TOWARDS THE DEVELOPMENT OF A LARVAL FEEDING STRATEGY FOR THE WHITE-MARGINED SOLE (DAGETICHTHYS MARGINATUS).

THESIS

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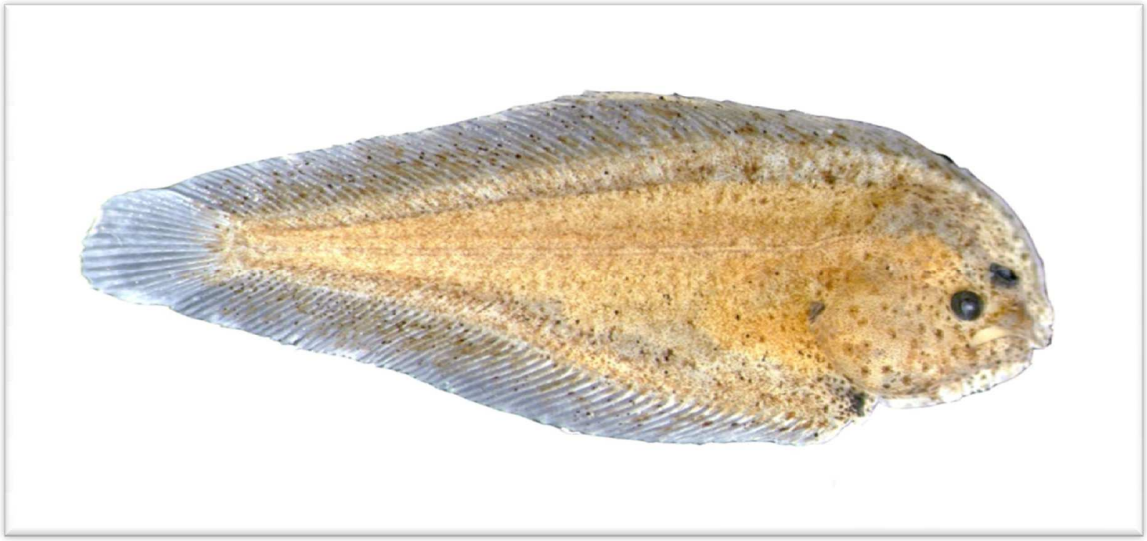

Settlement stage larva (10.53 mm BL) of the white-margined sole, *Dagetichthys marginatus* (Boulenger, 1900)(Soleidae) reared under laboratory conditions.

Photograph: Ernst Thompson

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Abstract

The major bottleneck during the domestication of the white-margined sole *Dagetichthys marginatus* in South Africa has been low larval survival. The cause of this is not clear but considering current literature on flatfish culture and more specifically soleid culture, nutritional deficiencies are hypothesized to be the main possible cause. Following the importance of nutrition, the first aim of the research was to use an ontogenetic developmental approach to develop a species specific larval feeding strategy. Ontogenetic development of *D. marginatus* showed that weaning will take place at much later ages than other soleids currently being farmed. This makes the partial replacement of *Artemia* with a suitable inert diet in co-feeding strategies very important to cut the cost associated of live food production. This leads on to the second aim, in which an '*in vitro*' approach was used to model the digestibility of *Artemia*, which could ultimately contribute towards designing inert feeds with similar digestibility characteristics to *Artemia* in the future.

Obtaining nutrients from food is closely linked to the functional status of the digestive tract, the support organs and the external morphological characteristics required for the ingestion of live or inert feeds. Considering both morphological and physiological ontogenetic development, it is clear that *D. marginatus* follow a similar pattern to other soleids. Larvae can successfully feed on *Artemia* as early as 3 days after hatching but exhibit a slow metamorphosis into the juvenile stage when compared to other soleids. The absence of any detectable acidic protease activity during the first 45 days of development and the importance of exogenous enzymes from *Artemia* all points to limited capacity to digest artificial diets.

Prior to modelling the '*in vitro'* digestion of *Artemia*, digestive enzyme activity at different pH's were modelled using functional forms from the normal distributive category of functions. As there is no substantiated information for the general effects of pH on enzyme activity in the literature for finfish larvae, three species

occurring in the same bio-geographical region of *Dagetichthys marginatus*, namely *Sarpa salpa*, *Diplodus sargus capensis* and *Argyrosomus japonicus* were used to investigate this effect. The fitted parameters, namely the optimal pH and sigma (the slope around the optimal pH) showed two interesting results. When using a negative log likelihood ratio test to test for differences between species for a particular enzyme, the optimal pH for alkaline proteases (7.67), lipase (8.03), amylase (7.69) and phosphatase (9.84) activity was the same for all three species. Furthermore, the study illustrated the potential to detect dietary shifts during ontogenetic development based on changes in enzyme activity around the optimal pH using the sigma parameter. *Sarpa salpa* showed increased amylase activity and a decrease in protease activity around the optimal pH with increased size, corresponding to a change in diet from zooplankton to algae.

The '*in vitro*' modelling approach taken in this study was based on known enzyme interactions and dynamics which makes the results very interpretable. Reasonable predictability of the degree of protein and carbohydrate digestion from *Artemia* is achieved based on gut evacuation time and enzyme levels. This '*in vitro*' study furthermore clearly indicates the importance of exogenous enzymes from *Artemia,* contributing as much as 54 % to protein digestion and 64 – 72 % to carbohydrate digestion. This was however, only an initial investigation, and further expansion of the model is required to achieve a complete understanding of *Artemia* digestion and ultimately partial replacement with artificial diets.

A feeding strategy for *D. marginatus* should therefore follow those of other farmed soleids, although there will be a general delay in implementation due to slower development. Problems can thus be solved and improvements made by transferring technology from other soleids to *D. marginatus*.

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Chapter 1: General Introduction

The worldwide development of marine finfish aquaculture has been immense over the last two decades, especially the selection and domestication of new species. While South Africa has not followed this worldwide increase in finfish aquaculture production, various indigenous species have been selected for aquaculture and domestication is underway. The abalone farming sector is the main driving force behind this diversification in South Africa, and is looking at farming various finfish species.

The total aquaculture output (including seaweeds) in South Africa for 2011 was 7700 MT and contributed less than 0.01 % to the total global aquaculture production. Mariculture contributed 4800 MT to the production, of which only 8 MT came from finfish. Abalone farming made up over a 1000 MT of the mariculture production, making it the biggest contributor to volume produced (excluding seaweeds) and economic benefit (Anon 2012). The 8 MT finfish produced consists exclusively of dusky kob (*Argyrosomus japonicas*), while a pilot scale production facility for the Yellowtail (*Seriola lalandi*) is in operation (Schoonbee & Bok 2006). Other finfish species for which domestication research is underway includes the White Stumpnose (*Rhabdosargus globiceps*), Spotted Grunter (*Pomadasys commersonnii*) and the Yellowbelly Rockcod (*Epinephelus marginatus*) (Anon 2012). Further screening for high value finfish species is ongoing.

Flatfish (Pleuronectiformes) have consistently attracted high prices on the European market (Howell 1997, Brown 2002, Imsland *et al.* 2010) and are generally considered to be well adapted for intensive culture conditions (Slaski 1999a, b, Le François *et al.* 2010). This makes it a favourable group for aquaculture, as is evident in the strong representation of flatfish species on the list of “new species for aquaculture” (Brown 2002), as well as the host of species being investigated for domestication.

Various flatfish species are being investigated worldwide as potential aquaculture

candidates (Imsland *et al.* 2010). Many of these species have been successfully reared through their larval stages, and include the winter flounder, *Pleuronectes americanus* (Litvak 1994, 1996) in Canada, the southern flounder, *Paralichthys lethostigma* (Luckenbach *et al.* 2003, Daniels *et al.* 2010) in North America, the Caribbean flounder, *Paralichthys tropicus* (Rosas *et al.* 1999), the Pacific halibut, *Hippoglossus stenolepis* (Stickney & Liu 1999) in British Columbia, the New Zealand turbot, *Colistium nudipinnis* and brill, *C. guntheri* (Tait & Hickman 2001) and the greenback flounder, *Rhombosolea tapirina* (Barnett & Pankhurst 1998) in Tasmania. Recent spawning and rearing success of the white margined sole, *Dagetichthys marginatus* has propelled this species to be one of the most important candidates for aquaculture in South Africa (Thompson *et al.* 2008).

The current demand for flatfish in South Africa is estimated, by industry, to be in excess of 2 500 MT. This cannot be met by local trawlers, which land around 570 MT / year (Anon. 2011). The shortfall is currently imported from Namibia (*A. microlepis*), Pakistan and India (*Cynoglossid spp*.). Very little is known about the state of the stocks in Namibia and Pakistan, while there has been some evidence of over-exploitation from India (Nair 2011). This is mainly because these species form part of the by-catch, and is not directly targeted. Industry is of the opinion that the current supply of flatfish for the South African market will not be sustained. This makes the culture of flatfish in South Africa an attractive alternative.

Thompson (2004) screened all the flatfish species in southern Africa for suitability, using various biological and economical selection criteria and species comparisons from Le François *et al.* (2002) and Quéméner *et al.* (2002). The soleid, *Dagetichthys marginatus* was selected as the most likely candidate for mariculture in South Africa (SA) from a list of three suitable species due to its wide distribution around the SA coast and the accessibility of broodstock. *D. marginatus* (Boulenger 1900), formerly *Synaptura marginata* (Vachon *et al*. 2008) is endemic to the east and south coasts of Southern Africa (Smith & Heemstra 1986, Vachon *et al.* 2008). It is mainly targeted by recreational fishermen on intertidal and subtidal

sandbanks, where densities are not high enough to sustain the commercial harvest of this sought after species. Research on *D. marginatus* is still in its infancy, but includes work on the cryopreservation of sperm (Markovina 2008, Markovina & Kaiser 2009) and cranial ontogeny with consideration of the feeding ability of larvae and early juveniles (Ende 2008, Ende & Hecht 2010). The demography and natural biology for *D. marginatus* is summarised below (Thompson 2004).

A Von Bertalanffy growth model, based on age estimates from sectioned otoliths, with an absolute error structure best describes the growth for this species. The model parameters were: L_∞ = 429.5 mm TL, K = 0.24 and t_0 = -1.79 years. Analysis of gut contents showed that *D. marginatus* feed exclusively on polychaete worms, predominantly of the genus *Morphysa*. It shows a protracted summer spawning season of six months, from October to April. Size at 50% and 100% sexual maturity for females was calculated to be 235 mm TL and 300 mm TL (ca. 1.5 – 2.5 years of age) respectively, while all males > 154 mm TL were mature. *D. marginatus* is a batch spawner, releasing a minimum of 3 batches of eggs per year. Relative fecundity is reasonably high (34000 eggs per year / kg) in comparisons to other flatfish species (Thompson 2004).

Currently, flatfish aquaculture is dominated by the production of around 40925 MT / year and 3977 MT / year of Bastard halibut (*Paralichthys olivaceus*) in Korea and Japan respectively, and 68890 MT / year of turbot (*Scophthalmus maximus*), mainly in China, Spain, Portugal and France (Fishstat$^+$, Aquaculture Production, FAO 2010). Other farmed flatfish species of commercial importance include Atlantic halibut (*Hippoglossus hippoglossus*) in Iceland, Norway and UK (1821 MT / year), sole (*Solea, S. senegalensis*) in Spain and Portugal (244 MT / year), and various flounder species, e.g. *Paralichthys dentatus* in North America (Brown 2002, FAO 2010). It is clear from these FAO statistics that there is still limited production of soleids, despite experimental rearing success as early as the 1980's (Dinis 1986, 1992, Drake *et al.* 1984) and being considered promising candidates since the nineties (Howell 1997, Dinis *et al.* 1999).

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Disease, weaning, abnormalities, diet and long and variable grow-out periods are the main factors currently constraining the production of noteworthy quantities of soleid species worldwide (Aragão *et al.* 2008, Howell *et al.* 2009). Qualitative and quantitative dietary imbalances are one of the biggest factors contributing to the problems with flatfish culture (Liao *et al.* 2001, Conceição *et al.* 2007) and has been linked to compromised immune systems leading to disease (Howell *et al.* 2009). There is an increasing volume of research on this topic, often exposing what is known as insufficient for the purpose of larval rearing (Hoehne-Reitan & Kjorsvik 2004).

The major bottleneck in most aquaculture ventures, especially those of finfish, is the limitation of a poor or unsteady supply of fry (Bromage 1995, Dhert *et al.* 1998, Lee & Ostrowski 2001, Liao *et al.* 2001, Yúfera & Darias 2007b). This is mainly due to low larval survival during periods of stress, such as the switch from endogenous to exogenous feeding (Gulbrandsen 1993, Jähnichen & Kohlmann 1999, Yúfera & Darias 2007b) and weaning. Research has shown that the switch from endogenous to exogenous feeding does not in general present a problem in soleid culture, although this might not be the case for other flatfish families (Shields et al. 1999). Weaning is the biggest bottleneck in soleid larviculture (Howell 1997, Dinis *et al.* 1999, Conceição *et al.* 2007, Bonaldo *et al*. 2011) and also appears to be the case in rearing trials of *D. marginatus* (pers. obs.).

Traditionally, live food such as rotifers, *Brachionus* sp. and brine shrimp, *Artemia* sp. are supplied as a food source during first feeding of marine fish larvae (Das *et al.* 2012). Supplying a large commercial finfish farm with sufficient live food is expensive (Kolkovski 2001) and can contribute 50 % of production costs in the first three months although it only contributes 1.6 % to the required total dry food weight (Person-Le Ruyet *et al.* 1993). Variable supply and nutritional inconsistencies of live organisms present further problems (Kolkovski 2001). Fish larvae are thus weaned onto inert diets as soon as they are physiologically capable of digesting and obtaining the required nutrients from the artificial food.

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Recent advances in larval nutrition, especially technological improvements in artificial diets, have significantly reduced the pre-weaning period in various species (Cahu & Zambonino Infante 2001). The most important of these advances is the technology to produce microdiets. The development of microagglomerated diets containing protein hydrolysates (Day *et al.* 1997, 1999, Tonheim *et al.* 2005, de Vareilles *et al.* 2012) has reduced weaning problems in Dover sole, *Solea* (Howell 1997, Day *et al.* 1999a, b) and Senegal sole, *Solea senegalensis* (Engrola *et al.* 2007). Producing consistently good weaning results in *S. senagalensis* remains a problem (Engrola *et al.* 2007). This is attributed to the early metamorphosis and occurrence of a 'peculiar non-proactive bottom-feeding behaviour' (Conceição *et al.* 2007).

Various attempts have been made to design and rear finfish larvae exclusively on artificial diets (Fernandez-Diaz & Yufera 1997, Rosenlund *et al.* 1997, Cahu *et al.* 1998, Southgate & Partridge 1998), but a general trend of poor larval growth and development has been observed (Cañavate & Fernández-Díaz 1999, Robin & Vincent 2003, Curnow *et al.* 2006a). While some studies have linked specific nutrient deficiencies to certain problems, like Ascorbic acid to skeletal deformities (Cahu *et al.* 2003), these larval problem are generally attributed to a combination of various factors including nutritional deficiencies in the diet and an underdeveloped digestive system that is not able to digest artificial diets (Cahu & Zambonino Infante 2001)." There is thus a need for co feeding strategies with a gradual transition from live to artificial food (Das *et al.* 2012). Cahu & Zambonino Infante (1994) amongst others, suggests that the live food, when supplied in such co-feeding strategies, contributes to the enzyme activity levels in the larvae, making artificial food more digestible. Acceptable survival and growth rates have been achieved in some instances, but there is still a vast difference in growth when live food is introduced even with economic considerations (Cahu & Zambonino Infante 2001, Sorgeloos *et al.* 2001). This is also the case for soleids (Bonaldo *et al.* 2011). Co feeding with *Artemia* gives results close to that of feeding *Artemia* alone (Howell 1998, Day *et al.* 1999b), but extended periods of feeding *Artemia* show increased difficulty to wean off *Artemia* completely.

During co-feeding strategies the gut shows improved digestive maturity, but this does not necessary translate to improved growth (Engrola *et al.* 2009, Mai *et al.* 2009). All this highlights the importance of live food in finfish larval rearing and it is generally accepted as an integral part of larval rearing until metamorphosis (Kolkovski 2001, Aragão *et al.* 2004, Conceição *et al.* 2010, Herrera *et al.* 2010).

In soleids, like all other flatfish, larval rearing problems are also associated with the dramatic metamorphic transformation from bilateral larvae to asymmetric juveniles (Inui & Miwa 2012). This transformation is accompanied by a complex habitat shift and behavioural change from a planktonic to a benthic form (Keefe & Able 1994, Ribeiro *et al.* 1999a, Fernández-Díaz *et al.* 2001). The complex shift in soleids during metamorphosis will change the acceptability of different live and inert food particles at different stages of transformation (Keefe & Able 1994). Prior to and during metamorphosis, sole larvae are less efficient in catching prey items due to their very radical transformation. There is therefore a need to hydrolyze stored lipids during this period to supply the larvae with its metabolic energy requirements while other potential energy rich nutrients, like protein, are redeployed to other part of the body and used for metamorphic changes within the larvae rather than energy (Yufera *et al.* 1999; Martinez *et al*. 1999). Hence, supplying sole larvae with an acceptable exogenous food source with the right nutritional value (Næss & Lie 1998, Estevez *et al.* 1999, Sargent *et al.* 1999, Shields *et al.* 1999, Hamre *et al.* 2007) is of utmost importance to ensure increased survival during this transition. This is especially the case for dietary lipids (Howell *et al.* 2009, Dâmaso-Rodrigues *et al.* 2010).

Malpigmentation, skeletal deformities and incomplete eye migration are often related to diet or nutritional problems during early larval development, although it appears that these can be mitigated by proper diet formulation (Solbakken *et al*. 1999, Bolker & Hill 2000, Sæle *et al.* 2002, Fernández *et al.* 2009, Howell *et al.* 2009, Piccinetti *et al.* 2011). Furthermore, these problems usually only manifest themselves during and after metamorphosis (Pittman *et al.* 1998, Gavaia *et al.* 2002). Similar problems have been experienced with Atlantic halibut, *Hippoglossus hippoglossus* (Pittman *et al.* 1998,

Sæle *et al.* 2002), olive flounder, *Paralichthys olivaceus* (Seikai 1985), turbot, *Scophthalmus maximus* (Shields 2001) and the senegal sole, *Solea senegalensis* (Gavaia *et al.* 2002, Soares *et al.* 2001, Engrola *et al.* 2005, Villalta *et al.* 2005a, Fernández *et al.* 2009). Gavaia *et al.* (2002) summarise other possible causes of the above problems, the principal one of which appears to be unfavourable abiotic conditions (Faustino & Power 1999). Extensive research has shown that these problems can be reduced to a point where they no longer restrict the production of some flatfish species (Seikai 1985, Pittman *et al.* 1998, Shields 2001, Gavaia *et al.* 2002, Sæle *et al.* 2002, Makridis *et al.* 2009). Several other teleosts show abnormalities during larval rearing that can be linked to nutritional problems (Deplano *et al.* 1991b, Boulhic & Gabaudan 1992, Rueda & Martinez 2001, Fernández & Gisbert 2010, 2011).

The importance of amino acids (AA) in larval rearing has largely been ignored in the early literature, but subsequent research has established their importance (Aragão *et al.* 2008, Rønnestad & Conceição 2012). Amino acids are the principle building blocks for protein synthesis, are important energy substrates and are involved in specific physiological functions. Free amino acids (FAA) are an important source of energy in early larval stages (Rønnestad & Fyhn 1993, Finn *et al.* 2002). Increased levels of FAA are beneficial for first feeding larvae (Fyhn & Serigstad 1987, Fyhn 1989) and stimulate gut maturation (Cahu and Zambonino-Infante 2001). It is also clear that changes in the AA profile during development effects the dietary requirements for these AA (Conceição *et al.* 1997, 1998a, Tulli & Tibaldi 1997, Aragão *et al.* 2004). There is a more marked change in AA profile in soleids like *Solea senegalensis* due to the metamorphic switch (Aragão *et al.* 2004). This research has indicated that *Artemia* does not have a balanced AA profile, and is limited in histidine, sulphur AA, arginine, lysine and valine (Aragão *et al.* 2004).

Essential fatty acids (EFA) are important in larval quality and rearing in both marine and fresh water species (Sargent *et al.* 2002). Unlike freshwater species that can satisfy their EFA requirements with highly unsaturated fatty acids (HUFA's), marine

species require longer chained HUFA's (20+ carbon groups) also referred to as long-chain polyunsaturated fatty acids (LC-PUFA's) to fulfil their needs (Tocher & Ghioni 1999). The three LC-PUFA's that are critically important for all marine species are 22:6n-3; docosahexaenoic acid (DHA), 20:4n-6; arachidonic acid (ARA) and 20:5n-3; eicosapentanoic acid (EPA). Various pleuronectid species can however be reared on DHA deficient diets, without affecting growth and survival, provided a high enough level of EPA is supplied. This is in contrast with other marine species and includes *Solea senegalensis* (Conceicao *et al.* 2007), *Solea* (Howell & Tzoumas 1991) *Paralichthys olivaceus* (Izquierdo *et al.* 1992) and *Pleuronectes platessa* (Dickey-Collas & Geffen 1992). Villalta *et al.* (2005b) hypothesize that this phenomena is due to the predominance of EPA rather than DHA in the benthic fauna. Flatfish species with short pelagic stages and fast settlement might therefore explain the need for the low exogenous DHA demand. At the same time it is clear that increased levels in ARA or high ARA: EPA also affect the incidence of malpigmentation (McEvoy *et al.* 1998, Næss & Lie 1998, Estevez *et al.* 1999, Sargent *et al.* 1999, Shields *et al.* 1999, Hamre *et al.* 2007, Lund *et al.* 2007).

The physiological and biochemical importance of these EFA and AA fall outside the scope of this particular dissertation, and will not be discussed any further, other than specific references.

Sorgeloos *et al.* (2001) describes the reasons for favouring *Artemia* as a live food organism in finfish larval rearing, despite the necessity of rotifers in fish species with very small larvae like the Cod, *Gadus morhua* (Olsen *et al.* 2004). *Artemia* can be grown to larger sizes, which ensures a better energy balance in food intake and assimilation. Furthermore, its palatability induces a good and fast feeding response. The use of bio-encapsulation techniques also enhances the quality of on-grown *Artemia*. The relatively large size of soleid larvae makes the need of smaller live food organisms like rotifers unnecessary, since they can ingest *Artemia* at first feeding (Conceicao *et al.* 2007, Ende 2008). Rotifers remain an important diet organism, and are still used as part of the rearing technology for many other species including soleids. This is done to ensure higher levels of highly unsaturated fatty acids and

amino acids in the diet (Cañavate & Fernández-Díaz 1999, Aragão *et al.* 2004, Conceicao *et al.* 2007).

The general low nutritional value of live foods at older ages, like larger *Artemia,* makes the need for enrichment very important (Conceição *et al.* 2010). *Artemia* can be enriched with various micro-algae or commercially produced enrichment media like INVE Selco and AlgaMac-2000. Coutteau & Sorgeloos (1997) reviewed the literature on the effectiveness of different enrichment methods to modulate essential fatty acids in live prey, while Aragão *et al.* (2004) studied the modulation of amino acids. Enrichment of live foods has dramatically improved larval rearing success over the last two decades and forms an integral part of larviculture.

Despite this success and the development of species specific enrichments, very little is known about the absolute nutritional requirements for different fish species, and the acquisition rate of these nutrients from the prey from different enrichments (Boglino *et al.* 2012, Rønnestad *et al.* 2012).

The absorption factor, also known as the digestibility coefficient, is essential in evaluating fish diets in terms of growth and survival (Lovell 1998, Martinez- Montano & Lazo 2012) and by extension live food. Getting a direct measure of digestibility of food in larvae is extremely difficult, so digestive enzyme activity and digestive tract ontogeny is used as a surrogate for its estimation. The effectiveness of live food and the enrichment thereof for any particular fish species is very different and is dependent on ontogenetic development of larval morphological, physiological and functional complexity as well as bioavailability of nutrients in *Artemia* (Favé *et al.* 2004, Martinez-Montano & Lazo 2012).

The determination of digestive enzymes activity in larvae can also be helpful in the selection of feed ingredients (Lan & Pan 1993), allowing the onset of *in vitro* assays for the evaluation of commercial feeds (Alarcón *et al.* 1999). As protein is the major ingredient in inert larval diets, most of the biochemical studies have been oriented to

the characterization of protease activity in finfish species (Haard 1992, Moyano *et al.* 1998, Noori *et al.* 2012). Other enzymes, like carbohydrases and lipases, have been much less studied, although several authors have reported the presence of a noticeable carbohydrase activity in the gut of freshwater and marine fish species (Chiu & Benitez 1981, Ugwumba 1993, Munilla-Moran & Saborido-Rey 1996, Hidalgo *et al.* 1999). There is also some evidence for this in *S. senegalensis* which seems to utilise plant proteins well, offering a good prospect for a high level of fish meal replacement (Howell *et al.* 2009).

This study aims to investigate the digestive capacity of *Dagetichthys marginatus* larvae and how this ultimately affects the digestibility of and availability of nutrients from *Artemia* as a live food organism. This was achieved by using a systematic approach to integrate all the factors that may influence digestion, including the morphological, physiological and functional complexity of the larvae throughout its development.

The general materials and methods used in this study are described in Chapter 2, while specific methods are described in the relevant chapter. Chapter 3 describes the external morphological development of *D. marginatus* and Chapter 4 the physiological development using a histo-chemical approach. The larval stage is a critical period in aquaculture, in which ontogeny causes important structural and functional changes in the body tissues, organs and systems. The success and progress of larviculture therefore depends on a thorough understanding of the development of such elements, in order to adjust culture conditions and feeding protocols to the ontogenetic status of the larvae. Chapter 3 and 4 represent an initial step towards the determination of the functional capabilities, and thus the physiological requirements needed for optimal larval growth and development.

Digestion in fish larvae is a function of several physiological factors which in turn can be affected by environmental factors. These physiological factors include the enzyme system, a tangible measure of the developmental status of the digestive tract, and gut

evacuation time. Chapter 5 characterises the effect environmental factors have on the activity of the major digestive enzymes. Chapter 6 presents an '*in-vitro*' protocol that can be used to estimate the digestibility of proteins, lipids and carbohydrates from *Artemia franciscanis* in marine fish larvae, using a holistic view of '*in vivo*' digestion. All the findings and the implications of the results are summarised in Chapter 7.

