THE EFFECT OF DIET AND AGE-AT-WEANING ON GROWTH AND SURVIVAL OF CLOWNFISH *Amphiprion percula* (PISCES: POMACENTRIDAE)

Submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE

of Rhodes University

by

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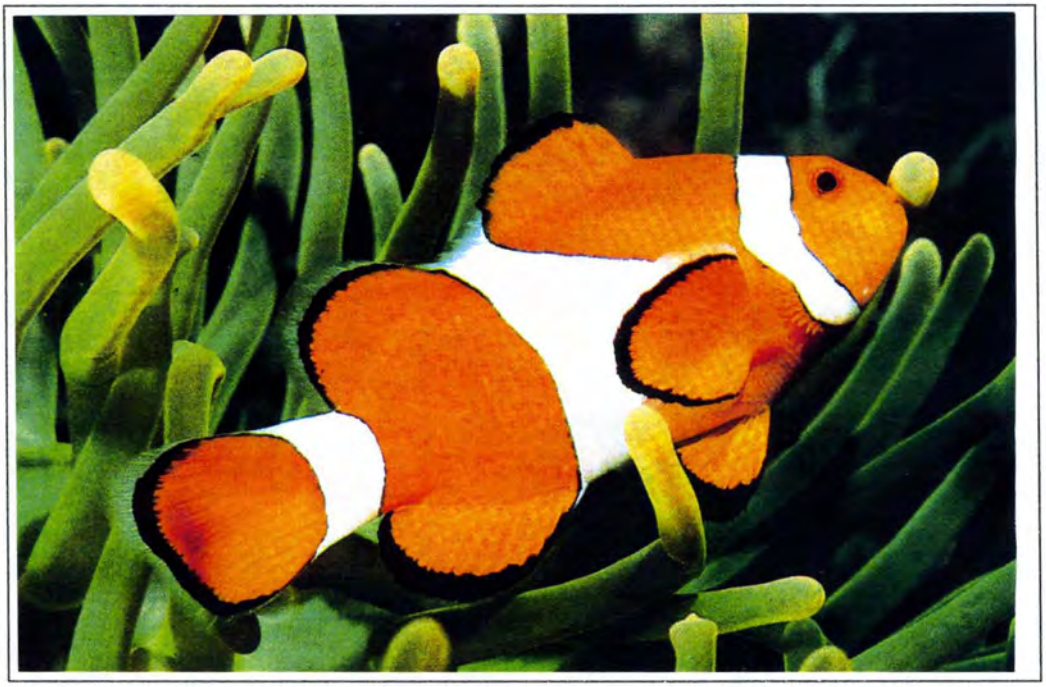
January 1999



Frontispiece: The common clownfish, *Amphiprion percula*

Photographed by H. Hall

Source: Ikan Agency, Frankfurt, Germany

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Acknowledgements

Thanks go to my supervisors Prof. Tom Hecht and Dr. Horst Kaiser for their guidance and advice. Also to Dr. Pieter Britz for constructive criticism of earlier manuscripts.

Rob Cross and Shirely Pinchuck of Rhodes University Electron Microscopy Unit, Prof. Ric Bernard of the Department of Zoology, Rhodes University, and Niall Vine must be thanked for technical advice they provided during the histological study.

To the following I'm grateful for feeding and caring for fish when I couldn't: Ferdie Endemann, Gavin Johnston, Cliff Jones, Larry Oellermann, Lucy Scott and Niall Vine.

This study was funded through the SANCOR Sea and Coast Program by the Foundation for Research and Development, the Department of Environmental Affairs and Tourism, and the Industrial Development Corporation.

Final thanks go to Terence and Ingrid Gordon.

Abstract

The aim of this study was to improve the rearing of common clownfish *Amphiprion percula*, by weaning juveniles from a live feed to a formulated feed as early as possible, while still maintaining good growth and survival.

The growth response of *A. percula* to a formulated dry feed was initially investigated. There was no difference in growth rate of juveniles fed a formulated feed, and the formulated feed supplemented with either *Artemia* or a *Donax serra* / *Penaeus indicus* combination. *Amphiprion percula* readily consumed the formulated feed, and the fishmeal/casein combination appeared an acceptable protein source.

As the amount of protein included in a diet can have a profound effect on growth, the optimal dietary protein level for juvenile *A. percula* was investigated by feeding semi-purified diets containing graded levels of protein, ranging from 40-65%. There was no difference in the growth rates of juveniles fed the various diets, however all diets promoted good growth with an average weight gain of 419%, and thus for the purposes of this study the diet formulation was deemed adequate.

The histological study of the digestive system of larval *A. percula* revealed that the alimentary canal was advanced at hatching and that larvae start exogenous feeding immediately. Three days after hatch (DAH) the yolk sac is completely absorbed. In the hind-gut epithelium of 5-day-old larvae small supranuclear inclusion vacuoles appear, suggesting pinocytotic digestion, and by 7 DAH gastric glands are established in the

epithelium of the stomach. Nine DAH supranuclear inclusion vacuoles appear in the epithelium of the mid-gut, indicating extracellular digestion and absorption across the lumen. As pinocytotic digestion of protein is less efficient than extracellular digestion, especially in the case of formulated feeds, it was hypothesised that the digestive system of *A. percula* could only effectively digest formulated feeds 9 DAH onwards.

The two weaning experiments designed to test this hypothesis revealed that *A. percula* was able to utilise the formulated feed, without reduction in survival, from 7 DAH onwards. However, in terms of growth, the optimal time to wean juveniles from the live feed to the formulated dry feed was between 15 to 20 DAH.

As *A. percula* accept a formulated feed and can benefit nutritionally from it, the dependence of larvae and juveniles on live feed can be reduced. This study has shown that the rearing of *A. percula* can been simplified and improved by weaning from 7 DAH with no reduction in survival, and from 15 to 20 DAH with no reduction in growth.

Chapter 1

Introduction

In 1985 the value of the international trade in marine aquarium fish was estimated to be US$ 24-40 million annually (Wood 1985). In 1992 imports into the United States of America and the European Union were valued at US$ 8.9 million (Chapman *et al.* 1997) and US$ 11 million (Bassleer 1994) respectively. Trade figures on aquarium fish imports and exports in the U.S.A. indicate that the industry is expanding. During the period 1982 to 1987 aquarium fish imports increased in value by approximately 34%, and export values more than tripled between 1986 to 1992 (Chapman *et al.* 1997).

Marine species are becoming increasingly popular in the aquarium market (Andrews 1990, Hoff 1996). In the United Kingdom freshwater fish imports doubled from 1977 to 1989, while marine fish imports trebled for the same period. During this period, the proportion of marine fish imported into the U.K. as a percentage of total aquarium fish imports rose from 11.4% to 17% (Wood 1992).

Approximately 90% of the freshwater fish available in the aquarium trade are captive bred (Andrews 1990). For marine species such an estimation is not possible, as there is no supporting data, but it is generally agreed that virtually all the marine species traded are captured in the wild (Andrews 1990, Chapman *et al.* 1997). Fish are caught using nets, or poisons such as sodium cyanide, rotenone and quinaldine (Wood 1985). Sodium cyanide (NaCN) is a very broad spectrum poison acting against the enzyme systems responsible for respiratory metabolism (Rubec 1988). The capture of fish using

sodium cyanide is highly destructive to coral reef habitat. Reef invertebrates and coral heads are killed when repeatedly exposed to sodium cyanide (Rubec 1988). Corals are also damaged by fishers who trample or smash them in the process of herding fish into nets (Wood 1985). Rubec (1988) estimated that, of the fish exposed to the poison, only 10% are selected and captured by the collector, 50% die entombed in coral, while the remaining 40% are consumed by predators. The fish which survive the initial cyanide exposure, also exhibit delayed mortalities. Cyanide exposure has been shown to cause internal damage to the fishes' liver, intestine and reproductive organs (Rubec 1988). Of the fish captured, more than 80% die during the journey from the collector to the marine hobbyist (Rubec 1988). Fish imported from Indonesia and Sri Lanka to South Africa have been found to have a survival rate of less than 2% (Vine & Hecht 1998). The fish which are exposed to the poison and manage to escape, probably suffer the same fate. Concern over the destruction of coral reef habitat (Rubec 1988, Medley *et al.* 1993) and the overexploitation and resulting depletion of certain species of marine aquarium fish (Wood 1985) has presented aquaculture with an opportunity of entering a new market, i.e. to provide a consistent supply of marine aquarium fish to an increasing market through the development of captive breeding programs.

Clownfish are the most popular species of fish in the marine aquarium trade (Hoff 1996). Of the 4000-4500 marine fish imported into South Africa monthly, clownfish make up the largest proportion, ca. 30% (Vine & Hecht 1998). Clownfish are also considered a good reference model for scientific research, especially in nutritional studies and in the determination of egg and larval quality (Delbare *et al.* 1995). It is

principally for these reasons that the common clownfish, *Amphiprion percula*, was chosen for this study.

Amphiprion percula, (see frontispiece) is one of 28 species belonging to the subfamily Amphiprioninae (Family Pomacentridae). Twenty seven species of this subfamily belong to the genus *Amphiprion* and one species to the genus *Premnas*. Members of the Amphiprioninae are commonly known as clownfish, or anemonefish because of their symbiotic relationship with large sea anemones. *Amphiprion percula* is found in association with three anemones; *Heteractis magnifica* (Quoy & Gaimard 1833), *Stichodactyla gigantea* (Forsskal 1775), and *Heteractis crispa* (Ehrenberg 1834) (Fautin & Allen 1992).

Amphiprion percula is normally bright orange with three vertical white bars across the body. The anterior bar is found immediately behind the eye, the mid-body bar has a pronounced forward projecting bulge, and the posterior tail-bar occurs in the precaudal region. All bars have a thick black margin. In some specimens associated with *Stichodactyla gigantea*, the black margins have expanded considerably resulting in a melanistic variety of this species. *Amphiprion ocellaris* is identical to *A. percula* in appearance, but is distinguished by having 11 dorsal spines instead of 10 (Fautin & Allen 1992).

Under natural conditions, adult *Amphiprion* species spend the greatest proportion of their time foraging and feeding, and appear to be omnivorous, opportunistic feeders (Allen 1972). Allen (1972) examined the stomach contents of 74 specimens. Species

examined included *A. chrysopterus*, *A. melanopus*, *A. tricinctus* and *A. perideraion*. By volume, 91.8% of their food consisted of 34.8% copepods, 33.0% algae, 9.7% polychaetes, 6.4% crustacean fragments, 5.2% tunicates, and 2.7% amphipods. The remaining 8.2% consisted of a wide range of food items including; isopods, barnacle nauplii, *Amphiprion* eggs, shrimps, crustacean larvae, barnacle appendages, unidentified eggs, crabs, nematodes, ostracods and polychaete remains. Ingested copepods included calanoid, cyclopoid and harpacticoid species. A wide diversity of algae were also consumed, including diatoms, red algae, blue green algae, brown algae, and green algae.

Like other Amphiprioninae, *A. percula* is a protandrous hermaphrodite, manifested by a discrete social hierarchy. The largest and socially dominant fish is the female. The next largest fish is a reproductively active male whose gonads function as testes but also possess non-functioning or latent ovarian cells. A number of smaller reproductively inactive males may also inhabit or associate with the host anemone. If the dominant female is removed the dominant male changes sex. Simultaneously, the largest non breeding male becomes the functional and reproductively active male. (Fautin & Allen 1992, Hattori 1994).

In the tropics, clownfish species spawn throughout the year, but in the subtropics reproductive activity is limited to spring and summer (Allen 1972). Spawning in *A. perideraion*, *A. chrysopterus* and *A. clarkii* has been strongly correlated with lunar cycles (Allen 1972, Ochi 1985). Spawning activity peaks around the 1st and 3rd quarter of the lunar cycle, and consequently hatching activity peaks near full and new moon. It

has been suggested that this correlation occurs because of the stronger currents associated with spring tides at new and full moon which would aid larval dispersal (Allen 1972).

Allen (1972) reported that captive *A. ocellaris* spawned every two weeks. A similar spawning frequency has been observed for *A. ocellaris*, *A. percula*, *A. clarkii* and *Premnas biaculeatus* in the marine hatchery of the Department of Ichthyology and Fisheries Science, Rhodes University. Spawning under natural conditions generally occurs 2-3 hours after sunrise and lasts for 30 minutes to 2 hours (Allen 1972, Ross 1978). The number of eggs laid range from 100 to 1000, with an average clutch size of 200-400 eggs (Allen 1980, L. Oellermann, Rhodes University, pers. comm.). Eggs are elliptical in shape and 3-4 mm long. During the incubation period the batch is guarded and attended by the male who fans the eggs at frequent intervals with his pectoral fins, while also removing debris and dead eggs (Allen 1972). Hatching occurs 1-2 hours after sunset between the sixth to eighth day after eggs are laid (Allen 1972, Hoff 1996, L. Oellermann, Rhodes University, pers. comm.).

Clownfish have the shortest larval period of the Pomacentridae, ranging between 8-12 days (Fautin & Allen 1992). During this time they are planktonic - living in the surface waters of the ocean, where they are passively transported by currents. It has been suggested that the short larval period has contributed to the localised nature of the distribution pattern of *A. percula* in northern Queensland, the islands of New Guinea, New Britain, New Ireland, Vanuatu, and the Solomon Islands (Fautin & Allen 1992).

Larvae of *Amphiprion percula* possess very little energy reserves in the form of yolk and oil deposits, and if unable to feed exogenously will die within three days of hatching (Hoff 1996). Their small size at hatching (3.8-4.2 mm)(Allen 1972), and the need for an immediate exogenous food source, presents problems to the aquaculturalist as a very small dietary item has to be supplied almost immediately. Larval and early juvenile clownfish have been reared successfully on highly integrated and diverse feeds (Frakes & Hoff 1983), consisting initially of rotifers, small particulate dry feed, and small *Artemia*, and then progressing to larger *Artemia*, krill meal, larger particulate dry feed and a gelatine mixture of natural feeds (chicken heart, fish roe, krill meal, fish flesh, shrimp tails and clam meats) (Hoff 1996). This highly integrated and complex diet and feeding practice is costly, time consuming and impractical in large scale commercial larviculture.

Improvements in finfish larviculture have been achieved by refining environmental factors like tank colour, photoperiod, light intensity, and by simplifying and improving the feeding sequence of larvae (Chatain 1997). These improvements have increased the survival of larvae and reduced the costs of producing fingerlings. For example, in the larval rearing of France's two most important finfish; the sea bass *Dicentrarchus labrax* and seabream *Sparus auratus*, survival rates have been increased from an average of 5% for both species to 50% for *D. labrax* and 30% for *S. auratus*. This has been achieved by simplifying the weaning procedure for these fish. Initially, weaning of these two species started four months after hatch and involved a long and complicated food supply sequence of live adult *Artemia*, frozen copepods, frozen adult *Artemia*, minced meat and then formulated feed pellets. The larviculture of these two species

now involves weaning from 1.5 months old, over a period of 3 days from live *Artemia* to pelleted food (Chatain 1997).

