Aquaculture* 84: 345-353.
- Cho, S. H., Hur, S. B. and Jo, J.-Y. (2001). Effect of enriched live feeds on survival and growth rates in Korean rockfish, *Sebastes schlegeli* Hilgendorf. *Aquacult. Nutr.* 32: 199-208.
- Chong, A., Hashim, R., Lee, L.-C. and bin Ali, A. (2002). Characterisation of protease activity in developing discus *Symphysodon aequifasciata* larva. *Aquacult. Res.* 33: 663-672.
- Conceicao, L. E. C., Grasdalen, H. and Rønnestad, I. (2003). Amino acid requirements of fish larvae and post-larvae: new tools and recent findings. *Aquaculture* 227: 221–232.
- Conceicao, L. E. C., Ozorio, R. O. A., Suurd, E. A. and Verreth, J. A. J. (1998). Amino acid profiles and amino acid utilization in larval African catfish (*Clarias gariepinus*): effects of ontogeny and temperature. *Fish Physiol. Biochem.* 19: 43–57.
- Cordova-Murueta, J. H. and Garcia-Carreno, F. L. (2002). Nutritive value of squid and hydrolyzed protein supplement in shrimp feed. *Aquaculture* 210: 371–384.
- Cowey, C. B. (1994). Amino acid requirements of fish: a critical appraisal of present values. *Aquaculture* 124: 1-11.
- Dabrowski, K. (1984). The feeding of fish larvae: Present <<state of the art>> and perspectives. *Reprod. Nutr. Develop.* 24(6): 807-833.
- Dabrowski, K. (1986). Ontogenetical aspects of nutritional requirements in fish. *Comp. Biochem. Physiol.* 85A: 639-655.
- Dabrowski, K. and Glogowski, J. (1977). A study of the application of proteolytic enzymes to fish food. *Aquaculture* 12: 349-360.
- Dabrowski, K., Lee, K.-J. and Rinchar, J. (2003). The Smallest Vertebrate, Teleost Fish, Can Utilize Synthetic Dipeptide-Based Diets. *133(12): 4225-4229.*
- Dabrowski, K. and Rusiecki, M. (1983). Content of total and free amino acids in zooplanktonic food of fish larvae. *Aquaculture* 30: 31-42.

- Day, O. J., Howell, B. R. and Jones, D. A. (1997). The effect of dietary hydrolysed fish protein concentrate on the survival and growth of juvenile Dover sole, *Solea solea* (L.), during and after weaning. *Aquacult. Res.* 28(12): 911-921.
- DeSilva, S. and Anderson, T. A. (1995). *Fish nutrition in aquaculture*. London, Chapman & Hall.
- Dhert, P., Rombaut, G., Suantika, G. and Sorgeloos, P. (2001). Advancement of rotifer culture and manipulation techniques in Europe. *Aquaculture* 200: 129–146.
- Dosanjh, B. S., Higgs, D. A., McKenzie, D. J., Randall, D. J., Eales, J. G., Roeshandeli, N., Roeshandeli, M. and Deacon, G. (1998). Influence of dietary blends of menhaden oil and canola oil on growth, muscle lipid composition, and thyroidal status of Atlantic salmon (*Salmo salar*) in sea water. *Fish Physiol. Biochem.* 19: 123-134.
- Douglas, S. E., Gawlicka, A., Mandla, S. and Gallant, J. W. (1999). Ontogeny of the stomach of winter flounder: characterisation and expression of the pepsinogen and proton pump genes and determination of pepsin activity. *J. Fish Biol.* 55: 897-915.
- Eales, J. G. (1985). The peripheral metabolism of thyroid hormones and regulation of thyroidal status in poikilotherms. *Can. J. Zool.* 63: 1217-1231.
- Eales, J. G. (1995). Regulation and measurement of thyroidal status in fish. *Neth. J. Zool.* 45(1-2): 175-180.
- Eales, J. G., Higgs, D. A., Uin, L. A., MacLatchy, D. L., Bress, O., McBride, J. R. and Dosanjh, B. S. (1990). Influence of dietary lipid and carbohydrate levels on chronic 3, 5, 3'-triiodothyronine treatment on thyroid function in immature rainbow trout, *Oncorhynchus mykiss*. *Gen. Comp. Endocrin.* 80: 146-155.
- Eales, J. G., MacLatchy, D. L., Higgs, D. A. and Dosanjh, B. S. (1992). The influence of dietary protein and caloric content on thyroid function and hepatic thyroxine 5'-monodeiodinase activity in rainbow trout, *Oncorhynchus mykiss*. *Can. J. Zool.* 70(8): 1526-1535.
