Improvement of Culture Techniques

for the Seahorse *Hippocampus* sp.

Thesis submitted by

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James Cook University

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ABSTRACT

The culture requirements of broodstock and juvenile seahorse *Hippocampus* sp. were investigated in four experiments: (1) the influence of dietary fatty acids on growth and survival of juvenile seahorses; (2) the effects of varying dietary fatty acid content on reproductive performance of broodstock and juvenile quality; (3) the effects of ambient calcium level on growth and survival of juvenile seahorses; and (4) the combined effects of temperature and salinity on the growth and survival of juvenile seahorse.

In the first experiment, three commercially available fatty acid enrichment emulsions (DC Selco, DC DHA Selco and DC Super Selco) were used to enrich *Artemia* nauplii fed to juvenile seahorses. The emulsions varied in their *n*-3 highly unsaturated fatty acid (HUFA) composition. Total *n*-3 HUFA content ranged from 200-450 mg g^{-1} while levels of eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3) ranged between 47-220 mg g^{-1} and 80-190 mg g^{-1} , respectively. Survival and growth of seahorses at the end of the 30-day growth trial were greater in treatments receiving enriched *Artemia.* Seahorses receiving *Artemia* enriched with DC DHA Selco and DC Super Selco showed significantly ($p < 0.05$) greater mean survival (71.6 ± 6.0 % and 78.3 ± 6.0%, respectively) than those receiving unenriched *Artemia* (48.3 ± 6.0%). Mean standard length was also significantly greater ($p < 0.05$) for fry fed DC DHA Selco and DC Super Selco enriched *Artemia* (20.2 ± 0.3 mm and 19.7 ± 0.3 mm, respectively) compared to those fed unenriched *Artemia* (18.1 ± 0.3 mm). The results show that dietary *n*-3 HUFA are essential for optimal growth and survival of *Hippocampus* sp. and, based on the fatty acid compositions of the enriched *Artemia* used in this study, a level of dietary DHA

supporting optimal growth and survival was indicated to be greater than 9.3 mg DHA g^{-1} DW.

In the second experiment, unenriched and enriched (DC DHA Selco) *Artemia* (Prime *Artemia* cysts, Great Salt lakes USA) were used to improve the nutritional quality of pelagic schooling shrimp, *Acetes sibogae,* to determine the effectiveness of nutritional enrichment on the fecundity and fertility of breeding seahorses. Six pairs of seahorses were fed either enriched or unenriched *Acetes* for a period of 45 days. Mean standard lengths, weights and number of newborns were counted for each clutch and fatty acid analysis was conducted on newborn seahorses and *Acetes* diets. Dietary quality of *Acetes* was effectively improved by feeding with enriched *Artemia*; (n-3) / (n-6) HUFA level increased from approximately 5:1 in the unenriched treatment to 7:1 in the enriched treatment and the DHA/EPA ratio increased from 0.76 in the control treatment to 0.92 in the enriched treatment. As a result, the weight at birth of newborn seahorses was significantly increased, demonstrating the importance of HUFA's in *Hippocampus* sp. broodstock diet.

In the third experiment, juvenile seahorses were subjected to four levels of ambient calcium to determine the effects of varying calcium levels on growth and survival during a 30-day period. Concentrations of calcium tested were: 489 ± 15.43 ppm, 520.83 ± 11.62 ppm, and 583 ± 10.21 ppm in the Low, Medium, and High treatments, respectively. Natural seawater was used as a control treatment with a calcium concentration of 432.67 ± 13.44 ppm. Under these treatments, final survival (% ± SE) ranged from 56.67 ± 12.03 % to 66.67 ± 12.03 %, with no significant differences observed between treatments. Final mean dry weights (mg ± SE) of juvenile seahorses in the treatments ranged from 10.67 ± 0.53 to 11.09 ± 0.82 with no

significant differences observed between the treatments. It was concluded that increasing ambient calcium levels above levels found in natural seawater did not significantly affect the growth or survival of juvenile seahorses examined in the study.

In the final experiment, a 4 x 4 factorial analysis of temperature and salinity combinations was investigated to determine their individual and combined effects on survival and growth of juvenile *Hippocampus* sp. Seahorses were stocked in sixty-four 7 L buckets at a density of 4 seahorses per bucket. Mean length, dry weight and survival were compared after 28 days at temperatures of 20^{o}C, 23^{o}C, 25^{o}C and 29^{o}C and salinities of 20 ppt, 25 ppt, 30 ppt and 35 ppt. All seahorses were fed DC DHA Selco enriched *Artemia* to satiation. Growth significantly increased with temperature; however, salinity had no significant effect on growth. At all salinities tested, a water temperature of 20^{o}C was lethal for juvenile *Hippocampus* sp. Survival was generally improved at lower salinities (20 ppt and 25 ppt). The combined effects of temperature and salinity significantly affected growth but not survival. These results indicate that growth of *Hippocampus* sp. during the newborn-juvenile stage may be maximized through appropriate adjustment of water temperature and salinity.