As a result this study focused on refining the feeding practices for the large-scale rearing of *A. percula* larvae and juveniles. The aim was to simplify the diet and feeding protocol, but still ensure that the diet was nutritionally adequate to maintain good growth and survival. Experiments were designed to investigate whether and how a formulated dry feed could be used for the successful rearing of larval and juvenile *A. percula*.

Formulated diets have been less efficient than live food for the rearing of marine fish larvae and juveniles. Problems include the difficulty in inducing larvae and juvenile fish to accept an artificial feed, the failure of formulated diets to meet the nutritional requirements of the larvae, and the inability of the larval gut to efficiently digest formulated feeds (Segner & Rosch 1990, Le Ruyet *et al.* 1993).

The acceptability of a particle of feed by a fish is controlled by its sensory responses. These include visual, electro-, mechano- (sound and turbulence), and chemoreceptive responses (Pigott & Tucker 1989). For example, Dendrinos *et al.* (1984) reported that first feeding Dover sole *Solea solea* larvae ate more *Artemia* stained black than those stained four other colours or unstained, concluding that the black ones were more visible. Appelbaum (1985) observed that the larvae of *S. solea* at first feeding may sense and adapt to catching and ingesting live and mobile food more readily than inert feeds. Appelbaum *et al.* (1983) inferred that final selection of food particles in *Solea*

solea larvae was chemoreceptive, occurring in the mouth where the particles were either swallowed or rejected. The authors attributed this response to taste buds found in the buccal cavity, and entrance to the oesophagus in 4-5mm larvae.

In addition to these sensory responses, different species have various preferences or habits for consuming food. The position of the fish in the water column when feeding (benthic, mid water, and surface) will dictate the optimal density of the feed. Thus the stimulus to feed can be affected by the size, movement, shape, density, texture, colour and contrast, and odour of the feed particles (Dabrowski 1984, Pigott & Tucker 1989, Le Ruyet *et al.* 1993). For this reason it was necessary that the first experiment in this study was designed to test whether *A. percula* juveniles would accept, ingest and derive nutritional benefit from a formulated dry feed (Chapter 3).

Poor growth and survival of larvae fed formulated feeds has been attributed to the inability of these feeds to meet the absolute nutritional requirements of most larval fishes (Jones *et al.* 1993). Investigating specific nutritional requirements of larvae will only be possible when a water stable compund diet is accepted, ingested, digested and assimilated at rates comparable to live feeds (Jones *et al.* 1993). Thus, at present, compound diets for larvae are formulated empirically (Abi-Ayad & Kestemont 1994). As nothing is known about the nutritional requirements of larval or juvenile *A. percula*, an experiment was designed to test the dietary protein requirements of early juvenile *A. percula* (Chapter 4). A diet formulated for *A. percula*, or for any other marine species for that matter, must maximise growth. The animal's needs for maintenance and growth are supplied by energy derived from the food (Cho & Bureau 1995). Energy in the diet

is supplied by protein, carbohydrate and lipids. However, fish have a limited ability to digest carbohydrate and therefore utilise protein and lipids for energy (Wee 1992). The energy content of the diet should not be set too low, nor should the protein level be allowed to fall below a certain level if maximal growth is to be achieved (Steffens 1989). If the energy in the diet is too low, the fish will utilise protein as an energy source, and if there is insufficient protein in the diet then the protein that is available will be used for basal metabolic requirements and not for somatic growth (Cho & Bureau 1995). For this reason it is important to determine the optimal protein level for growth and survival of the fish (Tucker 1992a).

Initially the larval fish's digestive tract is undifferentiated and gut length is relatively short, usually 0.5 standard body length (Lauff & Hofer 1984). After first exogenous feeding, changes in the digestive tract occur, with mucosal folds developing and the intestine becoming regionally differentiated (De Silva & Anderson 1995). The gut is sterile at first feeding and it can take several days or weeks before there is an increase in proteolytic activity (Lauff & Hofer 1984, Dabrowski & Culver 1991). The poor performance of larvae on formulated feeds can thus be attributed to a combination of both inefficient morphological and digestive functions in the larval gut. For example, the short larval gut length results in short gut retention time of food. As a result, there is insufficient time for extracellular digestion by tryptic enzymes to achieve complete hydrolysis of protein (De Silva & Anderson 1995). In addition, proteolytic enzymes have a low affinity for the processed proteins in formulated diets, resulting in insufficient hydrolysis and low food utilisation (Dabrowski & Culver 1991).

The inability of the larvae's incompletely developed gut to digest formulated dry feeds has resulted in poor growth and survival for most larvae (Le Ruyet *et al.* 1993). Although it is economically advantageous to wean larvae onto dry feeds as early as possible (Le Ruyet *et al.* 1993, Hayashi 1995), few marine species have been successfully reared from first feeding exclusively on artificial diets (Jones *et al.* 1993). In general, freshwater larvae can adapt to dry feed relatively easily, with *Coregonus lavaretus* (Champigneuille 1988), *Cyprinus carpio* (Charlon & Bergot 1984), *Clarias gariepinus* (Uys & Hecht 1985) and *Micropterus dolomieui* (Ehrlich *et al.* 1989) larvae all having have been reared exclusively on dry diets from first feeding with good growth and survival. However, with marine larvae total replacement of conventional live feeds with formulated feeds has resulted in very poor survival and growth rates (Table 6.1)(Jones *et al.* 1993).

It is unclear why, so far, there has been more success rearing freshwater larvae than marine larvae exclusively on formulated feeds. There are suggestions that, as freshwater larvae are larger at hatch (12-25 mm) than marine larvae (2-3 mm), their larger mouth size allows them to ingest formulated feed particles, which are larger than many live feeds. Furthermore their digestive system is often more advanced at a comparative age, allowing better digestion of the formulated feed by proteolytic enzymes (Jones *et al.* 1993). To determine when the necessary organs associated with the digestion of formulated feed develop in larval and early juvenile *A. percula*, a histological study was first conducted allowing an estimation of the time when weaning from a live feed to a dry feed could take place (Chapter 5). Two weaning experiments were then undertaken. An early weaning trial to test when *A. percula* larvae could

ingest and benefit nutritionally from a formulated feed, and a late weaning trial to test exactly when *A. percula* juveniles could be weaned off the live feed to a formulated feed, while still maintaining good growth and survival (Chapter 6). In the early weaning trial, larvae were weaned to a formulated feed 4, 7 or 10 DAH and in the late weaning experiment juveniles were weaned 10, 15, 20, 25 and 30 DAH.

The successful commercial rearing of *A. percula* would involve reducing production costs and increasing outputs of juveniles. This would be achieved through an understanding of the nutritional requirements of *A. percula* juveniles and through implementing a simple but effective rearing procedure. Thus, this study hopes to have fundamental relevance, adding to the understanding of the nutritional requirements of this species by conducting the protein requirement experiment and the histological study. It will also have practical relevance, improving the rearing procedure and simplifying the feeding practice of *A. percula* by determining the optimal time to wean juveniles off live feed to a formulated feed.

Chapter 2

Materials and methods

Origin of experimental fish

Larval and juvenile *A. percula* were obtained from the marine hatchery, Rhodes University. The two pairs of *A. percula* brood stock were each held in 400L rectangular tanks connected to a 4000L recirculating system. On the evening of their anticipated hatch the eggs were removed from the broodstock tank and placed into a 90L conical larval rearing tank.

Larval rearing tanks contained green water (*Nannochloropsis* sp.) at a concentration of 10000 cells.ml^{-1} and were maintained at a temperature of 28 ± 1°C and a salinity of 35ppt. Besides the addition of the greenwater during the first five days after hatch (DAH), water in the tank was kept stagnant. From 5 DAH water from the recirculating system was passed through the larval rearing tank at a flowrate of 0.22L.min^{-1}.

For the first 7 DAH larvae were fed rotifers (*Brachionus* sp.). From 5 DAH 1st instar *Artemia franciscana* (Jamaican strain) were fed to the fish until they were moved into the experimental tanks.

System design and management

The experiment to evaluate the suitability of a formulated dry diet (Chapter 3) and the two weaning experiments (Chapter 6) were conducted in a bucket system (Figure 2.1). Twelve 25L dark green buckets (height 36cm, diameter 32cm) were connected to a

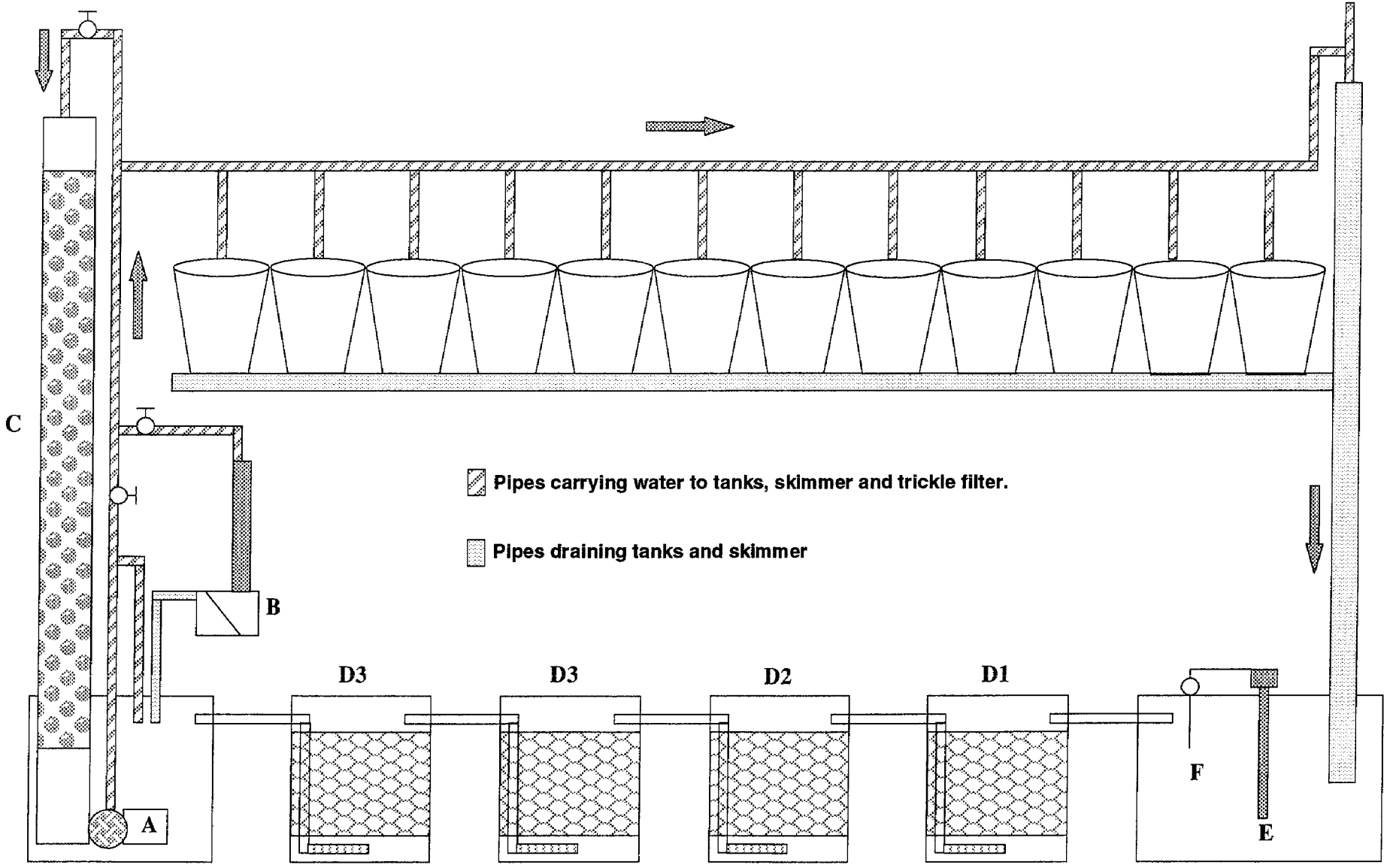

Figure 2.1 Experimental bucket system (arrows indicate direction of water flow). Pump (A); foam fractionator (B); trickle filter (C); mechanical filter (D1); shell buffer (D2); biological filter (D3); heater (E); thermostat (F).

1200L recirculating system with biological and mechanical filtration. Dark green buckets were used because Johnston (1997) showed that *Amphiprion clarkii* larvae reared in vessels with dark backgrounds displayed 80% survival in comparison with less than 4% survival for larvae reared in vessels with light backgrounds. The filtration system consisted of a 350L settlement tank and four 90L flooded filter compartments, a 2.5m x 200mm diameter trickle tower and a foam fractionator. Two of the wet filter compartments were filled with shredded plastic, one with oyster shells and one with synthetic filter wool. The trickle filter was filled with synthetic filter wool and coiled plastic. Temperature was kept constant at 26 ± 1°C by means of a 0.6KW thermostatically controlled submersible heater. This temperature lies within the ideal temperature range of 26-28°C for most *Amphiprion* species (Juhl 1992, Hoff 1996). Water was circulated through the system by means of a 0.3KW pump. Flow rate into the buckets was kept constant at 0.35L.min^{-1}. Water that was not fed to the buckets flowed through the trickle tower and back into the filtration system. Photoperiod was maintained at 12L : 12D by means of a time switch. Buckets were illuminated by a 2m biolux fluorescent tube mounted to the ceiling, and two 100 watt incandescent bulbs positioned 1m above the water surface. Light intensity at the water surface was measured with a QSL-100 laboratory quantum scalar irradiance meter and remained constant at 0.4x10^{15} quanta.sec^{-1}.cm^{-2}.