- Eales, J. G. and Shostak, S. (1985). Correlations between food ration, somatic growth parameters and thyroid function in Arctic charr, *Salvelinus alpinus* L. *Comp. Biochem. Physiol.* 80A(4): 553-558.
- Eggum, B. O., Beames, R. M. and Knudsen, K. E. (1985). The effect of provision of the first-limiting amino acid, gastrointestinal microbial activity and the level of nitrogen intake on protein utilization and energy digestibility in rats. *Br J Nutr.* 54(3): 727-739.
- Eguia, R. V., Kamarudin, M. S. and Santiago, C. B. (2000). Growth and survival of river catfish *Mystus nemurus* (Cuvier & Valenciennes) larvae fed isocaloric diets with different protein levels during weaning. *J. Appl. Ichthyol.* 16: 104-109.

- Eid, A. E. and Matty, A. J. (1989). A simple *in vitro* method for measuring protein digestibility. *Aquaculture* 79: 111-119.
- Ellingsen, T. E. and Mohr, V. (1987). Biochemistry of the autolytic processes in Antarctic krill *post mortem*. *Biochem. J.* 246: 295-305.
- El-Zibdeh, M. K., Tachihara, K., Tsukashima, Y., Tagawa, M. and Ishimatsu, A. (1996). Effect of thyronine injection of broodstock fish on seed production in cultured seawater fish. *Suisanzoshoku* 1996-H8: 487-496.
- Evjemo, J. O., Reitan, K. I. and Olsen, Y. (2003). Copepods as live food organisms in the larval rearing of halibut larvae (*Hippoglossus hippoglossus* L.) with special emphasis on the nutritional value. *Aquaculture* 227: 191–210.
- Farbridge, K. J., Flett, P. A. and Leatherland, J. F. (1992). Temporal effects of restricted diet and compensatory increased dietary intake on thyroid function, plasma growth hormone levels and tissue lipid reserves of rainbow trout *Oncorhynchus mykiss*. *Aquaculture* 104: 157-174.
- Fernandez-Diaz, C., Pascual, E. and Yufera, A. P. M. (1994). Feeding behaviour and prey size selection of gilthead seabream, *Sparus aurata*, larvae fed on inert food and live food. *Mar. Biol.* 118: 323-328.
- Fink, A. L., Calciano, L. J., Goto, Y., Kurotsu, T. and Palleros, D. R. (1994). Classification of acid denaturation of proteins: Intermediates and unfolded states. *Biochemistry* 33: 12504-12511.
- Fontagne, S., Robin, J., Corraze, G. and Bergot, P. (2000). Growth and survival of European sea bass (*Dicentrarchus Labrax*) larvae fed from first feeding on compound diets containing medium-chain triacylglycerols. *Aquaculture* 190: 261-271.
- Fruton, J. S. (1971). Pepsin. *The Enzymes*. Boyer, P. D. LA, Academic Press. **III** pp. 120-164.
- Fuchs, J. and Nedelec, G. (1991). Larval rearing and weaning of of seabass, *Lates calcarifer* (Bloch), on experimental compound diets. France, IFREMER Actes de Colloque 9.
- Fyhn, H. and Serigstad, B. (1987). Free amino acids as energy substrate in developing eggs and larvae of the cod *Gadus morhua*. *Mar. Biol.* 96(3): 335-341.
- Fyhn, H. J. (1989). First feeding of marine fish larvae: Are free amino acids thee source of energy? *Aquaculture* 80: 111-120.
- Garcia-Ortega, A., Kaussoulaki, A., Boer, H. and Verreth, J. (2000a). *In vitro* protein digestibility of *Artemia* decapsulated cysts and nauplii, and of microbound diets for larval fish. *Aquacult. Res.* 31: 475-477.

- Garcia-Ortega, A., Verreth, J., Van Hoornycck, A. and Segner, H. (2000b). Heat treatment affects protein quality and protease activity in decapsulated cysts of *Artemia* when used as starter food for larvae of African catfish *Clarias gariepinus* (Burchell). *Aquacult. Nutr.* 6(1): 25-31.
- Gavlik, S., Albino, M. and Specker, J. L. (2002). Metamorphosis in summer flounder: manipulation of thyroid status to synchronize settling behavior, growth, and development. *Aquaculture* 203: 359–373.
- Gaylord, T. G., MacKenzie, D. S. and D.M. Gatlin, I. (2001). Growth performance, body composition and plasma thyroid hormone status of channel catfish (*Ictalurus punctatus*) in response to short-term feed deprivation and refeeding. *Fish Physiol. Biochem.* 24: 73–79.
- Geiger, J. G. (1983). A review of pond zooplankton production and fertilisation for the culture of larval and fingerling striped bass. *Aquaculture* 35: 353-369.