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STATEMENT ON SOURCES

DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

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CHAPTER 1

1.1 Introduction

1.1.1 General

Aquaculture supplies 20-25% of the total production from fisheries (Ratafia, 1995). Aquaculture continues to grow, however, a general lack of information on the culture requirements of new species is a constraint to further development (Laubier and Barnabe, 1990). Development of finfish aquaculture, in particular, requires attention, as 75% of global production comes from molluscs (Laubier and Barnabe, 1990). The number of new species of finfish being cultured is expected to increase dramatically in the near future, as unsustainable fishing practices are currently depleting natural stocks.

When examining a new species for aquaculture, it is essential to investigate market demand, biology and life history of the target species. Culture conditions for the species should closely mimic natural conditions in which the species is found. These conditions may be simulated in culture through knowledge of an organisms reproduction, dietary preferences and natural distribution (water quality requirements). Previous literature presents a valuable source of information for investigating these factors and therefore helps determine culture requirements.

Recently, western society has recognized the economic and social importance of seahorses in Eastern medicine and the detrimental effects of the demand for seahorses on their natural populations worldwide. In Traditional Chinese Medicine (TCM), seahorses are dried and ingested with other animal and plant ingredients to

aid sexual function and other health problems (Bensky and Gamble, 1993). An estimated 45 tonnes of seahorses are consumed annually for this purpose (Vincent, 1996). In response, large-scale commercial aquaculture of seahorses has become a potential conservation tool as seahorse populations dwindle in over-fished areas. Although the first recorded large-scale seahorse aquaculture attempts were carried out in the early 1980's in China, few studies on the fundamental biology and reproductive requirements of seahorses, and how these factors relate to culture requirements, have been reported. This represents a large gap in information required for the sustainable aquaculture of seahorses.

1.1.2 Reproduction

In seahorses the male produces sperm and the female eggs; however, what sets seahorses apart is that the female, using an ovipositor, deposits her eggs into a brood pouch possessed by the male. Each egg is embedded in the inner epithelium and the brood pouch becomes a honeycomb like structure with each egg occupying one compartment (Nan'er, 1984). Eggs are fertilized within the broodpouch, which is also responsible for the subsequent roles of incubation, osmoregulation, and aeration of the embryos during development (Fig. 1-1). Incubation of the eggs occurs for 2-3 weeks depending on the species (Hubbs, 1943; Lourie *et al.*, 1999) and water temperature (Binsheng, 1992; Lourie *et al.*, 1999). Newborn seahorses are completely developed and ready to feed immediately after birth; subsequently, no parental care is exhibited. This unique method of sexual reproduction has led to the inference that sex roles in seahorses are reversed, or females compete more intensely for access to males. However, Vincent (1994) demonstrated that seahorses actually exhibit conventional

sex roles; males compete more intensely for females on the first and final days of courtship.

The natural breeding season of most seahorses in the wild occurs from mid spring to late summer when water temperatures and plankton availability are at their peak (Strawn, 1958; Nan'er, 1984; Vincent, 1995). Previous studies have shown captive bred seahorses are able to spawn-year round under controlled temperature and photoperiod, lending to their potential as an aquaculture species (Nan'er, 1984). The number of young produced by the male is dependent on age/size (Nan'er, 1984), the number of previous successive spawns (Nan'er, 1984) and species (Vincent, 1994; Masonjones and Lewis, 1996; Lourie *et al.,* 1999).