The experiment to determine the optimum protein level for growth (Chapter 4) was conducted in a 1500L recirculating system (Figure 2.2). The system consisted of eighteen 40L glass aquaria, which were filled to a capacity of 36L. Each of these tanks

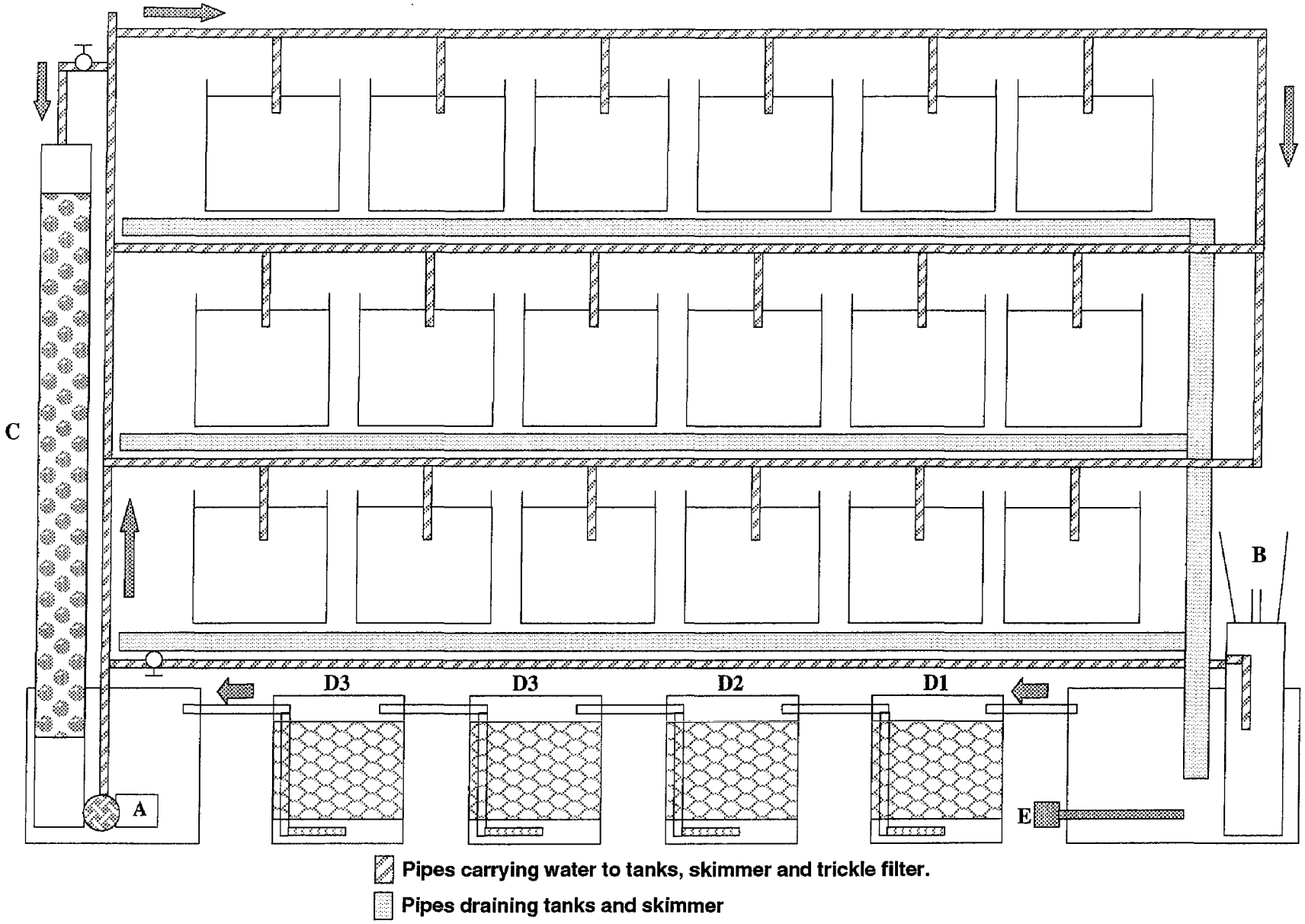

Figure 2.2 Glass aquaria experimental system (arrows indicate direction of water flow). Pump (A); foam fractionator (B); trickle filter (C); mechanical filter (D1); shell buffer (D2); biological filter (D3); heater (E).

was supplied with 2 water inlet pipes and aerated by means of a single air stone. The combined overflow from all tanks drained into a 500L settlement tank. From the settlement tank the water passed through a four-stage (4 x 90L) submerged biological filter (identical to the filter of the system described in the above paragraph) to the sump (90L). A 0.3 KW submersible pump circulated the system water. Fifty percent of the water not pumped to the tanks was passed through a foam fractionator in the sedimentation tank while the other half drained through a 2m high trickle filter (250mm diameter). The rate of inflow into the tanks was set at 0.6Lmin^{-1}. The water was kept at a constant temperature of 26 ± 1°C by means of a thermostatically controlled 0.4KW immersion heater in the settlement tank. Photoperiod was set to 12L : 12D with a time switch. Groups of three tanks were illuminated with a 1m Biolux fluorescent tube. The light intensity at the water surface was measured with a QSL-100 laboratory quantum scalar irradiance meter and remained constant at 6x10^{15} quanta.sec^{-1}.cm^{-2}.

Water quality for both systems was monitored weekly. Salinity was kept constant at 34 ± 1ppt with the addition of freshwater. Salinities lower than 35ppt are recommended as they may reduce the chance of disease as well as providing a buffer against rising salinity due to evaporation (Allen 1980, Hoff 1996). Ammonia (NH_4^+/NH_3) was measured by means of the Salicylate method (Bower & Holm-Hansen 1980), nitrite (NO_2^-) using the Diazotisation method (Boyd & Daniels 1988), and nitrate (NO_3^-) by means of an aquarium test kit (Interpet Nitrate Test). NH_4^+/NH_3 values ranged from 0 - 0.01mg.L^{-1}; NO_2^- values ranged from 0.0 - 0.01mg.L^{-1} and NO_3^- levels remained below 25 mg.L^{-1}. pH and dissolved oxygen were measured weekly using a Horiba pH meter F-

8 L and an OxyGuard^{R} Handy MK III respectively. pH ranged from 8.1 - 8.3 and dissolved oxygen from 90 - 100 %.

Feed preparation

The formulated feeds used in experiments to test the acceptability and suitability of an artificial feed (Chapter 3), optimal protein requirement (Chapter 4) and the optimal weaning time (Chapter 6) for *A. percula* juveniles were prepared according to guidelines for experimental feed preparation described by Lovell (1989). The dry ingredients were weighed and then thoroughly mixed to ensure homogenous distribution of all ingredients. After mixing the dry ingredients the oil was evenly dribbled over the dry ingredients. Finally, distilled water was slowly added while mixing, until a stiff dough was formed. The dough was placed in a commercial food mixer and mechanically kneaded for 10 minutes. It was then extruded through a 2 mm die. The strands of extruded diet were sliced into small pellets. These pellets were left to air dry, then hammer milled and sieved to the desired size range of 100 - 320μm for the weaning experiments (Chapter 6), and 320 - 500μm for the dry feed acceptability and suitability trial (Chapter 3), and optimal protein requirement trial (Chapter 4).

The experimental formulated feeds contained the same ingredients with the exception of dextrin, which was not included in the diet used to test the suitability and acceptability of the dry feed (Chapter 3). Dextrin was included in the other formulations to keep the diets isocaloric. Tables showing the test diet formulations appear in the materials and methods sections of the relevant chapters.

The natural feed used in the formulated feed acceptability and suitability trial (Chapter 3) was prepared by mincing equal quantities, on a wet weight basis, of sand-mussel (*Donax serra*) and shrimp (*Penaeus indicus*). This mix was frozen in small ice cube trays (1cm x 1cm).

The live feed control used in all experiments was enriched (Super Selco - INVE Aquaculture NV) *Artemia franciscana* (Jamaican strain). Cysts were hydrated, decysted, and hatched according to the method outlined in Sorgeloos *et al.* (1986).

Feeding protocols

Amphiprion percula did not feed off the bottom of the tank and did not readily take food from the water surface. Food was only taken in the water column. The formulated dry feed and the natural feed were consequently fed in excess, allowing the fish as much access to feed in the water column as possible. This feeding behaviour did not allow for controlled satiation feeding. Furthermore, waste could not have been equal under the different feeding regimes as dietary combinations contained different amounts of live, natural and dry feeds. Nevertheless, growth responses such as protein efficiency ratio (PER) and food conversion ratio (FCR) were calculated and used on a comparative basis between treatments.

Artemia were always provided to the fish at a density of 500 *Artemia*.L^{-1} . This density has been found to promote optimal survival in *Amphiprion clarkii* larvae (Johnston 1997).

Response monitoring

Growth is the most important criterion for measuring responses in nutritional studies and has been shown to be a sensitive and practical indicator of a particular nutrient or energy level (Lovell 1989). In this study, growth was assessed using the differences in the initial and final weights and lengths of the fish in each treatment. Food conversion ratio was calculated from:

FCR = Food Intake (dry mass) / Body Mass Gain (wet mass)

and PER from:

PER = Body Mass Gain (wet mass)/ Protein Ingested (dry mass)

The condition factor (CF) is a good indicator of the general "well-being or fitness" of an animal (Bolger & Connolly 1989). The higher the magnitude of the CF the greater is the weight in relation to unit length (Tesch 1968). Condition factor is calculated from the relationship:

$$CF = \text{Body mass (wet weight mg)}/ \text{Length (mm)}^{3.4}$$

This relationship was determined from a length/mass regression of *A. percula* larvae between the ages of 4 and 17 DAH, where $Y = -11.7941 + 3.4X$ ($r^2 = 97\%$, $n = 45$). Larvae used for this regression were reared in the marine hatchery, Rhodes University, on a live food combination of rotifer and *Artemia* nauplii.

In a trial weaning experiment, larvae or very young fish often died during the weighing and measuring process. As a result, only the final weights and lengths were recorded in the two weaning experiments. A sample of larvae were sacrificed for the initial

weighing and measuring. However, in experiments conducted on older juveniles (> 17 days after hatch), weighing and measuring was conducted fortnightly.

Prior to handling, the fish in each tank were anaesthetised in a solution of 0.2 ml 2-phenoxyethanol.L^{-1}. Anaesthetising fish regularly with 2-phenoxyethanol has been shown not to affect growth (Deacon *et al.* 1997). Fish in each replicate were weighed together (inividual weighing would have increased stress on the fish) and then photographed on graph paper to measure total length (0.1 mm).

Statistical analysis

In Chapters 3 and 4 slopes of the growth curves for length and weight were compared using an analysis of covariance (ANCOVA, Zar 1996) followed by Tukey's Multiple Range Test. In Chapter 3, 4 and 6 mean weight, length, FCR, PER and CF were analysed using an analysis of variance (ANOVA). The Kolmogorov-Smirnov test was used to test if data were normally distributed at $P < 0.05$. In all experiments size variance was analysed using Bartlett's test for homogeneity of population variances (Ott 1988).

Chapter 3

The suitability of a formulated dry feed for the feeding of juvenile *Amphiprion percula*

Introduction

The current practice of rearing of juvenile *Amphiprion percula* in the marine hatchery at Rhodes University involves feeding live feed until approximately 30 days after hatching (DAH). At this time the fish are transferred to the growout tanks, and fed on a general commercial marine flake (Wardley^{R}, Total Marine^{TM}). Presently, there is no formulated dry feed that has been specifically formulated for clownfish growout (Hoff 1996). The prolonged use of live feed is expensive and time consuming, and consequently formulated feeds are becoming popular as substitutes for live foods (Hayashi 1995).

Yet, marine species which are reared on formulated feeds from first feeding, always exhibit lower growth rates than their conspecifics fed live or natural feeds (see Table 6.1). The specific reasons for natural feeds promoting superior growth to inert formulated feeds are unproven, however a number of possibilities has been identified. Problems include the difficulty inducing larval and juvenile fish to accept formulated feeds (Le Ruyet *et al.* 1993). The suitability of a dry feed can be assessed on the basis of whether the fish recognises the particles as food, and is able to ingest, successfully digest and assimilate the feed in sufficient amounts to promote good growth and survival (Holt 1993). The acceptability of a feed particle to a fish is mediated by the fish's sensory systems. A formulated feed, therefore, only becomes acceptable when it

The second hypothesis tested was the effect of the formulated feed on the growth responses of *A. percula* juveniles:

H_O - *Amphiprion percula* juveniles fed on the formulated dry feed show growth rates comparable to juveniles fed diets of formulated dry feed supplemented with live or natural feeds.

H_A - *Amphiprion percula* juveniles fed only the formulated dry feed show inferior growth to juveniles fed diets of formulated dry feed supplemented with live or natural feeds.

The formulated dry feed chosen was one previously found optimal for the rearing of juvenile spotted grunter *Pomadasys commersonnii* (Haemulidae)(Irish 1997). This particular diet was selected because it is a relatively general formulation, providing *A. percula* with the fundamental nutrients required by most marine species. Furthermore it is formulated for a juvenile marine fish, and contains high quality protein sources that are readily accessible to the aquaculturist. Clownfish juveniles were divided into three treatments and fed three diets that differed according to appearance (texture, shape, movement) and protein source. The three diets consisted of the formulated dry feed, the formulated dry feed supplemented with live feed (*Artemia*) and the formulated dry feed supplemented with finely grated shrimp (*Penaeus indicus*) and sand mussel (*Donax serra*).

H_O - The formulated dry feed was not readily ingested by *A. percula* juveniles.

H_A - The formulated dry feed was recognised and ingested in adequate amounts to promote good growth and survival.

The second hypothesis tested was the effect of the formulated feed on the growth responses of *A. percula* juveniles:

H_O - *Amphiprion percula* juveniles fed on the formulated dry feed show growth rates comparable to juveniles fed diets of formulated dry feed supplemented with live or natural feeds.

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Materials and methods

Origin of the experimental fish and culture system

Juveniles were obtained from our marine hatchery and transferred to 25L dark green buckets (bucket height 36cm, diameter 32cm) connected to a 1200L recirculating system with biological filtration, at a flow rate of 0.35L.min^{-1}. Salinity was kept constant at 34ppt ± 1ppt and temperature at 26°C ± 1°C.

Diet combinations

One hundred and eight 29-day old juveniles were weaned in the marine hatchery onto the formulated dry feed over a three day period. Weaning consisted of feeding dry feed at 09h00 and 17h00, while *Artemia* was still fed at 13h00. After the three day period fish were moved to the experimental system, divided into three treatments of three replicates each (at 12 fish per replicate), randomly assigned a bucket and fed the three experimental diets for six weeks.

The experimental diets consisted of a dry diet formulated for juvenile spotted grunter *Pomadasys commersonnii* (Irish 1997) (Table 3.1) with a particle size range of 320 - 500µm; a combination of the dry diet and enriched (Super Selco - INVE Aquaculture NV) 1st instar *Artemia franciscana* (Jamaican strain), at 500 *Artemia*.L^{-1}; and a combination of the dry diet supplemented with a natural diet consisting of equal amounts on a wet weight basis of finely grated shrimp (*Penaeus indicus*) and sand mussel (*Donax serra*).

The formulated dry feed was presented to the fish in all treatments at 9.00 am and 5.00 pm. At 1.00 pm, treatment 1 received the dry formulated feed, treatment 2 received enriched *Artemia*, and treatment 3 was fed on the natural feed. To prevent the escape of *Artemia* the outflow of the buckets were covered with a 285µm mesh for two hours after feeding. Fish in all treatments were fed in excess of their requirements to ensure sufficient access to food so that food availability was not a growth-limiting factor.

At the beginning of the study and every two weeks thereafter, all fish were anaesthetised with 2-phenoxyethanol at $0.02ml.L^{-1}$ to obtain weight and length data. Anaesthetising fish regularly with 2-phenoxyethanol has been shown not to affect growth (Deacon *et al.* 1997). The twelve fish of each replicate were weighed together and then photographed on graph paper to measure total length (0.1mm).

Slopes of the growth curves for length and weight were compared using an analysis of co-variance (ANCOVA, Zar 1996). Size variance was analysed using Hartley's test for homogeneity of population variances (Ott 1988).

Proximate analysis and energy content of the formulated dry feed, *Artemia*, *P. indicus* and *D. serra* was determined at the Department of Animal Science and Poultry Science, University of Natal using standard methods (Table 3.1 & 3.2). The dry weight of the formulated feed, *Artemia*, and *P. indicus* / *D. serra* combination was determined at the beginning and end of the experiment. Proximate analysis of the diet combinations is presented in Table 3.3.