Chapter 2: General Materials and Methods

This chapter provides an outline of materials and the general methods used in this study. The specific methods used in each of the ensuing suite of experiments are described in detail in each of the relevant chapters.

SAMPLING AREA AND BROODSTOCK CAPTURE

Fish were collected between Port Elizabeth (33º57'S; 25º38'E) and Great Fish River Point (33º31'S; 27º06'E) on the south east coast of South Africa (Figure 2.1). The topography of this coastline is highly variable. The shores of Algoa Bay are generally sandy with rocky ledges at Woody Cape and Cape Padrone. The area from Cape Padrone to Great Fish Point consists of rocky outcrops alternating with sandy beaches (Hydrographer S.A. Navy 1985). The intertidal and subtidal rocky outcrops are temporal and are often buried under sand and then washed open again by currents. These currents are mainly determined by the prevailing winds (Lutjeharms 1998). The main collection sites along this stretch of coast included Cape Recife, Cannon Rocks, Port Alfred, Kleinemonde and Great Fish River Point (Figure 2.1).

Thompson (2004) tested various techniques used for flatfish capture, including the use of seine nets, feike nets, baited traps, diving and spearing. Spearing (with barbless prongs) was the only successful method and hence had to be employed. This was achieved by wading on shallow sandbanks and spearing (blindly) with a four-pronged fork. This technique is also used by all recreational sole fishermen in the area. These forks come in a wide range of shapes and sizes, but all forks have three basic parts to them; the head, prongs and handle (Figure 2.2). The prongs are barbed and are made of steel or steel alloy, and are attached to the head. The head is attached to the handle, usually a wooden broom stick. The barbs of the soling forks used in this study were filed away such that the fork could be easily removed from the fish.

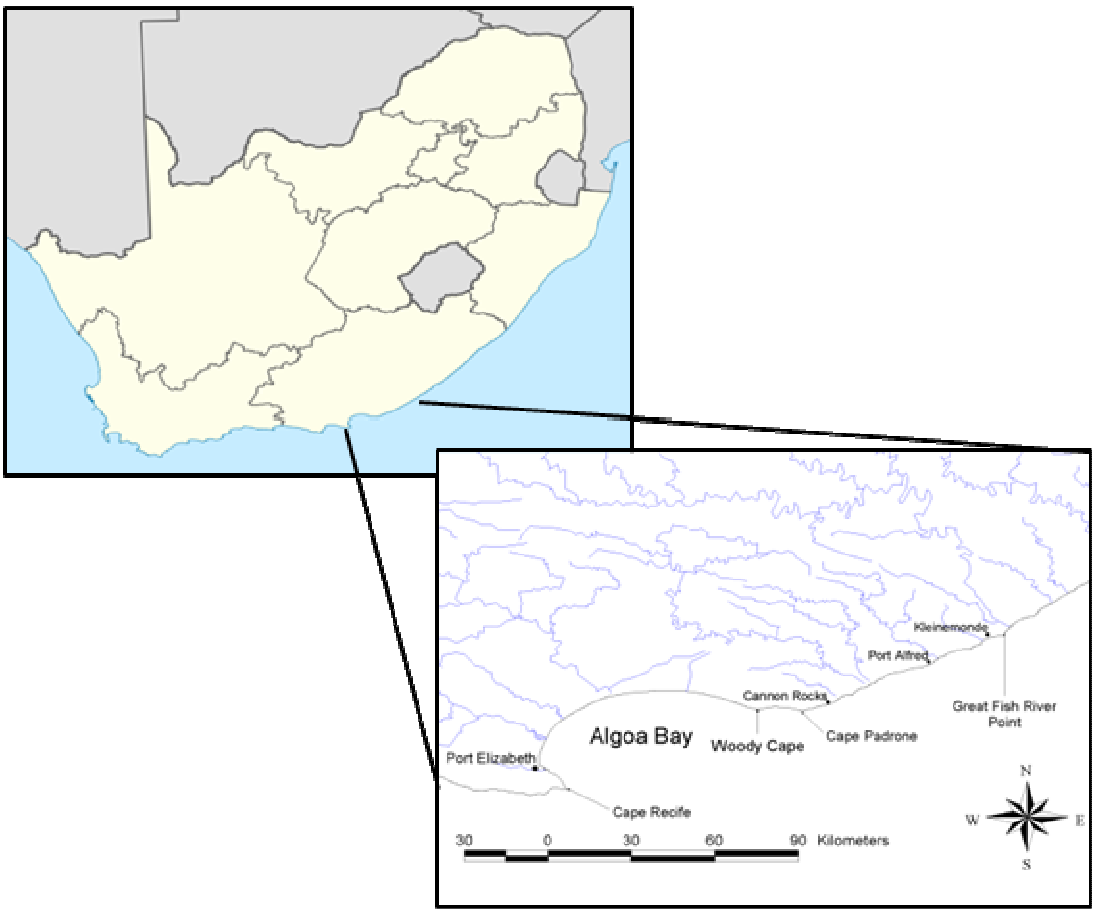


Figure 2.1: Map of the South East Cape coastal area showing the main sampling sites.

D. marginatus are generally more abundant on newly formed sandbanks (0 to 1.5 meters deep) with dispersed rocks covered by the green seaweed, *Caulerpa filiformis*. Sampling took place during the extended spawning season (October to March) (Thompson 2004), two days before and after spring tide (five days in total) for a period of about two hours before and after low tide during the day, when sandbanks were easily accessible.

Figure 2.2: Typical 4-barbed fork used to capture *D. marginatus* on the south east coast of South Africa.

BROODSTOCK TRANSPORT AND HUSBANDRY

All the captured fish were inspected for severity of the wounds and maturity, based on size (Thompson 2004). The fish that were too small or speared through any vital organ were discarded. Adult fish were transported to the marine hatchery at Rhodes University, Grahamstown in 200 litre dark, plastic bins containing 40 litres of well aerated sea water and a layer of sand. Prior to placing fish in broodstock systems, all the wounds were treated with an antiseptic tincture (Speelmanskop Biobalsam – 10 % propolis tincture) to prevent primary and secondary infections. The survival rate of the fish after capture, transport and treatment in such a manner was 89 %.

The fish were placed in one of two completely independent but identical recirculating broodstock systems in the marine hatchery at Rhodes University. The two systems

allowed for flexibility in conditioning and quarantining of certain fish. Each system consisted of rectangular (3 m x 1.8 m x 1.2 m) holding tank made from plastic canvas, a 1 m^3 sump, a mechanical sand filter filled with diatomaceous earth and a trickle filter. A plastic container (1.2 m x 0.95 m x 0.8 m) half filled with oyster shells and shredded plastic was used as the biological trickle filter. The total system volume was 8 m^3 and had a flow rate to ensure a complete water exchange every 2 to 3 hours in the holding tank. Once a week a total of 800 litres (10% of total volume) was replaced with fresh seawater that was mechanically filtered to 10 µm. The water in the system was continuously aerated.

Environmental conditions, under which fish were kept, were closely monitored. Temperature and salinity were maintained at 19 ± 1 ºC and 35 ± 1 ppt respectively, while the diurnal light cycle was adapted, varying between 12 and 16 hours of daylight, according to the natural light cycle. Water quality was monitored bi-weekly. Oxygen saturation was maintained above 7.4 ppm, ammonia (NH_3) below 0.014 mg/l and nitrite (NO^-) below 0.026 mg/l within each system.

Stocking densities of mature fish never exceeded more than 50 % bottom coverage, which is considered a medium stocking density for other soleids like *Solea* (Schram *et al.* 2006). Fish were fed shelled sand mussel (*Donax serra*) and squid (*Loligo vulgaris reynaudi*) three times a week *ad libitum*, following a similar regime proposed by Dinis (1986). Excess food was removed prior to next feeding.

SPAWNING AND EGG INCUBATION

Females in the final stages of oocyte maturation, identified on the basis of external morphological evidence of swelling, were induced to undergo final stages of vitellogenisis and ovulation using Aquaspawn ®, a GnRH analogue (Millar's Clinical Laboratories, Touws River, South Africa) at a dosage of 0.5 ml per kg of body weight. Eggs were obtained by strip spawning the females between 24 and 48 hrs after a

single injection. Males were sacrificed and the testes surgically removed. Testes were then homogenized in a small volume (1ml) of saline solution (0.9 ppt) and added to the eggs to allow fertilization while stirring with a primary bird feather. This process was completed with sterilised equipment and in the absence of any water.

After 20 minutes, one litre of fresh seawater, filtered to 10 micron at a temperature of 19 ºC was slowly added to the fertilised eggs while stirring continuously with the feather. An additional 20 minutes was allowed for hydration and hardening off eggs before the eggs were washed and placed into 60 L black, cylindro-conical fibre glass incubators at a density of approximately 20 eggs/ L. Eggs were incubated in the dark for the first 72 hours, to avoid egg mortality and reduced hatching rates (pers. obs.). These tanks also served as the larval rearing tanks.

LARVAL REARING AND SYSTEM DESIGN

The 12 identical cylindro-conical tanks were linked to a recirculating system, which consisted of a biological trickle filter (500 L), mechanical sand filter filled with diatomaceous earth, sump (1 m^3). Water inflow was from the bottom such that an upwelling system was created with the outflow at the top (Figure 2.3). A500 µm screen was placed halfway in the tank to serve as a circular false bottom, due to bottom associated feeding behaviour of larvae undergoing flexion. Flow rates were maintained at 5 L/h during incubation and 30 L/h during larval rearing. Each tank was supplied with gentle aeration to ensure a high level of oxygen saturation. 10 % of the total system volume was replaced with filtered sea water (10 µm) every week. Water quality parameters were monitored bi-weekly. Temperature and salinity were kept constant at 19 ± 1ºC and 35 ± 1ppt respectively, oxygen saturation above 7.4 ppm, ammonia (NH_3) below 0.011 mg/l and nitrite (NO_2) below 0.023 mg/l.

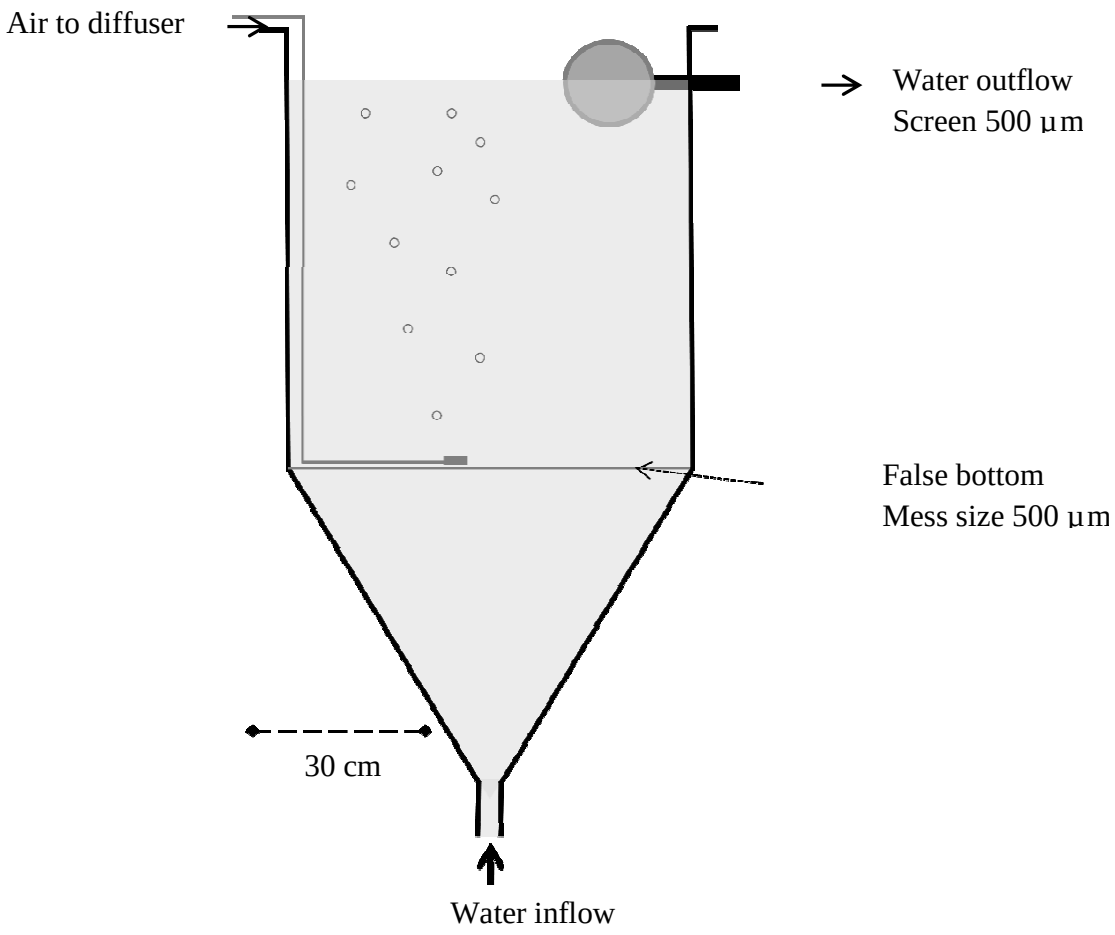


Figure 2.3: Schematic illustration of egg incubation and larval rearing tank with and inserted false bottom.

Larvae and early juveniles were fed twice each day at 09:00 and 17:00 according to the protocol illustrated in Figure 2.4. Larvae were fed on newly hatched *Artemia* franciscanis nauplii from 4 days after hatching (dah) until 16 dah at a density of >5 ind. / ml. From 16 dah until 35 dah the larvae and early juveniles were fed enriched *Artemia* metanauplii and enriched, frozen *Artemia* metanauplii. A new batch of enriched nauplii was provided every 24 hrs at a density of approximately five individuals / ml. The onset of weaning to an inert diet began at 25 dah. Larvae were co-fed on live *Artemia* metanauplii, frozen *Artemia* nauplii and the manufactured diet until 35 dah when live *Artemia* metanauplii and frozen *Artemia* nauplii were withdrawn. The pelleted diet was

supplied at a concentration of 6 mg / ml of water (Imsland et al. 2003). Uneaten pellets, dead larvae and *Artemia* where siphoned out twice each day, prior to next feeding.

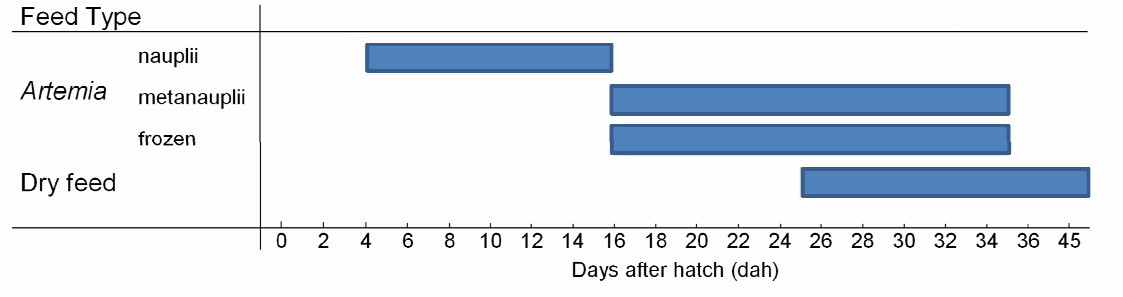


Figure 2.4: Diagram illustrating the feeding protocol used for the rearing of *D. marginatus* larvae.

ARTEMIA PRODUCTION

One batch of *Artemia franciscanis* cysts (Batch number 9540), EG: HE > 240 000 npl/g (INVE, Belgium) was used throughout the experiments. Cysts were prepared and incubated according to the method described by Hoff & Snell (1987). They were hydrated for one hour in conical plastic flasks (1 L) containing fresh water under strong aeration at 25°C. The hydrated *Artemia* cysts were then decapsulated with unflavoured liquid bleach (approximately 10 ml of liquid bleach per gram of cysts) until cysts reached a bright orange colour, after which they were thoroughly rinsed with fresh water. Decapsulated cysts were transferred into conical plastic flasks (1 L) containing filtered seawater (10 µm) kept at a constant temperature and salinity (25°C and 35 ppt) under strong aeration. Constant illumination at light intensities of 2000 lux was applied by using light tubes (Osram either fed to sole larvae DHA Selco® accordingL18/W72). Cysts hatched after 24 h and were then or reared to the metanauplii stage and enriched with the method described by the producer (Artemia Systems, INVE). Approximately 0.6 g of dry DHA Selco® (Batch number 09 27 B) was homogenised in 25 mL of seawater and added continuously, drop by drop, to the Artemia metanauplii culture over a period of 24 h. The enriched metanauplii were thoroughly rinsed with

fresh water and either fed to sole larvaeor frozen for later use. Care was taken to separate un-hatched cysts and egg shells from the nauplii.

INERT DIET COMPOSITION

The inert diet was an extruded pellet that was prepared in the laboratory. The composition of the diet is shown in Table 2.1. Squid and sand mussel meal used in the diet were made by freeze-drying fresh material to prevent protein denaturation and milled down to an average particle size of 50 µm. Larger particles was removed using a 100 µm sieve. All dry ingredients were stored at –20℃, until extrusion. The ingredients were weighed and mixed thoroughly with water. The mixture was put into a pasta extruder (ICME Bologna) and extruded to form 2mm diameter strands. These strands were oven dried at 38℃ for 12 h. The dried strands were milled and sieved to provide particles ranging in size from 100 to 400 µm (average = 250 µm). The proximate composition of the diet was determined using methods set out by Thompson (2004).

Table 2.1: Ingredients and proximate analysis (g / 100 g or %) of the formulated inert diet used for weaning of the sole larvae.

ıredients	**%**
hmeal (AAA Danish low temperature)	53 (dry matter)
nd Mussel meal (Donax serra)	8 (dry matter)
uid meal (Loligo vulgaris reynaudi)	8 (dry matter)
d liver oil	5
amin / Mineral mix	1
ırch binder	25
oximate Analysis	
otein	49.6
ude fat	17.3
h	14.8
isture	4

Chapter 3: Larval development of *Dagetichthys marginatus* (Family: Soleidae), obtained from hormone-induced spawning under artificial rearing conditions*.

***** Thompson, E.F., N.A. Strydom, and T. Hecht. – 2007. Larval development of *Dagetichthys marginatus* (Family: Soleidae), obtained from hormone-induced spawning under artificial rearing conditions. *Scientia Marina* **71(3)**: 421-428.

INTRODUCTION

Dagetichthys marginatus was identified as a suitable candidate species for aquaculture in South Africa, based on life history strategy, good natural growth rates and an established, lucrative market for flatfish (Thompson 2004). Wild broodstock were successfully induced to spawn and the larvae were reared through to metamorphosis under controlled laboratory conditions. These larvae were used to describe the early ontogeny of the species.

The white-margined sole, *Dagetichthys marginatus* (Boulenger 1900), formerly *Synaptura marginata* (Vachon *et al.* 2008), is one of 56 flatfish species that occur in southern African waters, 16 of which are soleids (Smith & Heemstra 1986). The new soleid genus *Dagetichthys* consists of five species, three occurring in the Western Indian Ocean (Vachon *et al.* 2008), namely *D. marginatus*, *D. albomaculatus* (Kaup 1858) and *D. commersonnii* (Lacepède 1802). The distribution of *Dagetichthys marginatus* is listed by Heemstra and Gon (1986) as extending from the Mozambique Channel southwards to Durban on the east coast of South Africa. However, Thompson (2004) recorded the distribution of *D. marginatus* to extend into temperate waters as far south as Gansbaai (34°35ʻS, 19°20ʻE), where it is the most abundant shallow water sole species on intertidal and sub-tidal sandbanks.

Nothing is known about the early life history of this species prior to the genus change. An isolated report on egg size (2.08 mm) (Ochiai 1966) was shown to be inaccurate

due to the misidentification of adult fish (Vachon *et al.* 2008), while a record of three *D. marginatus* larvae (2.8 – 3.2 mm) (Beckley 1986) in temperate South Africa remains unconfirmed. The only information for larvae of the 14 species in the genus *Synaptura* (Eschmeyer 1998) was an isolated description for *Synaptura kleinii* (Brownell 1979), which was subsequently moved to the genus *Synapturichthys* (Heemstra & Gon 1986). Hence there are no published larval descriptions for the new genus *Dagetichthys*.

Laboratory rearing of *D. marginatus* for aquaculture provided an ideal opportunity to study and describe larval development. Understanding the critical changes in morphology and behaviour of this species, as well as the timing and duration of each of these events, will facilitate larval rearing protocols.

Larval descriptions for coastal fish are generally lacking in South Africa (Strydom & Neira 2006). Descriptions are however, available for eight of the 16 soleid species occurring on the South African coast (Table 3.1). This paper presents the first description of the early larval development of the white- margined sole, *D. marginatus* (Boulenger 1900). This information will assist in the identification of this species in ichthyoplankton samples and in so doing, provide much needed information on spawning as well as larval and juvenile distribution for this species in coastal waters off South Africa.

MATERIALS AND METHODS

All broodstock fish were identified as *Dagetichthys marginatus* based on meristic counts given in Heemstra & Gon (1986) and Vachon *et al.* (2008). Male and female fish were collected with a hand-held, multipronged spear between Port Elizabeth (33º57'S; 25º38'E) and Great Fish River Point (33º31'S; 27º06'E) during the spawning season (October to March) (Thompson 2004) and transported to the marine hatchery at Rhodes University, Grahamstown. Females were induced to ovulate using Aquaspawn ®, a GnRH analogue (Millar's Clinical Laboratories, Touws River, South

Africa) at a dosage of 0.5 ml per kg of body weight. Eggs were obtained by strip spawning the females approximately 38 hrs after the single hormone injection. Males were sacrificed and the testes were homogenized in a small volume (1 ml) of saline solution (0.9 ppt). Eggs were fertilized with the testicular homogenate. After fertilization and hardening, eggs were thoroughly washed with seawater and divided among six 60 L black upwelling incubators at a density of 20 eggs / L for egg incubation and larval rearing. Hatching occurred between 42 and 49 hrs after fertilization and exogenous feeding began three days after hatching (dah). Larvae were fed twice daily on newly hatched brine shrimp, *Artemia franciscanis* nauplii from first feeding to 16 dah. After this, larvae were fed two-day old, Super Selco (INVE) enriched *A. franciscanis*. A new batch of enriched nauplii was provided every 24 hrs at a density of five individuals / ml. Temperature and salinity were kept constant at 19 ± 0.8℃ and 35 ppt respectively, for the duration of egg incubation and larval rearing.

Ten larvae were collected at specific intervals representing developmental endpoints after hatching. All samples were fixed in 5 % buffered formaldehyde for 24 hrs and then transferred into 70 % ethanol. Representatives of the larvae examined and described in this paper were lodged in the national fish collection at the South African Institute of Aquatic Biodiversity (SAIAB 77531).

All terminology pertaining to larval fish follows that of Neira *et al.* (1998). The term "larva" was used to designate all stages in the early life history from hatching to the attainment of a full fin ray complement, squamation and the subsequent loss of all larval characters, at which stage the "larva" becomes a "juvenile". Transforming stages, still in possession of isolated larval characters and in a planktonic state were considered as larvae. Newly transformed individuals were called “early juveniles” and were included in the study. The term "larva" was further subdivided into yolk-sac, preflexion, flexion and postflexion stages. The following body measurements were made for all developmental stages; body depth (BD), body length (BL), eye diameter (ED), head length (HL) and pre-anal length (PAL). All measurements were made to the nearest 0.1 mm using a dissecting microscope fitted with an eyepiece micrometer

for larvae < 10 mm and Vernier calipers for larger specimens. Body length (BL) represents notochord length in preflexion and flexion stage larvae, and standard length in postflexion larvae and early juveniles.

RESULTS

Age and size range of developmental stages

A total of 104 laboratory-reared larvae (2.09 – 14.75 mm BL) were examined to describe morphometrics, meristics and pigmentation. Newly hatched larvae (0h) ranged in size between 2.09 and 2.19 mm BL. The yolk-sac was completely absorbed after 4 days, while the oil globules persisted for approximately 12 hours. Yolk-sac larvae ranged from 2.09 to 3.41 mm BL (Table 3.2). The preflexion stage lasted for 6 days after yolk-sac absorption. During the preflexion stage the larvae ranged from 3.44 to 5.15 mm BL. Flexion of the notochord tip started 11 dah and was completed between 20 to 30 dah. Flexion stage larvae ranged from 5.00 mm (11 dah) to 7.06 mm BL (15 dah). The smallest postflexion larva measured 9.50 mm BL at 30 dah.

General morphology

Larvae are elongate (Figure 3.1A) in body shape during the yolk sac stage (mean BD = 13.61 %) becoming more moderate (Figure 3.1B) at the preflexion stage (mean BD = 26.59 %). Body depth increases at the onset of flexion as the gut enlarges and contorts to prepare for the settlement stage. Larvae remain moderately bodied until metamorphosis into juveniles (BD 28.44 – 50.00 %). The head is compressed and small during the yolk-sac stage (HL 14.63 %) becoming moderate in size during later developmental stages (HL 21.17 – 28.99 %). The snout is relatively short, giving the head a rounded, convex dorsal profile during preflexion and flexion (Figure 3.1). A dorsal hump is formed during the postflexion stage. This very distinct fleshy extension protrudes over the anterior, dorsal surface of the head and joins with the dorsal fin (Figure 3.1D). This hump fuses with the head after eye migration is complete. Both

body depth and head length, relative to body length, increase proportionally during development. Pre- anal length gradually decreases throughout development, from long to moderate (PAL 62.69 – 32.76 %) during early stages to short (mean PAL 24.53 %) at the settlement stage.