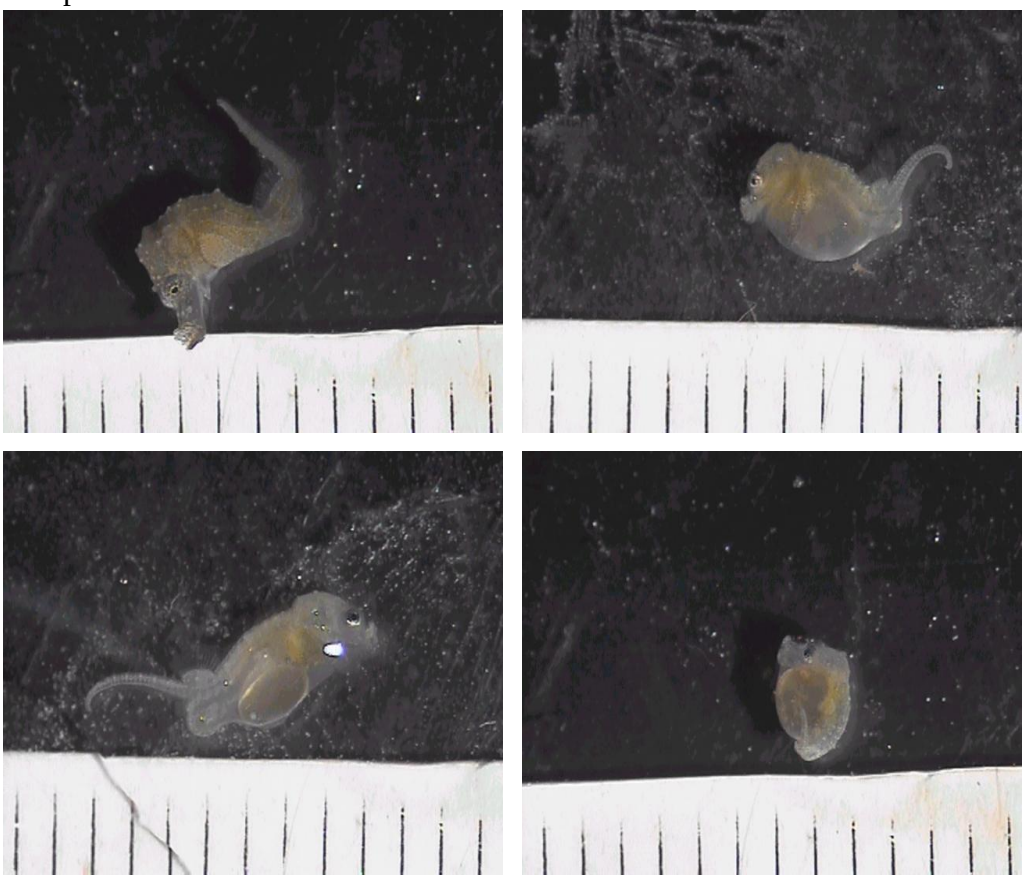
Nutrition is one of the most important factors affecting the reproduction of any aquaculture species. Among the constituents of broodstock diets, lipids or fatty acids are the chemical components that most effect the composition and therefore quality of eggs (Watanabe, 1985). The influence of fatty acids on the reproduction and spawning quality of seahorses has not been examined to date.

1.1.3 Diet and Nutrition

During the early stages of development, the yolk sac is the primary nutritive source for developing embryos in the brood pouch (Fig. 1-1). When the young are born, they are ready to feed on rotifers, copepods, and other small crustaceans in relatively large quantities (Binsheng, 1992). Most juvenile seahorses raised in captivity are fed *Artemia* nauplii (Wilson and Vincent, 2000) because of its relative ease of culture and its commercial availability. *Artemia* are typically enriched with an agent containing highly unsaturated fatty acids (HUFAs) as *Artemia* does not generally fulfill the (HUFA) requirements of most juvenile marine fish (Sorgeloos *et al*., 1986). Once a

seahorse reaches sub-adult size in captivity, it is usually fed live or frozen mysid shrimp (Wilson and Vincent, 2000).

Figure 1-1: Developing seahorse *Hippocampus* sp. during endogenous yolk absorption.[1]



[1]Scale in picture is millimeters

Seahorses are predators that rely on camouflage to ambush their prey (Hoang *et al.*, 1996). They require food items small enough to fit in their narrow snouts and prey that is moving (Table 1-1). This requirement for fresh, live foods with the correct nutritional profile, size, and behavior presents the main difficulty in keeping and breeding seahorses (Strawn, 1954; Binsheng, 1985; Zhang, 1994; Vincent, 1995).

Table 1-1: Comparison of wild diets reported for four different seahorse species

Species	Location	Wild diet	Author
H. zosterae	Florida, USA	harpacticoid copepods	Tipton & Bell, 1998
H. histrix	Vietnam	*Amphipoda, Tanaidacea, Caridae, Palaemonidae, Mysidae*	Hoang *et al*., 1996
H. trimaculatus	Vietnam	*Amphipoda, Tanaidacea, Caridae, Palaemonidae, Mysidae*	Hoang *et al*., 1996
H. kuda	Vietnam	*Amphipoda, Palaemonidae*	Ky, 1994

Previous seahorse aquaculture attempts in China have identified key factors that will improve the sustainability and economic viability of future commercial operations (Binsheng, 1992; Zhang, 1994). Lack of knowledge concerning the specific nutritional requirements of seahorses was identified to be one of the largest gaps in information required for research into the mass culture of live feeds and food technologies for seahorses. Dietary research may lead to the formulation of specific diet enrichment agents and pelleted feeds and will increase sustainability and profitability of seahorse aquaculture.