- Gelineau, A., Mambrini, M., Leatherland, J. F. and Boujard, T. (1996). Effect of feeding time on hepatic nucleic acid, plasma T3, T4 and GH concentrations in rainbow trout. *Physiol. Behav.* 59(6): 1061-1067.
- Gisbert, E., Williot, P. and -Orvay, F. C. (2002). Fully vitellogenic oocyte amino acid profile of Siberian sturgeon (*Acipenser baerii*, Brandt). *J. Appl. Ichthyol.* 18: 347–350.
- Grabner, M. and Hofer, R. (1989). Stomach digestion and its effect on protein hydrolysis in the intestine of rainbow trout (*Salmo gairdneri* Richardson). *Comp. Biochem. Physiol.* 92A(1): 81-83.
- Greenberg, N. A. and Shipe, W. F. (1979). Comparison of the abilities of trichloroacetic, picric, sulfosalicylic, and tungstic acids to precipitate protein hydrolysates and proteins. *J. Food Sci.* 44: 735-737.
- Grey, D. L. (1987). An overview of *Lates calcarifer* in Australia and Asia. Management of wild and cultured sea bass/barramundi (*Lates calcarifer*). Copland, J. W. and Grey, D. L. Canberra, Australian Centre for International Agricultural Research Proceedings. **20**.
- Guillaume, J. and Choubert, G. (1999). Digestive physiology and nutrient digestibility in fishes. *Nutrition and Feeding of Fish and Crustaceans*. Guillaume, J., Kaushik, S., Bergot, P. and Metailler, R. Chichester, UK, Springer-Praxis pp. 81-109.
- Harel, M., Koven, W., Lein, I., Bar, Y., Behrens, P., Stubblefield, J., Zohar, Y. and Place, A. R. (2002). Advanced DHA, EPA and ArA enrichment materials for marine aquaculture using single cell heterotrophs. *Aquaculture* 213(1-4): 347-362.
- Helland, S., Triantaphyllidis, G. V., Fyhn, H. J., Evjen, M. S., Lavens, P. and Sorgeloos, P. (2000). Modulation of the free amino acid pool and protein content in populations of the brine shrimp *Artemia spp.* *Mar. Biol.* 137(5-6): 1005-1016.



- Hey, J., Farrar, E., Bristow, B., Stettner, C. and Summerfelt, R. (1996). Thyroid hormones and their influences on larval performance and incidence of cannibalism in walleye *Stizostedion vitreum*. J. World Aquacult. Soc. 27(1): 40-51.
- Higgs, D. A., Dosanjh, B. S., Uin, L. A., Himick, B. A. and Eales, J. G. (1992). Effects of dietary lipid and carbohydrate levels and chronic 3,5,3'-triiodo-L-thyronine treatment on growth, appetite, food and protein utilisation and body composition of immature rainbow trout, *Oncorhynchus mykiss*, at low temperature. Aquaculture 105: 175-190.
- Higgs, D. A., Fagerlund, U. H. M., Eales, J. G. and McBride, J. R. (1982). Application of thyroid and steroid hormones as anabolic agents in fish culture. Comp. Biochem. Physiol. 73B(1): 143-176.
- Himick, B. A., Higgs, D. A. and Eales, J. G. (1991). The acute effects of alteration in the dietary concentrations of carbohydrate, protein, and lipid on plasma T<sub>4</sub>, T<sub>3</sub>, and glucose levels in rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrin. 82(3): 451-458.
- Huang, L., Miwa, S., Bengston, D. A. and Specker, J. L. (1998a). Effect of Triiodothyronine on Stomach Formation and Pigmentation in Larval Striped Bass (*Morone saxatilis*). J. Exp. Zool. 280: 231-237.
- Huang, L., Schreiber, A. M., Soffientino, B., Bengston, D. A. and Specker, J. L. (1998b). Metamorphosis of Summer Flounder (*Paralichthys dentatus*): Thyroid Status and the Timing of Gastric Gland Formation. J. Exp. Zool. 280: 413-420.
- Hutchinson, M. J. and Iwata, M. (1998). Effect of thyroxine on the decrease of aggressive behaviour of four salmonids during the parr-smolt transformation. Aquaculture 168: 169-175.
- Inui, Y., Yamano, K. and Miwa, S. (1995). The role of thyroid hormone in tissue development in metamorphosing flounder. Aquaculture 135(1-3): 87-98.
- Jany, K.-D. (1976). Studies on the digestive enzymes of the stomachless bonefish *Crassius auratus gibelio* (Bloch): Endopeptidases. Comp. Biochem. Physiol. 53B: 31-38.