Newly hatched larvae have a moderate to large, unsegmented yolk-sac (0.50-0.59 mm diameter). In excess of 50 oil globules are distributed in the yolk-sac and are clustered together in groups of eight or less situated at the posterior end of the yolk-sac. An additional dense cluster of about 30 oil globules is also situated at the posterior end of the yolk-sac. Prior to first feeding, the oil globules fuse to form one large globule situated in a posterior-dorsal position in the reduced yolk-sac. The eyes are indistinguishable and unpigmented in yolk-sac larvae and become fully pigmented and functional in early preflexion larvae. The mouth becomes functional at the same time as the gut is fully formed in preflexion larvae 3 dah (3.44 mm BL) and larvae started feeding on *Artemia franciscanis* 4 dah. Two to five small villiform teeth were first observed at 16 dah (6.60 ± 0.27 mm BL) on the blind and ocular side of the dentary. Villiform teeth are present on the premaxilla at 31 dah, while the number of teeth on the blind side dentary increases. At this stage, the teeth on the ocular side dentary disappear. No further teeth develop on the ocular side premaxilla at any stage during development (Ende & Hecht 2010). Preflexion larvae have a myomere count of 40, while the vertebral count is 42 after ossification. Myomeres could not be counted during any other larval stage due to heavy pigmentation. The air bladder was visible in isolated larvae 7 dah and not in older larvae due to heavy pigmentation. Larvae were however positively buoyant until flexion, after which they become substratum associated. This indicates the presence and use of an air bladder during the early stages of larval development. The gill membrane is also free from the isthmus at this stage. No head spination is present at any stage of development.

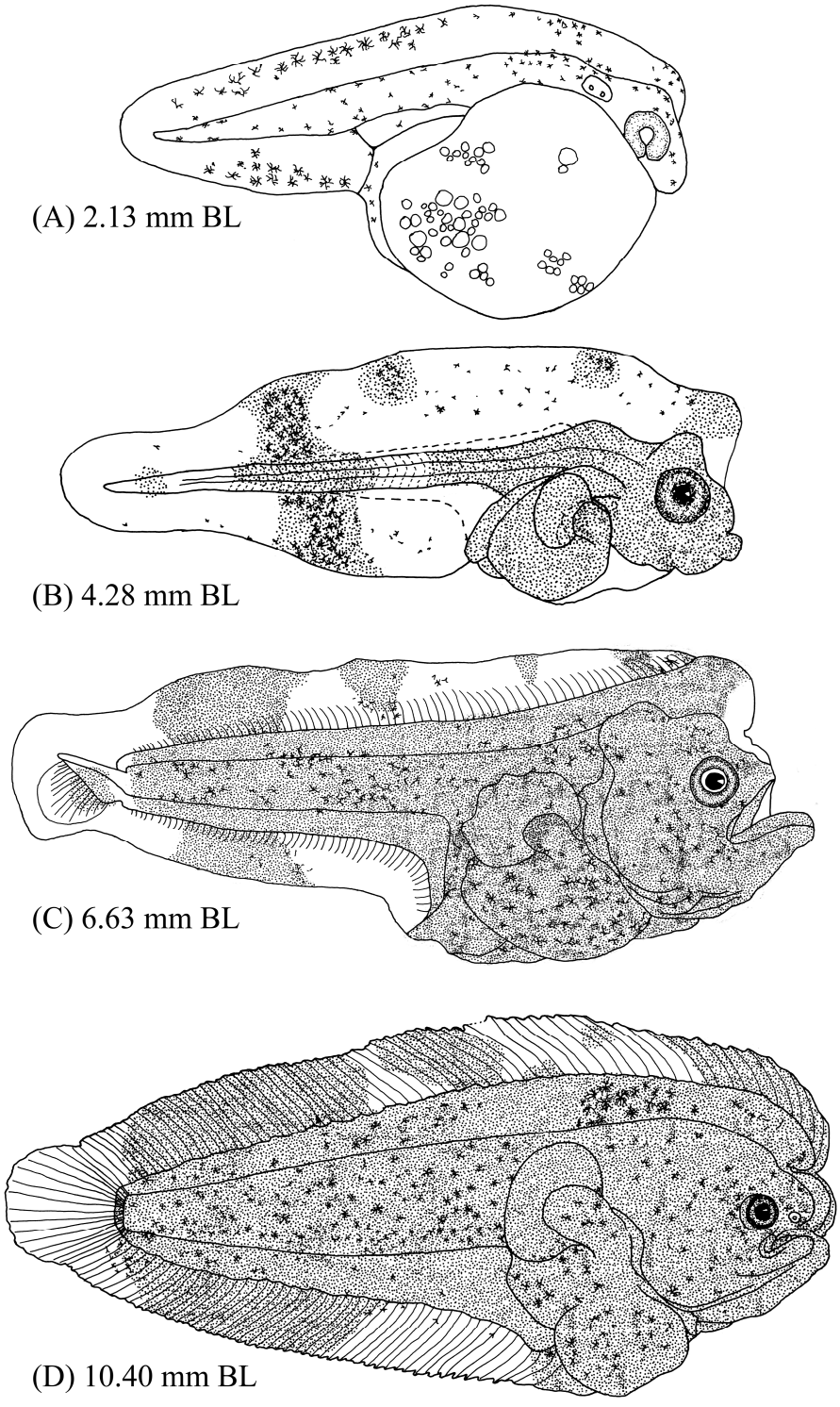


Figure 3.1: Larvae of *Dagetichthys marginatus* reared in laboratory. (A) newly hatched, yolk-sac. (B) preflexion. (C) flexion. (D) postflexion.

Development of fins

Pectoral fin buds appear in late yolk-sac or early preflexion larvae from 3.03 mm BL (2 dah). These increase in size throughout development up to settlement (9.50 mm BL, ± 30 dah), when the fins reduce in size and seven pectoral fin rays develop. Paired pelvic fin buds start developing during the late flexion stage from 6.50 mm BL (15 dah) and are fully developed in early postflexion larvae from 9.5 mm BL (30 dah), with 3 rays present. The dorsal, caudal and anal fin anlagen appear simultaneously in late preflexion larvae (± 4.72 mm BL). Incipient rays form during early flexion and a full adult ray complement (D 70, A 55, C18) is present by late flexion stages from 6.13 mm BL (15 dah). The dorsal, caudal and anal fin membranes remain fused throughout development and is characteristic in adults. No fin spines or extraordinary rays are present in *Dagetichthys marginatus* larvae during any stage of development.

Table 3.1: Meristic, morphological and pigmentation information on soleid species occurring in South African waters for which larval descriptions are available.

Species	Myomeres – range for preflexion and flexion	Vertebrae	Size at flexion	Pigment	Reference
Austroglossus microlepis	56 – 58	55 – 57	5.2 – 5.5	Dorsal and ventral midline; gut; lower jaw; behind eyes; pectoral fins	O'Toole 1977, Brownell 1979
Austroglossus pectoralis	50 – 58 (8-10 + 40-49)	58	3.5 – 3.8	Dorsal and ventral midline; fore- and hind-brain; snout; lower jaw; ventral and lateral gut; small spots on finfold	Wood 2000
Dicologossa cuneata	44 – 47 (9 + 35-38)	43 – 45	6.3 – 6.5	Dorsal and ventral midline; midbrain; hindbrain; finfold; swim bladder, gut; head	Lagardère & Aboussouan 1981
Heteromycteris capensis	39 – 41 (10 + 29-31)	40 – 43	6.2*	Midline body contour; finfold; ventral gut wall; lower jaw, behind eyes; lower pectoral fin margin	Brownell 1979
Monochirus lutens	36 – 38	36 – 40	5	Dorsal and ventral midline; midbrain; posterior tail (early); finfold; ventral abdominal wall	Nichols 1976 in Olivar & Fortuño 1991
Monochirus ocellatus	34 – 37 (8-9 + 26-28)	37 – 38	4	Three dorsal and two ventral concentrations of small spots on finfold; caudal tip; dorsal and ventral body contour; head, lower jaw; gut wall; swim bladder; pectoral fins	Palomera & Rubies 1971 in Olivar & Fortuño 1991
Pegusa lascaris	47 (9 + 38)	42 – 47	5.3	Many small melanophores scattered over head, body and fins. Heaviest concentrations over lateral and ventral gut surface and laterally on tail.	Clarke 1914 in Ahlstrom *et al.* 1984,
Synapturichthys kleini	42 – 45 (9-10 + 33-35)	46 – 47	6.5*	Densely packed stellate melanophores scattered over all body surfaces and finfold.	Russell 1976
Dagetichthys marginatus	40 (preflexion)	42	5 – 7.6	Three distinct clusters on the dorsal and one on the ventral finfold and later fins when the last dorsal and ventral cluster fuses to form a band over the body.	Brownell 1979 This Study

Table 3.2: Body length and body proportions of the larval and juvenile stages of *Dagetichthys marginatus* reared under laboratory conditions (n, number of larvae; BL, body length; HL, head length; BD, body depth; PAL, preanal length).

		Yolk-sac	Preflexion	Flexion	Postflexion	Juvenile
n		40	30	20	10	4
BL	**Range**	2.09 – 3.41	3.44 – 5.15	5.00 – 7.06	9.50 – 11.60	13.50 – 14.75
	Average	2.79	4.04	5.96	10.59	14.03
	Stdev	0.50	0.48	0.70	0.57	0.58
HL (%BL)	**Range**	12.35 – 17.14	17.86 – 25.23	22.02 – 28.04	25.47 – 30.77	26.32 – 31.19
	Average	14.63	21.17	24.50	27.95	28.99
	Stdev	1.12	2.04	1.45	1.80	2.04
ED (%BL)	**Range**	5.56 – 10.45	6.84 – 9.00	5.75 – 6.94	4.72 – 5.96	4.24 – 4.63
	Average	7.65	7.55	6.33	5.38	4.46
	Stdev	1.25	0.55	0.34	0.37	0.18
BD (%BL)	**Range**	10.09 – 19.12	22.22 – 34.23	28.44 – 36.28	39.62 – 50.00	30.70 – 33.05
	Average	13.61	26.59	31.65	44.99	32.30
	Stdev	3.02	3.28	1.96	3.73	1.10
PAL (%BL)	**Range**	42.20 – 62.69	40.00 – 48.65	42.59 – 49.56	32.76 – 46.32	22.88 – 25.93
	Average	51.54	43.52	46.60	39.65	24.53
	Stdev	6.84	2.55	1.89	4.03	1.26

Pigmentation

General pattern of development

Pigmentation increases from sparsely pigmented yolk-sac larvae to heavily pigmented postflexion larvae just prior to squamation. The general pattern of occurrence of melanophores on the head and trunk remains similar from first feeding (3 dah) to postflexion larvae, although intensity and melanophore type (e.g. punctate and/or stellate) increased with development. Xanthophores dominate the pigmentation of live yolk-sac larvae mirroring the melanophore pattern observed in four day old larvae. Xanthophores however decrease in number and intensity until they disappear completely around 4 dah and are replaced by branched, stellate melanophores.

Head pigmentation

Head pigmentation is characterised by melanophores on the lower lip (extending along the lower jaw line in later stages), preopercle, behind the eye and the isthmus in preflexion larvae, increasing in number and intensity in flexion larvae. In some flexion specimens, a melanophore ring appears around the eye. Postflexion larvae lose this general pattern of pigmentation, as the whole head region becomes covered with many random melanophores. An isolated group of internal melanophores is visible at the join of the lower and upper jaw, as well as on the dorsal hump of the head in preflexion larvae.

Trunk pigmentation

Trunk pigmentation in early preflexion larvae consists of three large clusters of melanophores or “blotches” on the dorsal finfold and one on the ventral finfold (Figure 3.1B). The first of the dorsal blotches is situated anterior to the nape, the second occurs opposite the anal opening (midway down the trunk) and the third, occurs two thirds along the length of the trunk. The ventral blotch is in line with the third dorsal blotch. This third dorsal and the ventral blotch expand laterally over the trunk, across the myomeres, until the two fuse and become an “hour shaped” band across the larva during late preflexion. This band widens during flexion to form one solid band and continues to be visible as a “dusky bar” on postflexion larvae. The first two dorsal blotches remain visible during and after fin ray

development. In live larvae, iridiophores overlay the melanophore pattern of the three dorsal blotches at the start of flexion and continue to exist until the end of flexion. The notochord, during preflexion, is sparsely pigmented with punctuate melanophores that become heavily stellate during flexion, covering almost the entire thickening of the myomeres. With this thickening during flexion, internal pigmentation appears on the ventral side along the notochord and above the gut, which become increasingly difficult to see as the external pigmentation develops. The gut is even, but lightly pigmented in preflexion larvae. This pigmentation pattern remains the same although individual melanophores expand and the pigmentation becomes heavy, concealing any internal pigmentation that might be present in and around the gut during flexion and later stages of the development. Pelvic fins become pigmented with melanophores during the postflexion stage and remain so through to the juvenile stage. The base of the pectoral fin becomes pigmented during preflexion stages.

Tail pigmentation

A cluster of melanophores appear on and around the notochord, anterior to the tip, during the preflexion stage and disappears at the end of flexion. Other than this cluster, the caudal peduncle and caudal fin remains free of melanophores.

DISCUSSION

The development of *Dagetichthys marginatus* larvae follows typical soleid development (Leis & Carson-Ewart 2000). Fin development as well as the lack of extraordinary rays and spines is characteristic. The anterior dorsal fin supports (pterygiophores and the proximal portion of rays) form a deep notch with the top of the snout and head of postflexion larvae. The eye migrates through this notch and the notch closes with increasing growth. This anterior extension of the dorsal hump on the head is however not common amongst soleids. The only other soleid species with a similar morphological feature is *Heteromycteris japonicus* (Ahlstrom *et al.* 1984).

All the larvae used for this description were laboratory reared from eggs, which according to Watson (1982) may result in heavier pigmentation. Laboratory reared larvae may also show slightly different meristic characteristics in comparison to those of wild caught individuals. Variable laboratory rearing conditions can manifest in those characteristics that are partially

controlled by environmental conditions, such as vertebral and fin ray counts (Hunter 1984). In this study however, fin ray and vertebral counts observed in laboratory reared larvae fell within the range for wild caught adult *Dagetichthys marginatus*; D 70 – 81, A 55 – 64, P 7, C 18, V 42 - 45 (Vachon *et al.* 2008).

Dagetichthys marginatus larvae are easily distinguished from other pleuronectiform larvae commonly found in temperate nearshore waters of South Africa. The larvae of the cynoglossid, *Cynoglossus capensis* (Brownell, 1979) are easily distinguishable from *D. marginatus* by four elongated anterior dorsal rays that only start disappearing in late postflexion stages. At this stage *C. capensis* is a left-eyed (sinistral) flatfish, while *D. marginatus* is right-eyed (dextral). The larvae of *Cynoglossus zanzibarensis*, another common cynoglossid found in coastal waters of South Africa, have not yet been described. Specimens lodged in the SAIAB collection were examined and are briefly described here. Larvae were lightly pigmented throughout development with two characteristic rows of evenly spaced melanophores along the dorsal and ventral body margin. The smallest preflexion larva measured (4.2 mm BL) had two elongated rays, unlike *C. capensis* with four, which start disappearing during the postflexion stages (7.8 mm BL) and are completely lost at the onset of eye migration (10.5 mm BL). Other characteristics that could be used to make a distinction between Cynoglossidae and *D. marginatus* are the higher meristic counts, a coiled gut and a single pelvic fin (Leis & Carson-Ewart 2000).

Except for *Arnoglossus capensis* and *Pseudorhombus arsius*, bothid larvae are not commonly found in ichthyoplankton samples in temperate coastal waters of South Africa (Strydom *pers. comm*.). The larvae of *A. capensis* have been partially described by Brownell (1979). Despite the paucity of descriptive information, *D. marginatus* can be distinguished from bothids by the presence of a continuous dorsal, caudal and anal fin membrane that remains fused until the fin rays ossify. Other notable bothid characteristics are the elongated dorsal fin rays during early larval stages and the fact that they are sinistral flatfish.

Soleids commonly encountered in temperate inshore waters in South Africa are *Heteromycteris capensis*, *Solea turbynei* (formerly *S. bleekeri*) and *Austroglossus pectoralis*. The larvae of *A. pectoralis* and *S. turbynei* are notably smaller than those of *D. marginatus*, reaching flexion at a size of 3.5 – 3.8 mm (Wood 2000) and ~ 3.5 – 3.9 mm (Strydom pers. comm.) respectively, in comparison to *D. marginatus* which range from 5 to 7.06 mm BL at

flexion. *A. pectoralis* and *H. capensis* larvae are light to moderately pigmented, *S. turbynei* larvae are moderate to heavily pigmented and *D. marginatus* larvae are heavily pigmented. Postflexion larvae of all these species can also be separated by fin ray counts. *Dagetichthys marginatus* can also be separated from *H. capensis* by the presence of the fused anal, caudal and dorsal fins. *Synapturichthys kleini* (Brownell 1979) has a very different melanophore arrangement to *D. marginatus*, although fin ray counts and sizes at different developmental stages are similar. *Synapturichthys kleini* have densely packed stellate melanophores scattered randomly over the body and finfold, while *D. marginatus* has a distinctive melanophore pattern with four characteristic melanophore blotches on the finfold.

The actual spawning habitat of *D. marginatus* has not been identified, mainly due to the lack of eggs, larvae and juveniles in shallow surf or nearshore plankton catches (Lasiak 1983, 1984, Strydom 2003, Watt-Pringle & Strydom 2003) and the lack of larval fish research in offshore waters of South Africa. Although there is no evidence to suggest a spawning migration given the prevalence of mature females intertidally, it is not unlikely among flatfish as Dagang *et al.* (1992) and Shuozeng (1995) showed this to be the case for most flatfish species in the Yellow Sea, China. Further research is required on soleid spawning strategies off temperate South Africa.

Chapter 4: Description of the histo-morphology development of the alimentary tract and specific activity of digestive enzymes for *Dagetichthys marginatus* (Soleidae) larvae.

INTRODUCTION

The absorption of nutrients from food by fish larvae is dependent on the specific activity of digestive enzymes present in the gut and is closely linked to the functional status of the digestive tract and the support organs (Martinez *et al.* 1999, Cahu & Zambonino Infante 2001, Kolkovski 2001, Gisbert *et al.* 2009). A comprehensive knowledge of the morpho-functional characteristics of the larval digestive tract at each developmental stage is therefore essential to predict the digestive capacity of larvae (Segner *et al.* 1994, Yúfera *et al.* 2000, Kolkovski 2001, Zambonino-Infante & Cahu 2001). This knowledge is crucial to determine appropriate weaning strategies (Jones *et al.* 1993, Person-Le Ruyet *et al*. 1993, Kolkovski *et al.* 1997a, b), as well as designing suitable weaning diets for different species (Kolkovski 2001).

The ontogeny of the digestive tract and associated organs during larval development has been described for a diverse group of commercially important marine finfish species, including turbot, *Psetta maxima* (Segner *et al.* 1994, Padrós & Crespo 1996); Senegal sole, *Solea senegalensis* (Sarasquete *et al.* 1996 & 2001, Ribeiro *et al.* 1999a); Atlantic cod, *Gadus morhua* (Kjørsvik *et al.* 1991, Morrison 1993); yellowtail kingfish, *Seriola lalandi* (Chen *et al.* 2006a); Japanese eel, *Anguilla japonica* (Kurokawa *et al.* 2004); gilthead seabream, *Sparus aurata* (Sarasquete *et al.* 1993a & 1995, Elbal *et al.* 2004); European seabass, *Dicentrarthus labrax* (García-Hernández *et al.* 2001); white seabream, *Diplodus sargus* (Ortiz-Delgado *et al.* 2003); common dentex, *Dentex* (Santamaría *et al.* 2004); brill, *Scophthalmus rhombus* (Hachero-Cruzado *et al.* 2009) and common pandora, *Pagellus erythrinus* (Micale *et al.* 2006). Moreover, Dabrowski (1984), Person-Le Ruyet (1989) and Zambonino-Infante & Cahu (2001) have described the important ontogenetic changes in the digestive tract of fish larvae. Sánchez-Amaya *et*

al. (2007) concluded that the basic mechanisms of organogenesis are similar in all teleosts, and differences are mainly in the relative timing of occurrence in ontogeny. This timing difference in organ development is generally related to the life history of a species, as well as abiotic and biotic factors such as water temperature or food availability (reviewed by Falk-Petersen 2005).

Enzymes are often used to give some indication to the digestive capacity of finfish larvae, and are often examined in support of a histological description of the digestive tract and the associated organs (Zambonino Infante & Cahu 2001). Enzyme activity studies in marine teleosts have been undertaken for various species, including *Solea senegalensis* (Ribeiro *et al.* 1999b), *Paralichthys olivaceus* (Bolasina *et al.* 2006), *Hippoglossus* (Gawlicka *et al.* 2000), *Scophthalmus maximus* (Tong *et al.* 2012), *Ompok bimaculatus* (Pradhan *et al.* 2012), *Limanda ferruginea* and *Pseudopleuronectes americanus* (Baglole *et al.* 1998); *Dicentrarchus labrax* (Zambonino-Infante & Cahu 1994), *Sparus aurata* (Moyano *et al.* 1996), *Seriola lalandi* (Chen *et al.* 2006b), *Thunnus albacores* (Alejandro Buentello *et al.* 2011), *Lutjanus guttatus* (Galaviz *et al.* 2012) and *Pagellus bogaraveo* (Ribeiro *et al.* 2005). These studies have detected activities for numerous digestive enzymes in fish larvae, but the activities of only a few are generally regarded as physiologically very important (see Chapter 5). These include the activities of alkaline and acid proteases, lipase, amylase and alkaline and acid phosphatase, and are therefore most often determined. The presence of these enzymes as well as their specific activity (referred to as an enzyme system) varies with ontogenetic development, within an individual and under different feeding regimes (Kolkovski 2001). Furthermore, digestive enzymes can be of endogenous nature; i.e. produced by the larval digestive system (Gisbert *et al.* 2004) or of an exogenous nature; i.e. introduced via live food (see Chapter 6) and enzymes produced by the intestinal microfauna or probiotics (Ringo & Birkbeck 1999, Ganguly & Prasad 2012, Ray *et al.* 2012).

There is currently no literature available on the functional development of the digestive system for the white margined sole, *Dagetichthys marginatus*. The chapter investigates and describes the major histological and enzymatic ontogenetic changes throughout

larval development of *D. marginatus*, especially those structures and enzymes related to the acquisition of nutrients.

MATERIALS AND METHODS

Larvae for this study were reared and collected in the marine hatchery at Rhodes University (Grahamstown, South Africa), and were not starved prior to collection of samples. Rearing protocols are described in Chapter 2. All the larvae were examined under a dissecting microscope to eliminate abnormally formed larvae from the sample and then euthanized with an overdose of 200 ppm Tricaine Methane Sulphonate or MS 222 (Alpharma, Animal Health Ltd, Fordingbridge, Hampshire). Larvae were randomly selected from all 12 rearing tanks at specific time intervals (days) representing developmental endpoints after hatching and include 3, 5, 8, 12, 15, 20, 25 and 30 days after hatching (dah). These developmental endpoints were established during the larval description (Chapter 3). Ten larvae were collected for histological sectioning (including a sample of 10 larvae immediately after hatching – 0 dah), and three pooled samples of between 10 to 50 larvae each were collected for the determination of enzyme activity (n=3) at each interval (Table 4.1). The difference in the number of larvae in each sample was due to size differences in larvae during development. An adequate number of larvae were collected at each time interval to ensure a large enough sample for crude enzyme extract to determine the specific activities of the digestive enzyme. All samples were stored in cross referenced 1.5 ml reaction vessels (Eppendorf tube).

Histology

Methods for histological preparation of larvae were adapted from Elbal *et al.* (2004) and Gisbert *et al.* (2004) and follow those described by Sumner & Sumner (1969), Bernard & Hodgson (1988), and Hinton (1990), and are briefly described here. Larvae were fixed in 10 % buffered formalin (pH 7.2) for 24 hours and then preserved in 70 % ethanol under zero light conditions. Prior to embedding the larvae in paraffin wax, they were dehydrated by passage through a series of ethanol solutions of increasing concentrations (80 %, 90

% and 2 x absolute ethanol) each for 10 minutes. Dehydration is essential prior to embedding the tissue in paraffin wax, as the wax will not penetrate tissue in the presence of water (Hinton 1990). To reduce and prevent cell shrinkage, the ethanol was then removed from the tissue by immersion in three solutions of 100% xylene at 40℃, each for 20 minutes. Impregnation of tissue with paraffin wax took place for an hour under a vacuum of 460 mm / Hg at 57 ℃ (Townsen & Mercer Vacuum Oven). The samples were then imbedded in paraffin wax in small moulds with pre-cast wax beds and left overnight to harden. These blocked samples were trimmed and mounted. Sections of 3 - 5 microns were cut with steel blades using a sliding microtome. The resulting ribbons were floated onto slides in a warm water bath (40 ℃) and attached using Haupt's adhesive. The slides were dried over night at 37 ℃. Serial transverse and sagittal sections were stained with Haematoxylin-eosin (H-E) for topographic observations, Periodic Acid Schiff reagent (PAS) to detect neutral mucosubstances, and Alcian blue (AB) at pH 2.5 to detect acid mucosubstances. The final sections were covered with DPX slide mountant and a coverslip. The slides were then examined and photographed under 40 - 400 x magnification in a random order to prevent possible interpretational bias associated with prior knowledge of collection time.

Enzyme activity determination

Pooled samples of 10 - 50 larvae were placed in a 1.5 ml Eppendorf tube and snap frozen in liquid nitrogen immediately after collection, and remained frozen at -80 ºC until crude enzyme extraction. All the larvae were blotted on drying towel to remove excess water prior to being frozen. Enzyme extraction was achieved by homogenising partially thawed larvae (4 ºC) in the 1.5 ml reaction vessel (approximately 35 mg. ml-1) with tevlon pestles in cold 50 mM Tris-HCL buffer, pH 7.5. This was followed by centrifugation (13500g for 15 min at 4 ºC). The supernatant was removed, placed in a new, appropriately referenced reaction vessel and put on ice. Assays for each enzyme (as described below) were done on the same day as extraction. Fresh chemicals were mixed daily and standard curves were also constructed every day. A substrate and extract blank was included for standardisation purposes.

Absorbances for all the assays were determined using the KC junior software and the 96 well spectrophotometer SPECTRAmax 190, Powerwave X, Biotek Instruments, USA. All reactions took place at a room temperature of 20 ºC and at the pH for optimal activity for each enzyme (see Chapter 5).