1.2 Market

In addition to biological factors affecting culture requirements, it is important to examine current markets for a species when considering it for aquaculture. Ideally, from a marketing viewpoint, this should be a high-value/low capital investment product, which has a high market demand and cannot be sustainably supplied by wild fisheries.

Internationally, traded seahorses are consumed as medicine, used as aquarium pets, and as curios. Commonly fished Indo-Pacific species include *H. kuda, H. histrix, H. kelloggi,* and *H. trimaculatus.* Seahorses are primarily consumed in TCM where they are used to "cure" a broad range of maladies. Research has indicated at least 32 nations are involved in trading seahorses with China being the largest consumer of seahorses (20 tonnes or six million seahorses) for use in the TCM market (Vincent, 1994). Taiwan (11.2 tonnes or 3 million), Hong Kong (10 tonnes), and Singapore (unknown) follow as the largest consumers in the world (Vincent, 1994). A total figure of 45 tonnes or 16-20 million seahorses has been estimated to be consumed annually. These estimates did not include black market or unorthodox trade routes and did not include other nations suspected to be major consumers such as Japan, Singapore, Malaysia and South Korea, making this figure a large underestimate. Prices for dried seahorses on the Chinese market range between US$264-880 per kilogram (Table 1-2).

Table 1-2: Retail prices ($US) per kg for dried seahorses in (Vincent, 1994)

Size	Price per kg
Small (50-100 mm, 3-4 g)	$264
Medium (100-150 mm, 5-8 g)	$572
Large (150-200 mm, 8-15 g)	$880

Price depends on quality which is measured by: (1) size-larger seahorses are considered higher quality; (2) species-although most TCM vendors cannot discern between species; spiny seahorses are considered of lower quality; (3) color-white bleached seahorses are usually considered higher quality. Prices for live seahorses in the ornamental/aquarium markets range from US$10-80 each. Prices depend on

factors such as: (1) species-colorful species such as *H. reidi*, *H. ingens*, and *H. subelongatus* are frequently traded at higher prices and; (2) size-larger specimens are traded at higher prices.

1.3 Conservation and Aquaculture

Currently, the demand for seahorses far exceeds the supply that wild populations can support (Vincent, 1994). Exploitation and increasing consumption of marine animals used in traditional medicine is threatening seahorse populations in heavily fished areas. Increased demand for medicines derived from wildlife and, increasing human populations, are leading to increased and unsustainable rates of exploitation (Vincent, 1994). For example, fishers in a village in the Philippines reported an average of 20-30 seahorses per night in 1983, but now catch fewer than 10 per night (Vincent, 1995). Fishermen in Java and Bali report a 50% decline in numbers caught over the past five years and a fishery in India reports a significant deterioration of the fishery as a result of merely two years of fishing (Vincent, 1995).

The solution for conservation is a difficult one because of unorthodox trade routes and because many seahorses are caught as by-catch. Also, many subsistence fishermen in areas such as the Philippines and Vietnam, depend on seahorse fishing. These fishermen will circumvent any bans placed on them if no compensation is given. In addition, placing a ban or restrictions on the seahorse trade may increase their market price and subsequently increase supplier's willingness to trade. Therefore, innovative conservation initiatives will need to be formulated to address these problems. Low-technology aquaculture is a viable option currently being examined; however, the lack of relevant information hinders its progress.

In Australia, 11 of the 35 species of seahorses have been identified to exist in coastal areas. The extent of exploitation in Australia is unknown, however, all syngnathids within Australian waters receive government protection; the export of all syngnathids requires a permit granted only for specimens bred in captivity or collected under an approved management scheme. It is apparent that the management scheme for the trade of seahorses in Australia must include aquaculture as a viable option; however, previous aquaculture attempts in China have identified several key problems associated with sustainable culture of seahorses (Binsheng, 1985). These include: (1) lack of information concerning dietary requirements at all stages of life, and (2) lack of information on general water quality parameters (i.e. temperature and salinity) to maximize growth and reproduction in captivity.

1.4 Research Aim and Approach

One objective of aquaculture is the production of a high-value product, which is not obtained in sufficient quantity from natural harvest (Barnabe, 1994). The success of aquaculture depends on the requirements of an organism in a culture environment, organisms react to their total environment rather than to single factors within it. This makes it extremely difficult to quantify every detail of the complex relationships between an organism and its environment. It is impossible to measure simultaneously all factors involved in the environment of aquatic organisms. However, we may begin by studying easily measured and controlled environmental factors which are assumed to have a significant biological effect on the species being examined.