- Jones, D., Kamarudin, M. and Le Vay, L. (1993). The potential for replacement of live feeds in larval culture. J. World Aquacult. Soc. 24(2): 199-210.
- Julshamn, K., Malde, M. K., Bjorvatin, K. and Krogedal, P. (2004). Flouride retention of Atlantic salmon (*Salmo salar*) fed krill meal. Aquacult. Nutr. 10: 9-13.
- Ketola, H. G. (1982). Amino acid nutrition of fishes: Requirements and supplementation of diets. Comp. Biochem. Physiol. 73B: 17-24.
- Khan, A. R. and James, M. N. G. (1998). Molecular mechanisms for the conversion of zymogens to active proteolytic enzymes. Protein Sci 7(4): 815-836.

- Kiel, B. (1971). Trypsin. The Enzymes. Boyer, P. D. LA, Academic Press. **III** pp. 250-277.
- Kim, B. G. and Brown, C. L. (1997). Interaction of cortisol and thyroid hormone in the larval development of Pacific threadfin. *Am. Zool.* 37: 470-481.
- Kolkovski, S. (2001). Digestive enzymes in fish larvae and juveniles--implications and applications to formulated diets. *Aquaculture* 200(1-2): 181-201.
- Kolkovski, S., Arieli, A. and Tandler, A. (1997a). Visual and chemical cues stimulate microdiet ingestion in sea bream larvae. *Aquacult. Int.* 5: 527-536.
- Kolkovski, S. and Tandler, A. (2000). The use of squid protein hydrolysate as a protein source in microdiets for gilthead seabream *Sparus aurata* larvae. *Aquacult. Nutr.* 6(1): 11-15.
- Kolkovski, S., Tandler, A. and Izquierdo, M. S. (1997b). The effect of live food and dietary digestive enzymes on the efficiency of microdiets for seabass (*Dicentrarchus labrax*) larvae. *Aquaculture* 148: 313-322.
- Kolkovski, S., Tandler, A., Kissil, G. W. and Gertler, A. (1993). The effect of dietary exogenous digestive enzymes on ingestion, assimilation, growth and survival of gilthead seabream (*Sparus aurata*, Sparidae, Linnaeus) larvae. *Fish Physiol. Biochem.* 12(3): 203-209.
- Kolkovski, S., Yackey, C., Czesney, S. and Dabrowski, K. (2000). The effect of microdiet supplementation of dietary digestive enzymes and a hormone on growth and enzyme activity in yellow perch juveniles. *N. Am. J. Aquacult.* 62: 130-134.
- Koven, W., Kolkovski, S., Hadas, E., Gamsiz, K. and Tandler, A. (2001). Advances in the development of microdiets for gilthead seabream, *Sparus aurata*: a review. *Aquaculture* 194: 107-121.
- Koven, W., Rojas-Garcia, C. R., Finn, R. N., Tandler, A. and Rønnestad, I. (2002). Stimulatory effect of ingested protein and/or free amino acids on the secretion of the gastro-endocrine hormone cholecystokinin and on tryptic activity, in early-feeding herring larvae, *Clupea harengus*. *Mar. Biol.* 140: 1241-1247.
- Kubitza, F. and Lovshin, L. L. (1997). The use of freeze-dried krill to train largemouth bass (*Micropterus salmoides*): feeds and training strategies. *Aquaculture* 148: 299-312.
- Kurokawa, T., Shiraishi, M. and Suzuki, T. (1998). Quantification of exogenous protease derived from zooplankton in the intestine of Japanese sardine (*Sardinops melanotictus*) larvae. *Aquaculture* 161: 491-499.
- Kvale, A., Harboe, T., Espe, M., Naess, T. and Hamre, K. (2002). Effect of predigested protein on growth and survival of Atlantic halibut larvae (*Hippoglossus hippoglossus* L.). *Aquacult. Res.* 33: 311-321.

- Lam, T. (1991). Hormones and egg/larval quality in fish. *J. World Aquacult. Soc.* 25(1): 2-12.
- Lam, T. J. (1980). Thyroxine enhances larval development and survival in *Sarotherodon (Tilapia) mossambicus* Ruppell. *Aquaculture* 21(287-291).
- Lam, T. J., Juario, J. V. and Banno, J. (1985). Effect of thyroxine on growth and development in post-yolk-sac larvae of milkfish, *Chanos chanos*. *Aquaculture* 46: 179-184.
- Lam, T. J. and Sharma, R. (1985). Effects of salinity and thyroxine on larval survival, growth and development in the carp, *Cyprinus carpio*. *Aquaculture* 44: 201-212.