Table 3.1 The composition of the formulated dry feed (g/100g)

Fishmeal	46
Casein	14
Pregelatinized starch	27
Fish oil	4.5
Sunflower oil	2.2
Mineral mix[1]	4
Vitamin mix[2]	2
Carophyll pink	0.1
Proximate composition	
Protein (%)	43.8
Fat (%)	8.4
Ash (%)	10.8
Moisture (%)	12.7
Gross energy (MJ/Kg)	18.0

[1] Mineral mix (g/kg) : 74g potassium; 516g vermiculite RSU; 14g salt; 0.05g ammonium chloride; 31g choline chloride; 0.31g cobalt; 0.15g copper; 1.5g iron; 0.05g iodine; 0,22g manganese; 41g magnesium; 1g zinc and traces of selenium.

[2] Vitamin mix (IU or g/kg) : 500 000 IU vitamin A; 400 00 IU vitamin D3; 10 000 IU vitamin E; 1g vitamin K3; 0.25g vitamin B1; 1.5g vitamin B2; 0.5g vitamin B6; 25g vitamin C; 2.5g Niacin; 0.09g Folic acid; 0.025g Biotin; 2.5g Calpan; 2.5g Inositol.

Table 3.2 Proximate composition of *Artemia*, *P. indicus* and *D. serra*.

	Protein (%)	Fat (%)	Ash (%)	Moisture (%)	Gross energy (MJ/Kg)
Artemia	53.4	15.8	9.1	86.8	20.8
P. indicus	91.3	2.3	8.2	78.6	20.4
D. serra	69.0	2.3	20.5	83.7	17.1

Table 3.3 Proximate analysis of the experimental diet combinations

	Protein (%)	Fat (%)	Ash (%)	Gross energy (MJ/Kg)
Dry diet	43.8	8.4	10.8	18.0
Dry/live	46.5	10.5	10.4	18.7
Dry/ natural	48.2	7.8	11.2	18.3

Results

On completion of the study, fish from treatments 1, 2 and 3 had received, by dry weight, 30.36 ± 0.47g, 30.22 ± 0.69g and 24.46 ± 0.46g of feed respectively. In treatment 2, the proportion of dry feed replaced by live feed was 27.85 ± 0.94%. While in treatment 3, the proportion of dry feed replaced with natural feed was 10.13 ± 0.25%.

Average survival rate over all treatments was 92.6 % (Table 3.4). Two fish died in treatment 1, one in treatment 2, five in treatment 3 (four of these as a result of an accident during the cleaning of tanks 71 DAH). On completion of the six-week growth trial, all fish had tripled their weight and many had quadrupled it (Table 3.4).

The slopes of the growth curves for both length (ANCOVA, $P > 0.05$, $F = 0.39$, d.f. = 426) and weight (ANCOVA, $P > 0.05$, $F = 0.59$, d.f. = 36) did not differ significantly between treatments (Figure 3.1). The data were pooled into one growth model for both weight and length. Growth was best expressed by linear models $y = 0.076 + 0.03937X$ ($r^2 = 91.02$, n= 36) for weight and $y = 13.4773 + 1.6345X$ ($r^2 = 56.15$, n = 426) for length, where y = weight or length and x = time in two week intervals. Food conversion ratios and PER did not differ significantly between treatments ($P > 0.05$, ANOVA).

Variance of size within each treatment increased significantly over the study period ($P < 0.05$, Hartley's Test) (Figure 3.1). However, size variation of the three treatments was not significantly different from one another. ($P > 0.05$, Hartley's Test).

Table 3.4 The effect of different diets on survival and growth of *A. percula* (± standard deviation).

	Dry diet	Dry/live	Dry/natural
Survival (%)	94.4 ± 9.6[a]	97.2 ± 4.8[a]	86.1 ± 17.3[a]
Initial mean weight (g)	0.09 ± 0.011	0.07 ± 0.010	0.08 ± 0.008
Final mean weight (g)	0.31 ± 0.048[a]	0.3 ± 0.050[a]	0.34 ± 0.041[a]
Initial mean length (mm)	13.5 ± 1.84	13.2 ± 1.71	13.8 ± 1.74
Final mean length (mm)	23.4 ± 3.68[a]	22.5 ± 4.60[a]	23.0 ± 4.09[a]
FCR	10.35 ± 1.44[a]	11.17 ± 1.87[a]	8.96 ± 1.96[a]
PER	0.22 ± 0.03[a]	0.20 ± 0.03[a]	0.24 ± 0.06[a]

Values in the same row sharing a common superscript are not significantly different ($P > 0.05$)

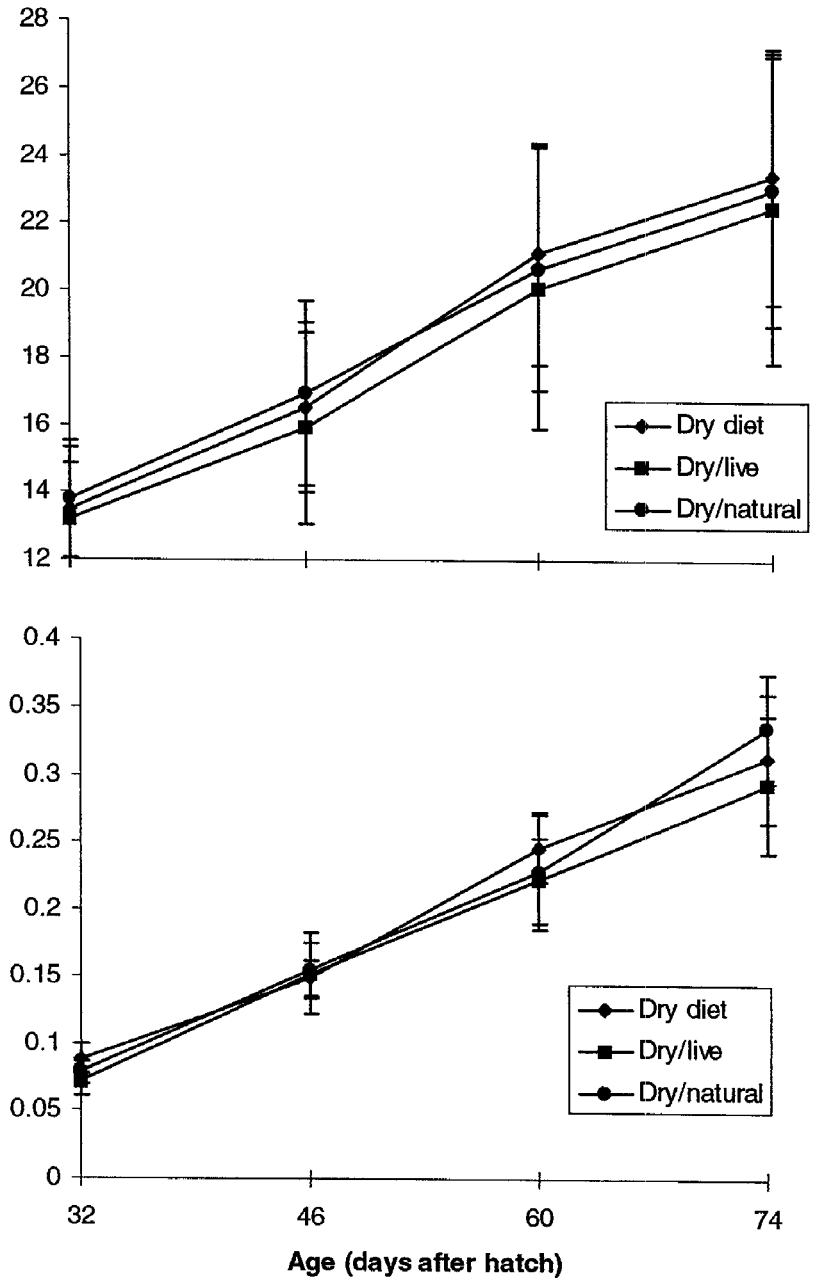

Figure 3.1 Growth curves for length (mm) and weight (g) (± Standard deviation) of *A. percula* juveniles.

Discussion

The supplementation of the fishmeal/casein based formulated diet with *Artemia* and the *P. indicus* / *D. serra* combination did not lead to an increased growth rate nor did it affect survival rates of *A. percula* juveniles. Furthermore the FCR of fish fed the supplemented diets were not significantly different from those fed only the formulated diets. This suggests that *A. percula* readily consumed the formulated feed and found it palatable. In addition, the size variance of fish fed the formulated diet was not significantly different from those fed the supplemented diets, indicating that the dry diet was accessible to even the smallest of the fish. In the case of the first hypothesis, testing the acceptance of the formulated dry feed, the H_O can be rejected. The formulated dry feed particles were recognised as food and readily ingested by *A. percula*.

As PER can reveal how well the protein source in the diet provides for the essential amino acid requirements of an animal, it can be used as a method for determining appropriate protein sources for fish diets (De Silva & Anderson 1995). The *A. percula* juveniles presented with the formulated feed showed comparable growth and survival rates, FCR and PER to those fed the diets supplemented with *Artemia* or *P. indicus* / *D. serra*. Therefore, the H_A of the second hypothesis was rejected. The comparable growth rates and similar PER values between treatments suggests that the fish were able to utilise the formulated feed (with fishmeal and casein as protein sources) as effectively as the protein sources of *Artemia* or *P. indicus* / *D. serra*.

This experiment has shown that 32 DAH juvenile *A. percula* readily accept and benefit nutritionally from a formulated dry feed. Furthermore, it is not necessary to supplement their diets with live or natural feeds. The development of a formulated feed is desirable as it will reduce the dependence on live and frozen natural feeds, saving time and money associated with collecting and producing these feeds. Given that a formulated feed must maximise growth, and as the dietary protein inclusion level can have a profound effect on growth (Khan *et al.* 1993), the dietary protein requirement of *A. percula* juveniles needed to be investigated. This has been done in the following chapter.

Chapter 4

The dietary protein requirements of juvenile *Amphiprion percula*

Introduction

The success of intensive marine aquaculture relies on the external feed input meeting the nutritional requirements of the fish species and promoting maximum growth at an acceptable cost (De Silva & Anderson 1995). Feed usually constitutes the highest proportion of the running costs in intensive aquaculture, and protein is the most expensive component in a diet. Furthermore, growth can be limited by too little (Cho & Bureau 1995) and in some cases too much protein in the diet (NRC 1977, Mazid *et al.* 1979). The growth depression observed in some fish fed diets exceeding their protein requirement has been attributed to the increase in the requirement of energy to rid the body of excess nitrogenous waste (Zeitoun *et al.* 1976, Phillips 1979), and to inadequate non-protein energy in isocaloric diets containing high protein levels, resulting in part of the dietary protein being metabolised and used for energy (Jauncey 1982, Santiago & Reyes 1991). Thus, economically and nutritionally, it is important to determine the optimal dietary protein inclusion level.

Although no data exists on the protein requirements of clownfish, it is known that carnivorous marine fish need relatively high protein levels in their diets. Dietary protein requirements for juvenile marine fish lie most often within the narrow range of 50-60% protein inclusion (Tucker 1992a)(Table 4.1). Therefore, it was assumed that *A. percula* would have a similar requirement.

Table 4.1. Dietary protein levels of feeds used for the successful rearing of juvenile marine fish.

Species	Weight of experimental fish (g)	Protein (%)	Reference
Epinephelus malabaricus	9	50	Shiau & Lan 1996
Sciaenops ocellatus	46	44	Daniels & Robinson 1986
Siganus guttatus	1	45	Parazo 1990
Oplegnathus fasciatus	6	45	Ikeda *et al.* 1988
Dicentrarchus labrax	31	50	Hidalgo & Alliot 1988
Sciaenops ocellatus	2	40	Serrano *et al.* 1992
Sparus aurata	2	61	Sabaut & Luquet 1973
Fugu rubripes rubripes	2	50	Kanazawa *et al.* 1980
Chanos chanos	0.004	40	Lim *et al.* 1979

Animals do not have a requirement for protein *per se,* but a requirement for amino acids, and more specifically essential amino acids. Proteins are essential ingredients in the diets of fish as sources of essential and non-essential amino acids (Wilson 1989). In Chapter 3 it was shown that fishmeal and casein were adequate protein sources and that the diet formulated for juvenile spotted grunter *Pomadasys commersonnii* (Table 3.1) was readily consumed by juvenile *A. percula.* A similar formulation was therefore used in this experiment to determine the optimal dietary protein inclusion level for *A. percula.* A hypothesis was posed:

H_o - There was no significant difference in growth rates of fish fed six diets ranging from 40-65% protein inclusion, increasing at 5% intervals.

H_A - There was a significant difference in growth rates of fish fed six diets ranging from 40-65% protein inclusion, increasing at 5% intervals.

Materials and methods

System design and management

This experiment was conducted in a 1500L recirculating system consisting of 18 glass aquaria. System design and management are described in Chapter 2. Water quality was monitored weekly and salinity kept constant at 35 ± 1ppt with the addition of dechlorinated tapwater. Oxygen saturation remained above 90%. Nitrite levels and total ammonia levels remained below 0.01 $mg.L^{-1}$. pH ranged from 8.1 - 8.3.

Experimental fish

At 17 days after hatch (DAH) 288 juvenile *A. percula* were moved from the departmental marine hatchery to the experimental system at night. Moving fish at night appeared to reduce the stress which had killed a sample of juveniles moved earlier that day. Sixteen fish were placed into each replicate tank, with three replicates for each of the six treatments. Replicates were randomly allocated amoung the 18 tanks.

Preparation of experimental diets

Six semi-purified diets were formulated with protein levels ranging from 40-65% (Table 4.2) according to methods outlined in Chapter 2. The protein content of each diet was calculated on a dry weight basis according to the total percentage protein in both protein sources, fishmeal (71.5% dry matter, Fiskernes Fiskeindustri, A.M.B.A.) and casein (92.7% dry matter). The diets were based on a diet which was found optimal for

Table 4.2. Formulation and proximate composition of the test diets.

Ingredient	Diet (g/100g)					
	1	2	3	4	5	6
Fishmeal	15.6	17.5	19.4	21.4	23.3	25.3
Casein	31.2	35	38.8	42.8	46.6	50.6
Pregelatinized starch	10	10	10	10	10	10
Fish oil	6	6	6	6	6	6
Mineral mix[1]	4	4	4	4	4	4
Vitamin mix[2]	2	2	2	2	2	2
Carophyll pink	0.1	0.1	0.1	0.1	0.1	0.1
Dextrin	31.1	25.4	19.7	13.7	8	2
Calculated protein level	40	45	50	55	60	65
Proximate composition						
Crude protein (%)	41.14	43.61	48.72	53.14	62.10	65.28
Fat (%)	7.13	7.61	7.43	8.01	7.48	7.54
Ash (%)	6.12	7.13	7.62	6.80	8.28	7.78
Moisture (%)	8.60	8.30	9.50	9.00	12.10	7.90
Gross energy (MJ/Kg)	19.06	19.15	19.16	19.63	19.36	20.31

[1] Mineral mix (g/kg) : 74g potassium; 516g vermiculite RSU; 14g salt; 0.05g ammonium chloride; 31g choline chloride; 0.31g cobalt; 0.15g copper; 1.5g iron; 0.05g iodine; 0,22g manganese; 41g magnesium; 1g zinc and traces of selenium.