The aim of this study is to increase the current knowledge of culture techniques for *Hippocampus* sp., thus providing a base for further research in

improving the sustainability of seahorse culture for economic and conservational purposes. To achieve this aim, a broad approach to the fundamental requirements of seahorses was adopted. Factors were selected that represented easily manipulated and measured variables that are considered critical to growth, survival and reproduction. Specifically, the parameters of diet and environmental conditions were examined in four separate experiments.

1.4.1 Dietary requirements

Previous studies on seahorse culture have identified a lack of information regarding the dietary requirements of broodstock and juvenile seahorses (Binsheng, 1992). In addition, gamete quality, which is strongly influenced by broodstock diet, is considered a bottleneck in the culture of most fish species (Dhert *et al.*, 1998). Fish require proteins, lipids, minerals, vitamins and growth promoting factors. The nutritional requirements of fish do not vary greatly between species except in their requirements for essential fatty acids and capacity to assimilate sugars (Barnabe, 1994). Therefore Chapters 3 and 4 examine the fatty acid requirements of juvenile and broodstock seahorses. Fatty acid content of the diet is quantified and resulting fatty acid content of seahorses and their growth are compared and discussed.

1.4.2 Environmental conditions

Despite the increasing importance of seahorses a cultured species, few studies have attempted to optimize environmental conditions which affect the growth and survival of juvenile seahorses. Chapters 5 and 6 examine the effects of varying ambient calcium levels on growth and survival (Chapter 5) and the combined effects of temperature and salinity on growth and survival (Chapter 6).

CHAPTER 2

GENERAL METHODS & MATERIALS

2.1 Study Area and Duration

This study took place in Townsville, North Queensland, Australia between March 1998 and March 2000. Dietary research (Chapters 3 and 4) was conducted at Reef HQ, Great Barrier Reef Marine Park Authority 'quarantine room'. Fatty acid analysis for dietary research was conducted at the Department of Primary Industries-Health and Nutritional Biochemistry Laboratory located in Yeerongpilly, Queensland. Water quality experiments (Chapters 5 and 6) took place at the James Cook University (JCU) aquaculture facility and water analysis was conducted at the JCU Advanced Analytical Center. Broodstock maturation and husbandry in Chapters 5 and 6 took place in the back yard of a residential property also located in Townsville, North Queensland.

2.2 Taxonomy and Species Description

2.2.1 Confusion of seahorse taxonomy

Seahorses belong to the family *Syngnathidae* and the genus *Hippocampus* (Lourie *et al.,* 1999). Seahorse taxonomy is currently under revision due to the confusing number of scientific names resulting from misidentification. Recently, genetic work has cleared much of the confusion. Currently 32 species have been positively

described (Lourie *et al.*, 1999). The species examined in the current study, has yet to be identified and named. Currently, this species is undergoing genetic identification to determine if it is related to any of the currently named species in the Indo-Pacific. The species used in the current study was reared in captivity, which may also lend to morphological differences when compared to wild specimens (Lourie, pers. com., 2000). Until further taxonomic research is completed the species in the current study will be referred to as *Hippocampus* sp.

Figure 2-1: Adult male *Hippocampus* sp. examined in study

2.2.2 Species description

All measurements are in mm to nearest 0.1 mm unless otherwise stated.

Measurements follow protocol described by Lourie *et al.* (1999).

Adult Height:	75-140 (to nearest 5 mm)
Trunk rings:	11
Tail rings:	36
Trunk length:	25-54 (to nearest mm)
Tail length:	46-80 (to nearest mm)
Coronet height:	5.5-7.3
Head length:	17.1-24.0
Snout length:	7.1-10.6
Snout depth:	2.2-2.9
Head depth:	9.6-11.8
Base of pectoral fin:	3.0-3.9
Trunk depth between 4th and 5th trunk rings:	5.8-8.8
Trunk depth between 9th and 10th trunk rings:	9.4-12.4
Trunk width between 9th and 10th trunk rings:	4.9-8.8
Trunk width at 9th trunk ring including spines:	6.5-10.4
Base of dorsal fin:	5.7-7.4
Keel index:	1-2
Coronet index:	2
Spininess index:	2
Chin shape index:	5-5/1
Pectoral fin rays:	14-15
Dorsal fin rays:	16-17

Morphological Features:

Double cheek spines; square ended spines; double spines under eye; dark edged keel; very small coronet (not much more than ridge, barely above ridge of head).