Table 4.1: Number of larvae collected at each time interval for determination of specific enzyme activity (n_{total} = 690 larvae)

Time (dah)	# Larvae per sample	Total # Larvae (n=3)
3	50	150
5	50	150
8	40	120
12	30	90
15	20	60
20	20	60
25	10	30
30	10	30

Alkaline proteases

The activity of alkaline proteases (mainly trypsin and chymotrypsin) was determined using the method of Anson (1938) and Walter (1984). The substrate was prepared by dissolving 0.2 g haemoglobin in 10 ml of 50 mM Tris-HCL buffer (pH 7.7). 400 µl of substrate and 100 µl of enzyme extract were pipetted into a reaction vessel, and incubated at room temperature for 30 minutes. Digestion of the substrate was stopped by the addition of 800 µl of Trichloro acetic acid (5g / 100 ml Milli-Q water), and left for 20 minutes to allow any precipitate to settle. Samples were centrifuged for 5 minutes at 13000*g*. Duplicate readings were pipetted into quartz microtitre plates, and the liberated product of l-tyrosine being read at 280 nm. Graded concentrations of l-tyrosine were used to make a standard curve. Specific enzyme activity was determined using the equation;

$$A = \frac{umol\ (l-tyrosine)\ liberated}{incubation\ time / ug\ protein_{sample} \times vol_{enzyme\ extract}}$$

Activity (A) was expressed as U / mg protein or umol of l-tyrosine liberated / minute / mg protein in sample.

α - Amylase (E.C. 3.2.1.1)

Amylase activity was determined using the Somogy-Nelson procedure described by Robyt & Whelman (1968). The substrate was made by dissolving 1 % soluble starch in a 50 mM Tris-HCL buffer (pH 7.7) by heating up the solution over a Bunsen burner. Milli-Q water was added to this substrate solution to account for evaporation during the heating process. Digestion started by combining 150 uL of the enzyme extract with 150 uL of the substrate and allowing the mixture to incubate for 5 min. 600 uL DNS (Dinitrosalicylic reagent – see Appendix A) reagent was added to stop the reaction and then incubated at 100 ºC in a heating block for 5 min. Duplicate samples was pipette into microtitre plates (Falcon flat bottom 96-well microplate) and absorbance measured at 540 nm. Maltose was used as a standard.

Specific enzyme activity was determined using the equation;

$$A = \frac{umol\ maltose\ liberated}{incubation\ time / ug\ protein_{sample} \times vol_{enzyme\ extract}}$$

Activity was expressed as umol maltose liberated / minute / mg protein in sample or U / mg protein.

Lipase (E.C. 3.1.1.-)

Lipase activity was measured according to Iijima *et al.* (1998). Lipases was activated by

incubating 40 ul of the enzyme extract with 600 ul of sodium cholate (5.2 mM) in 50 mM Tris-HCL buffer (pH 8.0) and 20 ul of 10 mM 2-methoxyethanol at room temperature for 15 minutes. 40 ul of the substrate, p-nitrophenyl myristate (10 mM p-nitrophenyl myristate dissolved in 100% ethanol) was added to the reaction vessel and allowed to digest for 2 hours at room temperature. The reaction was stopped by the addition of 800 ul acetone heptane mixture (5:2) and was then centrifuged at 13500g for 2 min at 4 ºC. Duplicate absorbance readings were then recorded at 405 nm for each sample.
Specific enzyme activity was determined using the equation;

$$A = \frac{umol\ (p-nitrophenol)\ liberated}{incubation\ time\ /_{ug\ protein_{sample}} \times vol_{enzyme\ extract}}$$

The standard curve was made from a stock solution of 5 mM *P*-nitrophenol in sodium cholate Tris-HCL buffer and 2-methoxyethanol in same ratios as per the assay.
Activity was expressed as umol *p*-nitrophenol liberated / minute / mg protein in sample or U / mg protein.

Phosphatase (E.C. 3.1.3.1)

Phosphatase activity was determined by combining 150 ul of the enzyme extract, 150 ul of 12 mM p-nitrophenyl phosphate (substrate) and 600 ul of 50 mM Tris-HCl buffer (pH 9.8) in an reaction vessel and allowing to incubate for 20 minutes. This procedure was adapted from Walter & Schuett (1974). The change in absorbance was recorded after 20 minutes at 405 nm.

Specific enzyme activity was determined using the equation;

$$A = \frac{umol\ (p-nitrophenol)\ liberated}{incubation\ time\ /_{ug\ protein_{sample}} \times vol_{enzyme\ extract}}$$

The standard curve was made from a stock solution of 5 mM *P*-nitrophenol in Tris-HCl

buffer. Activity was expressed as umol *p*-nitrophenol liberated / minute / mg protein in sample or U / mg protein.

Other enzymes

Pepsin or acid protease activity was determined using exactly the same assay method as for alkaline proteases, except that the Tris-HCl buffer was buffered to a pH of 2. Similarly, acid phosphatase was determined with the method for alkaline phosphatase described above, where the Tris-HCl buffer was buffered to a pH of 2.

Protein

The soluble protein content of the homogenates was analysed using Bradford's assay technique (Bradford 1976) with bovine serum albumin as a standard. In a microtitre plate, 250 µl of Bradford reagent (Coomassie Blue) was added to 5 µl of enzyme extract and allowed to incubate at room temperature for 5 minutes after which absorbance was read at 595 nm. Each sample was done in triplicate and the average taken as being the representative value for the sample.

RESULTS

Morphologically the digestive tract appeared as a straight undifferentiated tube laying dorsally to the yolk sac at hatching (0 dah), closed at the mouth and anus. The digestive tract Anlage increased in length throughout development over the next couple of days, showing a slight bend in the posterior region. The mouth and anus opened 3 dah at which point an intestinal loop was clearly visible in the mid and posterior intestine. Pigmentation around the gut region increased in intensity from this point onwards to a stage where any morphological description of the larvae gut under a microscope was impossible. During this period of increased pigmentation, there were no visible changes in the morphological arrangement of the digestive tract (Chapter 3).

The digestive tract was histologically undifferentiated along its entire length at hatching (0 dah), but developed rapidly up to the opening of the mouth and anus at 3 dah. Three constrictions separated the digestive tract into four different sections at mouth opening: the oesophagus, the posterior part of the foregut or stomach anlage, anterior intestine, and the posterior intestine (Figure 4.1). The first *Artemia* was also visible in the gut 3 dah. The oesophagus consisted of cubic epithelium surrounded by a muscular layer. The stomach anlage appears as a pouched shape extension immediately posterior to the oesophagus with a slight indent of the smooth muscle layer pointing to a constriction and a reduction of the lumen. Very little difference was detected in the cubic epithelium cells forming the stomach to that found in the oesophagus, other than the presence of large, centrally located, ellipsoid nuclei in the epithelium of the stomach in some histological sections. The anterior and posterior intestine also showed some separation with the initiation of a valve. Both these sections of the intestine were lined with columnar epithelium with basophilic nuclei and prominent nucleolus. The anterior intestine showed more development than the posterior with the presence of folds in the lumen, a brush border, dense granules and more basophilic cytoplasm with apical vesicles.

From 3 dah onwards, there was very little change regarding the arrangement of the digestive tract and the support organs other than an increase in length / size until 15 dah. This supports morphological observations made of the gut. This period corresponded to an increase in the number of vesicles, dense granules and also thickening of the brush border in the anterior intestine. Large numbers of supra-nuclear vacuoles was present in the enterocytes along the anterior portion of the gut (Figure 4.2). These persisted until 15 dah, corresponding to the onset of flexion and the start of metamorphosis. Small groups of vacuoles were also visible in the posterior part of the gut during this period and persisted until 30 dah. The increase in mucosal folding of the gut continued throughout development to increase the absorption surface and the brush border appeared PAS positive.

The appearance of the first mucous cells/goblet cells (Figure 4.2), which stained positive with the PAS for neutral mucosubstances, in the oesophagus occurred 5 dah

together with longitudinal folds. The number of mucous cells increased throughout development, while the longitudinal folds increased in size and there was a thickening of the muscle layer around the oesophagus. At 5 dah the first mucous cells was also visible in the buccal cavity and the anterior part of intestine. The number of mucous cells increased throughout development. The presence of acid mucosubstances could not be detected with the Alcian blue stain.

Metamorphosis started at 16 dah and the digestive tract contorted to reach the final adult like positioning of the organs. This is a gradual process until the larvae settle at about 30 dah and eye migration is complete. At 16 dah the visceral cavity had a similar shape and arrangement as at 3 dah, with the stomach still poorly developed but increasing in size and showing deep mucosa rugae. During contortion, the anterior intestine folds over itself to move from an anterior to posterior position in the abdominal cavity and the posterior intestine from a posterior to an anterior position. This process is completed around 30 dah at which point the larvae had acquired a benthic way of life. The visceral cavity showed a triangular shape which was wider near the head, becoming thinner towards the posterior part. The stomach appeared as a large sac like structure occupying the wider part of the cavity. At this age the epithelial cells of the stomach mucosa had a wider apical portion than basal.

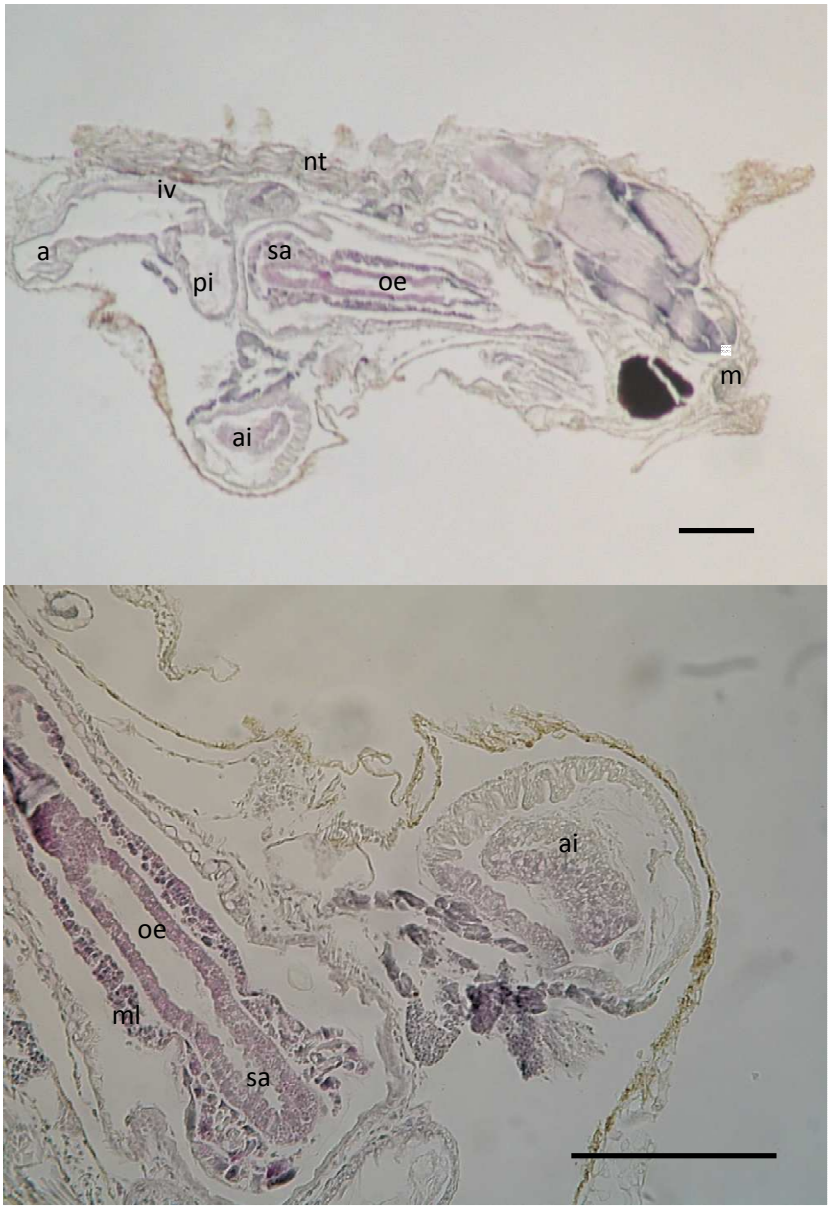


Figure 4.1: Histo-morphological sections (4 µm) of *Dagetichthys marginatus* larvae at 5 dah. Bar = 200 µm. a – anus; ai – anterior intestine; oe – oesophagus; ie – intestinal epithelium; iv – intestinal valve; m – mouth; ml – muscular layer; nt – notochord; pi – posterior intestine; sa – stomach.

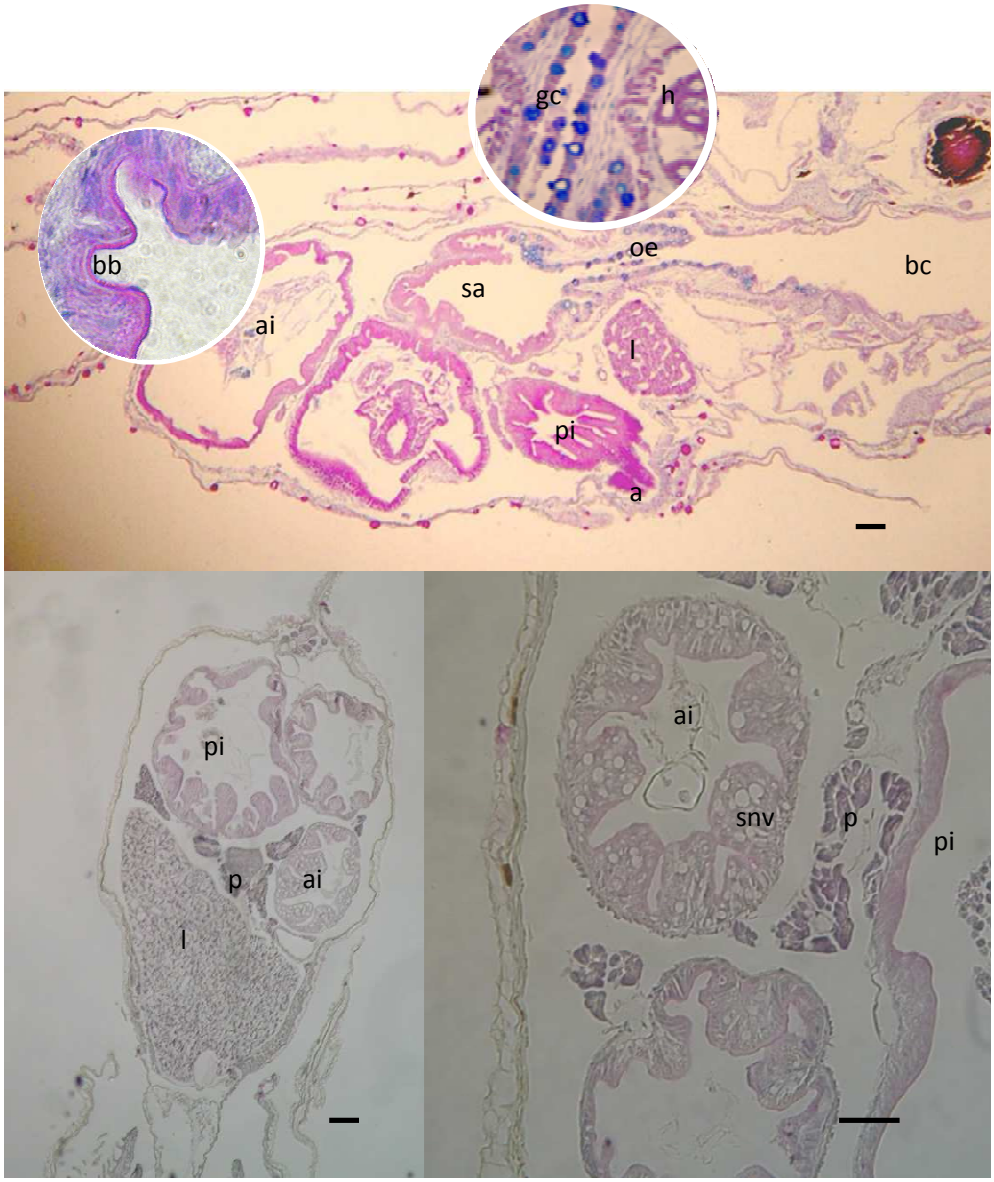


Figure 4.2: Histo-morphological sections (4 µm) of *Dagetichthys marginatus* larvae at 20 dah. Bar = 200 µm. a – anus; ai – anterior intestine; bb – brush border; bc – buccal cavity; gc – goblet cells; h – hepatocytes; l – liver; oe – oesophagus; p – pancreas; pi – posterior intestine; sa – stomach Anlage; snv – supranuclear vacuoles.

Table 4.2: Specific digestive activities (±SD) for alkaline proteases, lipase, alkaline phosphatase (expressed as mU/mg protein) and amylase (U/mg protein) in Dagetichthys marginatus throughout larval development. (n=3)

	Larval Age (dah)							
	3	5	8	12	15	20	25	30
Lipase	13.2±0.3	4.7±0.1	7.1±0.3	9.4±0.1	12.9±0.3	17.1±0.5	22.4±0.3	32.6±0.4
Amylase	5.0±0.7	12.6±0.8	7.8±0.5	4.7±0.4	3.8±0.1	2.9±0.2	2.1±0.1	1.8±0.2
Alkaline Phosphatase	28.0±6.0	48.3±3.5	26.3±4.5	63.7±3.8	43.3±1.5	102.7±5.0	605.3±5.7	1114.3±13.9

The specific enzyme activity at different ontogenetic stages are summarised in Table 4.2. It is clear from these results that there is a general pattern of an increase in activity with development up to 30 dah, except for amylase that showed a steady decline in activity up to completion of metamorphosis. Alkaline proteases had a specific activity ranging between 66.3 ± 2.1 at 5 dah and 145.7 ± 5.0 mU / mg protein at 30 dah (Figure 4.3). Similarly, lipase showed a minimum activity of 4.7±0.1 at 5 dah and maximum activity of 32.6 ± 0.4 mU / mg protein at 30 dah (Figure 4.4). Alkaline phosphatase showed the lowest activity at 8 dah of 26.3±4.5 mU / mg protein, while a sudden order of magnitude increase took place at 20 dah and which peaked at 30 dah with an activity of 1114.3 ± 13.9 mU / mg protein (Figure 4.6). Amylase, however, had a maximum activity of 12.6 ± 0.8 U / mg protein at 5 dah and minimum activity of 1.8±0.2 U / mg protein at 30 dah (Figure 4.5). No acidic proteases or phosphatase were detected throughout the 30 days of ontogenetic development.

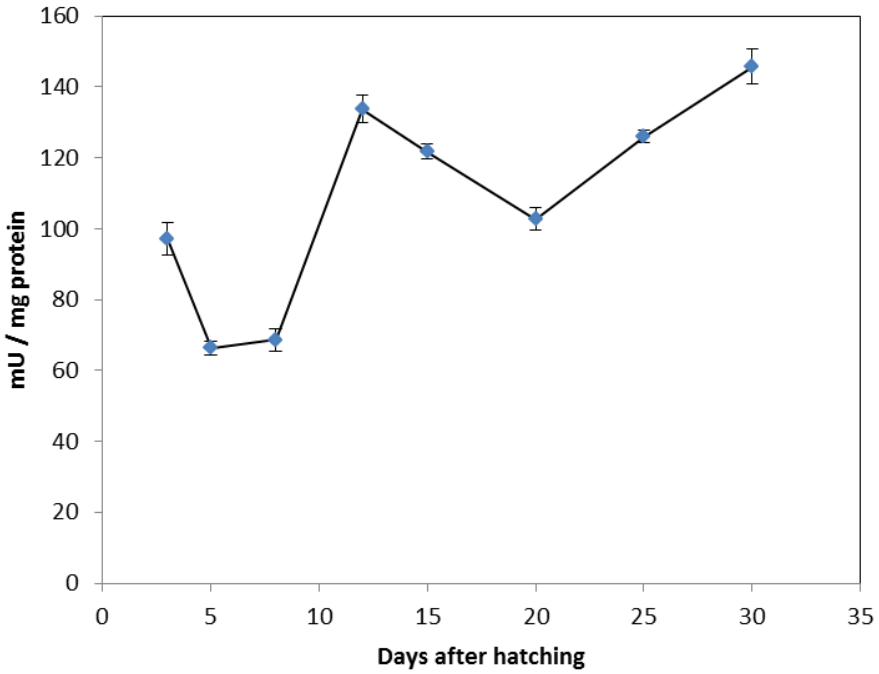


Figure 4.3: Specific activity of alkaline proteases during the first 30 dah for *Dagetichthys marginatus* larvae. Error bars represent standard deviation of the mean (n=3)

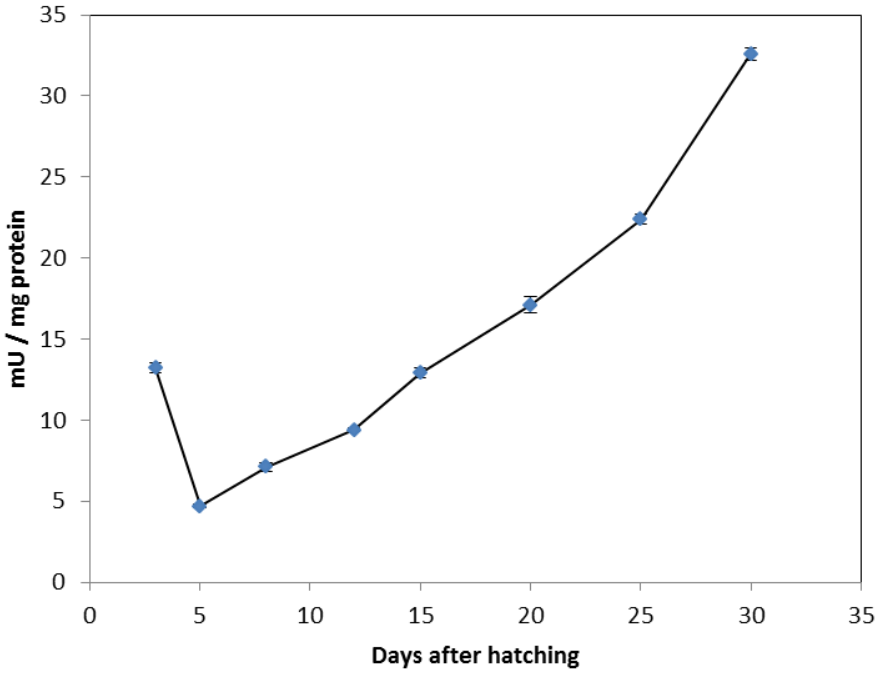


Figure 4.4: Specific activity of lipase during the first 30 dah for *Dagetichthys marginatus* larvae. Error bars represent standard deviation of the mean (n=3).

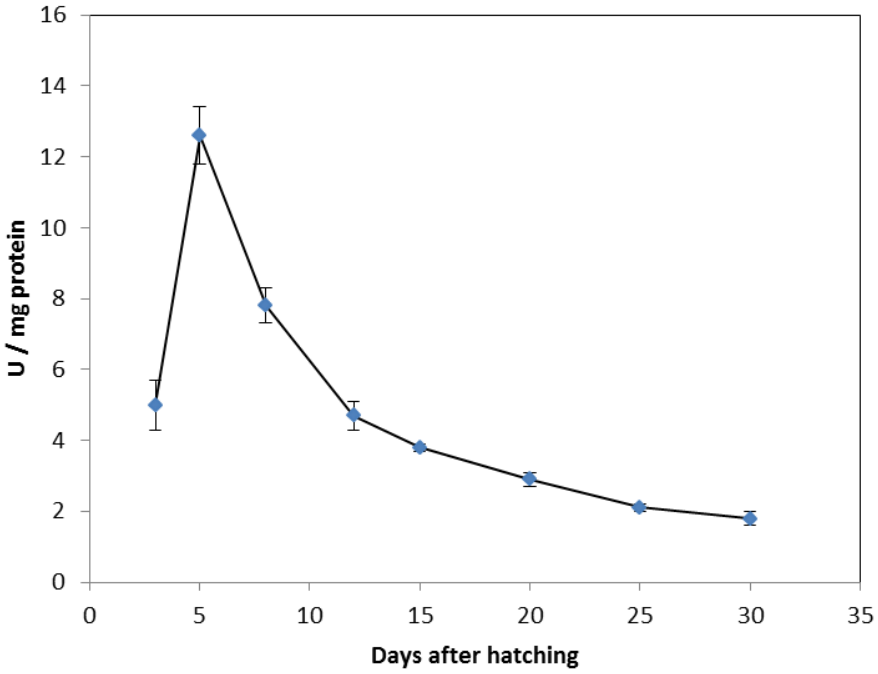


Figure 4.5: Specific activity of amylase during the first 30 dah for *Dagetichthys marginatus* larvae. Error bars represent standard deviation of the mean (n=3).

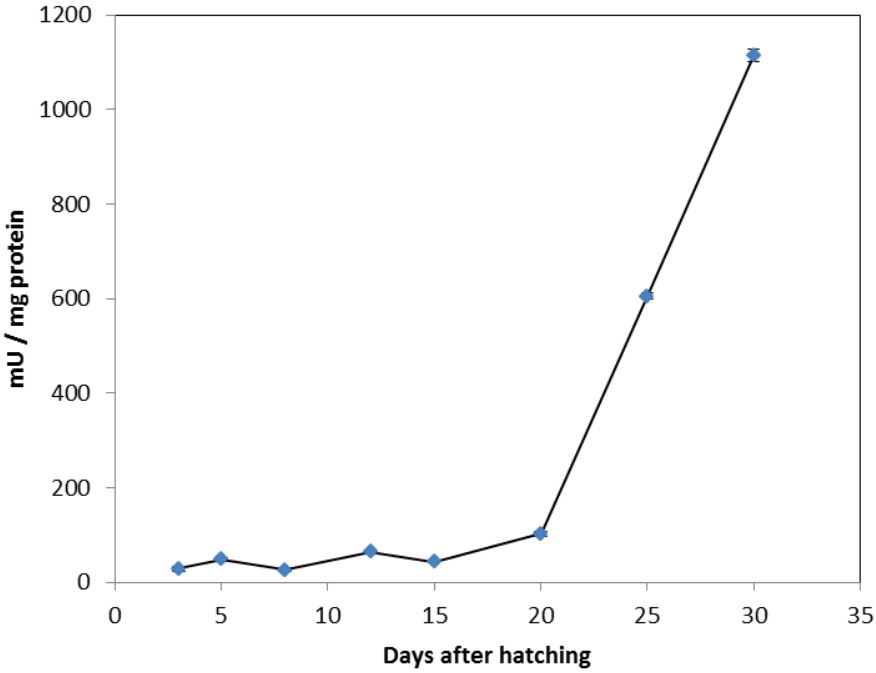


Figure 4.6: Specific activity of phosphatase during the first 30 dah for *Dagetichthys marginatus* larvae. Error bars represent standard deviation of the mean (n=3).