- Lauff, M. and Hofer, R. (1984). Proteolytic enzymes in fish development and the importance of dietary enzymes. *Aquaculture* 37: 335-346.
- Leatherland, J. F., Cho, C. Y. and Slinger, S. J. (1977). Effects of diet, ambient temperature and holding conditions on plasma thyroxine levels in rainbow trout, (*Salmo gairdneri*). *J. Fish. Res. Board Can.* 34: 677-682.
- Lee, C.-S. (2003). Biotechnological advances in finfish hatchery production: a review. *Aquaculture* 227: 439-458.
- Lee, P., Southgate, P. and Fielder, D. (1996). Assessment of two microbound artificial diets for weaning Asian sea bass (*Lates calcarifer*, Bloch). *Asian Fish. Sci.* 9(2): 115-120.
- Lee, S., Jeon, I. G. and Lee, J. Y. (2002). Effects of digestible protein and lipid levels in practical diets on growth, protein utilization and body composition of juvenile rockfish (*Sebastes schlegeli*). *Aquaculture* 211(1-4): 227-239.
- Lemm, C. A. and Lemarie, D. P. (1991). Survival and growth of larval striped bass (*Morone saxatilis*) fed *Artemia* enriched with highly unsaturated fatty acids (HUFA). *Aquaculture* 99: 117-126.
- Lidner, P., Eshel, A., Kolkovski, S., Tandler, A. and Harpaz, S. (1995). Proteolysis by juvenile sea bass (*Dicentrarchus labrax*) gastrointestinal enzymes as a method for the evaluation of feed proteins. *Fish Physiol. Biochem.* 14(5): 399-407.
- Liu, G. and Xiong, Y. L. (2000). Electrophoretic pattern, thermal denaturation, and in vitro digestibility of oxidised myosin. *J. Agric. Food Chem.* 48: 624-630.
- Liu, Y. W. and W. K. Chan (2002). Thyroid hormones are important for embryonic to larval transitory phase in zebrafish. *Differentiation* 70(1): 36-45.
- López-Alvarado, J., Langdon, C. J., Teshima, S. and Kanazawa, A. (1994). Effects of coating and encapsulation of crystalline amino acids on leaching in larval feeds. *Aquaculture* 122: 335-346.

- Lubzens, E., Tandler, A. and Minkoff, G. (1989). Rotifers as food in aquaculture. *Hydrobiologia* 186/187: 387-400.
- MacKenzie, D. S., VanPutte, C. M. and Leiner, K. A. (1998). Nutrient regulation of endocrine function in fish. *Aquaculture* 161: 3-25.
- Mackinnon, M. R. (1987). Rearing and growth of larval and juvenile barramundi (*Lates calcarifer*). Management of wild and cultured seabass/barramundi (*Lates calcarifer*). ACIAR Proceedings.
- MacNiven, A. M. and Little, D. C. (2001). Development and evaluation of a stress challenge testine methodology for assessment of Nile tilapia (*Oreochromis niloticus* Linn.) fry quality. *Aquaculture Research* 32: 671-679.
- Mambrini, M. and Guillaume, J. (1999). Protein Nutrition. *Nutrition and Feeding of Fish and Crustaceans*. Guillaume, J., Kaushik, S., Bergot, P. and Metailler, R. Chichester, UK, Springer-Praxis pp. 81-109.
- Meyer, G. and Fracalossi, D. M. (2004). Protein requirement of jundia fingerlings, *Rhamdia quelen*, at two dietary energy concentrations. *Aquaculture* 240(1-4): 331-343.
- Mihelakakis, A., Yoshimatsu, T. and Tsoikas, C. (2001). Spawning in captivity and early life history of cultured red porgy, *Pagrus pagrus*. *Aquaculture* 199(3-4): 333-352.
- Moore, R. Spawning and early life history of barramundi, *Lates calcarifer* (Bloch), in Papua New Guinea. *Aust. J. Mar. Freshw. Res.* 33: 647-661
- Moullac, G. L., Goyard, E., Saulnier, D., Haffner, P., Thouard, E., Nedelec, G., Goguenheim, J., Rouxel, C., Cuzon, G. and Aquacop (2003). Recent improvements in broodstock management and larviculture in marine species in Polynesia and New Caledonia: genetic and health approaches. *Aquaculture* 227: 89-106.
- Murillo-Gurrea, D. P., Coloso, R. M., G., B. and Serrano, J. A. E. (2001). Lysine and arginine requirements of juvenile Asian sea bass (*Lates calcarifer*). *J. Appl. Ichthyol.* 17: 49-53.
- Nacario, J. F. (1983). The effect of thyroxine on the larvae and fry of *Sarotherodon niloticus* L. (*Tilapia nilotica*). *Aquaculture* 34: 73-83.