[2] Vitamin mix (IU or g/kg) : 500 000 IU vitamin A; 400 00 IU vitamin D3; 10 000 IU vitamin E; 1g vitamin K3; 0.25g vitamin B1; 1.5g vitamin B2; 0.5g vitamin B6; 25g vitamin C; 2.5g Niacin; 0.09g Folic acid; 0.025g Biotin; 2.5g Calpan; 2.5g Inositol.

the rearing of juvenile grunter *Pomadasys commersonnii* (Irish 1997)(Table 3.1). The energy content of the diets was maintained at 19.44 ± 0.47 MJ/Kg with the supplementation of dextrin and assumed isocaloric.

Feeding

During the first three days of the experiment the fish were weaned from an *Artemia* diet to the formulated diet. The fish were initially fed 3 times daily in excess of their requirements. After two weeks it was observed that all fish had lost weight and were smaller than their conspecifics in the hatchery system. Consequently the feeding frequency was increased to five times per day.

Data collection and statistical analysis

Fish from each treatment were weighed and measured fortnightly by anaesthetising them with 2-phenoxyethanol, according to methods described in Chapter 2. Slopes of the growth curves for length and weight were compared using an analysis of covariance (ANCOVA, Zar 1996) followed by Tukey's Multiple Range Test. Final body weight and length, survival rate, FCR and PER were analysed using analysis of variance (ANOVA).

Results

The average survival rate for all treatments was 93.9% (S.D. ± 8.3%). Percentage weight gain of fish in all treatments was not significantly different (ANOVA $p > 0.05$), with average percentage weight gain of 419% (Table 5.3). The slopes of the growth curves for both length (ANCOVA, $P > 0.05$, $F = 2.49$, d.f. 1212) and weight (ANCOVA,

P > 0.05, F = 1.14, d.f. 90) did not differ significantly between treatments. Food conversion ratio and PER of fish in all treatments was not significantly different (Table 4.3).

Table 4.3. Effect of dietary protein levels on survival (± S.D.), percentage weight gain (± S.E.), FCR and PER (± S.D.) of juvenile common clownfish *A. percula* fed diets containing graded levels of protein for 8 weeks.

Diet	1	2	3	4	5	6
Protein level (%)	41	44	49	53	62	65
Survival rate (%)	92 ± 9.5[a]	94 ± 6.3[a]	86 ± 14.8[a]	96 ± 7.2[a]	98 ± 3.6[a]	98 ± 3.6[a]
Initial total length (mm)	10.2± 0.3	10.2± 0.3	10.2± 0.3	10.2± 0.3	10.2± 0.3	10.2± 0.3
Final total length (mm)	16.8 ± 2.6[a]	15.9 ± 2.9[a]	16.1 ± 2.4[a]	17.5 ± 3.1[a]	16.2 ± 2.7[a]	16.1 ± 2.5[a]
Initial body weight (g)	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Final body weight (g)	0.10 ± 0.01[a]	0.08 ± 0.03[a]	0.08 ± 0.01[a]	0.11 ± 0.02[a]	0.08 ± 0.02[a]	0.08 ± 0.00[a]
Percentage weight gain	455.2 ± 19.1[a]	384.1 ± 95.2[a]	382.0 ± 36.6[a]	504.0 ± 68.2[a]	393.4 ± 42.4[a]	388.6 ± 10.8[a]
FCR	12.70 ± 1.07[a]	14.27 ± 6.57[a]	15.19 ± 3.63[a]	11.47 ± 3.82[a]	14.02 ± 4.29[a]	13.53 ± 1.90[a]
PER	0.20 ± 0.02[a]	0.16 ± 0.09[a]	0.14 ± 0.03[a]	0.18 ± 0.05[a]	0.12 ± 0.03[a]	0.12 ± 0.02[a]

Values in the same row sharing a common superscript are not significantly different (P > 0.05)

Discussion

Mazid *et al.* (1979) outlined two trends regarding the effect of various levels of dietary protein on growth rate. Firstly, there is an increase in growth rate which is proportional to increased dietary protein up to a threshold level, whereafter growth declines with increasing protein. This has been found in plaice *Pleuronectes platessa* (Cowey *et al.* 1972), grouper *Epinephelus malabaricus* (Chen & Tsai 1994) and juvenile spotted grunter *Pomadasys commersonnii* (Irish 1997). The second trend shows no decrease in growth rate after the protein threshold is reached. This has been shown for gilthead seabream *Sparus aurata* (Sabaut & Laquet 1973), milkfish *Chanos chanos* (Lim *et al.*1979), and red drum *Sciaenops ocellatus* (Serrano *et al.* 1992). Clownfish in this experiment fed protein levels from 40-65% inclusion did not differ significantly in weight gain, FCR or PER, indicating that their growth response was not sensitive to the range of dietary protein presented. However, the average weight gain of 419% and high PER of fish in all treatments, suggests that there was sufficient protein available in all diets. Thus it appears that *A. percula* responds according to the second trend outlined by Mazid *et al.* (1979) and that the protein inclusion levels tested in this trial have exceeded the level which promotes maximum growth rate in juvenile *A. percula*. If this hypothesis is valid, then the protein requirements of *A. percula* would fall below the typical requirements of most juvenile marine fish (Table 4.1).

Although there is no information available on the diet of *A. percula*, Allen (1972) examined the stomach contents of 72 specimens of clownfish from a variety of species. By volume, 91.8% of their food consisted of an average of 34.8% copepods, 33.0% algae, 9.7% polychaetes, 6.4% crustacean fragments, 5.2% tunicates, and 2.7%

amphipods. From the proximate composition of the above prey items an attempt was made to determine the dietary protein requirement of adult clownfish (Table 4.4). Copepoda have a protein content of 45% (Fyhn *et al.* 1995). The algae species *Ulva pertusa*, *Grateloupia sparsa* and *Undaria pinnatifida* have protein contents of 23.2, 17.2 and 25.5% respectively (Floreto *et al.* 1996), and thus an average protein content of 21.9% for algae was used. The protein content for Terebellidae tentacles is 64.3% (Vine 1998), and was used as a general figure for the polychaete protein content. However, proximate composition of amphipods and tunicates could not be attained from the literature. As a result, amphipods and crustacean fragments were given a general protein content of 51.1%, based on the protein contents of three crustaceans; mysid *Mesopodopsis slabberi* (58%), mud prawn *Upogebia africana* (41.9%) and *Artemia* (53.4%) (Irish 1997). Although tunicates may provide some specific amino acids to clownfish, their high moisture content and low representation in the diet suggest they have little influence on the protein content consumed. As a result tunicates were eliminated from the calculation. Of the 33.8% protein consumed by adult clownfish, Copepoda contributed 15.7%, algae 7.2%, polychaetes 6.2%, and Amphipoda / crustacean remains 4.7% protein (Table 4.4). The remaining prey items not included in the calculation made up, by volume, 13.4% of stomach contents. They included isopods, tunicates, barnacle nuplii, *Amphiprion* eggs, shrimps, crustacean larvae, barnacle appendages, unidentified eggs, crabs, nematodes, ostracods and polychaete remains. Thus from the proximate composition of the above prey items the dietary protein requirement of adult clownfish was estimated to be at least 33.8% (Table 4.4). However, it must be acknowledged that it is the amino acids incorporated in

each each protein source that is of most importance. Unfortunately this information was unnattainable from the literature.

Table 4.4. Protein content of wild adult clownfish prey items and an estimation of adult clownfish protein requirement.

Prey item	% Volume of the diet	Protein content of prey (%)	Contribution to protein consumed (%)
Copepoda	34.8	45	15.7
Algae	33.0	21.9	7.2
Polychaetes	9.7	64.3	6.2
Amphipoda and crustacean remains	9.1	51.1	4.7
miscellaneous	13.4	?	?
Protein requirement of wild adult clownfish, at least...			33.8

The high percentage volume of algae in clownfish stomachs suggests that they are not strict carnivores, and that carbohydrate may be an important dietary component. Digestive enzyme activity is often related to feeding habits. For example, in rainbow trout (*Oncorhynchus mykiss*), amylase activity increases when fish are fed a protein-rich diet. In tilapia (*Oreochromis mossambicus*), amylase activity increases when fish are fed a starch-rich diet (De Silva & Anderson 1995). Thus, dietary enzyme activity in *A. percula's* gut may become more active with increasing levels of carbohydrate in the diet, resulting in improved digestion and, as a result, growth.

Although the percentage weight gain of fish between treatments was not significantly different, the large standard deviation suggests that percentage weight gain of fish within treatments varied considerably. This result suggests that some environmental or behavioural aspect might have affected the growth of fish in specific tanks. However the culture system was situated within a controlled environment room, and the various diets were randomly assigned between tanks. Furthermore, tanks received the same lighting and waterflow, and water quality parameters were maintained at acceptable limits throughout the experiment. In addition, the feeding behaviour of the fish did not appear to differ between tanks, and presentation of the diet to all tanks was identical. The consumption of the formulated feed was good, and all fish readily adapted to the diet. Genotype could not have affected food utilisation as fish were all from the same hatch. Niether could sex have played a role, as clownfish are protandrous hermaphrodites and thus all fish would have been males. It thus seems unlikely that abiotic or behavioural factors could have been responsible for the varying weight gains of fish in some tanks.

Reducing the protein level for economic reasons is less important in the rearing of clownfish than in the rearing of food fish. The value of a formulated feed for clownfish lies more in promoting maximum growth. As algae make up a large proportion of the diet of wild clownfish, further studies on the nutritional requirements of *A. percula* should focus on the possibility of increasing the inclusion of carbohydrate and reducing the inclusion of protein.

For the purposes of this study the formulated feed promoted adequate growth. As a result emphasis was shifted to determining the optimal time to wean *A. percula* off live feeds to formulated feed.

Chapter 5

Histology of the digestive system in *Amphiprion percula* larvae

Introduction

The change from an endogenous food source to external feeding is a critical stage in the rearing of larvae (Sarasquete *et al.* 1995). Reducing mortality at this time is dependent on the physical and nutritional properties of the feed meeting the requirements of the larvae (Pigott & Tucker 1989). The problems of appearance, acceptability and nutritional content of formulated feeds have been discussed in Chapter 1, and in Chapter 3 an experiment was conducted to determine the acceptability of a formulated diet by juvenile *A. percula*. However, in larvae, the physical and nutritional problems of formulated feeds are compounded by the inability of the undeveloped digestive system to utilise formulated feeds once ingested (Le Ruyet *et al.* 1993). The incompletely developed digestive tract in the early stages of larval growth results in low digestive enzyme activity causing inefficient digestion of protein (Dabrowski 1984, Lauff & Hofer 1984). Furthermore, the short undifferentiated larval gut results in short gut retention time of food. The result is insufficient time for extracellular digestion by tryptic enzymes to achieve complete hydrolysis of protein (De Silva & Anderson 1995).

The better growth of larvae fed live feeds compared to formulated feeds has been attributed to the soft consistency of live foods improving digestibility in the larval gut (Dabrowski & Glogowski 1977), the higher ingestion rate of larvae fed live foods (Kolkovski *et al.* 1993) and the exogenous enzymes introduced into the gut by live

foods (Appelbaum 1985). Digestive enzymes introduced by live prey aid digestive processes in the gut and play a role in activating the endogenous enzymes by cleaving the inactive zymogen forms (Dabrowski & Glogowski 1977, Kolkovski *et al.* 1993) resulting in better feed digestion. Formulated dry feeds usually possess no exogenous enzymes (Kolkovski *et al.* 1993) and require that the larval gut is fully functional; that digestive glands are active and are secreting enzymes to break down the ingested food, and that the gut epithelial cells are able to absorb the digested matter.

As the ability of the larval gut to digest formulated feed appears to increase with age and the development of digestive organs, an understanding of the ontogeny of the digestive system and its functional capabilities in relation to age is necessary to gain an understanding of when the larval gut is functionally ready to digest formulated feeds (Sarasquete *et al.* 1995).

Besides a description of the embryonic development of *A. chrysopterus* (Allen 1972), little is known of the early life history of *Amphiprion* species. The aim of this study was to describe the development of the digestive system and to use this information to infer the age at which *A. percula* larvae are able to ingest food, and capable of digesting and assimilating a formulated dry feed.

Materials and methods

The material for this study was obtained from the departmental marine hatchery. A sample of five embryos was obtained six days after fertilisation, then samples of five larvae were obtained just after hatching, and then 1, 3, 5, 7 and 9 days after hatching

(DAH). Post hatch larvae were maintained in a 90L conical tank, containing green water (*Nannochloropsis* sp.) at a concentration of 10000 cells.ml^{-1} . Temperature was kept constant at 28 ± 1°C and salinity at 35ppt. Besides the addition of the greenwater during the first five days after hatch (DAH), water in the tank was kept stagnant. From 5 DAH water from the recirculating system was passed through the larval rearing tank at a flow rate of 0.22L.min^{-1}. For the first 7 DAH larvae were fed rotifers (*Brachionus* sp.) and from 5 DAH onwards 1st instar *Artemia franciscana* (Jamaican strain).

The embryos and larvae were fixed in Bouin's solution and then stored in 60% propyl alcohol. Samples were dehydrated through a series of increasing alcohol concentrations, cleared in xylene and impregnated with paraffin wax (Bancroft & Stevens 1990). The wax impregnated material was sectioned between 7 and 10 µm using a Lipshaw Rotary Microtome (Model 45). Sections were stretched and mounted onto glass slides (Bancroft & Stevens 1990) and stained using standard Gill's haematoxylin (a protein stain), and eosin (a cytoplasmic stain) procedures. After drying, coverslips were placed over the slides using DPX mounting medium (Humason 1979). Light microscopy was used to examine the stained sections.

Results

Development of the digestive system

Six days after fertilisation. While still in the egg the jaws of the embryos have ossified, and the mouth has opened. The buccal cavity is lined with stratified squamous epithelium. The alimentary canal has differentiated into oesophagus, rudimentary stomach, intestine and rectum. The oesophagus is muscular and is lined with simple

cuboidal cells. Within the epithelium of the oesophagus a few mucous secreting cells have already developed (Figure 5.1a). The rudimentary stomach is sac like and composed of stratified cubiodal epithelium with centrally located nuclei. The intestine appears well developed, and is composed of stratified cuboidal epithelium with basally located nuclei, and a well developed brush border. The yolk sac with oil globule is present and located ventral to the intestine. The liver and spleen have differentiated from the gut (Figure 5.1b).

Newly hatched larvae. The only difference between the newly hatched larvae and the embryo is the increased number of mucous cells in the oesophagus. The yolk sac and oil globule, although reduced in size are still present. All other systems (e.g. cells and organs) associated with the digestive tract are still the same, and if not mentioned in the rest of the results section should be assumed not to have developed further.

1-day-old larvae. Larvae were feeding on exogenous food. The lumen of both the mid- and hind-gut has increased in size to accommodate this ingested matter (Figure 5.2a). Yolk sac and oil globule very much reduced.