DISCUSSION

This study provides the first description of the ontogeny of the digestive tract of *Dagetichthys marginatus*. The general observed pattern of ontogenetic development in *Dagetichthys marginatus* was however similar to what is has been found for other teleosts (Dabrowski 1984, Person-Le Ruyet 1989, Zambonino Infante & Cahu 2001). At 3 dah *Dagetichthys marginatus* larvae were sufficiently developed for successful first feeding, and *Artemia* was detected in the gut. Intense organogenesis occurred during the first three days (yolk-sac stage) in preparation for exogenous feeding. All the major organs of the digestive system were developed at this point. From first feeding (3 dah) the digestive tract and associated organs only increase in size and complexity, although major positional shifts of the digestive system takes place during settlement. This is a common characteristic shared by all pleuronectids (Cousin & Baudin-Laurencin 1985, Boulhic & Gabaudan 1992, Bisbal & Bengston 1995, Sarasquete *et al.* 1996,

Baglole *et al.* 1997, Ribeiro *et al.* 1999a).

Vacuoles were present in the enterocytes of the anterior intestine after two days of exogenous feeding in *D. marginatus*. This has also been found in various other species (Stroband *et al.* 1979, Cousin & Baudin-Laurencin 1985, Boulhic & Gabaudan 1992). It has been suggested that the presence of vacuoles signify lipid absorption and temporary storage within the enterocytes (Stroband & Dabrowski 1979, Watanabe & Sawada 1985, Deplano *et al*. 1991b, Sarasquete *et al.* 1995, Diaz *et al.* 2002, Chen *et al.* 2006a). This is most likely caused by an inability to mobilize lipids during these early developmental stages (Kjørsvik *et al.* 1991, Loewe & Eckmann 1988). A peak in the presence of lipid vacuoles was observed between day 6 and 15. With the onset of flexion there was a noticeable reduction in lipid vacuoles. This is largely due to the rapid development of the enterocytes with an increased ability for lipoprotein synthesis (Deplano *et al.* 1991b).

Supra-nuclear inclusions were also observed in the posterior intestine of *D. marginatus* larvae once feeding began. These inclusions persist until the end of the 30 day description period, although they decrease in number from 12 dah onwards. This decrease corresponds to a sudden increase in alkaline protease activity. These supranuclear inclusions are the result of pinocytosis of proteins as was demonstrated with the use of peroxidase (Stroband *et al.* 1979, Stroband & Kroon 1981, Watanabe 1984, Georgoupoulou *et al.* 1986, Govoni *et al.* 1986) and have been described in larvae and adults of various other teleosts (Govoni *et al.* 1986, Deplano *et al.* 1991a, Boulhic & Gabaudan 1992, Segner *et al.* 1994, Sarasquete *et al.* 1995, Elbal *et al.* 2004, Gisbert *et al.* 2004, Chen *et al.* 2006a). O'Connell (1981) also showed that these inclusions can contain lipids, but no evidence for this was found with the staining method used in this study.

Mucous secreting goblet cells were either absent or scarce at mouth opening in the oesophagus and buccopharynx in *D. marginatus*, and only appeared at 5 dah. A similar pattern has also been found in species such as gilthead seabream, *Sparus aurata*

(Sarasquete *et al.* 1995, Calzada *et al.* 1997); haddock, *Melanogrammus aeglefinus* (Hamlin *et al.* 2000); yellowtail kingfish, *Seriola lalandi* (Chen *et al.* 2006a) and California halibut, *Paralichthys californicus* (Gisbert *et al.* 2004), in which wall abrasion or the desquamation of the oesophageal epithelium and the absence of mucous secretion may be observed (Yúfera & Darias 2007b). Wall abrasion may also promote bacterial infections under stressful conditions (Gisbert *et al.* 2004). On the other hand, mucos cells have been detected at first feeding in Senegal sole, *Solea senegalensis* (Ribeiro *et al.* 1999a); Dover sole, *Solea solea* (Boulhic & Gabaudan 1992); summer flounder, *Paralichthys dentatus* (Bisbal & Bengtson 1995); yellowtail flounder, *Limanda ferruginea* (Baglole *et al.* 1997) and clownfish, *Amphiprion percula* (Gordon & Hecht 2002).

Throughout the ontogenetic development of *D. marginatus* larvae in this study, digestion occurred in an alkaline environment. Specific activity of enzymes in *D. marginatus* generally increased with the development of the digestive tract and its associated organs. Amylase, however shows a general decreasing pattern throughout development. The initial high levels could be as a result of exogenous enzymes originating from *Artemia* (Chapter 6). The presence of alkaline protease, lipase and amylase activity imply that larvae can digest an exogenous food source like *Artemia*, while strong alkaline phosphatase from 20 dah onwards suggests an increased capacity of the larvae to absorb nutrients (Gawlicka *et al.* 1995). The activity of pancreatic enzymes (trypsin, lipases and amylase) has been biochemically detected at first feeding and even before the mouth opening in many marine fish (Zambonino- Infante & Cahu 1994, Oozeki & Bailey 1995, Moyano *et al.* 1996, Martínez *et al.* 1999, Ribeiro *et al.* 1999b, Hoenhe-Reitan *et al.* 2001, Ma *et al.* 2001, Zambonino-Infante & Cahu 2001, Cara *et al.* 2003, Ma *et al.* 2005, Alvarez-Gonzalez *et al.* 2006, Bolasina *et al.* 2006, Chen *et al.* 2006b).

The last phase of digestive development, prior to metamorphosis, is the proliferation of gastric glands in the stomach. This is accompanied with acid digestion by pepsin (Tanaka *et al.* 1972, Stroband & Dabrowski 1979, Govoni *et al.* 1986, and Baragi &

Lovell 1986, Sarasquete *et al.* 1993b) and is normally the stage at which weaning onto inert diets can occur successfully. *Dagetichthys marginatus* show no pepsin activity throughout the first 30 dah. This corresponds to the absence of gastric glands or acidic mucosubstances in histo-chemical sections of the stomach, which is fully delineated at 12 dah. Clark *et al.* (1986) also found an absence of pepsin activity in *Solea solea* until 200 dah, although gastric glands were observed by 27 dah (Ribeiro *et al.* 1999a). It is noteworthy that gastric digestion develops early in most cultured sparids (Ortiz-Delgado *et al.* 2003, Roo *et al.* 1999), with exceptions like *S. aurata* (Sarasquete *et al.* 2001).

Physiological parameters like gut pH and relative enzyme activities are strongly correlated to the developments in the gut. With respect to gut pH levels *Solea senegalensis* shows a different pattern to most other farmed species. In general, there is a drop in pH values from around 7 to 4 throughout development and this is correlated to the appearance of gastric glands, specifically in the stomach. This pattern has been observed in *Sparus aurata* (Elbal & Agulleiro 1986), *Pagrus pagrus* (Darias *et al.* 2005), *Paralichthys olivaceus* (Rønnestad *et al*. 2000), *Lates calcarifer* (Walford & Lam 1993) and *Psetta maxima* (Hoehne-Reitan *et al.* 2001). Yúfera & Darias (2007a) found that the gastric pH of *S. senegalensis* never drops below 6, even in adult fish, despite the appearance of gastric glands.

This baseline data on the functional status of the alimentary tract throughout development is crucial for the successful larviculture of *Dagetichthys marginatus*. It can further be useful to detect morphological and physiological adaptations in future research when varied feeding protocols and feed ingredients are considered (Kolkovski 2001, Bonaldo *et al.* 2006, Escaffrea *et al.* 2007, Papadakis *et al.* 2009). This study has shown that *D. marginatus* can successfully feed on exogenous food sources like *Artemia* 3 dah. Furthermore, the lack of a completely developed stomach and an assumed inability for acid digestion points to a lack in capacity to digest complex artificial food prior to 30 dah. Artificial diets containing protein hydrolysates (Day *et al.* 1997 & 1999) has shown to work for other soleids (Chapter 1) and should also be the case as early as 20 dah with *D. marginatus*, where pinocytosis and high levels of

alkaline phosphatase should enable the absorption of nutrient from diets containing protein hydrolysates.

Chapter 5: Characterisation of alkaline digestive enzyme activity at different pH levels for larvae of three warm temperate marine teleosts.

INTRODUCTION

Nutrition is an important aspect for consideration in larval rearing (Conceição et al. 2010). This is apparent for a host of economic and biological reasons, all of which are well documented (Chapter 1). Understanding the digestion of larval feeds, or the mechanism by which nutrients are acquired for growth and metabolic processes, is therefore essential to solve problems commonly associated with current rearing techniques (Chapter 1). As digestive enzymes are primarily responsible for the hydrolysis of feed into available nutrients, it is clear that studying digestive enzymes has a wide range of potential interest in aquaculture (Fernandez et al. 2001). Studies measuring enzyme activity, either qualitatively and quantitatively are often used as a tool to understand basic digestive processes, predicting for example, the correct time of weaning and feed formulation (Chapter 4 & 6).

In fish larvae, the potential capacity of the intestinal digestive enzymes to hydrolyse a substrate is set in DNA coding (Cahu & Zambonino Infante 2001, Kolkovski 2001). Measuring enzyme activity in digestion studies is however by itself not a fair representative of actual enzyme activity in the gut, as the effect of environmental parameters on enzyme activity is ignored or not clearly validated. This presents a clear problem when comparisons between species are made, limiting the results for comparative purposes. As this problem form the fundamental reason for completing this chapter, the example below is crucial to understand the problem, the modelling methods used and the interpretation of results from this study.

Considering only two of a multitude of studies, the pH's used to determine the enzyme activities for *Solea senegalensis* by Martinez *et al.* (1999) and *Hippoglossus hippoglossus* by Gawlicka *et al.* (2000) are clearly different. Martinez *et al.* (1999) calculate enzyme activities for all the digestive enzymes at a pH7.5, while Gawlicka *et al.* (2000) determine enzyme activity at a pH ranging from 7 for amylase to 7.8 for lipase.

Any comparison of enzyme activity between these two species will therefore be questionable, and consequently also the use of digestibility coefficients that might, for example be used for feed formulation. No attempts have been made to quantify the extent of this problem in aquaculture.

Historical research on the effect of environmental conditions on enzyme activity comes from enzyme kinetic - and enzyme characterisation studies. Although both contribute to our understanding of the effect of environmental parameters on enzyme activity, their intended outcome and application tend to be vastly different. Enzyme kinetic studies most often deal with biotechnological applications or the industrial use of fish enzymes, in for example the food industry (Shahidi & Kamil 2001). Studies on industrial applications deal with optimising the environmental conditions under which the enzyme will achieve maximum activity. Enzyme kinetic studies are not often done in aquaculture studies, and although the reasons for this is not well defined, one could argue that it is as a result of the complexity of enzyme kinetics models and the subsequent loss of applicability to a biological system or the organism (Hofmeyer & Comish-Bowden 1997). In most aquaculture studies, as is the case with the majority of the literature referenced below, enzyme characterisation is more commonly used to establish the effect of environmental parameters on enzyme activity relative to the maximum enzyme activity obtained under optimal environmental conditions for the fish or fish larvae.

The majority of the enzyme kinetic and characterization studies on digestive fish enzymes focus on proteases. This is mainly due to the importance of protein and amino acids as the primary energy source in fish larvae (Fyhn 1989, Haard 1992, Dias *et al.* 1998, Moyano *et al.* 1998, Rønnestad *et al.* 1999), in synthesizing body protein to ensure normal growth and development (Tacon & Cowey 1985, Tacon 1992, Martinez-Montano & Lazo 2012) and the role of proteases, more specifically trypsin, in activating other pancreatic digestive enzyme from their inactive zymogen forms (Corring 1980, Hjelmeland *et al.* 1984). Shahidi & Kamil (2001) reviewed the literature on the characterisation and kinetics of proteases in fish. More recent studies on proteases in

finfish include investigations on walleye pollock, *Theragra chalcogramma* (Kishimura *et al.* 2008); red drum, *Sciaenops ocellatus* (Lazo *et al.* 2007); jacopever, *Sebastes schlegelii* and elkhorn sculpin, *Alcichthys alcicornis* (Kishimura *et al.* 2007); skipjack tuna, *Katsuwonus pelamis* (Klomklao *et al.* 2007); Orange-spotted grouper, *Epinephelus coioides* (Liu *et al.* 2012) and the Asian bony tongue, *Scleropages formosus* (Natalia *et al.* 2004). While all the aforementioned literature calculate the optimal pH for highest enzyme activity in a particular species, none make any comparison between the different species to give any indication on the inter species similarity in enzyme characterisation.

Investigations into the characterisation of other digestive enzymes have been restricted to the characterisation of lipases in species such as anchovy (*Engraulis mordax*), striped bass (*Morone saxatilis*) and pink salmon (*Oncorhynhcus gorbuscha*) (Patton *et al.* 1975, Leger 1985); cod, *Gadus morhua* (Gjellesvik *et al.* 1989); red sea bream, Pagrus *major* (Iijima et *al.* 1998) and red drum, *Sciaenops ocellatus* (Lazo *et al.* 2007), and studies on α-amylase in *Pagrus pagrus*, *Pagellus erythrinus*, *P. bogaraveo*, *Boops boops* and *Diplodus annularis* (Fernández *et al.* 2001). This literature is further limited mainly to larger fish. Very little work has been undertaken on enzyme characterisation and kinetics in larval fish. The most important studies are those by Applebaum *et al.* 2001, Lazo *et al.* 2007).

The major environmental parameters that affect enzyme activity in the digestive system of fish are either physiologically (pH and osmolality or ionic strength) or environmentally (temperature) controlled (Garrett & Grisham 1995). The temperature at which maximum enzyme activity is most often achieved is in excess of 50 ºC. This is much higher than the normal environmental temperatures experienced by fish (Fernández *et al.* 2001, Lazo *et al.* 2007, Kishimura *et al.* 2008). Conducting digestion trials at the temperature for optimal enzyme activity therefore has very little biological applicability. For the purpose of this study, it was decided to standardise the temperature to 20 ºC. This temperature approximates the average temperature experienced by warm temperate marine fish species in South Africa, and is also a

practical temperature to maintain when conducting enzyme assays in a laboratory environment.

The objective of this investigation was to establish baseline knowledge, which was required to address those experiments discussed in Chapters 4 and 6. To this end, this paper has two aims. Firstly, to establish a standard set of condition under which to determine alkaline enzyme activity for the sole, *Dagetichthys marginatus* (Chapter 4), as there is no universally proposed and/or accepted standard set of environmental parameters under which qualitative and quantitative enzyme activity in fish must be determined (Tipton 2002). Secondly, characterisation of digestive enzymes is crucial for the development of a robust *in vitro* digestion model (Alarcón *et al.* 1999) against which *D. marginatus* could be tested (Chapter 6).

To achieve these aims, the larvae of three warm temperate species, from the same biogeographical region as *D. marginatus*, namely the strepie (*Sarpa salpa,* Sparidae), blacktail (*Diplodus sargus capensis,* Sparidae) and dusky kob (*Argyrosomus japonicus,* Sciaenidae) were used for alkaline enzyme characterisation. The use of these three species was necessitated due to the large amount of larval material required to run characterisation studies and the limited number of sole larvae available.

MATERIALS AND METHODS

Larvae of *S. salpa*, *D. sargus capensis* and *A. japonicus* ranging in length between 2.8 and 17.6 mm were used in this study. *S. salpa* and *D. sargus capensis* larvae were collected from the wild along the coast at Port Alfred, South Africa (33º36'42.35"S; 26º53'23.02"E) throughout the year. This was achieved by seining the surf zone with a modified larval beach-seine net. The larvae were carefully washed out from the cod-end of the net with 5 µm filtered sea water into a 20 l bucket, which was supplied with an air stone and returned to the Department of Ichthyology's Marine Laboratory, Port Alfred. In the laboratory only live larvae were transferred to Petri dishes using a transparent glass beaker, identified, rinsed in fresh water, dried on blotting paper, bottled and snap-

frozen in liquid nitrogen. Bottles were carefully labelled for later reference. Larvae of *A. japonicus* were obtained from Espadon Marine (Pty) Ltd., a commercial enterprise currently farming this species. These were collected from rearing tanks at pre- and post-flexion stages and snap frozen as described above in liquid nitrogen. The frozen larvae were then stored in a cryo-flask filled with liquid nitrogen at -80^{o}C until enzyme extraction.

In the lab, larvae were pooled to obtain sufficient biological material per sample to complete each assays (Table 5.1) and ensure an extraction at a dilution of 35 mg larval material to 1 ml buffer. Nine samples were assayed, three for each species. The three samples for each species were made up of distinct size classes, making them independent replicates (Table 5.1). This was based on the assumption that quantitative and qualitative enzyme activities change throughout ontogenetic development (see Chapter 4).

Table 5.1: The size range and larval stage for each sample assayed.

nple name	**val stage**	**e range (mm)**
argus 1	-flexion	– 4.8
argus 2	t-flexion	– 9.8
argus 3	t-flexion	) – 13.6
alpa 1	-flexion	– 4.5
alpa 2	t-flexion	– 8.9
alpa 3	t-flexion	. – 17.6
aponicus 1	-flexion	– 3.0
aponicus 2	-flexion	– 5.7
aponicus 3	t-flexion	– 11.3

Enzyme extractions and assays for alkaline proteases, lipase, amylase and alkaline phosphatase on each sample were done as outlined in Chapter 4, unless otherwise stated. Enzyme activity was expressed as a relative measure (%) and not as an absolute value of product liberated/min/mg protein. Relative enzyme activity at different

pH values was tested by replacing the Tris - buffer used in Chapter 4 in each assay with a universal buffer, buffering in the pH range of 2 to 12. The pH range used for alkaline proteases, lipase, amylase and phosphatase was 6-11, 6-10, 2-12 and 2-12 respectively. The protracted pH range tested for alkaline proteases and lipase was due to substrate instability at extreme pH's (Gjellesvik *et al.* 1989). The interval between pH values tested was smaller around the hypothesised optimal pH for maximum enzyme activity to ensure better amplification and more predictive power.

Each sample was assayed in triplicate for each enzyme and the average taken as being representative of the sample. Further to this, a substrate blank and an enzyme blank (samples where either the substrate or enzyme extract was not added to the assays) was done for each sample, to elucidate between the contribution of the substrate-buffer solution and enzyme extract on the absorbance readings from the contribution resulting in the hydrolysis of the substrate by the enzyme. The true change in absorbance due to enzyme activity was then calculated by subtracting the blanks from the absorbance reading for each sample assay.

Data analysis and modelling

The absorbance readings for each sample were plotted against the natural logged pH value as the independent variable. The normal, skewed normal and Gamma distribution plots were fit to the observed data as the best supported models due to the normal distributive pattern of this data.

Normal distribution plot

$$Abs = {e^{-(pHmax - pH)^2}}/{2\sigma^2}$$

Skew normal distribution plot

$$Abs = {e^{-(pHmax-pH)^2}}/{2\sigma^2}\left\{1-\frac{\alpha\sigma^{3/2}}{2}\left[\frac{pHmax-pH}{\sigma}-\frac{(pHmax-pH)^2}{3\sigma^3}\right]\right\}$$

Gamma distribution plot

$$Abs = \left({pH\,max}/{pH}\right)^{pH/p}.e^{(pH-pH\,max)/p}$$

Where

$$p = 0.5\left[\sqrt{pH^2+4\sigma^2}-pH\right]$$

The dependant variable *Abs* is the absorbance at a specific *pH*. Absorbance is directly linked the enzyme activity, so higher absorbance readings indicate higher enzyme activities. Furthermore, *pH max* is the *pH* at which enzyme activity is predicted to be the highest, while σ describes the shape or slope of the bell shaped curve.

The two parameters used in the models are very relevant in answering the specific question posed by this chapter. The *pH max* represents the best pH to use when enzyme activity is determined as a surrogate for the digestibility coefficient, as is most often done. The *σ* will indicate how quickly enzyme activity drops at pH's around the optimal pH, or *pH max*. This value is important as it can be used to determine how comparable enzyme activity results between species are, especially considering the example used in the introduction where the enzyme activity was determined at different pH's.

The three models were fitted to the data and the sum of squares minimized. The Akaike Information Criterion (AIC) statistic, considering both the fit and complexity of the model, was then calculated according to Johnson and Omland (2004) and the model

with the lowest AIC statistic was chosen as being representative for that enzyme.

$$AIC = -2\ln(L) + 2p$$

; where *L* is the maximized value of the likelihood function for the estimated model, and *p* is the number of model parameters.

The full model was then solved for each of the nine samples using the selected model from the AIC statistic for each enzyme. This was achieved by minimizing the sum of squares in errors. A likelihood ratio test was then used to determine if there was a significant difference in the model parameters for each enzyme or different size classes for each species, combining all the samples into a reduced model. The log-likelihood of the full and reduced model was then compared.

$$\lambda = 2(lnL_{full} - lnL_{reduced}) \approx X^2(df = \Delta p)$$

; where L_{full} and $L_{reduced}$ are the maximized value of the likelihood function for the full and reduced estimated model respectively, and p is change in the number of parameters from the full and the reduced estimated models.

If there was no significance difference in the model parameters between the full and reduced model, the solution of the reduced model was accepted for that enzyme.

RESULTS

A summary of model selection for each enzyme is presented in Table 5.2. The Normal plot, Skewed Normal plot, Normal Plot and Gamma plot were selected for alkaline proteases, lipase, amylase and phosphatase respectively. This selection was based on the lower average AIC statistic for each enzyme.

Alkaline Proteases

The likelihood test showed a significant difference between the full and reduced model

for alkaline proteases when all nine samples are included in the reduced model ($A = 28.1$, $p < 0.005$ – Table 5.3). This was due to significant difference in the reduced model for the three *S. salpa* samples ($A = 8.5$, $p = 0.01$), as well as the reduced model for all the *D. sargus capensis* samples when tested against the *A. japonicas* samples ($A = 19.1$, $p < 0.005$). The reduced model for the *D. sargus capensis* samples ($A = 2.4$, $p = 0.30$) and the *A. japonicus* samples ($A = 1.1$, $p = 0.59$) showed no difference.

Table 5.2: Summary of the AIC results that were used for model selection. The average AIC stat and the number of samples showing a best fit to a particular model is given for each of the enzymes.

	Proteases		Lipase		Amylase		Phosphatase	
	Average	#	Average	#	Average	#	Average	#
Normal plot	**60,4**	**4**	33,8	0	121,3	1	**80,6**	**9**
Skewed normal plot	67,2	1	**32**	**9**	120,5	0	86,6	0
Gamma plot	60,9	4	33,7	0	**117,2**	**8**	82,6	0

Numbers in bold indicate best model for each enzyme

Table 5.3: Results of the likelihood test for alkaline proteases. The A - statistic and p - value of the reduced models for the different samples and model parameters (σ and pH max) are given.

	All parameters equal					
	A	p	A	p	A	p
All species	20.9	<0.005*	0.4	0.55	28.1	<0.005*
D. sargus	1.3	0.26	0.4	0.53	2.4	0.30
S. salpa	7.1	<0.05*	0.3	0.58	8.5	0.01*
A. japonicus	0.8	0.37	0.1	0.75	1.1	0.59
D. sargus vs *A. japonicus*	13.3	<0.005*	0.2	0.75	19.1	<0.005*

(* denotes significant differences)

Based on the results from the likelihood tests (Table 5.3), it was accepted that the samples of *D. sargus capensis* and *A. japonicas* can be presented by a single reduced model each, while the full models for each S*. Salpa* sample were maintained. The parameters and the Sum of Squares for the errors (SSe) for each of the above models are summarized in Table 5.4. Figure 5.1 shows the observed relative enzyme activity values at different pH values as well as the values predicted from the normal plot models for *D. sargus capensis*, *A. japonicas* and the three *S. salpa* samples.

Considering Table 5.3, it was clear that the differences detected in the full and reduced models for the nine samples was as a result of differences in the slope (σ) of the curve, as there was no difference in the predicted pH max ($A = 0.4$, $p = 0.55$) between any of the samples in the reduced model (pH max equal). The optimal pH for maximum alkaline proteases activity was thus accepted to be the same for all nine samples at a predicted pH max value of 7.67 (Table 5.7).The differences detected between the *S. salpa* samples ($A = 7.1$, $p < 0.05$) correspond to a drop in σ values, or a reduction in the effective pH range for enzyme activity, and match up to an increase in larval size from *S. salpa* 1 to 3 (Table 5.1).

Table 5.4: Model parameters predicted by the normal distribution plot for alkaline proteases activity at different pH values for the three species and/or samples. The SSe for each model is also presented.