- Nankervis, L., Matthews, S. and Appleford, P. (2000). Effect of dietary non-protein energy source on growth, nutrient retention and circulating insulin-like growth factor I and triiodothyronine levels in juvenile barramundi, *Lates calcarifer*. *Aquaculture* 191(4): 323-335.
- Nayak, P., Mishra, T. and Mishra, J. (2000). Combined application of cortisol and thyroxin in the culture of larvae of freshwater catfish, *Heteropneustes fossilis*. *J. Aquacult. Trop.* 15(4): 323-328.

- Nugegoda, D., Walford, J. and Lam, T. (1994). Thyroid hormones in the early development of seabass (*Lates calcarifer*) larvae. *J. Aquacult. Trop.* 9(4): 279-290.
- Partridge, G. and Southgate, P. (1999). The effect of binder composition on ingestion and assimilation of microbound diets (MBD) by barramundi *Lates calcarifer* Bloch larvae. *Aquacult. Res.* 30(11-12): 879-886.
- Partridge, G. J. (1996). The development of a species-specific weaning diet for Barramundi (*Lates calcarifer*, Bloch) larvae. Dept. of Marine Biology and Aquaculture. Townsville, James Cook University.
- Penaflorida, V. D. (1989). An evaluation of indigenous protein sources as potential component in the diet formulation for tiger prawn, *Penaeus monodon*, using essential amino acid index (EAAI). *Aquaculture* 83: 319-330.
- Péres, A., Cahu, C. L., Zambonino Infante, J. L., Le Gall, M. M. and Quazuguel, P. (1996). Amylase and trypsin responses to intake of dietary carbohydrate and protein depend on the developmental stage in sea bass (*Dicentrarchus labrax*) larvae. *Fish Physiol. Biochem.* 15(3): 237-242.
- Person Le Ruyet, J., Alexandre, J. C., Thébaud, L. and Mugnier, C. (1993). Marine fish larvae feeding: Formulated diets or live prey? *J. World Aquacult. Soc.* 24: 211-224.
- Plohman, J. C., Dick, T. A. and Eales, J. G. (2002). Thyroid of lake sturgeon, *Acipenser fluvescens* II. Deiodination properties, distribution, and effects of diet, growth and a T3 challenge. *Gen. Comp. Endocrin.* 125: 56-66.
- Rimmer, M. A., Reed, A. W., Levitt, M. S. and Lisle, A. T. (1994). Effects of nutritional enhancement of live food organisms on growth and survival of barramundi, *Lates calcarifer* (Bloch), larvae. *Aquacult. Fish. Man.* 25: 143-156.
- Rimmer, M. A. and Russel, D. J. (1998). Aspects of the biology and culture of *Lates calcarifer*, Academic Press.
- Ringø, E., Olsen, R. E., Mayhew, T. M. and Myklebust, R. (2003). Electron microscopy of the intestinal microflora of fish. *Aquaculture* 227: 395-415.
- Ritar, A. J., G. A. Dunstan, M. M. Nelson, M. R. Brown, P. D. Nichols, C. W. Thomas, E. G. Smith, B. J. Crear and S. Kolkovski (2004). Nutritional and bacterial profiles of juvenile *Artemia* fed different enrichments and during starvation. *Aquaculture* 239(1-4): 351-373.
- Rodgers, L. J. and Barlow, C. G. (1987). Better nutrition enhances survival of barramundi larvae. *Aust. Fish.* July 1987(46): 30-32.
- Rodriguez, C., Perez, J. A., Izquierdo, M. S., Lorenzo, A. and Palacios, H. F. (1994). The effect of n-3 HUFA proportions in diets for gilthead seabream (*Sparus aurata*) larval culture (abstract). *Aquaculture* 124: 284.

- Rolando, G. F. (2003). Bioencapsulation in *Artemia*: II. Influences of the particle concentration in the enrichment process. *Aquaculture* 216(1-4): 143-153.
- Rønnestad, I., Conceicao, L. E. C., Aragao, C. and Dinis, M. T. (2000). Free amino acids are absorbed faster and assimilated more efficiently than protein in postlarval Senegal sole (*Solea senegalensis*). *J. Nutr.* 130(11): 2809-2812.
- Rønnestad, I., Thorsen, A. and Finn, R. N. (1999). Fish larval nutrition: a review of recent advances in the roles of amino acids. *Aquaculture* 177: 201– 216.
- Rønnestad, I., Tonheim, S. K., Fyhn, H. J., Rojas-Garcia, C. R., Kamisaka, Y., Koven, W., Finn, R. N., Terjesen, B. F., Barr, Y. and Conceicao, L. E. C. (2003). The supply of amino acids during early feeding stages of marine fish larvae: a review of recent findings. *Aquaculture* 227: 147–164.