3-day-old larvae. Mucous secreting cells increase in number and are concentrated in the anterior section of the oesophagus. The gut, although still straight, has differentiated further as cells in the hind-gut have become columnar. The lumens of both the mid- and hind-gut have become more folded, and villi are now well developed in the mid-gut. The liver and spleen have increased in size. The liver has increased into the space left by the yolk sac which is now completely absorbed.

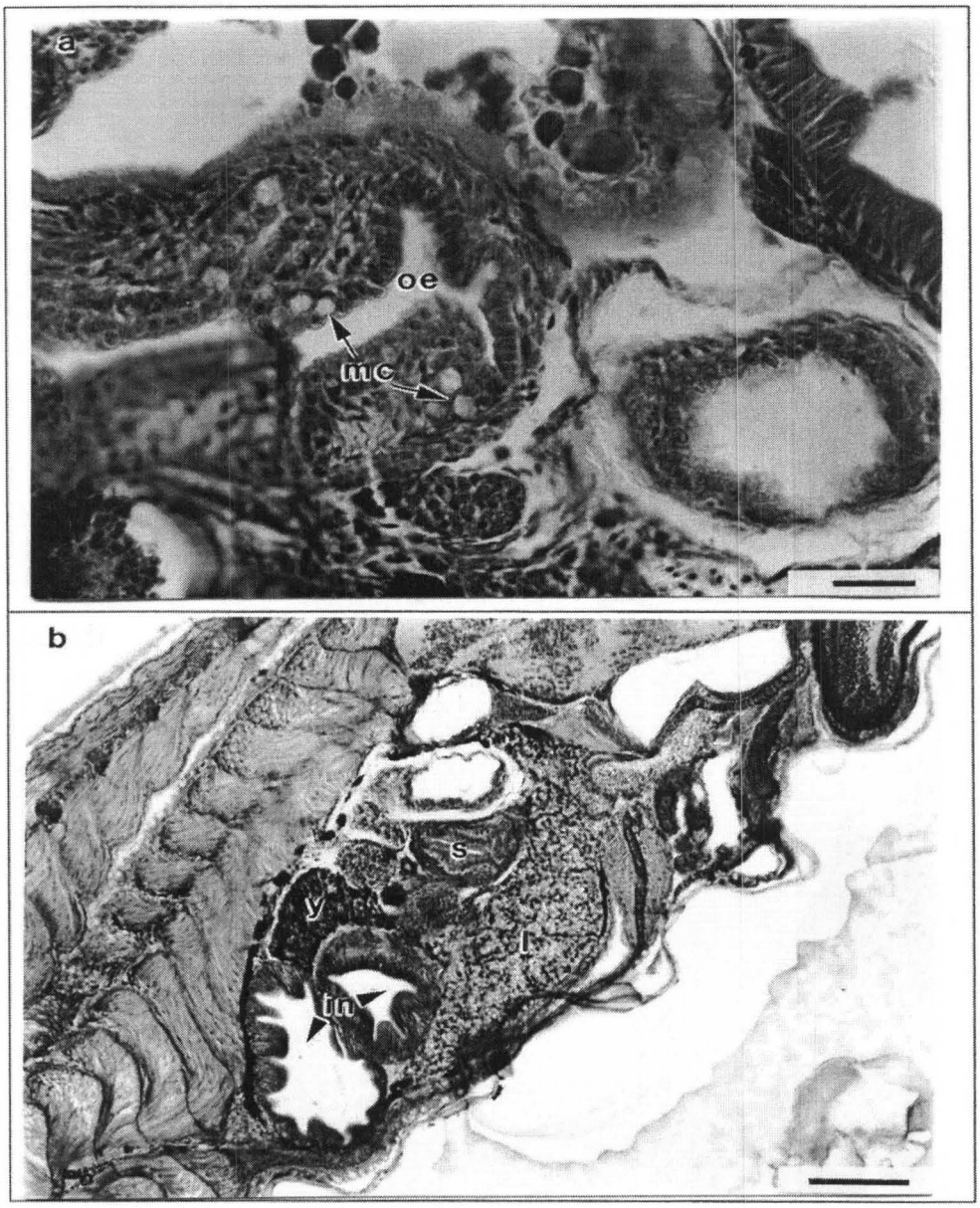

Figure 5.1 : Photomicrographs showing saggital sections of *A. percula* embryo six days after fertilisation. (a) Muscular oesophagus (oe) is lined with simple cuboidal cells and mucous secreting cells (mc) have appeared, magnification 40X, scale bar = 20 µm. (b) Rudimentary stomach (s) with well developed intestine (in). Yolk sac (y) and oil globule are located ventral to the intestine and the liver (l) has differentiated from the gut, magnification 10X, scale bar = 100 µm (Figure 5.1b).

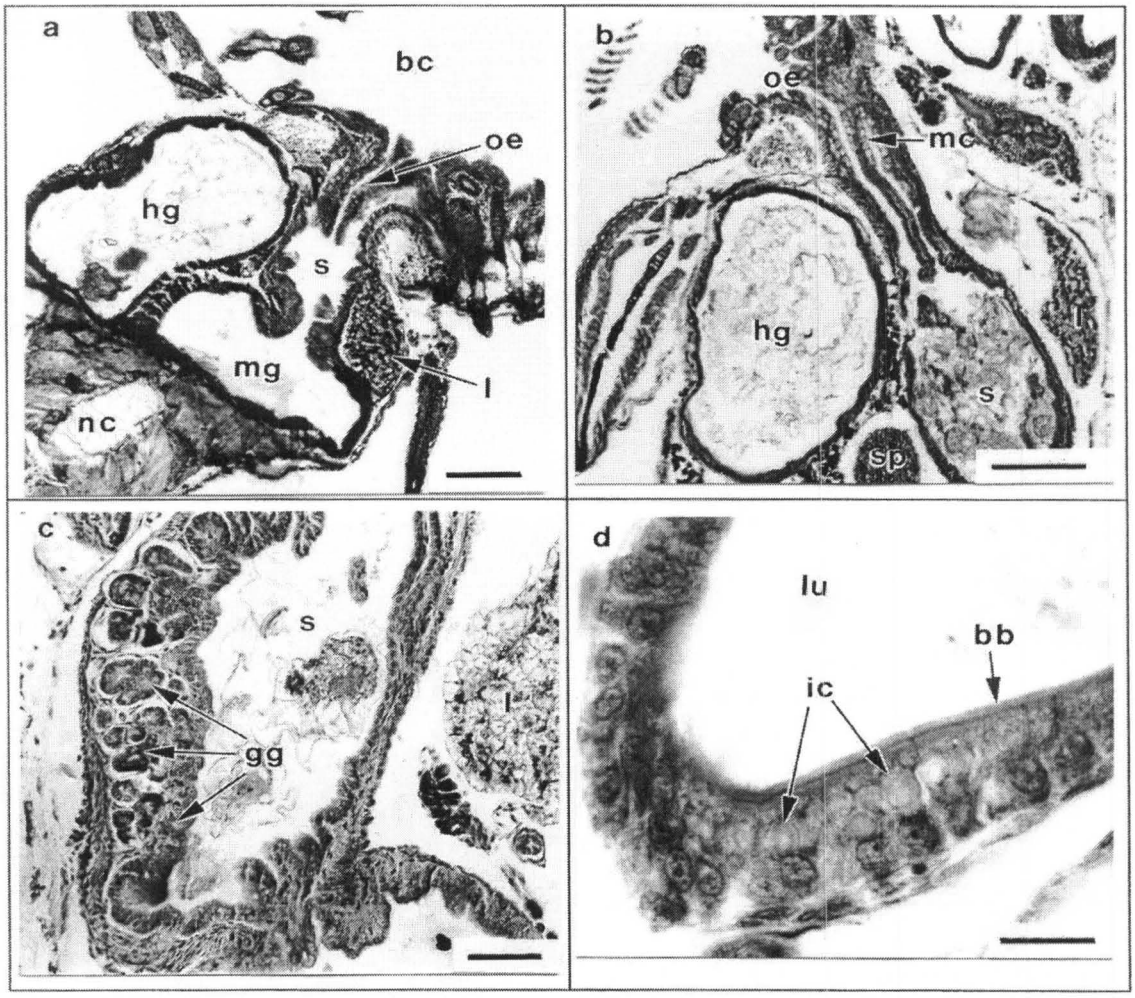

Figure 5.2 : Photomicrographs of *A. percula* larva. (a) Horizontal section of 1-day-old larvae showing the digestive tract and ingested material, 4.2 mm TL, SD ± 0.1 mm, magnification 40X, scale bar = 20 μm (b) Sagittal section of 5-day-old larvae showing the differentiation of the stomach and development of rudimentary gastric glands, 5.8 mm TL, SD ± 0.8 mm, magnification 10X, scale bar = 100 μm (c) Sagittal section of 7-day-old larvae showing the establishment of gastric glands in the stomach, 5.8 mm TL, SD ± 0.6 mm, magnification 20X, scale bar = 40 μm (d) Sagittal section of 7-day-old larvae showing an increase in the number of supranuclear inclusions in the hind-gut epithelium, 5.8 mm TL, SD ± 0.6 mm, magnification 100X, scale bar = 10 μm: (bb) brush border, (bc) buccal cavity, (gg) gastric glands, (hg) hind-gut, (ic) supranuclear inclusions, (l) liver, (lu) lumen, (mc) mucous secreting cells, (mg) midgut, (nc) notocord, (oe) oesophagus; (s) stomach, (sp) spleen.

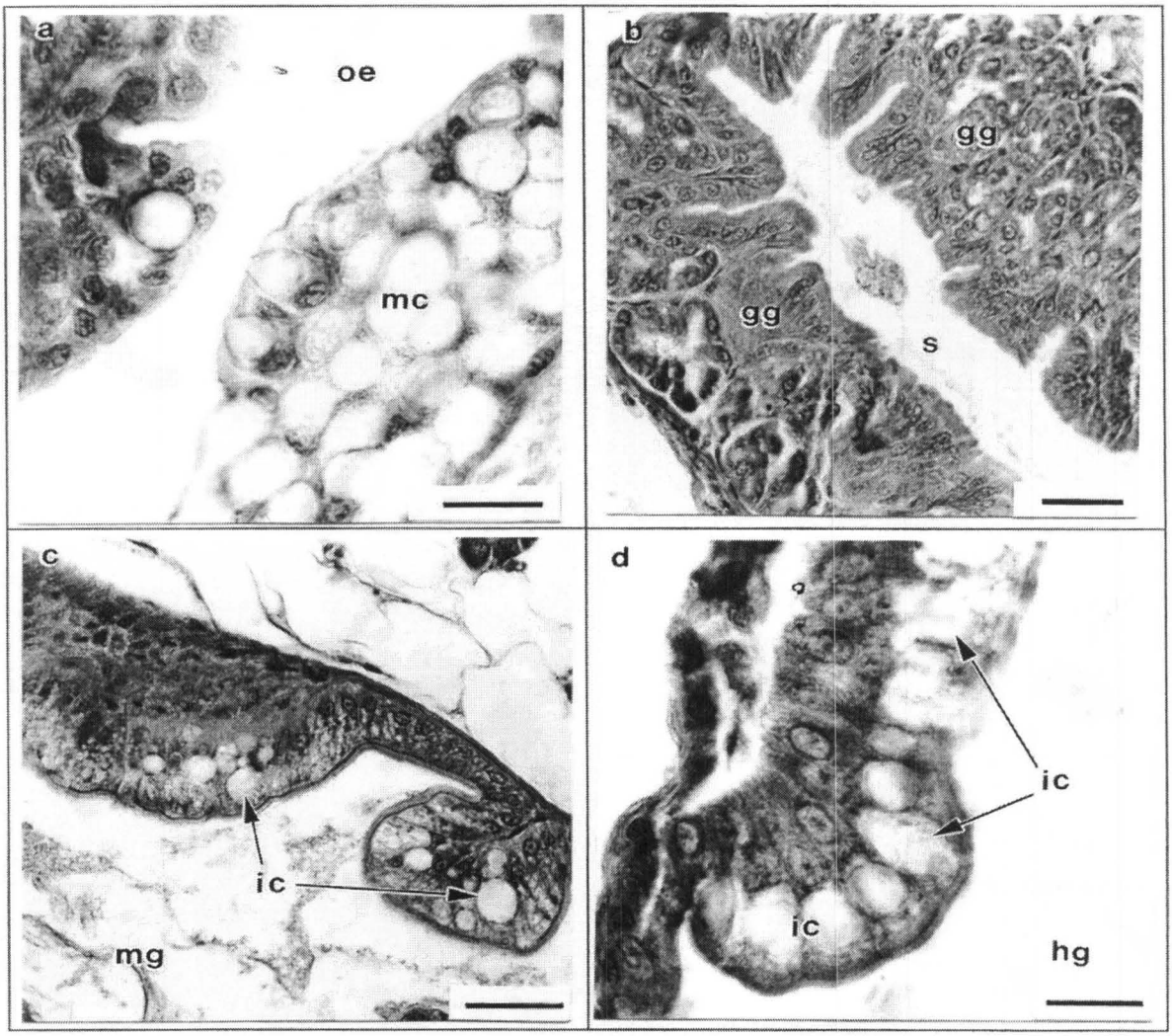

Figure 5.3 : Photomicrographs showing sagittal sections of 9-day-old *A. percula* juveniles. (a) Large number of mucous secreting cells (mc) in the anterior of the oesophagus (oe), 7.0 mm TL, SD ± 0.6 mm, magnification 100X, scale bar = 10 µm (b) Gastric glands (gg) well established in the stomach (s), 7.0 mm TL, SD ± 0.6 mm, magnification 40X, scale bar = 20 µm (c) Appearance of supranuclear inclusion vacuoles (ic) in the mid-gut (mg) epithelium, 7.0 mm TL, SD ± 0.6 mm, magnification 100X, scale bar = 10 µm (d) Increased number and diameter of supranuclear inclusion vacuoles (ic) in the hind-gut (hg) epithelium, 7.0 mm TL, SD ± 0.6 mm, magnification 100X, scale bar = 10 µm.

5-day-old larvae. The epithelium of the stomach has started to differentiate; simple cuboidal cells still line the tract from the oesophagus into the stomach, but toward the terminal end the epithelium is thickening and changing to columnar cells, where rudimentary gastric glands appear to be developing (Figure 5.2b). In the hind-gut a distinct brush border has developed, and small supranuclear inclusion vacuoles have appeared in the epithelium.

7-day-old larvae. At this stage the larvae are undergoing metamorphosis prior to settlement. Gastric glands are now well established in the epithelium of the stomach (Figure 5.2c), and there is an increased number of supranuclear inclusions in the epithelium of the hind-gut (Figure 5.2d).

9-day-old juvenile (post metamorphosis). There has been an increase in the number of mucous secreting cells in the anterior of the oesophagus (Figure 5.3a). Gastric glands are now well established in the stomach (Figure 5.3b). Supranuclear inclusion vacuoles have appeared in the epithelium of the mid-gut (Figure 5.3c). The supranuclear inclusion vacuoles in the hind-gut have increased in number and diameter (Figure 5.3d).