	σ	pH max	SSe
D. sargus	0.77	7.64	0.24
S. salpa 1	0.51	7.50	0.23
S. salpa 2	0.27	7.69	0.29
S. salpa 3	0.18	7.69	0.11
A. japonicus	0.25	7.67	0.86

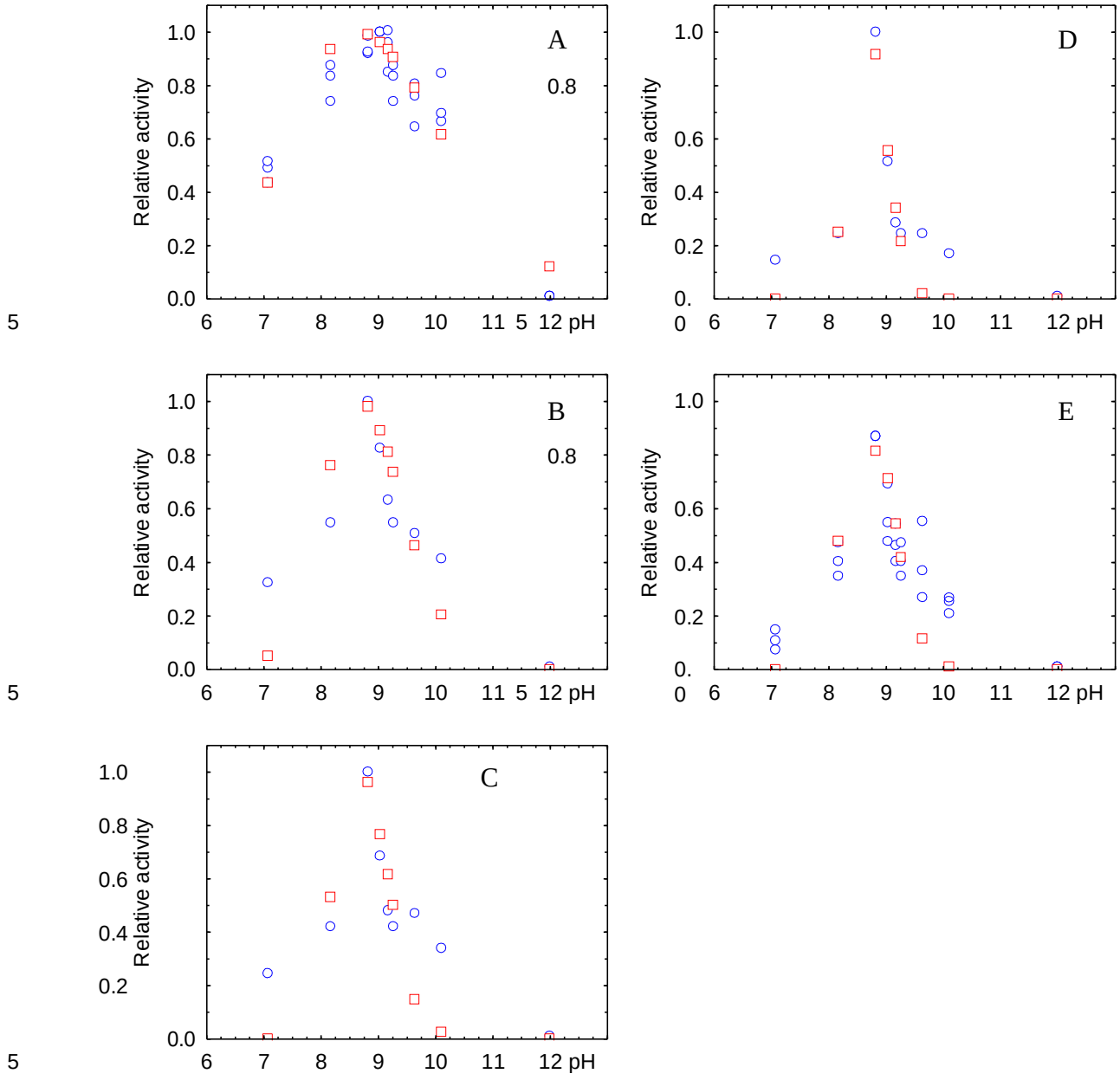


Figure 5.1: Observed (blue circles) and predicted (red squares) values of relative activity at different pH's for alkaline proteases. A) *D. sargus capensis*; B) *S. salpa* 1; C) *S. salpa* 2; D) *S. salpa* 3 and E) *A. japonicus*.

Lipase

The full and reduced models (σ and pH max equal) describing lipase activity at different pH values for the different samples showed no significant difference when compared with the likelihood test (A = 2.1, p = 0.55). The optimal pH for the highest lipase activity was accepted to be the pH max for the reduced model, and was predicted to be at a pH value of 8.03. Figure 5.2 shows the observed relative enzyme activity at different pH values for all the samples, as well as the relative enzyme activity values predicted by the skewed normal plot. The parameters for the reduced model are; σ = 0.39, α = -0.39 and pH max = 8.03 (SSe = 3.23).

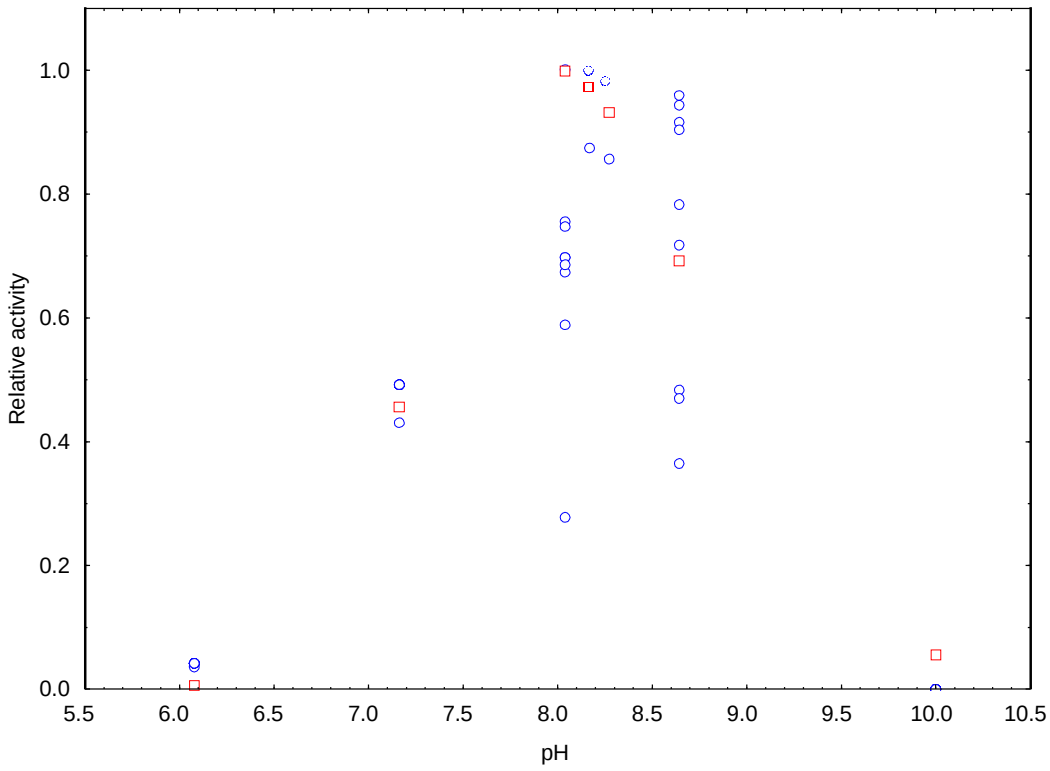


Figure 5.2: Observed (blue circles) and predicted (red squares) values of relative lipase activity at different pH values for the reduced model of all the samples.

Amylase

The reduced model (pH max and σ equal) for amylase showed a significant

difference from the full model between the samples (A = 7.4, p = 0.01 – see Table 5.5). This was due to the significant difference between the *S. salpa* samples (A = 12.9, p < 0.001). No further differences were detected between any other samples, once *S. salpa* was removed from the reduced model (A = 1.3, p = 0.51). There was, however, no difference in the predicted pH max values (A = 0.5, p = 0.46) between any of the samples in the reduced model (pH max equal) even if the *S. salpa* samples were included (Table 5.5). The optimal pH for maximum amylase activity was accepted as 7.69, the pH max value predicted by the reduced gamma distribution model (Table 5.7).

Based on the results from the likelihood tests (Table 5.5), it was accepted that the samples of *D. sargus capensis* and *A. japonicas* can be presented by a single reduced model each, while the full models for each *S. salpa* sample were maintained. The parameters and the SSe for each of the above models are summarized in Table 5.6. Figure 5.3 shows the observed relative activity values at different pH values as well as the values predicted from the gamma distribution plot models for *D. sargus capensis*, *A. japonicus* and the three *S. salpa* samples. Similarly to alkaline proteases, the differences detected in the *S. salpa* samples were as a result of changes in the shape (σ) of the curve which can be explained by a change in relative activity of amylase around pH max for optimal enzyme activity (σ equal; A = 6.4, p = 0.01). Unlike alkaline proteases activity, differences in σ for amylase activity for the three *S. salpa* samples correspond to an increase in σ (Table 5.6) with an increase in the size of larvae between samples.

Table 5.5: Results of the likelihood test for amylase. The A - statistic and p - value of the reduced models for the different samples and model parameters (σ and pH max) are given.

	σ equal		pH max equal		parameters equal	
	A	p	A	p	A	p
All species	6.4	0.01*	0.5	0.46	7.4	0.03*
D. sargus	1.3	0.25	0.2	0.63	1.7	0.43
S. salpa	12.4	<0.005*	0.1	0.75	12.9	<0.001*
A. japonicus	0.7	0.39	0.2	0.65	0.9	0.65
D. sargus vs *A. japonicus*	1.1	0.30	0.3	0.61	1.3	0.51

(* denotes significant differences)

Table 5.6: Model parameters predicted by the gamma distribution plot for amylase activity at different pH values for the three species and/or samples. The SSe for each model is also presented.

	σ	pH max	SSe
D. sargus	1.08	7.66	0.27
S. salpa 1	0.70	7.74	0.32
S. salpa 2	1.11	7.81	0.02
S. salpa 3	1.10	7.73	0.02
A. japonicus	1.04	7.63	0.01

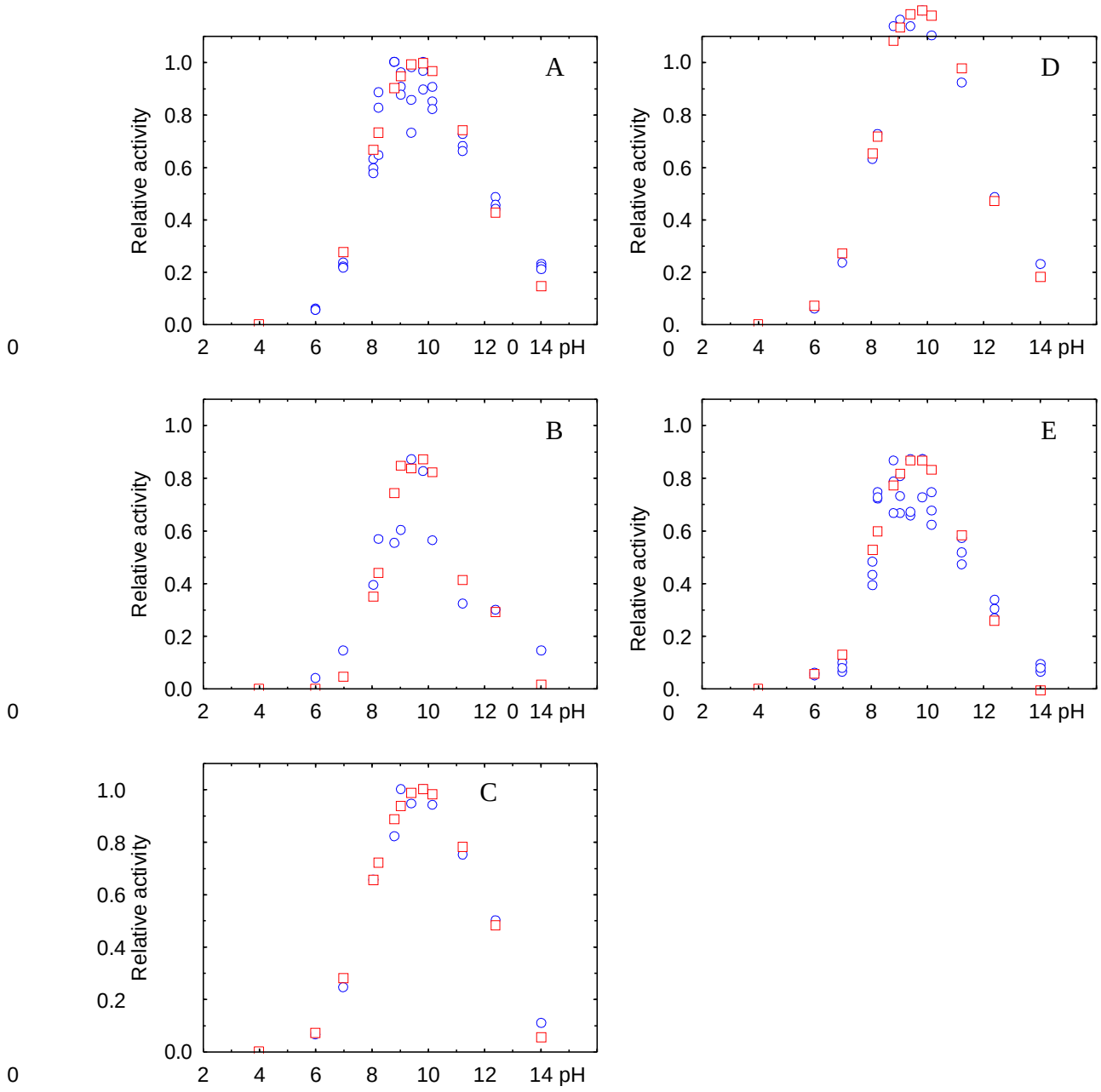


Figure 5.3: Observed (blue circles) and predicted (red squares) values in relative activity at different pH's for alkaline proteases. A) *D. sargus capensis*; B) *S. salpa* 1; C) *S. salpa* 2; D) *S. salpa* 3 and E) *A. japonicus*.

Phosphatase

The full model describing phosphatase activity at different pH values for the different samples showed no significant difference when compared to the reduced model (σ and pH max equal) for all nine samples (A = 0.86, p = 0.65). The reduced model was therefore accepted as a suitable model to describe phosphatase activity at different pH levels for all nine samples. The parameters predicted by the normal distribution plot for the reduced model were; σ = 0.74 and pH max = 9.84 (SSe = 1.86). The optimal pH for maximum phosphatase activity was therefore accepted to be 9.84. Figure 5.4 shows the observed relative activity values at different pH values for all the samples as well as the values predicted from the normal plot.

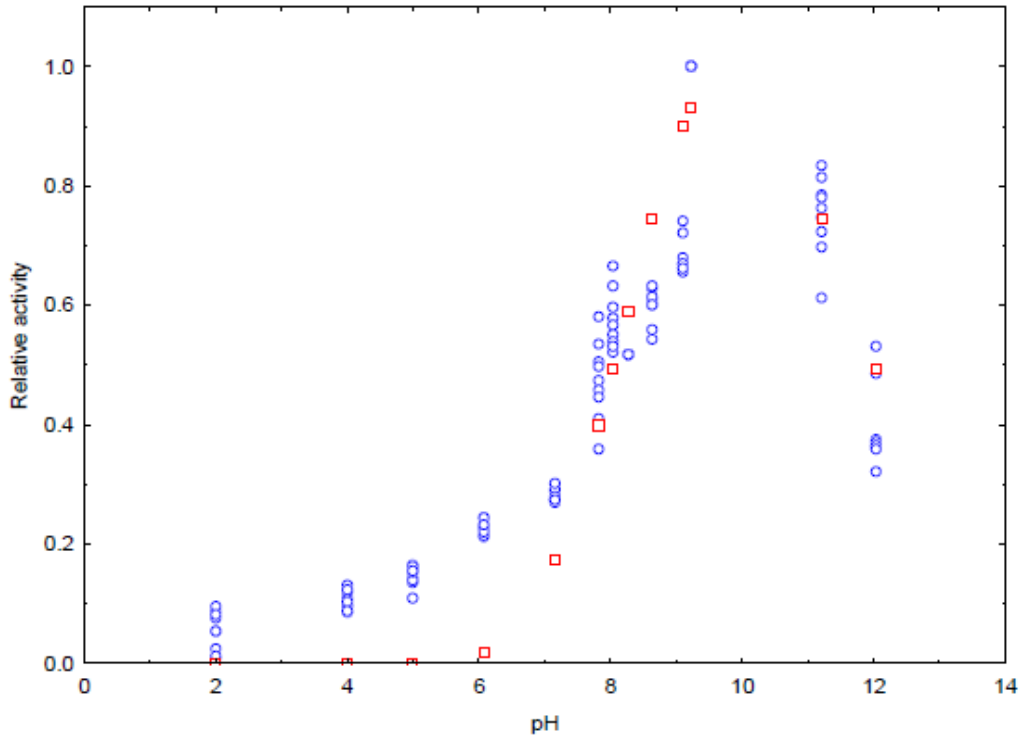


Figure 5.4: Observed (blue circles) and predicted (red squares) predicted values of relative activity at different pH's for the reduced model of all the samples for phosphatase.

Table 5.7: Model selection and the predicted optimal pH for each enzyme.

Enzyme	Model	Optimal pH
Alkaline proteases	Normal plot	7.67
Lipase	Skewed normal plot	8.03
Amylase	Gamma plot	7.69
Phosphatase	Normal plot	9.84

DISCUSSION

Studies that quantitatively and qualitatively determine enzyme activity in fish larvae have played an important role in understanding larval digestion and nutrition. Most of these studies however, take a simple one dimensional outlook towards enzyme activity, without elucidating the effect of environmental parameters on enzyme activity. The result is that a host of similar studies on quantitative and qualitative enzyme activity are being undertaken (Chapter 4) for various species that are being considered for their aquaculture potential, without an adequate capacity for comparisons between different species. An incomplete physiological understanding of enzyme activity or characterisation under different environmental conditions has limited our ability to predict and extrapolate more general trends in enzyme activity amongst fish species, and also dictates the boundaries for experimental work by researchers, like that attempted in Chapter 6.

The degree to which comparisons can be made in enzyme activity between species is principally dictated by genetics (Cahu & Zambonino Infante 2001, Kolkovski 2001), as DNA transcribes the activity level of digestive enzymes under different environmental conditions. The evolutionary rate of functionally important regions of DNA, like those that produce enzymes is much slower than in less important regions. Important enzymes are thus mostly conserved throughout evolution (Ridley 1996). To what degree this is the case for finfish species (globally, phylogentically, geographically, dietary etc.) remains to be determined. This chapter takes some basic, initial steps

towards understanding the effect of pH on enzyme activity for different fish species and alkaline digestive enzymes.

The pH at which alkaline digestive enzyme activity was optimal at 20 ºC was determined for *S. salpa*, *D. sargus capensis* and *A. japonicas*. These three warm temperate species were also used as a substitute for *D. marginatus*. As mentioned earlier this was necessitated due to the lack of larval material for *D. marginatus*. Although the models were not identical in enzyme activity over the whole pH range tested, these three species showed the same pH level for optimal enzyme activity for each of the four digestive enzymes tested. This suggests that the use of the *pH max* values predicted by the models will be suitable for calculating enzyme activities for the purpose of comparing digestibility coefficients between different species.

Alkaline proteases accounts for all the proteolytic activity in larval fish (Smith 1989, Zambonino Infante & Cahu 2001). The major contributing enzymes to this proteolytic activity are serine type, endoproteases like trypsin, chymotrypsin and also aminopeptidases (LAP). The optimal pH for maximum trypsin activity in fish is generally found to be in the pH range of 8 to 9 (Overnell 1973, Hjelmeland & Raa 1982, Shahidi & Kamil 2001) and for maximum chymotrypsin activity at a pH of around 7.8 (Shahidi & Kamil 2001). LAP has a maximum enzyme activity between a pH of 7.4 and 8.3 (Overnell 1973, Khablyuk & Proskuryakov 1983, Lazo *et al.* 2007). Considering that chymotrypsin remains the highest contributor to proteolytic activity during the early stages with an increase in trypsin and LAP activity during the latter stages in physiological development (Applebaum *et al.* 2001, Zambonino Infante & Cahu 2001), the optimal pH of 7.7 found in this study for maximum alkaline protease activity is close to that expected for chymotrypsin.

Maximum lipase activity was reached at a pH of 8.03 and this is close to the pH of 8 normally recorded for fish (Gjellesvik *et al.* 1989, Iijima *et al.* 1998, Lazo *et al.* 2007). The pH of 7.7 for maximum amylase activity found in this study varies from the neutral value of 7 that is normally recorded for freshwater and marine fish (Ugwumba 1993,

Munilla-Morán & Saborido-Rey 1996). Research on the characterisation of amylase activity at different pH values is however limited. Recently, Fernández *et al.* (2001) showed varied results, illustrating multiple peaks (some as high as at pH 9) for optimal amylase activity in three sparids. This indicates the possible existence of iso-enzymes, or enzymes that differ in amino acid sequence but catalyse the same chemical reaction. The existence of iso-enzymes was further supported in Fernández *et al.* (2001) by zymograms made from the extracts. Interestingly all three species tested in this study showed only one optimal peak for maximum amylase activity.

Although the pH levels for optimal enzyme activities as reported here compares well to that of other studies, a further two points need to be considered. Firstly, all the literature quoted above is based on work undertaken on late juvenile and adult fish rather than larval fish. Larvae have simple, undifferentiated intestinal tracts and have not yet developed the full suite of digestive enzymes associated with complex digestion (Chapter 3). Secondly, some questions can be raised about possible weaknesses in the methods employed in other studies determining the pH for optimal enzyme activity. For example, in the studies by Alarcón *et al.* (1995), Shahidi & Kamil (2001), Lazo *et al.* (2007) predictions of optimal pH were made by visual observations of scatter plots, with very little amplification around the optimal pH, hence reducing their predictive power. Modeling the relative activity of enzymes at different pH values and reducing the pH interval around the optimal pH, as in this study, is a clear improvement. Multiple peaks in activity, as previously discussed, will however not be detected and be incorporated into the model as variance. A visual assessment of the scatter plot is therefore still required.

Sigma (σ) signifies the rate at which relative activity drops around the pH for optimal enzyme activity. Based on the sigma values obtained from the models, it is clear that enzyme activity does not drop off very quickly around pH max and is maintained at activity levels close to the maximum enzyme activity. This suggests a small impact on enzyme activity measured at differences in pH values for different studies, with the exception of alkaline phosphatase. A cautious approach should be taken when

comparisons between species are made for alkaline phosphatase.

It is furthermore clear from this study that there is more to glean from modeling the relative activity of the digestive enzymes than just determining the pH at which the digestive enzyme will show maximum activity. There was a significant decrease in the slope, σ for *S. salpa* around the pH value for maximum alkaline proteases activity and an increase around the pH for maximum amylase activity with ontogenetic development. This indicates a change in enzyme "behaviour" with larval development in *S. salpa*. This may be associated with the dietary shift of *S. salpa* above 10mm TL from copepods and amphipods to an algal dominated diet (Christensen 1978). Protease activity is associated with high protein diets like copepods and amphipods, and with the relative importance of these organisms being reduced, one might also expect a change in protease "behaviour". A similar argument can be constructed for amylase with the increase of algae in the diet. It is not clear if the change in σ is as a result of this dietary shift or if this quantitative aspect of enzyme activity is genetically controlled and further research is required to test this hypothesis. The possible use of σ as an indicator of dietary shift, especially where the diet of a species during the larval stages is unknown, also needs to be investigated.

Chapter 6: Modelling the digestibility of *Artemia franciscanis* ‘in vitro’ during the early larval stages of marine finfish – A novel approach.

INTRODUCTION

Various attempts have been made to design and rear finfish larvae exclusively on artificial diets (Fernandez-Diaz & Yufera 1997, Rosenlund *et al.* 1997, Southgate & Partridge 1998, Kolkovski 2001), but a general trend of poor larval growth and development has been observed (Cañavate & Fernández-Díaz 1999, Robin & Vincent 2003, Curnow *et al.* 2006a). These difficulties are attributed to nutritional deficiencies in designed diets (Verreth 1994), a lack of visual stimuli as from live feed (Kolkovski *et al.* 1995), an underdeveloped larval digestive system (Cahu & Zambonino Infante 2001) and suggestions that live food contribute to the digestion and assimilation process through a direct or indirect contribution (Dabrowski 1984, Lauff & Hofer 1984, Kolkovski *et al.* 1993, Cahu & Zambonino Infante 1994). *Artemia* and other live food organisms are generally accepted as an integral part of larval rearing, playing an important part in co-feeding strategies (Person-Le Ruyet 1989, Person-Le Ruyet *et al.* 1993, Kolkovski 2001, Aragão *et al.* 2004, Stottrup & McEvoy 2008, Das *et al.* 2012). There is an increasing volume of research on this topic, often exposing what is known as insufficient for the purpose of larval rearing (Hoehne-Reitan & Kjorsvik 2004, Roennestad *et al.* 2012). Understanding the digestion of live food organisms by larvae is important for successful larviculture and could ultimately contribute towards to our knowledge to designing improved replacement diets. This is further highlighted for soleids that can currently only be weaned at very late stages of ontogenetic development (Chapter 1).

Only a few studies deal with larval digestion, despite the importance. Cook *et al.* (2008) and Johnson *et al.* (2009) investigated the digestion of an artificial diet and *Artemia* in Atlantic cod, *Gadus morhua* using rare earth oxides as an inert digestibility marker. Indicator methods, like earth oxides are rarely used due to the error associated with faecal collection in the aquatic environment and larval size (Johnson *et al.* 2009). More

complicated *in vivo* techniques have been developed in recent years to overcome the limitations of faecal collection. Conceição *et al.* (2007b) reviewed the literature using these techniques and include the incorporation of radioactivemarkers (Rønnestad *et al.* 2001, Morais *et al.* 2004 a & b, Tonheim *et al.* 2004, Hovde *et al.* 2005, Kvåle *et al.* 2006), auto-fluorescence (Kelly *et al.* 2000), differences in stable isotope ratios (Schlechtriem *et al.* 2004, 2005), and visible dyes (Werner & Blaxter 1980, Önal & Langdon 2004).

In vitro studies have also been used for diet formulation of larval feeds and contrasting nutrient availabilities (Alarcón *et al.* 1999, García-Ortega *et al.* 2001, Moyano & Savoie 2001, Chong *et al.* 2002, Lazo *et al.* 2002, Tonheim *et al*. 2007, Hamdan *et al.* 2009, Martinez-Montano & Lazo 2012), in which most of the limitations of *in vivo* studies are overcome. There is however an inherent complexity of enzyme systems and digestion *in vivo* that cannot be accounted for during *in vitro* studies, leading to certain assumptions and as a result a source of error (see framework). Despite this simplification of digestion *in vitro* research, various studies have found that *in vitro* studies correlate well with the digestibility measured *in vivo* (Hsu *et al.* 1977, Saterlee *et al.* 1979, Eid & Matty 1989, Lan & Pan 1993, Tonheim *et al.* 2007, Lazo *et al.* 1998).