- Russell, D. J., O'Brien, J. J. and Longhurst, C. (1987). Barramundi egg and larval culture. *Aust. Fish.* July, 1987: 26-29.
- Salhi, M., Izquierdo, M. S., Hernbdez-Cruz, C. M., Gonzalez, M. and Fernbdez-Palacios, H. (1994). Effect of lipid and n-3 HUFA levels in microdiets on growth, survival and fatty acid composition of larval gilthead seabream (*Sparus aurata*). *Aquaculture* 124: 275-282.
- Shimizu, C., Ibrahim, A., Tokoro, T. and Shirakawa, Y. (1990). Feeding stimulation in seabream, *Pagrus major*, fed diets supplemented with Antarctic krill meals. *Aquaculture* 89: 43-53.
- Sivalonganathan, B., Walford, J., Ip, Y. K. and Lan, T. J. (1998) Free amino acids and energy metabolism in eggs and larvae of seabass *Lates calcarifer*. *Marine Biology* 131:695-702
- Soffientino, B. and Specker, J. L. (2001). Metamorphosis of Summer Flounder, *Paralichthys dentatus*: Cell Proliferation and Differentiation of the Gastric Mucosa and Developmental Effects of Altered Thyroidal Status. *J. Exp. Zool.* 290(1): 31–40.
- Solbakken, J. S., Berntssen, M. H. G., Norberg, B., Pittman, K. and Hamre, K. (2002). Different iodine and thyroid hormone levels between Atlantic halibut larvae fed wild zooplankton or *Artemia* from first exogenous feeding until post-metamorphosis. *J. Fish Biol.* 61: 1345-1362.
- Solbakken, J. S., Norberg, B., Watanabe, K. and Pittman, K. (1999). Thyroxine as a mediator of metamorphosis of Atlantic halibut, *Hippoglossus hippoglossus*. *Env. Biol. Fish.* 56(1-2): 53-65.
- Sorgeloos, P., Dhert, P. and Candreva, P. (2001). Use of the brine shrimp, *Artemia* spp., in marine fish larviculture. *Aquaculture* 200: 147–159.

- Southgate, P. C. and Lee, P. S. (1993). Notes on the use of microbound artificial diets for larval rearing of sea bass (*Lates calcarifer*). *Asian Fish. Sci.* 6: 245-247.
- Southgate, P. C. and Lou, D. C. (1995). Improving the n-3 HUFA composition of *Artemia* using microcapsules containing marine oils. *Aquaculture* 134: 91-99.
- Southgate, P. C. and Partridge, G. J. (1998). Development of artificial diets for marine finfish larvae: problems and prospects. *Tropical Mariculture*. DeSilva, S. London, Academic Press pp. 151-169.
- Storebakken, T. (1988). Krill as a potential feed source for salmonids. *Aquaculture* 70: 193-205.
- Tachihara, K., El-Zibdeh, M. K., Ishimatsu, A. and Tagawa, M. (1997). Improved seed production of goldstripe amberjack *Seriola lalandi* under hatchery conditions by injection of triiodothyronine (T3) to broodstock fish. *J. World Aquacult. Soc.* 28(1): 34-44.
- Tagawa, M. and Hirano, R. (1987). Presence of thyroxine in eggs and changes in its content during early development of chum salmon, *Oncorhynchus keta*. *J. Exp. Zool.* 257: 360-366.
- Tanaka, M., J. Tanangonan, M. Tagawa, E. de Jesus, H. Nishida, M. Isaka, R. Kimura and T. Hirano (1995). Development of the pituitary, thyroid and interrenal glands and applications of endocrinology to the improved rearing of marine fish larvae. *Aquaculture* 135(1-3): 111-126.
- Tengjaroenkul, B., Smith, B. J., Smith, S. A. and Chatreewongsin, U. (2002). Ontogenetic development of the intestinal enzymes of cultured Nile tilapia, *Oreochromis niloticus* L. *Aquaculture* 211: 241-251.
- Trotter, A. J., Battaglione, S. C. and Pankhurst, P. M. (2003). Effects of photoperiod and light intensity on initial swim bladder inflation, growth and post-inflation viability in cultured striped trumpeter (*Latris lineata*) larvae. *Aquaculture* 224(1-4): 141-158.
- Trotter, A. J., Pankhurst, P. M. and Hart, P. R. (2001). Swim bladder malformation in hatchery-reared striped trumpeter *Latris lineata* (Latridae). *Aquaculture* 198(1-2): 41-54.