Coloration:

Pale brown/tan from 3 weeks to 10 weeks of age. After 10 weeks, coloration may change to (1) bright yellow with orange spots, (2) muddy orange/red color (3) dark brown (4) white or (5) black. Black or pale white 'saddles' across their dorso-lateral surfaces are present in some specimens especially those with yellow coloration. In general, males have black 'saddles' and females have white or no 'saddles' present.

Life History in captivity:

Reef HQ/Great Barrier Reef Marine Park Authority supplied seahorses used in the current study. Specimens were collected from the Townsville region, approximately 5 years previously and have since been bred in captivity. Specimens used in the current study were captive bred for an estimated 3-4 generations prior to the current project. The effects of inbreeding or captive breeding seahorses have not been well studied, however anecdotal evidence suggests maximum size of seahorses decrease with successive generations of captive breeding. The current study took this into consideration and comparisons were only made between progeny of the same generation. The Juveniles reared during the course of the experiment exhibit benthic foraging and settlement behaviour. This species is able to produce viable young at 3 months of age. Typically, broodsize is 243 ± 52 (n=44) with a gestation period of about 23 days. This species is non-monogamous in captivity.

2.3 Culture Techniques and Materials

2.3.1 Broodstock system design and husbandry

Broodstock *Hippocampus* sp. were kept outdoors in a 1200-L circular tank (Fig. 2-2). Cover was provided using a tarpaulin to reduce light intensity by 80% and exposure to rainfall. Salinity was maintained between 33-36 ppt, temperature was maintained between 22-30^{o}C and pH was maintained around 8.0. Biological filtration was provided using coral rubble as bio-media and a venturi-type protein skimmer was used to remove dissolved organics. Broodstock were fed *Acetes* sp. shrimp, (*ad libitum*) collected from the wild at the Townsville breakwater using a large, 1 mm mesh dipnet. Supplementary diet consisted of live adult *Artemia* fed micro-algae (*Nannochloropsis occulata*) and brewers yeast.