There is currently no standard method for conducting *in vitro* trails or modelling larval digestion studies, as the experimental framework will vary to suit the specific objectives of a particular study. This study aims to set up and test a model, based on simple achievable biochemical assays, for the digestibility of newly hatched and enriched *Artemia* in first feeding marine finfish larvae using *in vitro* approach. A framework was set up to achieve this goal and is discussed below.

Framework and assumptions for "*in vitro*" digestibility trials

The procedure to determine and model *Artemia* digestion was as follows; whole bodied larval enzyme extracts of various species were allowed to digest *Artemia* for different times (analogous to gut evacuation time) in a buffered environment to produce the digesta in a reaction vessel. The use of larval extracts of the species of interest is more

suitable, because the enzyme complex obtained from the extract catalyzes digestion and different to single enzyme reactions and will be more accurately reflect digestion in the larval intestine (Alarcón *et al.* 2002). Several authors have observed important differences in molecular structures, digestive physiology, catalytic efficiency, and specificity to substrates between digestive enzymes of different phyla (Moyano & Savoie 2001, Alarcón *et al.* 2002, Chong *et al.* 2002, Chapter 5). Quantitative estimates were made for the degree of lipid, protein and carbohydrate digestion in the digesta using colorimetric methods, comparable to the degree of hydrolysis used by Martinez- Montano & Lazo (2012). Digestion was modelled (see Model Construction) using specific enzyme activity of the extracts, the preparation method of *Artemia* and incubation time as independent variables, and the degree of digestion as dependant variable. This model takes a novel approach by considering multiple enzymes and products in one experimental digestion. This approach further ensures that the procedural framework closely approximates the natural system or *in vivo* digestion, contrary to simple single enzyme; single product approaches.

Enzyme extracts were obtained from whole larvae homogenates for which the specific enzyme activity of alkaline proteases, lipase and amylase was predetermined. These three enzymes and enzyme group were chosen as they represent the physiological most important alkaline enzymes in crude digestion of substrates (Chapter 1). These enzymes are by no means the only enzymes that will contribute to the digestion measured in this *in vitro* study, and are only specific indicator enzymes. Larval enzyme extracts were obtained from three warm temperate finfish species showing variable feeding strategies or natural prey selection during the early juvenile and adult stages. These species includes the blacktail; *Diplodus sargus capensis* (Sparidae), the strepie; *Sarpa salpa* (Sparidae), and the dusky kob; *Argyrosomus japonicus* (Sciaenidae). An enzyme extract from the white margined sole, *Dagetichthys marginatus* (Pleuronectidae) was used to test the ability of the model to predict *in vitro* digestion for other species. Importantly, characterisation of relative enzyme activity was done in Chapter 5 at different pH to ensure that species differences can be incorporated and accounted for in the model if necessary. This further served as a basis for the selection of an optimal pH for the determination of specific enzyme activities and *in vitro* trails in this study (Chapter

5).

Both the two common forms of *Artemia*, newly hatched and enriched, used in larviculture were included as a categorical predictor in the experimental framework. The number of *Artemia* nauplii used in a single digestion was chosen to ensure that the substrate was not a limiting factor during digestion and that the amount of product measured using the colorimetric method was amplified and high enough to detect small differences between treatments in digestibility, thereby reducing the associated error. Appropriate blanks (where the substrate, *Artemia* or the larval enzymes extract was left out of the digestion, to account for their contribution to final result) and treatments were prepared to account for the contribution of exogenous enzymes from *Artemia* to the digestion, as the specific enzyme activity was not determined for *Artemia*.

It is also important to maintain a homogenous environment for digestion in the reaction vessel as it will effect that rate of digestion. This can be solved by constantly mixing the digestive tube. This however increased the mechanical breakdown of the *Artemia*, increasing the digestion. Very little mechanical digestion takes place in fish larvae, which is evident from histological sections from the gut (Chapter 4). Both the above concerns were detected in the lab, and increased variability between replicates (pers. obs.). We found by simply allowing the reaction to take place in a reactor vessel laid on its side with an even distribution of a single layer *Artemia* in the extract showed the least variability. A single layer of *Artemia* nauplii should logically reduce the potential surface area loss for enzyme reactions that would be associated with multiple nauplii layers. As the loss of surface area is highly variable depending on the orientation of *Artemia* nauplii and the number of layers in the reaction vessel (which is impossible to control), the results will be more variable as observed in this study.

MATERIALS, METHODS AND MODEL CONSTRUCTION

Source of enzymes

The larvae of *Diplodus sargus capensis*, *Sarpa salpa*, *Argyrosomus japonicus* were

used to make up 8 independent enzyme extract samples used to construct a model for protein and carbohydrate digestion. *Dagetichthys marginatus* larvae were used to test the model. All the larvae used as an enzyme source for *in vitro* trails ranged in length between 4 and 13 mm. Specific collection details are not important in this study, as the larval extracts were only used as a source of enzymes.

All the larvae were rinsed and blotted dry before being stored in liquid nitrogen. Samples remained frozen at -80 ºC until extraction. Enzyme extraction was achieved by homogenising partially thawed larvae (4 ºC) in cold 50 mM Tris-HCL buffer, pH 7.7 (35 mg larval tissue . ml-1). This was followed by centrifugation (13500g for 15 min at 4 ºC). The supernatant was removed and used to determine specific enzyme activity and conduct *in vitro* trials. Enough larvae were combined to give an enzyme extract sample of at least 15 ml, which was required to complete all the necessary assays.

Enzyme assays

All the assays (Chapter 4 and 5) were carried out in triplicate and the absorbencies were measured using the KC junior software and the 96 well spectrophotometer SPECTRAmax 190, Powerwave X, Biotek Instruments, USA. All reactions took place at a room temperature of 20 ºC (Chapter 5).

Alkaline proteases activity was determined using a modified method from Anson (1938). The enzyme extract was incubated for 30 min with a heamoglobin substrate (0.2 g heamoglobin in 10 ml of 50 mM Tris-HCL buffer, pH 7.7). Activity was determined by measuring the change in l-tyrosine concentration at an absorbance of 280 nm. Amylase (E.C. 3.2.1.1) was determined using the Somogy-Nelson procedure described by Nelson (1944) and Robyt & Whelan (1968). The substrate was prepared by dissolving % soluble starch in a 50 mM Tris-HCL buffer (pH 7.7) by heating the solution. The reaction started by combining the enzyme extract with the starch substrate and allowing it to incubate for 5 min. The DNS reagent (Dinitrosalicylic reagent) was added to stop the reaction and then incubated at 100 ºC in a heating block for 5 min. Reducing sugars were determined by measuring the change in absorbance at 540 nm. Lipase (E.C. 3.1.1.-) activity was measured according to Iijima *et al.* (1998). The enzyme extract was allowed to incubate

with 5.2 mM sodium cholate in 50 mM Tris-HCL buffer (pH 8.0) and 10 mM 2-methoxyethanol at room temperature for 15 min prior to addition of substrate. 10mM p-nitrophenyl myristate dissolved in 100% ethanol was added as a substrate and allowed to incubate for another 2 h. The reaction was stopped by the addition of an acetone:heptane mixture (5:2). The change in absorbance was recorded at 405 nm for p-nitrophenyl to determine the degree of substrate hydrolysis.

The soluble protein fraction content of the homogenates was determined using the Bradford's assay technique (Bradford 1976) with bovine serum albumin as a standard.

Specific enzyme activity was expressed as U/mg protein where U represented the amount of product liberated during 1 min of hydrolysis.

Artemia preparation

Artemia franciscanis cysts, EG:HE > 240 000 npl/g (INVE, Belgium) were prepared and incubated according to the method described by Hoff & Snell (1987). Cysts hatched after 24 h and were either used as newly hatched nauplii, or reared to the metanauplii stage and enriched with DHA Selco® according to the method described by the producer (*Artemia* Systems, INVE). Both the newly hatched nauplii and enriched metanauplii were thoroughly rinsed with fresh water, concentrated and separated into reaction vessels for the *in vitro* trails. The same volumetric volume of 200 uL concentrated *Artemia* was pipette into each reaction vessel to factor in size differences between newly hatched and enriched *Artemia*. Five subsamples of 10 ul each was counted under a dissecting microscope and used to determine the number of nauplii in each reaction vessel to further validate and standardise between reaction vessels and *Artemia* sizes. Each reaction tube was centrifuged for 3 minutes at 4000 g or until pellet formation to remove excess water prior to the addition of the enzyme extract.

In vitro digestion

The *Artemia* nauplii were re-suspended by the addition of 500 uL of the enzyme extract,

which signified the start of *in vitro* digestion. The extracts were obtained from whole tissue homogenates as described above. The enzymes were allowed to hydrolyse the substrate / *Artemia* for 15, 30, 45, 90, 120, 150, 210 and 270 min. 500 uL of cold (4 ºC) MilliQ water was then added to minimise and stop subsequent enzyme activity until centrifuging. This was followed by centrifuging the reaction vessels for 10 min at 12 000 rpm to separate undigested *Artemia* from the digesta. An extract blank for each of the abovementioned incubation times served as a negative control and a substrate blank helped to account for the degree of hydrolysis in the enzyme extract.

The digesta (supernatant) was tested for the degree of protein, fat and carbohydrate digestion. The degree of protein digestion was measured using a modified Ninhydrin colourimetric method. Ninhydrin binds to the amine group of amino acids and peptides, and give an accurate measure of the degree of protein hydrolysis. Ninhydrin reagent (0.35 g Ninhydrin dissolved in 100 ml ethanol) was added to a sample of the digesta and covered to prevent the loss of the solvent. This solution was allowed to react for 7 min at 90 ºC with gentle stirring after which it was cooled and the change in absorbance was measured at 570 nm. Glycine was used as a standard amino acid to determine the degree of protein digestion. The digestion of carbohydrates was determined using a modified Somogyi – Nelson method for reduced sugars as previously explained. Lipid digestion was determined using the Korn method (Whiteley *et al.* 2003) for glycerol.

Glycerol is a product liberated when lipase cut the fatty acids from the glycerol backbone of triglycerides. Glycerol is thus used as an indirect measure of fatty acids hydrolysed. Using this method is problematic as not all fats in *Artemia* are in a triglyceride form (other forms like steroids, phospholipids are also present), but to maintain the simplicity of the *in vitro* protocol for future studies, the Korn method was favoured and it was assumed that it will give an indirect measure of total fat digestion.

The Korn method was achieved as follows; 10 uL H_2SO_4 (5M) and 25 uL of sodium periodate ($NaIO_4$ – 0.1M) were added to the digesta, vortexed and allowed to react for 5 min. This was followed by the addition of 25 uL of $NaHSO_3$ and 250 uL of Chromotropic acid reagent. Once again the chemical mixture (digesta) was vortex and incubated at 90

ºC for 1h. The samples where cooled and the absorbance read at 570 nm.

Each reading was done in triplicate, while a standard curve of absorbance vs. product concentration was used to determine the concentration of product in the assays.

Model construction

In biochemistry, there has been significant interest in the appropriate mathematical models for describing and predicting the rate (v) of enzyme-catalysed reactions. The general reaction scheme for enzyme-catalysed conversion in digestion from the substrate (S) to the product (P) by enzyme (E) can be described as follows;

$$E + S \dashrightarrow E.S \dashrightarrow E.P \dashrightarrow E + P$$

The rate of an enzyme catalysed reaction (v) can be described by the Michaelis-Menten kinetic model and describes the kinetics of enzyme-catalysed reactions as follows;

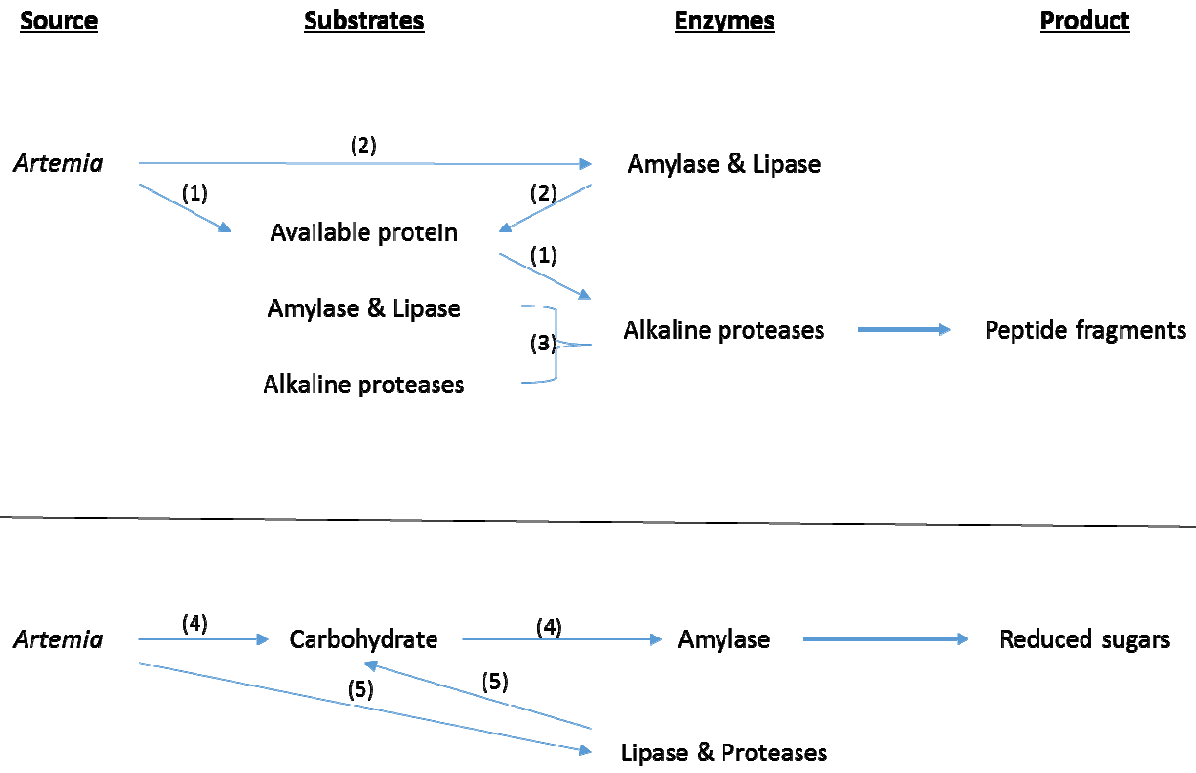
$$v = \frac{v_{max}[s]/K_m}{1 + [s]/K_m}$$

V = reaction rate. Vmax = maximum reaction rate, Km = Michaelis constant and s= concentration of substrate.

It is clear from the reaction scheme of the enzyme-catalysed conversion above, that many reactions occur with the formation of reactive intermediates (*E*·*S* and *E*·*P*), even in a single enzyme – single substrate/product approach. The *in vitro* protocol followed for digestion in this study was not of a simple nature, with a number of different enzymes digesting a complex substrate like *Artemia*. The decision on which model to use depends completely on the type and complexity of reaction being modelled, the environment it takes place in and the type of data being collected based on the experimental design of the study. Despite the obvious complexities of the general reaction scheme applicable to this *in vitro* study (see theoretical models below), it can be represented as a series of elementary reactions. The rate equation for each individual step can be combined so that the overall rate equation can be derived from the

individual steps. These steps point to the use of a dynamic metabolic network of single models using a system modelling approach. To achieve this system modelling approach for digestion, a metabolic network was built that consists of various linked functions, combining to form a kinetic model or a coupled reaction network based on the mechanism or theoretical model described below. Similar constructed models were used by Rønnestad & Conceição (2010).

Theoretically the degree of protein and carbohydrate digestion can simply be described by the following schematic illustration and include various allosteric interactions that are numbered and described below.



The alkaline proteases hydrolyse proteins in *Artemia* into peptide fragments (1). The degree of protein digestion was determined by the number of peptide fragments, using the Ninhydrin method explained in materials and methods. The proteases can also auto-digest itself and hydrolyse other enzymes like amylase and lipase, as enzymes consist of proteins (3). Furthermore, lipase and amylase can also digest *Artemia* that

can indirectly make proteins in the *Artemia* more or less available for proteases to digest (2).

Amylase hydrolyses glycogen and other carbohydrate chains in *Artemia* into simple reduced sugars (4). Furthermore, lipase and alkaline proteases can also digest *Artemia* that can indirectly make carbohydrates in the *Artemia* more or less available for amylase to digest (5). This theoretical model for carbohydrate digestion was however not implemented as amylase had no effect on the hydrolysis of carbohydrates (see Results and Discussion).

The Korn method for triglyceride digestion yielded no positive results for glycerol in the digesta, despite attempts to modify the digestive liquid to contain an emulsifier and was subsequently ignored (see Discussion).

Based on the theoretical model described above for protein digestion, the metabolic processes (metabolic control and regulation) governing digestion was modelled using the following four equations and is based on the Michaelis-Menten model for enzyme kinetics.

a)

$$Protease'(t) = \frac{-kcat4 \times {protease(t)^2}/{kprotease}}{1 + {protease(t)}/{kprotease} + {lipase(t)}/{klipase} + {amylase(t)}/{kamylase}}$$

b)

$$Lipase'(t) = \frac{-kcat3 \times {lipase(t)}/{klipase} \times {protease(t)}/{kprotease}}{1 + {protease(t)}/{kprotease} + {lipase(t)}/{klipase} + {amylase(t)}/{kamylase}}$$

c)

$$Amylase'(t) = \frac{-kcat2 \times {\text{amylase}(t)}/{k\text{amylase}} \times {\text{protease}(t)}/{k\text{protease}}}{1 + {\text{protease}(t)}/{k\text{protease}} + {\text{lipase}(t)}/{k\text{lipase}} + {\text{amylase}(t)}/{k\text{amylase}}}$$

$$Protein'(t) = \frac{-kcat1 \times {\text{protein}(t)}/{k\text{protein}} \times {\text{protease}(t)}/{k\text{protease}}}{1 + {\text{protease}(t)}/{k\text{protease}} + {\text{lipase}(t)}/{k\text{lipase}} + {\text{amylase}(t)}/{k\text{amylase}}} + 2a + 2b + 2c$$

; where *k*protease, *k*protein, *k*amylase, *k*lipase are binding constants and *kact* 1 – 4 are the catalytic constants, while Protein'(t) refers to the degree of protein digestion over time, and can also be described as the slope or first order derivative describing the protein signal in the digesta. Multiplying equations a, b and c with two before the addition to the degree of protein digestion (eqn. d), signifies the doubling of the peptide fragments with every hydrolysis of an enzyme or peptide chain.

A further variable was built in to account for differences between newly hatched and enriched *Artemia* (*prot 1* and *2* respectively). This was achieved by splitting the time course into two sections, the second of which will incorporate a higher value for starting protein when *Artemia* was enriched. This event was placed between the 90 and 120 min sampling times for digestion, and was decided upon after visual inspection of the observed data and consideration that the maximum level of free amino acids is reached in dead *Artemia* around 120 min (Gulbrandsen *et al.* 2009).

The model constants were then solved by minimizing the sum of squares in errors between the observed and expected data for the eight samples. The predictive power of this model was then tested by calculating the fit of the observed data from the sole, *Dagetichthys marginatus* to the model.

Data analyses

Other than specifically mentioned, the degree of protein and carbohydrate digestion is based on total digestion and not only the contribution of the larval extracts to digestion. Descriptive statistics were done using *Statistica v9*, StatSoft; while model building, simulation and solving was achieved using *Mathematica 7.0*, Wolfram.

RESULTS

The number of *Artemia* used for each in vitro digestion was 2215±390 nauplii for un-enriched and 1543±243 meta-nauplii for enriched. This number of *Artemia* was standardised for model construction so that the degree of digestion was always for 1000 newly hatched and 700 enriched nauplii. This maintained the volumetric ratio (weight to volume ratio) of 70 % of newly hatched to enriched *Artemia* found in this study to ensure that a relative constant weight of substrate was maintained. Enzyme activities used during the model construction (samples #1 - #8) ranged between 52 – 76 mU/mg protein, 2.7 – 4.1 mU/mg protein and 5.9 – 10.8 U/mg protein for alkaline proteases, lipase and amylase respectively (Table 6.1). Sample #9 & #10 were taken from *D. marginatus* larvae 8 and 25 dah respectively, and were used to test the predictive power of the model. One should note that specific enzyme activity of sample #10 for alkaline proteases and lipase fell outside of the range for which the model was constructed (Table 6.1).

Table 6.1: Specific enzyme activity and method of *Artemia* preparation used for each sample used in model construction (#1 - #8) and model testing (#9 & #10). The fit of the model to each sample for the degree of protein digestion is represented by R^2.

Sample	Artemia Preparation Method	Protease Activity mU/mg protein	Amylase Activity U/mg protein	Lipase Activity mU/mg protein	R^2 Protein signal
#1	Newly hatched	75.6	10.1	3.9	0.90
#2	Newly hatched	54.5	9.6	3.2	0.37
#3	Newly hatched	64.2	5.9	3.8	0.84
#4	Newly hatched	63.9	7.1	3.9	0.67
#5	Enriched	73.5	10.6	3.9	0.76
#6	Enriched	52.2	10.8	2.7	0.60
#7	Enriched	58.3	5.9	2.8	0.75
#8	Enriched	65.2	7.8	4.0	0.92
#9	Newly hatched	68.7	7.8	7.1	0.76
#10	Enriched	126.0	2.1	22.4	0.58

Exogenous enzymes from *Artemia* contributed 54.5 ± 2.4 % and 53.6 ± 2.1 % to total protein digestion, and 64.0 ± 5.6 % and 72.2 ± 5.1 % to total carbohydrate digestion for un-enriched and enriched *Artemia*, respectively and were based on the degree of digestion within the digesta without the presence of the larval extract. This contribution was significantly higher for enriched than un-enriched *Artemia* for carbohydrate digestion ($t = -6.14$, $df = 62$, $p < 0.0001$) but not protein digestion ($t = 1.52$, $df = 62$, $p = 0.134$).

None of the endogenous larval enzymes included in the study as predictors for the model, including amylase, had any effect on the rate of carbohydrate digestion from *Artemia*, due to the strong linear trend that is presented below. For this reason the system model approach was dropped for carbohydrates, and only a simple linear model was used to predict the degree of carbohydrate digestion, DCD (see Discussion).

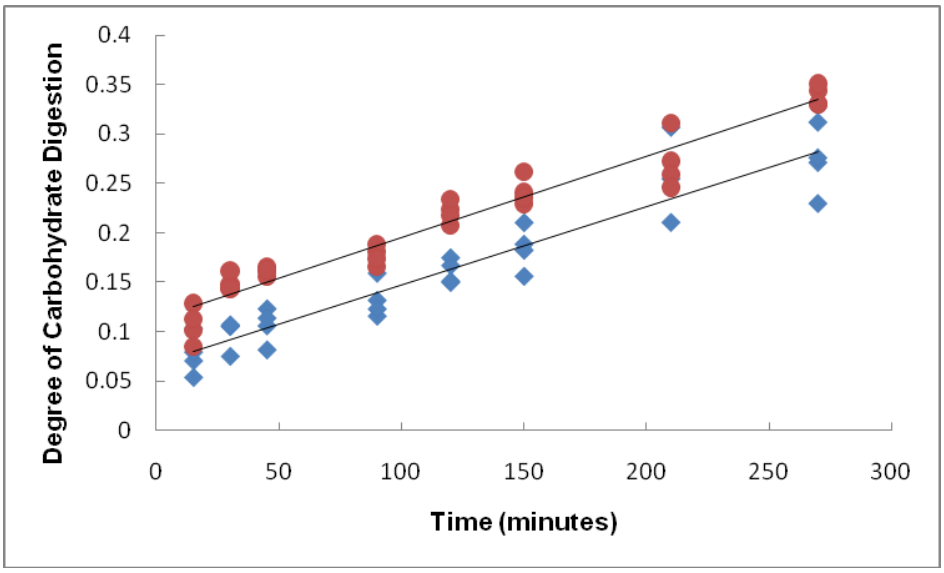


Figure 6.1: Graph presenting the degree of carbohydrate digestion for newly hatched (diamonds) and enriched *Artemia* nauplii (circles) at different digestion times for sample #1 - #8, showing a linear regression of y=0.0008x+0.0682 (R2=0.90) and y=0.0008x+0.1132 (R2=0.94) respectively.

A repeated measures ANOVA for carbohydrate digestion showed that although the pattern of digestion over time stays the same ($F = 1.718$, $p = 0.13$), that by enriching the *Artemia* a significantly higher amount of carbohydrates were available for digestion ($F = 36.47$, $p = 0.0009$) throughout the digestion period.

The solution for the constants in the model for protein digestion or the protein signal is 0.22 $mN.min^{-1}$, 0.01 $mN.min^{-1}$, 0.07$mN.min^{-1}$, 0.001 $mN.min^{-1}$, 5.55 N^{-1}, 4.1 N^{-1}, 3.3 N^{-1}, 2 N^{-1}, 0.01 and 0.11 for kact1, kact2, kact3, kact4, Kprotease, Kprotein, Klipase, Kamylase, prot1 and prot2 respectively. From these results it is clear that the binding constants (Kprotease, Kprotein, Klipase and Kamylase) or the capacity of protease to the different protein sources in the digestion is relatively constant, being in the same order of magnitude. The degree of protein digestion also increases by an order of magnitude between newly hatched and enriched *Artemia* after 90 min of digestion in the in vitro system (prot1 = 0.01 and prot2 = 0.11). Furthermore, the catalytic constant (kcat1) for the hydrolysis of protein by proteases to peptides contribute much more than any of the allosteric interaction of the hydrolysis of protease, lipase and amylase by protease. One

should note however, that despite the fact that the model was optimised there are still some visual pattern differences when comparing between the observed and model predicted values (Figure 6.2). These differences are especially obvious when one consider the pattern of protein digestion over the first 90 min of digestion of enriched Artemia. There appears to be a very sudden increase in the degree of protein digestion, which is not predicted by the digestive model. This highlights the potential shortcomings of the current model.