- Tucker, J. W., Russel, J. D. and Rimmer, M. A. (2002). Barramundi Culture: A success story for aquaculture in Asia and Australia. *World Aquacult.* 33(3): 53-59.
- Van der Plancken, I., Delattre, M., Indrawati, Loey, A. V. and Hendrickx, M. E. G. (2004). Kinetic study on the changes in the susceptibility of egg white proteins to enzymatic hydrolysis induced by heat and high hydrostatic pressure pretreatment. *J. Agric. Food Chem.* 52: 5621-5626.
- Venkatachalam, M. and Sathe, S. K. (2003). Phaseolin in vitro pepsin digestibility: role of acids and phenolic compounds. *J. Agric. Food Chem.* 51: 3466-3472.

- Volden, H., Mydland, L. T. and Olaisen, V. (2002). Apparent ruminal degradation and rumen escape of soluble nitrogen fractions in grass and grass silage administered intraruminally to lactating dairy cows. *J. Anim. Sci.* 80: 2704–2716.
- Walford, J. and Lam, T. J. (1993). Development of digestive tract and proteolytic enzyme activity in seabass (*Lates calcarifer*) larvae and juveniles. *Aquaculture* 109(2): 187-205.
- Walford, J., Lim, T. and Lam, T. (1991). Replacing live foods with microencapsulated diets in the rearing of seabass (*Lates calcarifer*) larvae: Do the larvae ingest and digest protein-membrane microcapsules?. *Aquaculture* 92(2-3): 225-235.
- Wan, L., Heng, P., Chan, L., Lee, C., Ho, E., Ngoh, W., Walford, J. and Lam, T. (1997). Effect of feeding with microspheres containing thyroid hormone on larval growth and development in tilapia, *Oreochromis mossambicus*, and on larval growth in seabass, *Lates calcarifer*. *J. Aquacult. Trop.* 12(3): 191-199.
- Watanabe, T. (1993). Importance of docosahexaenoic acid in marine larval fish. *J. World Aquacult. Soc.* 24(2): 152-161.
- Watanabe, T. and Kiron, V. (1994). Prospects in larval fish dietetics. *Aquaculture* 124: 223-251.
- Watanabe, T., Oowa, F., Kitajima, C. and Fujita, S. (1980). Relationship between dietary value of brine shrimp *Artemia salina* and their content of omega 3 highly unsaturated fatty acids. *Bull. Jap. Soc. Sci. Fish.* 46(1): 35-41.
- Wright, I. (2003). Fish Hydrolysate: More than just a flavour enhancer? *International Aquafeed* 6(3): 8-12.
- Yamano, K., Takano-Ohmuro, H., Obinata, T. and Inui, Y. (1994). Effect of Thyroid Hormone on Developmental Transition of Myosin Light Chains during Flounder Metamorphosis. *Gen. Comp. Endocrin.* 93(3): 321-326.
- Yúfera, M., Fernández-Díaz, C., Pascual, E., Sarasquete, M. C., Moyano, F. J., Díaz, M., Alarcón, F. J., García-Gallego, M. and Parra, G. (2000). Towards an inert diet for first-feeding gilthead seabream *Sparus aurata* L. larvae. *Aquacult. Nutr.* 6: 143-152.
- Yufera, M., Kolkovski, S., Fernandez-Diaz, C., Rinchar, J., K.J., L. and Dabrowski, K. (2003). Delivering bioactive compounds to fish larvae using microencapsulated diets. *Aquaculture* 227: 277–291.
- Zambonino Infante, J. L. and Cahu, C. (1994a). Development and response to a diet change of some digestive enzymes in sea bass (*Dicentrarchus labrax*) larvae. *Fish Physiol. Biochem.* 12(5): 399-408.



- Zambonino Infante, J. L. and Cahu, C. (1999). High dietary lipid levels enhance digestive tract maturation and improve *Dicentrarchus labrax* larval development. *J. Nutr.* 129(6): 1195-1200.
- Zambonino Infante, J. L. and Cahu, C. L. (1994b). Influence of diet on pepsin and some pancreatic enzymes in sea bass (*Dicentrarchus labrax*) larvae. *Comp. Biochem. Physiol.* 109A: 209-212.
- Zambonino Infante, J. L., Cahu, C. L. and Peres, A. (1997). Partial substitution of Di- and tripeptides for native proteins in sea bass diet improves *Dicentrarchus labrax* larval development. *J. Nutr.* 127(4): 608-614.
- Zilberg, D., Ofir, R., Rabinski, T. and Diamant, A. (2004). Morphological and genetic characterization of swimbladder non-inflation in angelfish *Pterophyllum scalare* (Cichlidae). *Aquaculture* 230(1-4): 13-27.