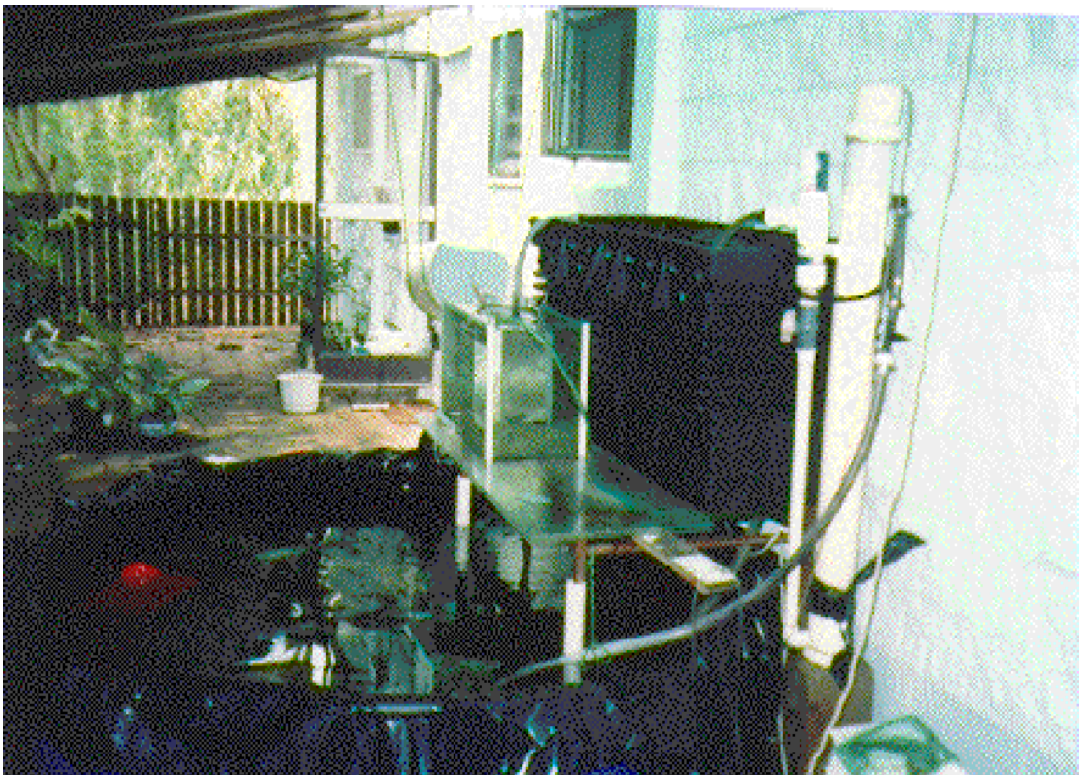


Figure 2-2: 1200-L pond used to house broodstock seahorses

2.3.2 General rearing protocol for newborn and juveniles

Newborn *Hippocampus* sp. were reared in 6 x 100 L square glass aquaria, with all sides and the bottom of the tank covered in black plastic. All tanks recirculated to a 250-L sump supplied with biological filtration (coral rubble) and a protein skimmer. Bottom siphoning to remove detritus was conducted daily while 25% water changes were conducted weekly. Newborn seahorses were fed newly-hatched *Artemia* (Prime *Artemia* cysts, Salt Lake City USA) provided twice daily for 3-7 days. Juvenile seahorses were then weaned to DC DHA Selco (INVE Aquaculture, Belgium) enriched *Artemia* (Section 3.2.1) starting on day 3 and completing the diet change-over on day 7.

2.4 Measuring Standard Length of Juveniles

Standard length of newborn and juvenile seahorses was measured from the tip of the snout to the tip of the tail following the protocol for measuring adult seahorses described by (Lourie *et al.*, 1999). A specialized video image processing system was used to determine standard lengths of juveniles due to their delicate nature and a need for accurate growth measurements. The system components included a dissecting microscope (Olympus SZ-CTV 40) and a video camera (Panasonic WV-CP410/A) linked to a portable computer (Acer Travelmate 720 TX) using a 'frame grabber' (Snappy 3.0). Other components included 2 light sources, black background, a portion of a ruler and a petri dish.

Lighting and focus were optimized prior to data collection. Juvenile seahorses were then placed, one at a time, in a petri dish containing about 4 mm of sea water

from the respective culture tank (just enough to keep seahorse flat and non-mobile). The petri dish was placed under the microscope with a piece of black paper as a background and the ruler piece was placed in the water for calibration of measurements. An image was obtained, digitized and stored on computer file (jpeg) for later analysis (Fig 2-3). Analysis of standard length derived from computer images was accomplished using graphics analysis software (ImageTools 2.0) which measured the image to the nearest 0.01 mm. Handling time ranged from 5-10 seconds per seahorse. Preliminary trials using this method found 0-2% mortality following the day of measurement indicating stress-induced mortality using this method is minimal.

The advantages of this system, when large numbers of small animals must be measured accurately, is apparent. Labor savings, elimination of manual data transcription errors, reduced fish handling and the option of storing images for later analysis are all benefits.

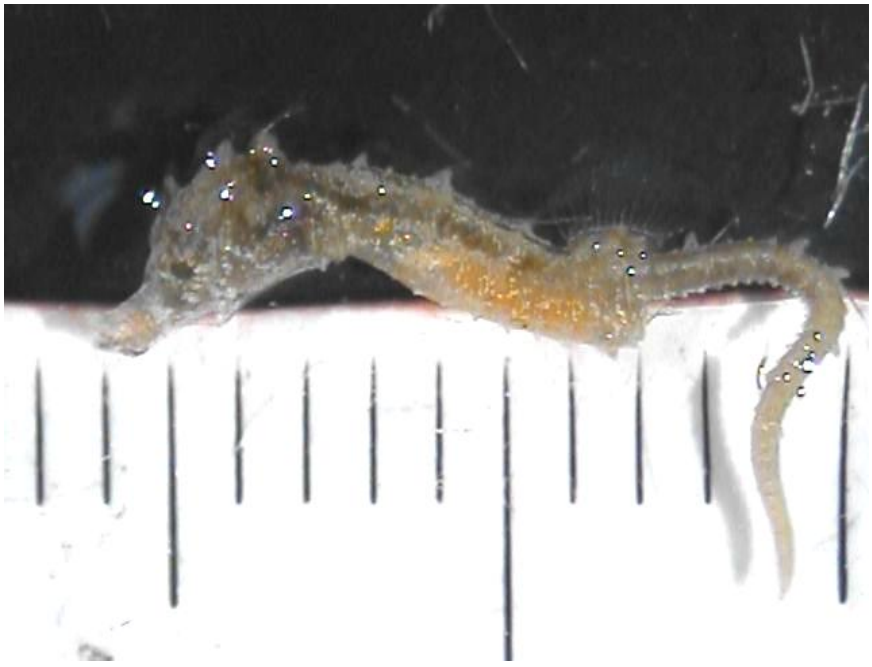


Figure 2-3: Seven-day old seahorse measured using a video image processing procedure.

CHAPTER 3

EFFECTS OF LIVE FEED ENRICHMENT ON GROWTH AND SURVIVAL OF *Hippocampus SP.*, JUVENILES.