The model constructed with samples #1 - #8 showed good predictive power for the DPD and DCD in *D. marginatus* larvae (samples #9 and #10). Sample #9 showed a R^2 = 0.985 for the digestion of carbohydrates of unenriched *Artemia* when compared to the constructed model, while sample # 10 showed a R^2 = 0.870 for enriched *Artemia* (Figure 6.3). The model also showed good predictive power for the degree of protein digestion in *D. marginatus* (Table 6.1 and Figure 6.4)

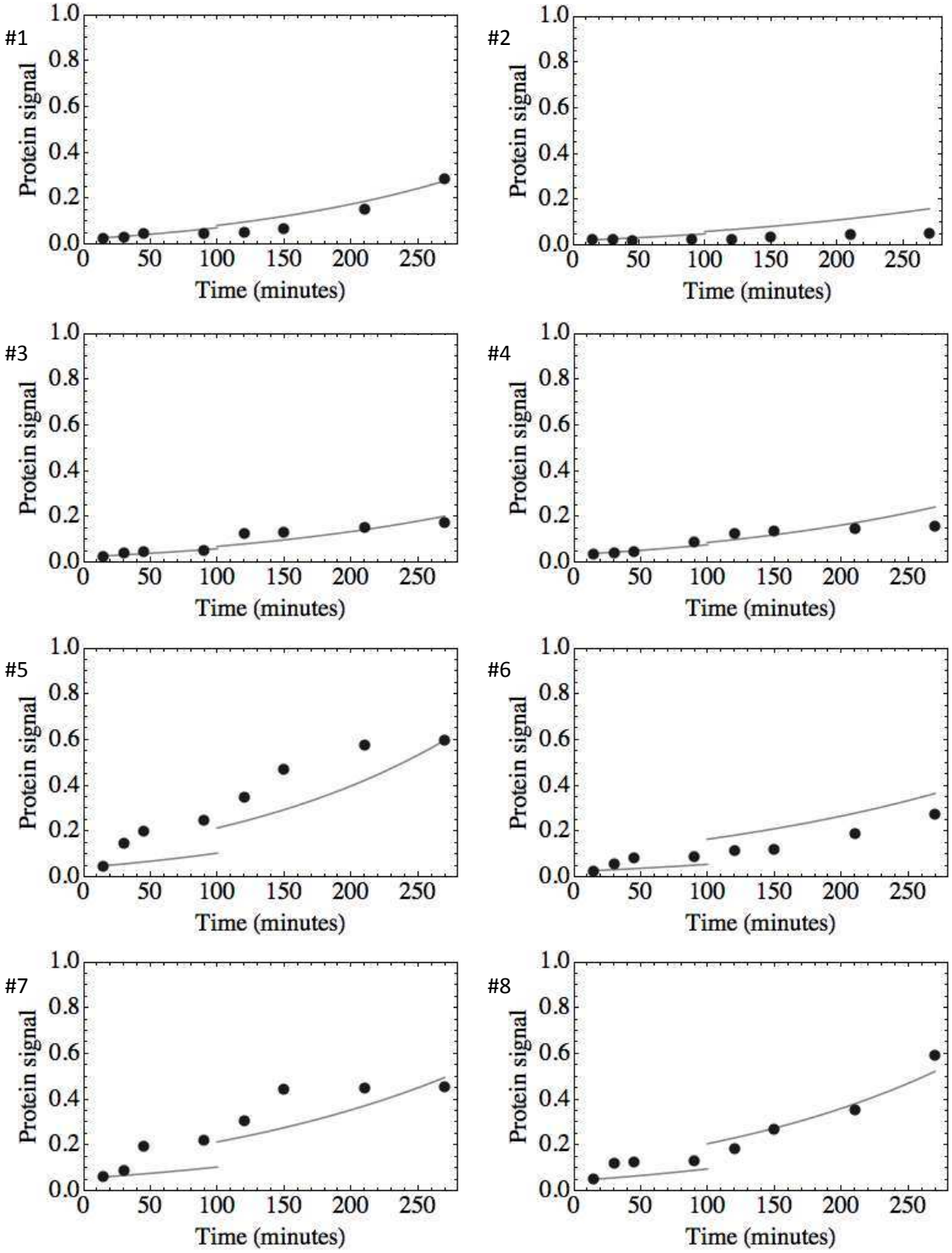


Figure 6.2: The observed and expected protein signals or DPD for samples #1 - #8 used for model construction. The R^2 of the model to the observed data are given in Table 6.1.

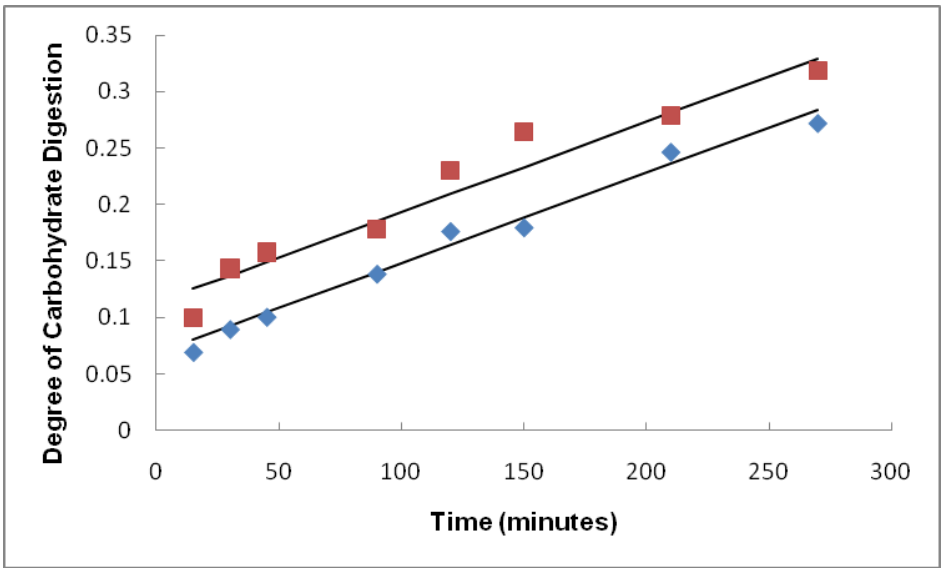


Figure 6.3: Graph presenting the degree of carbohydrate digestion for newly hatched (diamonds) and enriched *Artemia* nauplii (squares) at different digestion times for sample #9 and #10 respectively. This shows a good fit to the linear regression models constructed for unenriched samples #1 - #4 of y=0.0008x+0.0682 (R^2=0.985) and enriched samples #5 - #8 of y=0.0008x+0.1132 (R^2=0.870) respectively.

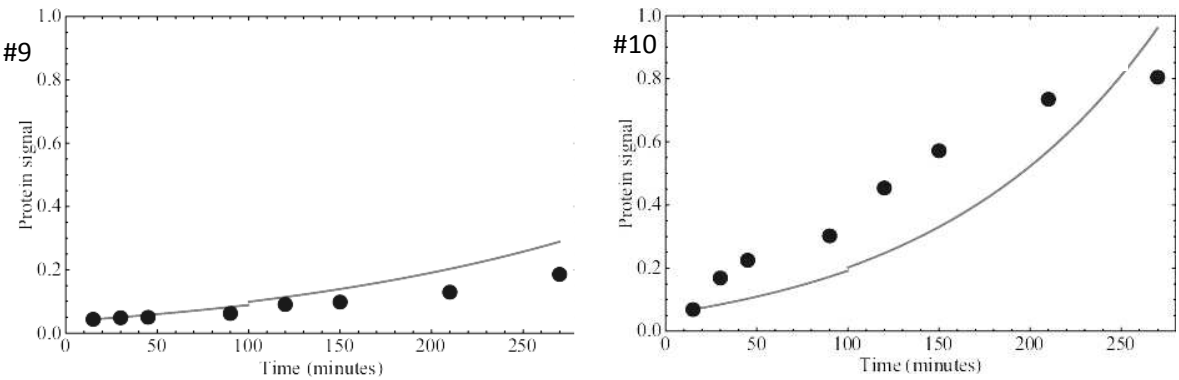


Figure 6.4: The observed and expected protein signals or DPD based on constructed model for Dagetichthys marginatus samples #9 and #10. The R^2 of the observed data to the model are given in Table 6.1.

DISCUSSION

Live food organisms like *Artemia* remain an integral part of larval rearing at great costs to finfish farmers (Kolkovski 2001, Stottrup & McEvoy 2003, Aragão *et al.* 2004), despite attempts to eliminate them from first feeding larval strategies (Fernandez-Diaz & Yufera 1997, Rosenlund *et al.* 1997, Cahu *et al.* 1998, Southgate & Partridge 1998). Understanding the mechanism by which larval enzymes and gut evacuation times affect the digestion of *Artemia* will result in improved species specific enrichment formulations and rearing protocols for *Artemia*. It will further give valuable insight into the potential capacity of finfish larvae to digest artificial food sources and ultimately feed design.

This paper attempts to elucidate some of the general factors and mechanisms contributing to *Artemia* digestion *in vivo* by describing *Artemia* digestion using a novel *in vitro* approach. This is however, only an initial attempt at understanding the processes governing the digestion of *Artemia* using a biological meaningful integrated *in vitro* and modelling approach. No comparisons can thus be drawn with regards to predicted model constants, and those found by other authors. Nonetheless, some crucial information can be taken from this study.

Various authors have shown that *Artemia* contribute a large portion of enzyme activity (40 – 80 %) during digestion through exogenous enzymes (Jancarik 1964, Dabrowski & Glogowski 1977a,b, Dabrowski 1984, Lauff & Hofer 1984, Kolkovski *et al.* 1993, Cahu & Zambonino Infante 1994, Oozeki & Bailey 1995, Gawlicka *et al.* 2000; Lazo *et al.* 2000). Research has also shown that exogenous enzymes can induce an increase in endogenous enzyme activity from fish larvae (Pedersen & Hjelmeland 1988) and that the autolysis of live food can stimulate endogenous enzyme secretions through neurohormonal factors (Chan & Hale 1992, Hjelmeland *et al.* 1993, Person-Le Ruyet *et al.* 1993).

On the contrary, other studies show a very small contribution by exogenous enzymes and suggest that it may be negligible (Oozeki & Bailey 1995, Moyano *et al.* 1996, Zambonino-Infante *et al.* 1996, Cahu & Zambonino-Infante 1997, Kurokawa *et al*. 1998,

Lazo *et al.* 2000). Research on the Japanese sardine, *Sardinops melanoticus* larvae (Kurokawa *et al.* 1998) and herring larvae, *Clupea harengus* (Pedersen *et al*. 1987, Pedersen & Hjelmeland 1988) showed similar results. They concluded that exogenous enzymes from rotifers and copepods contributed less than 1% and 0.5% to protease activity respectively and suggests that live feed contribute to the digestion and assimilation process other than a direct contribution to enzyme activity. Support for this came from Kolkovski *et al.* (1997a) and Koven *et al.* (1998) that showed that lipid fractions extracted from *Artemia* lipids, enhance assimilation of microparticulate diets in gilthead seabream larvae, *Sparus aurata*.

Data from this study suggest that exogenous enzymes from *Artemia* contribute to about 54% of protein digestion and 64 – 72% of carbohydrate digestion. These results were based on the contribution of *Artemia* to digestion when it was allowed to auto-digest itself, and not the contribution of activity levels of the digestive enzymes as is most often the case in the above referenced literature. The results from this study do not preclude any of the hypotheses described above, but present indirect evidence that supports the hypothesis that the low contribution of larval enzymes was due to the hypothetical contribution of exogenous enzymes from *Artemia*. Since the exogenous enzyme activity for *Artemia* was not measured in this study, there is no direct measure to support this hypothesis. Using the actual enzymes activities as a direct measure for the contribution of exogenous enzymes to larval digestion presents its own possible problems. Amylase, used as an indicator enzyme for carbohydrate digestion in this study, played no role in determining carbohydrate digestion from *Artemia*. This, together with the strong linear trend observed in the results for carbohydrate digestion, suggests that another possible enzyme from an exogenous source had a bigger overriding effect. These can include carbohydrases like maltase, chitinase and chitobiase (Clark *et al.* 1984). The latter two has been shown to occur at very low levels in larvae, and originate mostly from exogenous enzymes from *Artemia* (Clark *et al.* 1984). Care should therefore be taken in the selection of the indicator enzymes when one considers the digestibility of *Artemia*.

This presents contradictory results, as this study also suggested that only between 64

and 72 % of carbohydrate digestion can be accounted for by exogenous enzymes from *Artemia*. This discrepancy can only be explained by various possible allosteric interactions of endogenous and exogenous enzymes put forward in the hypothetical models or other unknown hypothetical contributions hinted towards by various other studies (Oozeki & Bailey 1995, Moyano *et al.* 1996, Zambonino-Infante *et al.* 1996, Cahu & Zambonino-Infante 1997, Kurokawa *et al*. 1998, Lazo *et al.* 2000).

The constructed models for carbohydrate and protein digestion showed reasonable predictive power for the sole, *Dagetichthys marginatus*. This suggests that *D. marginatus* digest *Artemia* in a very similar manner to other teleosts. This is contradictory to what was found for the Dover sole, *Solea solea* (Morais *et al.* 2004). The methods followed by Morais *et al.* (2004) that resulted in this conclusion have been criticised by subsequent research, showing that the digestibility of *Artemia* may increase with a decreased evacuation rate caused by a single meal followed by starvation (Conceição *et al.* 2007).

The degree of fat digestion could not be determined, despite the importance of fats in larval development (Chapter 1). The Korn method used to determine fat digestion was selected due to the high prevalence of triglycerides (50 – 72 mg/g dry weight) in *Artemia* (García-Ortega *et al.* 1998). No glycerol was however assayed in the digesta, illustrating that the Korn method was unsuitable to determine the degree of fat digestion. This is contrary to what is suggested by Koven *et al.* (1997), and can only be explained by the incomplete hydrolysis of triglycerides to di- and monoglycerides. A more appropriate assay needs to be used to include fat digestion of *Artemia* in future studies of this nature.

Care should however be taken when using a system modelling approach, as it does make the assumption that the theoretical model used to predict digestion *in vitro* is fundamentally true. This is not always the case, as is clearly illustrated by the fact that amylase did not have any impact on carbohydrate digestion. Careful consideration of model predictors and inspection of data is therefore required to ensure that any assumption is based on a true effect and not a theoretical effect. This does not imply that the theoretical model is wrong, but rather the predictors selected to model the

system is not of any significant importance.

Conventional modelling, using techniques like general linear models (Hardin *et al.* 2003, Giménez *et al.* 2007, Hua & Bureau 2009) might have given better fits to the observed values, but these would have had little biological meaning in an enzyme catalysed system. For this reason the model was based on enzyme kinetic principles, as it represent biological significant values that can be used to describe the mechanism by which larvae digest *Artemia* in various studies. This modelling approach also only model known enzyme-substrate interactions in the digestive system, and does not look for interactions based on a search for significant interactions like the other modelling techniques. It is also likely that further experimentation, while building more complexity into the *in vitro* model, will show very good fits with a high degree of predictability. It can also be adapted to include specific outputs like the release of specific amino and fatty acids.

The validity of an *in vitro* approach to describe a very complicated process remains to be determined, although a good fit of the data to the hypothetical model, based on *in vivo* process shows a strong potential. This ’*in vitro’* study can thus be used as a base case, while increasing the complexity of the hypothetical model to make more accurate predictions.

Chapter 7: General Discussion

The white-margined sole, *Dagetichthys marginatus* is considered a promising new candidate species for aquaculture in South Africa (Thompson 2005, Thompson *et al.* 2008). The aims of this research were twofold. Firstly, to understand the early larval ontogeny of *D. marginatus* with specific focus on aspects of larval development directly linked to nutritional physiology (Chapter 1). Secondly, to model the physiological environment under which the digestion of feed in the larval gut of *D. marginatus* takes place. In the context of this thesis, the *in vitro* approach to model *Artemia* digestion in the gut of *D. marginatus* was of particular interest. The methods followed and developed give a mechanistic view of digestion that could shed light on a number of problems currently prevalent in larviculture.

The discussion attempts to advance the current understanding of nutritional physiology in finfish larvae by critically examining the pertinent findings of Chapters 3 to 6. Furthermore, the methods developed and the results from chapter 5 & 6 are evaluated and the possible implications for the wider field of nutritional physiology in mariculture are assessed. The chapter concludes with suggestions for future research in larval nutrition.

Low survival of finfish larvae throughout development is a common feature in larviculture when all the nutritional and environmental requirements of the animals are not properly met and this is especially dramatic in research on new species or diets (Kaji *et al.* 1996, Fernández-Díaz & Yúfera 1997, Cahu *et al.* 1998, Hamlin & Kling 2000, Robin & Vincent 2003, Papandroulakis *et al.* 2005, Yúfera *et al.* 2005, Howell *et al.* 2009, Das *et al.* 2012). A study of this nature is presented with a conundrum, as it depends on rearing several batches of larvae successfully in the complete absence of proven, species specific, rearing technologies. A strategy was adopted to use rearing technologies from closely related species throughout the larval rearing of *D. marginatus*. This strategy showed “reasonable” larval survival and growth, which allowed the examination of the morphological and physiological aspects set out in the aims. The

most suitable species from which rearing technologies could be adopted for *D. marginatus*, were *Solea solea* and *S. senegalensis* (Chapter 1, Thompson 2005).

The digestive capacity, as well as nutritional requirements of larvae must be considered when designing feeding strategies to ensure the ingestion, digestion and assimilation of nutrients (Lan & Pan 1993, Lovell 1998, Cahu & Zambonino Infante 2001, Martinez-Montano & Lazo 2012, Meyer 2012). While no evidence was presented in this thesis to dismiss the possible impact of ingestion and assimilation on rearing success of *D. marginatus*, the lack of a completely developed stomach, the absence of any detectable acidic protease activity during the first 45 days of ontogenetic development (Chapter 4) and the importance of exogenous enzymes (Chapter 6) all points to limited digestive capacity. Engrola *et al.* (2007) and Day *et al.* (1999) also hypothesize that a reduced digestive capacity is the most likely cause of rearing problems in soleids, especially during weaning (Chapter 1). Further support for the lack of acid digestion in soleids, which limits the capacity to digest artificial diets was found by Yúfera & Darias (2007), who showed that the gastric pH of *S. senegalensis* never drops below 6 despite the appearance of gastric glands.

Determining the digestive capacity of fish larvae remains one of the ultimate goals of larval nutrition studies (Lan & Pan 1993, Kolkovski 2001). It is clear that an accurate estimate of digestive capacity will lead to better designed diets and feeding strategies (Lovell 1998, Cahu & Zambonino Infante 2001, Kolkovski 2001, Gisbert *et al.* 2009, Martinez- Montano & Lazo 2012). Digestion capacity is however a very complicated physiological process and is technically difficult to predict (Favé *et al.* 2004, Martinez-Montano & Lazo 2012). The scientific solution to this problem is to use a 'surrogate' method that will approximate digestive capacity. These 'surrogate' methods are widely used, and generally involve the determination of one the factors generally considered to be important in digestion.

The digestive capacity of finfish larvae is umongst others, dependent on the specific activity of digestive enzymes in the gut. This is, of course, closely linked to the functional

status of the digestive tract and the support organs at any point in time (Martinez *et al.* 1999, Cahu & Zambonino Infante 2001, Kolkovski 2001, Gisbert *et al.* 2009) and can further be linked to external morphological characteristics required for ingestion of prey (Ende & Hecht 2010). The chronological age of the larvae does however, not necessarily indicate its physiological age (Blaxter 1988). Therefore the physiological and histological description of the digestive tract was done on the basis of discernible external morphological characteristics, clearly defined in Chapter 3. This allows one to adapt a feeding strategy at appropriate times corresponding to the physiological readiness, and not at a chronological age.

This study has shown that *D. marginatus* can successfully feed on live food such as *Artemia* and rotifers as early as 3 days after hatching (dah). The first feeding stage, which is associated with mouth opening and eye pigmentation, is not generally regarded as a critical stage in soleid ontogeny (Shields et al. 1999), as it is with other teleosts (Gulbrandsen 1993, Jähnichen & Kohlmann 1999, Yúfera & Darias 2007). In soleids the weaning stage is the most critical (Howell 1997, Dinis *et al.* 1999, Conceição *et al.* 2007, Bonaldo *et al*. 2011). This is also the case with *D. marginatus*, as no post metamorphosed larvae could be weaned on to an artificial diet (Chapter 2).

A diverse range of weaning strategies has been tested for soleids. Gatesoupe & Luquet (1982) started weaning *Solea solea* on to artificial feed at 10 dah, Person Le-Ruyet *et al.* (1980) at 30–40 dah and Overton *et al.* (2010) at 20 – 22 dah. Dinis (1992) started weaning *S. senegalensis* larvae 30 dah using re-hydratable pellets, while Yufera & Darias (2007) started at 50 dah. Flos *et al.* (1995) started weaning *S. senegalensis* at 6 months. None of the above weaning strategies show an advantage over another, but rather show a general compromise in slower growth but lower mortality with delayed weaning (Bonaldo et al. 2011). The use of innovative feed ingredients to improve the digestibility of the diet does seem to improve weaning success. For example, diets containing protein hydrolysates have been shown to work for weaning soleids prior to metamorphosis (Day *et al.* 1997, 1999) due to the reduced digestive requirements for the absorption of hydrolysed proteins. Theoretically, this should also be the case for post flexion *D.*

marginatus larvae as early as 20 dah (10.6 ± 0.6 mm), where pinocytosis associated with high levels of alkaline phosphatase (Chapter 4) should enable the absorption of nutrients from diets containing protein hydrolysates. However, regardless of dietary improvements, the slow development of *D. marginatus* (Chapter 3 & 4) suggests a later age of weaning for this species. This becomes clear considering that *D. marginatus* starts metamorphosis at a size of 9 mm between 16 – 30 dah (Chapter 3). In comparison *S. senegalensis* starts metamorphosis at about 8 mm and an age of between 11 – 19 dah (Dinis et al. 1999).

A simple qualitative and quantitative determination of enzyme activity in the gut is a useful starting point for improving larval and early juvenile rearing techniques. Various studies suggest that the activity of certain digestive enzymes is a key indicator for fish larvae to survive on formulated feed (Yufera et al. 2000, Kolkovski 2001). Although enzyme activity studies are often employed for this purpose, Chapters 4, 5 and 6 present a clear case why this could be misleading. The underlying reasons seem to be related to; 1) the experimental conditions under which the digestive enzyme activity was determined in the lab, and 2) the inadequacies for an accurate measure of digestion when complex food items and enzyme systems are considered.

Digestibility is better understood when a holistic view of digestion is taken, either through *in vitro* or *in vivo* studies (Montano & Lazo 2012)(see Chapter 6) and may be a more accurate method with which to define feeding strategies and feed formulations (Chapter 1).

An *in vitro* protocol was designed to establish a mechanistic view of digestion physiology and this was used to model the digestion of enriched and unenriched *Artemia* by various fish larvae given their enzyme activity levels and gut evacuation rates. The experimental protocol and the system modeling approach allowed for a rapid and reasonable approximation of *Artemia* digestion and showed good predictability when larval extracts from *D. marginatus* were used to test the model. This, together with the results from the enzyme characterization work (Chapter 5), also showed that regardless of taxonomic relatedness or dietary niche, warm temperate species show

strong similarities in their digestive capacity.

Artemia remains an important part of co-feeding weaning strategies for soleids over extended periods of time (Chapter 1). It has been hypothesised that the digestibility of artificial diets is enhanced through the exogenous enzymes provided by *Artemia* (Appelbaum 1985). While there is ongoing debate on this issue (Jancarik 1964, Dabrowski & Glogowski 1977a,b, Dabrowski 1984, Lauff & Hofer 1984,Cahu & Kolkovski *et al.* 1993, Zambonino Infante 1994, Oozeki & Bailey 1995, Zambonino-Infante *et al.* 1996, Moyano *et al.* 1996, Cahu & Zambonino-Infante 1997, Kurokawa *et al*. 1998, Gawlicka *et al.* 2000; Lazo *et al.* 2000), strong evidence is presented in Chapter 6 to supports this hypothesis, at least in part. 'In vitro' digestion of *Artemia* clearly shows that carbohydrate digestion is almost entirely the results of amylase (or possibly another form of carbohydrate digesting enzyme) from an exogenous origin. Amylase from endogenous origin contributes very little to the *Artemia* digestion during the early stages of larval development, despite the fact that it is often measured as an indicator for digestion.

Accepting the hypothesis that exogenous enzymes contributes significantly to digestive capacity, understanding the actual mechanism by which *Artemia* digestion takes place and the impact of different enzyme sources, substrate complexity and enzyme interactions become crucially important to get a holistic and realistic view on digestion. The *in vitro* approach taken in Chapter 6 is an attempt at this, and may prove useful in the design and the implementation of improved enrichment protocols for *Artemia* and ultimately weaning diets for *Artemia* replacement with further refinement of the model.

Chapter 5 also presents some evidence for the possible use of enzyme characterisation as a tool to predict dietary shifts under natural conditions. *Sarpa salpa* exhibited a significant decline in alkaline protease activity around the optimal pH with ontogenetic development, while amylase showed the opposite trend. This change in enzyme activity around the optimal pH corresponds to a change in the natural diet of *S. salpa* larvae at this stage (Christensen 1978, Chapter 5). It was therefore hypothesised that digestive

enzyme 'behaviour' is related to the natural feeding habits of the larvae. The digestive physiology of different species exhibited closer correlations with diet than their taxonomic relatedness. If this hypothesis gains further support then enzyme characterization could perhaps be used as an indicator of dietary shifts and hence the critical stages during larviculture. Furthermore, it could also serve as an indicator for the type and level of the nutrients that should be included in a formulated diet (Alarcón *et al.* 1997).

Conclusion

In conclusion, the degree to which technology is transferable between species remains a fundamental question in aquaculture research. This is especially true in larviculture where increasing complex studies and methods are employed to solve very species specific questions. This study presents evidence that the three warm temperate species studied show a predictable digestive capacity for *Artemia*, and that any variation is due to species specific enzyme activity levels and gut evacuation rates. While a complex *in- vitro* protocol was used to predict digestion of *Artemia*, the study further confirms that basic enzyme activity assessments are a good measure of digestion. This is despite the simplistic view of a very complicated digestive system used in modelling digestion. A feeding strategy for *D. marginatus* should therefore follow those of other farmed soleids, although there will be a general delay in implementation due to slower larval development. Problems can thus be solved and improvements made by transferring technology from other soleids to *D. marginatus.*

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