Discussion

Larval fish have been divided into three groups according to alimentary tract morphology and enzyme secretion in the gut (Dabrowski 1984). The first group possess a functional stomach before changing from endogenous to exogenous feeding (e.g. Salmonidae and Cichlidae) (Jones *et al.* 1993). Fish from this group usually adapt

easily to a dry formulated diet. The second group are fish which do not have a functional stomach or gastric glands at the larval stage, but develop digestive organs at metamorphosis (e.g. cod *Gadus morhua*) (Pedersen & Falk- Petersen 1992). Generally fish in this group show poor growth and survival when reared solely on a dry diet during the larval stages. The third group are fish that remain stomachless throughout life (e.g. Labroidei and Bleniidae) (Kobegenova 1989).

The development of the alimentary tract in *A. percula* appears to place it between groups one and two. Although the alimentary canal is differentiated before the embryos hatch and appears functional before the yolk sac is completely absorbed, a stomach with functional gastric glands develops 5 DAH, two days before metamorphosis. Thus it seems impractical to categorise alimentary tract development into only three groups as there is a large variation in larval gut development between species. Furthermore, it is often incorrect to infer the ability of a species to digest a formulated feed based on the development of a stomach or gastric glands. In some species, such as catfish, *Clarias gariepinus* (Verreth *et al.* 1992) and turbot *Scophthalmus maximus* (Segner *et al.* 1993), stomach differentiation is a decisive event in larval nutrition physiology and can be used as an external marker for the start of weaning (Segner *et al.* 1988). However, in whitefish *Coregonus lavaretus*, the stomach develops very late in larval ontogeny, but larval coregonids from Lake Constance reared purely on a dry diet from first feeding attained comparable growth to those fed live feeds (Segner & Rosch 1992). The above examples show that the development of the alimentary tract in larval fishes is highly variable. Prediction of the functional capabilities of the gut in relation to age is therefore very unreliable. Histological studies of the early life history of each specific species

appears at present the most reliable way of determining when the gut of the larvae could functionally and enzymatically digest formulated feeds.

The pattern of development in alimentary canal of *A. percula* embryos and larvae did not appear to proceed at the same rate. The opening of the mouth, appearance of mucous secreting cells and development of the intestine all occurred just before hatch. The alimentary tract changed very little after hatching, until the rapid development of digestive glands in the stomach between 5 and 9 DAH, just prior to metamorphosis. The development of the alimentary canal of *A. percula* larvae appeared to follow a pattern similar to Balon's (1979) theory of saltatory development. This theory states that development is characterised by periodically rapid changes rather than by a continuous gradation. Different structures that together form a system (organ) grow and differentiate at different rates, but are completed and become functional at the same time enabling the individual to undergo a rapid change via a threshold, from one stabilised state to another (Balon 1986). In larval *A. percula* the various structures associated with the alimentary tract develop and differentiate between hatch and 7 DAH, whereupon the threshold is reached and by means of metamorphosis the fish moves from the larval state to the juvenile state (Figure 5.4).

Amphiprion percula larvae were able to ingest exogenous food particles 1 DAH. The main function of the gut, even in fish larvae, is the absorption of fat, protein and carbohydrates (Stroband & Dabrowski 1979). The first evidence that the particles were being digested was observed 5 DAH, when supranuclear inclusion vacuoles appeared in the epithelial cells of the hind-gut. Watanabe (1981) observed similar vacuoles in the

hind-gut mucosal epithelium and attributed them to the pinocytotic absorption of protein.

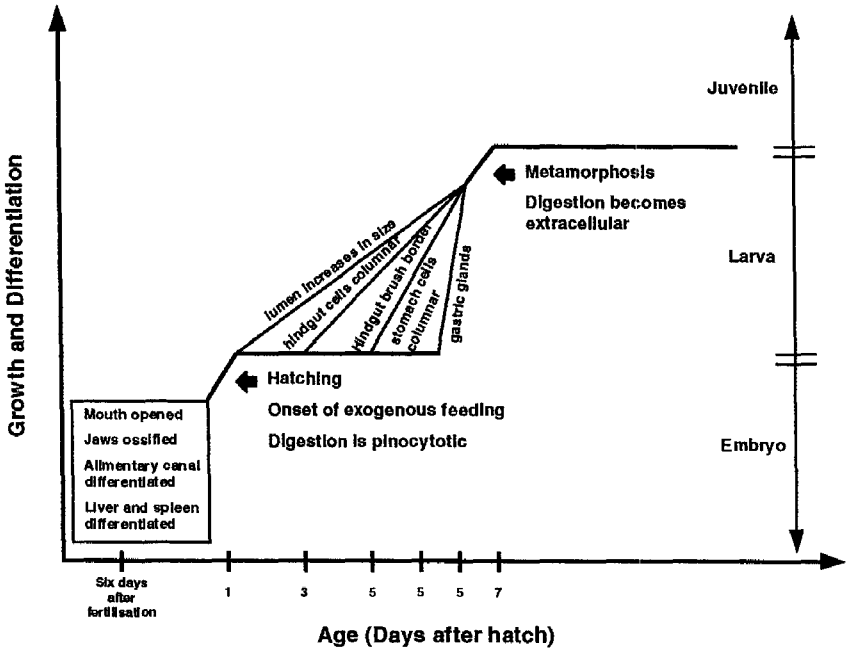

Figure 5.4 : Scheme of the consecutive steps in the ontogeny of the digestive system of *A. percula*, demonstrating saltatory development. The various structures of the alimentary canal grow and differentiate at different rates but are completed and functional at the same time, enabling the larvae to undergo rapid change (hatching and metamorphosis) from one stage or step to another. The steps in *A. percula* development have been divided into the embryo, larval and juvenile stage. There is no free embryo or eleutheroembryo stage (sensu Balon 1986) as the larvae begin exogenous feeding immediately after hatching and before the yolk sac is fully absorbed.

It is possible that the capacity to pinocytotically absorb proteins, although first observed histologically 5 DAH, could be occurring before the yolk is fully absorbed (3 DAH), such as in cherry salmon, *O. masou* and pond smelt, *Hypomesus transpacificus nipponensis* (Watanabe 1984). As in the cherry salmon, pinocytosis in larval *A. percula* continued after the development of gastric glands, whereas in pond smelt the mechanism of protein digestion and absorption changed from pinocytosis and intracellular digestion to extracellular digestion and membrane transfer with the development of gastric glands (Govoni *et al.* 1986).

Continued pinocytotic absorption and intracellular digestion of proteins is a possible advantage. This mechanism of digestion may compensate for incomplete digestion by allowing for the assimilation of macromolecular proteins (Stroband & Van der Veen 1981). Thus for *A. percula* the critical transition period from an endogenous food source to a purely exogenous one takes place from 3 DAH. At this time high mortalities could occur if the larvae do not receive a feed that they will readily ingest and that meets their immediate nutritional requirements.

By 9 DAH supranuclear inclusion vacuoles had appeared in the epithelial cells of the mid-gut. Similar vacuoles have been described for graying *Thymallus thymallus* (Eckmann 1987), whitefish *Coregonus fera* (Loewe & Eckmann 1988), gilthead seabream *Sparus aurata* (Sarasquete *et al.* 1995) and Pacific bluefin tuna *Thunnus thynuss* (Kaji *et al.* 1996). These vacuoles were reported to contain fat. The existence of these fat vacuoles is as a result of extracellular digestion of fat in the intestine by lipases, diffusion of fatty acids into the absorptive epithelial cells across the apical

membrane, and intracellular resynthesis of triglycerides to maintain the diffusion gradient for fatty acids (Loewe & Eckmann 1988). The appearance of vacuoles in the mid-gut co-insides with the establishment of gastric glands in the stomach suggesting that the digestive enzymes secreted by the gastric glands promote extracellular digestion.

This study has shown that the alimentary canal of *A. percula* is advanced at hatching and the larvae can immediately start exogenous feeding. Three DAH, when the yolk sac is completely absorbed, the larvae are fully dependent on an exogenous food source. At this time protein molecules are digested in the hind-gut by pinocytosis. This type of protein digestion is not very efficient and only by 9 DAH, when the digestive glands are functioning and extracellular digestion is occurring in the midgut, will the assimilation of ingested feed become an efficient process. This is especially the case with a formulated feed, while a live feed with its associated exogenous enzymes can be digested and assimilated before digestive glands are functioning. A formulated feed will theefore only be efficiently digested by *A. percula* juveniles older than 9 DAH.

Chapter 6

The weaning of larval and juvenile *Amphiprion percula*

Introduction

It is economically advantageous to wean larvae onto dry feeds (Le Ruyet *et al.* 1993, Hayashi 1995), as purchasing and producing live food is costly and labour intensive (Lee & Litvak 1996). Dry feeds are advantageous as they can be maintained cheaply, stored refrigerated or frozen, and dispensed by automatic feeders, thereby reducing labour costs. By contrast, production of live food can be uncertain, as cultures often crash. Furthermore the nutritional content of livefood organisms can vary considerably over time (Holt 1993), whereas dry feeds can be formulated to consistently meet specific nutritional requirements.

Marine fish larvae have, however, proved difficult to rear on formulated feeds (Table 6.1)(Jones *et al.* 1993). In general, greater success has been achieved with fresh water larvae, with *Coregonus lavaretus* (Champigneuille 1988), *Cyprinus carpio* (Charlon & Bergot 1984), *Clarias gariepinus* (Uys & Hecht 1985) and *Micropterus dolomieui* (Ehrlich *et al.* 1989) larvae all having have been reared exclusively on formulated diets from first feeding with good growth and survival. With marine larvae, however, total replacement of conventional live feeds with formulated feeds has resulted in very poor survival and growth. It is unclear exactly why freshwater larvae are reared more successfully than marine larvae on formulated feeds. There are a number of theories for their limited success and these are discussed in more depth in Chapter 1.

Table 6.1 Results of complete replacement trials of live feeds for first-feeding marine fish larvae with formulated diets (Jones *et al.* 1993)

Species	Feed replacement	Result	Author
Pleuronectes platessa	Microparticulate	poor growth and survival	Adron *et al.* 1974
Solea solea	Mircoparticulate/micr oencapsulated	poor growth and survival	Appelbaum 1985
Solea solea	Zein coated particles	poor growth and survival	Gatesoupe *et al.* 1977
Dicentrarchus labrax	Zein coated particles	poor growth and survival	Gatesoupe *et al.* 1977
Sparus aurata	Micro diet with/without exogenous enzymes	poor growth and survival	Kolkovsky *et al.* 1990
Lates calcarifer	Microcapsules	no survival after 10 days	Walford & Lam 1991
Gadus morhua	Microencapsulated roe	poor growth and survival	Garatun-Tjeldsoto *et al.* 1989
Clupea harrengus	Encapsulated cod roe	poor growth and survival	Fox 1990
Pagrus major	Microcapsules, zeincoated and microbound	poor growth and survival	Kanazawa *et al.* 1982

Essentially, the feeding behaviour of larvae may not be stimulated as the size, movement, shape, density, texture, colour and contrast, and odour of the formulated feed particles may be incorrect (Dabrowski 1984, Mearns 1986, Pigott & Tucker 1989, Jones *et al.* 1993, Le Ruyet *et al.* 1993). Furthermore, formulated diets may not meet the nutritional requirements of the larvae (Le Ruyet *et al.* 1993), the larval gut may not be developed enough to digest a formulated feed (Dabrowski 1984, Holt 1993), and the effect of water quality deterioration, caused by artificial feeds, may disrupt the feeding behaviour and affect survival and growth of larvae (Abi-Ayad & Kestemont 1994).

Suggestions on the best time to start weaning fish larvae vary considerably. Lee & Litvak (1996) suggest weaning winter flounder *Pleuronectes americanus* soon after larvae switch to exogenous feeding. Holt (1993) weaned red drum *Sciaenops ocellatus* from 5 days after exogenous feeding with good growth rates. The Dover sole *Solea solea* can be weaned with good survival rates from 10 days after hatch (Appelbaum 1985). The best time to wean sea bass *Dicentrarchus labrax* was at 3-4mm or 20 days after hatch (Le Ruyet et al. 1993). Verreth & Van Tongeren (1989) report weaning *Clarias gariepinus* larvae from *Artemia* to a commercial trout diet, with best growth rates, 4.1 days after exogenous feeding. However, Uys & Hecht (1985) using an optimal dry feed for *C. gariepinus* larvae found superior growth rates in larvae fed only the formulated diet from first feeding. Timing of weaning, therefore, appears to be very species specific.

Weaning in *A. percula* has not been investigated, although general suggestions for the weaning of clownfish have been published by Hoff (1996). Larvae of clownfish are reared on a wide variety of feeds that overlap considerably. In general clownfish larvae are initially fed on rotifers until 10 days after hatch (DAH). At 4 DAH dry food (50 - 100μ) is added to the diet, and particle size is increased over time. From 6 DAH *Artemia* are introduced into the diet, and are fed until 14 DAH. Shrimpmeal is fed from 8 DAH onwards (Hoff 1996). This complicated feeding protocol is labour intensive and therefore costly.

Thus two weaning experiments were designed with the aim of reducing the dependence of larvae on live feeds and simplifying the feeding regime. The objective was to wean *A. percula* onto an artificial feed as early as possible, while still maintaining good growth and survival. In the early weaning experiment larvae were weaned 4, 7 and 10 DAH and the following hypothesis was posed:

H_O - There is no significant difference in final weight or length of larvae weaned either 4, 7 or 10 DAH from a live feed to a dry feed, or a control live feed.

H_A - There is a significant difference in final weight or length of larvae weaned either 4, 7 or 10 DAH from a live feed to a dry feed, or a control live feed.

The same hypothesis, except for the specific number of DAH, was posed in the late weaning experiment during which juveniles were weaned 10, 15, 20, 25 and 30 DAH

Materials and Methods

Experimental fish and culture system

Larvae were obtained from our marine hatchery and transferred to 25L dark green buckets connected to a 1200L recirculating system. Water quality parameters and a description of the experimental system are found in Chapter 2.

Weaning procedure

The transition from live feed to formulated dry feed should be gradual enough to allow slow learners to survive the process (Tucker 1992b). Thus the weaning procedure adopted in both experiments lasted 3 days. On the first day fish were fed 75% *Artemia* / 25% dry feed, on the second day 50% *Artemia* / 50% dry feed and on the third day 25% *Artemia* / 75% dry feed. By the fourth day of the weaning period, fish were fully weaned onto dry feed.

Diets

Larvae used as control groups in the two experiments were fed enriched (Super Selco - INVE Aquaculture NV) *Artemia franciscana* nauplii (Jamaican strain). The formulated dry feed used to wean larvae was diet # 2, formulated for the dietary protein requirement experiment (see Table 4.2). As all diets from Table 4.2 promoted the same growth, any one could have been used for this experiment.

Early weaning experiment

On the evening of the 3rd DAH, larvae were removed from the marine hatchery to the experimental system, divided into four duplicate treatments which were randomly arranged, at 15 larvae per bucket. Weaning commenced on 4, 7, or 10 DAH for the respective treatments. Larvae were weaned from the enriched *Artemia* diet to the formulated dry feed, and control fish were fed enriched *Artemia* throughout the experiment. The experiment was conducted over a period of 18 days.

Late weaning experiment

On the evening of the 9th DAH, juveniles were removed from the marine hatchery to the experimental system, divided into six duplicated treatments, at 15 juveniles per bucket. Weaning commenced on 10, 15, 20, 25 and 30 DAH. Juveniles were weaned from the enriched *Artemia* diet to the formulated dry feed. Control fish were fed enriched *Artemia* throughout the experiment. The experiment was conducted over a period of 41 days.

Data analysis

At the beginning of each experiment 15 larvae were sacrificed, weighed and measured. Once a day fish in each bucket were counted to determine survival rate. At the end of the early weaning trial, fish from each replicate were weighed and measured individually according to methods described in Chapter 2. However, these fish did not survive the stress of being individually weighed. It was decided that in the late weaning trial fish from each replicate would be weighed together at the end of the experiment and an average weight for the replicate determined. This method of weighing did not

allow for rigorous statistical analysis. Mean weights and length data, and Condition Factor (CF) were analysed using an analysis of variance (ANOVA) if normally distributed, and Kruskal-Wallis test when data was non-parametric. The Kolmogorov-Smirnov test was used to test if data was normally distributed at $P < 0.05$.

Results

Early weaning

There was no significant difference in survival between the control group and fish weaned at 7 and 10 DAH (Kruskil-Wallis $P > 0.05$), with a mean survival rate of 79 ± 4.2% (Table 6.2). However, fish weaned at 4 DAH displayed a significantly lower survival rate (20%). Average final weights, lengths and CF of the control fish were significantly higher than those of fish weaned 4, 7 and 10 DAH (ANOVA $P < 0.05$) (Table 6.2).

Table 6.2 Final mean weight (mg), length (mm) and condition factor (± S.D.) of *A. percula* weaned at 4, 7, 10 days after hatch (DAH) or fed live food (control) throughout a growth period of 22 days.

	Day weaning started			Control
	4 DAH	7 DAH	10 DAH	
Survival (%)	20[a]	80[b]	73[b]	83[b]
Initial mean weight (mg)	0.75 ± 0.26	0.75 ± 0.26	0.75 ± 0.26	0.75 ± 0.26
Final mean weight (mg)	16.55 ± 5.31[a]	20.88 ± 8.06[a]	18.74 ± 6.44[a]	33.11 ± 6.29[b]
Initial mean length (mm)	3.84 ± 0.36	3.84 ± 0.36	3.84 ± 0.36	3.84 ± 0.36
Final mean length (mm)	10.0 ± 0.36[a]	10.5 ± 0.54[a]	10.4 ± 0.42[a]	10.9 ± 0.32[b]
Condition factor	0.006 ± 0.001[a]	0.007 ± 0.001[a]	0.006 ± 0.001[a]	0.010 ± 0.001[b]

Values in the same row sharing a common superscript are not significantly different ($P > 0.05$).

Late weaning

There was no significant difference in the survival rate of fish from all treatments, with a mean survival rate of 79 ± 14% (Kruskil-Wallis $P > 0.05$) (Table 6.3). Final weights of fish varied widely, but treatment means did not differ significantly (Kruskil-Wallis $P > 0.05$). The mean final length of fish weaned 10 DAH was not significantly different in mean final length from that of fish weaned 15 DAH, but was significantly lower than fish weaned 20, 25, and 30 DAH and the control fish (ANOVA $P < 0.05$). The condition factor did not differ significantly between treatments (ANOVA $P < 0.05$) (Table 6.3).

Table 6.3 Final mean weight (mg), length (mm) and condition factor (± S.D.) of *A. percula* weaned at 10, 15, 20, 25 and 30 days after hatch (DAH) or fed live food (control) throughout a growth period of 41 days.

	Day weaning started					Control
	10 DAH	15 DAH	20 DAH	25 DAH	30 DAH	
Survival (%)	70[a]	93[a]	66[a]	97[a]	80[a]	66[a]
Initial mean weight (mg)	5.85 ± 3.02	5.85 ± 3.02	5.85 ± 3.02	5.85 ± 3.02	5.85 ± 3.02	5.85 ± 3.02
Final mean weight (mg)	34.50 ± 3.54[a]	59.50 ± 28.99[a]	83.00 ± 18.39[a]	68.50 ± 14.85[a]	60.50 ± 35.40[a]	77.00 ± 32.53[a]
Initial mean length (mm)	6.90 ± 1.13	6.90 ± 1.13	6.90 ± 1.13	6.90 ± 1.13	6.90 ± 1.13	6.90 ± 1.13
Final mean length (mm)	11.9 ± 2.1[a]	14.1 ± 3.3[ab]	15.6 ± 2.6[b]	14.8 ± 2.8[b]	14.6 ± 2.7[b]	15.6 ± 2.3[b]
Condition factor	0.008 ± 0.000[a]	0.007 ± 0.001[a]	0.007 ± 0.002[a]	0.007 ± 0.000[a]	0.007 ± 0.000[a]	0.007± 0.000[a]

Values in the same row sharing a common superscript are not significantly different ($P > 0.05$)

Discussion

Amphiprion percula appear able, without a reduction in the rate of survival, to utilise a dry feed from 7 DAH onwards. The low survival of larvae weaned from 4 DAH suggests that *A. percula* were either unable to ingest the food particles because the size was to large and texture to hard compared to the live feed (Pigott & Tucker 1989, Le Ruyet *et al.* 1993, Dabrowski 1984), or were incapable of digesting and assimilating sufficient amounts of dry feed, as a result of an incompletely developed digestive system (Lauff & Hofer 1984, Smith 1989). It also cannot be discounted that the dry feed may not have possessed nutrients that were essential to larvae at that time (Le Ruyet *et al.* 1993).

In terms of length and weight gain, the optimal time to wean *A. percula* juveniles from the live feed to the formulated dry feed was between 15 and 20 DAH. Those weaned at 4, 7, and 10 DAH displayed significantly lower length and weight gain than the control fish fed on live feed. Segner & Rosch (1992) observed that live feeds presented to *Coregonus lavaretus* larvae provoked more intensive hepatic protein synthesis and stronger stimulation of overall hepatocellular metabolism than formulated feeds, resulting in improved growth. Poor growth rates of larvae fed formulated diets has been attributed to the low digestive enzyme activity in the gut of early stage larvae (Dabrowski 1984, Lauff & Hofer 1984). The digestive enzymes introduced by live prey aid digestive processes in the gut and play a role in activating the endogenous enzymes by cleaving the inactive zymogen forms (Dabrowski & Glogowski 1977, Kolkovski *et al.* 1993).

However, with the development of gastric glands in the stomach, there is potential for efficient digestion of proteins (Steffens 1989), but it can take several days or weeks before proteolytic enzymes becomes fully active (Lauff & Hofer 1984, Dabrowski & Culver 1991). This appeared to be the situation in the digestive system of *A. percula* larvae (Chapter 5). Although gastric glands developed in the stomach epithelium at 7 DAH, digestive enzyme secretion by the gastric glands only appears to become adequate for efficient digestion and assimilation of the formulated feed 15-20 DAH.

The development of the stomach and associated gastric glands has been correlated with the improved digestion of formulated feeds (Steffens 1989). Segner et al. (1993) reported that *Clarias gariepinus* larvae fed a formulated diet from first feeding onwards only showed comparable growth rates to a live fed control after stomach development. In *A. percula*, gastric glands became developed 7 DAH and larvae weaned at this time show improved survival rates. However, in terms of growth rates the best time to wean *A. percula* was between 15-20 DAH, long after the development of gastric glands. With the great number of factors affecting the efficient utilisation of a diet by larvae (behavioural, nutritional and gut physiology), determining the best time to wean a species to formulated feed cannot be purely based on the development of certain digestive organs, but can only be achieved by conducting experimental weaning trials.

Good growth was achieved in this experiment when fish were weaned from a purely live food diet to a purely dry food. However, situations of improved growth in sea bass *Lates calcarifer* (Walford & Lam 1993), turbot *Scophthalmus maximus* (Munilla-Moran *et al.* 1990), red drum *Sciaenops ocellatus* (Holt 1993), and white fish *Coregonus*

clupeaformis (Lauff & Hofer 1984) have occurred when fish were fed live prey in conjunction with an artificial diet. It has been suggested that exogenous enzymes (proteases) from the live prey act, firstly to aid digestive processes in the gut and, secondly, play a role in activating the endogenous enzymes (Dabrowski & Glogowski 1977). Thus further investigations conducted on *A. percula* larvae and juveniles, should focus on improving growth by supplementing the live feed with dry feed during the first 15 to 20 DAH, before weaning to a purely dry food takes place.

In conclusion, the null hypotheses of both weaning experiments were rejected. In the early weaning trial there was a significant increase in final mean weight and length in the control group where larvae were fed live food only. In the late weaning trial, the fish weaned 10 DAH had a significantly lower final length than fish weaned 20, 25 or 30 DAH or those fed on live food control, but were not significantly different from the fish weaned 15 DAH. Thus *A. percula* can be weaned from 7 DAH with no reduction in survival, and from between 15 to 20 DAH with no reduction in growth.

Chapter 7

General discussion

Many species of clownfish have been successfully reared on a variety of mixed live and dry feeds (Frakes & Hoff 1993, Hoff 1996). The collection and preparation of such feeds is expensive, very time consuming and impractical in large-scale commercial aquaculture. The aim of this study was to simplify the feeding of *A. percula*. The principal objective was to wean larvae and juveniles from live food to a formulated feed as early as possible, while still maintaining good growth and survival.

To achieve this goal a number of experiments were conducted in a logical sequence. The aim of the first experiment was to test if *A. percula* recognised and accepted the formulated feed as food, and if a combined protein source of fishmeal and casein promoted adequate growth. The second experiment attempted to improve the formulation by determining the optimal dietary protein requirement of *A. percula* juveniles. This was followed by a histological study of the digestive system of the larvae to determine when the digestion of a formulated feed could theoretically take place. Finally, two weaning experiments were conducted to determine the earliest time at which *A. percula* could be successfully weaned onto a formulated feed, while still maintaining good growth and survival.

Supplementing the fishmeal/casein based formulated feed with *Artemia* or a combination of shrimp (*Penaus indicus*) and sand mussel (*Donax serra*) did not have an effect on growth and survival rates or PER of *A. percula* juveniles. This suggested

that *A. percula* readily accepted the formulated feed, and that its nutritional content (with fishmeal and casein as protein sources) was as effective as the protein sources provided by *Artemia* or *P. indicus* and *D. serra* in meeting their nutritional requirements.

Eighteen day old clownfish fed on diets with protein levels ranging from 40-65% for 56 days showed no significant difference in their growth rate, indicating that their growth response was not sensitive to the range of dietary protein content. All diets promoted good growth with an average percent weight gain of 419% and a high PER, suggesting that sufficient protein was available in all diets to sustain growth. As particular attention must be paid to the protein, fatty acid and vitamin content when formulating feeds for carnivorous and omnivorous marine fish (Tucker 1992a), this experiment forms a good basis for future studies concerned with formulating a diet to meet the specific nutritional needs of *A. percula*. For the purposes of this study though, the recorded weight gains were highly satisfactory, and thus efforts were focused on determining when to begin weaning *A. percula* from live feed to a formulated feed.

The alimentary canal of *A. percula* is advanced at hatching and larvae can immediately start exogenous feeding. Three days after hatch (DAH) the yolk sac is completely absorbed and the larvae are fully dependent on an exogenous food source. Digestion of protein molecules at this time is pinocytotic. This type of protein digestion is not very efficient and only 9 DAH, when the digestive glands are functioning and extracellular digestion is occurring in the midgut, is the assimilation of protein likely to become an efficient process.

The optimal time to wean *A. percula* juveniles, in terms of growth, from live feed to formulated feed is between 15 to 20 DAH. However, *A. percula* can be reared from 7 DAH onwards without a reduction in survival. *Amphiprion percula* are fully dependant on exogenous food from 3 DAH, thus the low survival of larvae weaned from 4 DAH suggests that *A. percula* were either unable to ingest the food particles or that the formulated feed did not meet the nutritional requirements of the larvae at this time (Le Ruyet *et al.* 1993), or finally that the incompletely developed digestive system was incapable of digesting and assimilating sufficient amounts of feed (Lauff & Hofer 1984, Smith 1989). Thus it appears that the formulated feed tested in experiments is not suitable for rearing *A. percula* larvae before 7 DAH. However, further investigations on the optimal feeding frequency should be conducted before such a statement can be made with certainty.

Early juvenile *A. percula* (before 15-20 DAH) appear able to utilise live feed more efficiently than dry feed. Live feed is probably utilised more efficiently because of the introduction of digestive enzymes into the gut thereby supporting and accelerating the digestive processes (Dabrowski & Glogowski 1977). When gastric glands develop in the stomach epithelium, there is the potential for efficient digestion of proteins (Steffens 1989), but it can take several days or weeks before proteolytic enzymes becomes fully active (Lauff & Hofer 1984, Dabrowski & Culver 1991). In *A. percula*, gastric glands are well developed 9 DAH and extracellular digestion is taking place, but it is presumably only by 15-20 DAH that the digestive enzymes secreted by the gastric glands have reached a level allowing efficient digestion and assimilation of the dry feed.

This study has achieved its objective of determining the optimal time to wean *A. percula* from live feed to a formulated dry feed. The results have both practical and fundamental relevance. On a practical level, the feeding procedure used in rearing clownfish has been simplified, reducing the costs and time involved in producing and feeding large amounts of live feed.

Poor juvenile survival is still one of the main bottlenecks in expanding marine fish culture (Lubzens *et al.* 1997). Any study on the early-rearing of marine larval fishes is therefore of fundamental importance. The connection between the development of the digestive system and the survival and growth rates determined in the two weaning experiments has contributed to the understanding of digestion of formulated feeds by larval and early juvenile fishes. Furthermore, results from the dietary protein inclusion experiment have formed a basis for future studies of the nutritional requirements for this species.

Recommendations for future studies include investigating the improved growth and survival rates associated with co-feeding live and artificial diets. Dry feeds tend to support a higher daily intake of nutrients than live feed (Rosenlund 1995), whereas live feeds introduce exogenous digestive enzymes into the gut improving digestion (Dabrowski & Glogowski 1977). The importance of mixed diets in promoting improved growth has been demonstrated in freshwater species like goldfish *Carassius auratus* (Abi-Ayad & Kestemont 1994), carp *Cyprinus carpio* (Szlaminska & Przybil 1986), and the African catfish *Clarias gariepinus* (Awaiss & Kestemont 1998). And also demonstrated in marine species, milkfish *Chanos chanos* (Marte and Duray 1991), sea

bass *Lates calcarifer* (Walford & Lam 1993) and red drum *Sciaenops ocellatus* (Holt 1991). Maximising the growth rate is desirable as it would increase the turnover of clownfish juveniles within a hatchery and thus reduce the running costs needed to produce each fish.

Chapter 8

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