3.1 Introduction

3.1.1 Fatty acid requirements of marine fish larvae

It is now well established that the larvae of marine fish have a dietary requirement for *n*-3 highly unsaturated fatty acids (HUFA) and a number of recent studies have shown eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) to be essential dietary components (Webster and Lovell, 1990; Lemm and Lemarie, 1991; Rimmer *et al*., 1994). These essential fatty acids (EFA) have a critical role as membrane constituents; they are particularly important in early developmental stages (Ozkizilcik and Chu, 1994) and must be supplied in the diet to ensure optimal growth and survival.

3.1.2 *Artemia* as a food source for larval fish

Artemia (brine shrimp) are considered to be one of the best and, in many cases, the only available food source for rearing marine fish larvae and early juveniles (Bardach *et al*, 1972, Sorgeloos *et al*., 1986) and they are readily consumed by seahorses. However, many strains of *Artemia* are deficient in *n*-3 HUFA and therefore do not provide adequate nutrition for fish larvae (Watanabe *et al*., 1983; Wester and Lovell, 1990). To overcome this deficiency, *Artemia* nauplii can be 'enriched' by allowing them to ingest material rich in *n*-3 HUFA prior to feeding to fish larvae (Leger *et al*., 1986).

This process greatly increases the *n*-3 HUFA content of the nauplii and improves their nutritional value. Fatty acid enrichment is conducted routinely in marine finfish hatcheries using commercially available enrichment preparations.

3.1.3 Aims of the experiment

There is a paucity of information on the nutritional requirements of seahorses, in particular, the *n*-3 HUFA requirements of juvenile seahorse. The aims of this study were: (1) to establish whether juvenile *Hippocampus* sp. have a dietary requirement for *n*-3 HUFA as shown for other marine fish; and (2) to assess the effects of varying dietary *n*-3 HUFA composition on growth and survival of juvenile *Hippocampus* sp.

3.2 Materials and Methods

3.2.1 Preparation of *Artemia*:

Freshly hatched *Artemia* (Great Salt Lakes, USA; Primo Aquaculture, Brisbane) nauplii were enriched for 24 h using one of three commercially available enrichment preparations: (1) DC Selco; (2) DC DHA Selco; and (3) DC Super Selco (Inve Aquaculture, Dendermonde, Belgium). The three preparations varied in their fatty acid compositions, which are shown in Table 3-1. *Artemia* were enriched at the recommended level of 0.6 g L^{-1} of sea water in 2 L vats containing *Artemia* nauplii at a density of 80 nauplii mL^{-1}. Following enrichment, nauplii were harvested with a 100 μm sieve, rinsed with fresh water and resuspended in 400 mL of seawater.

Table 3-1: Nutritional composition of three commercial enrichment emulsions used in this study (manufacturer information).

Composition	DC Selco	DC DHA Selco	DC Super Selco
n-3 HUFA (mg/g DW)	200	200	450
EPA (mg/g DW)	110	47	220
DHA (mg/g DW)	80	139	190
DHA/EPA	0.73	2.96	0.87
Lipid (%)	65	65	65
Moisture (%)	30	30	30
Ash (%)	3	3	3
Vit A (Ul/kg)	1,500,000	1,500,000	1,500,000
Vit D3 (Ul/kg)	150,000	150,000	150,000
Vit E (Ul/kg)	2,000	2,000	2,000
Vit C(mg/kg DW)	400	400	400
Antioxidants	yes	yes	yes

3.2.2 Seahorse culture conditions

Three-day post-hatch *Hippocampus* sp. were held in 10 L aquaria supplied with air-lift sponge filtration. Each aquarium was stocked with 20 seahorses and randomly allocated to one of four treatments consisting of each of the three enrichment types and an unenriched control. Three replicate tanks were established for each treatment. The bottom of each aquarium was siphoned daily to remove detritus and 25 % water changes were conducted every-other day. Salinity and water temperature in aquaria were maintained at 31 ppt and 24-26 ^{o}C. Ammonia, nitrate, nitrite, and phosphates were maintained at low levels and pH was 8. Mean oxygen saturation was maintained above 85%. Light intensity was not measured but was kept at a low level. Mortalities were recorded daily.

3.2.3 Data collection and analysis

The experiment was terminated after 30 days when mortalities reached approximately 50% in the control treatment. Remaining seahorses in all treatments were counted, measured from snout to tail (Section 2.4), weighed (0.001g), and stored in liquid nitrogen prior to fatty acid analysis. The data were checked for normal distribution using residual plots (Appendix 3.2; 3.5, 3.8) and homogeneity of variances was tested with Levene's test (Appendix 3.1; 3.4; 3.7). Percent survival data were arcsin transformed and standard lengths were log transformed when necessary (Zar, 1984). Overall significant differences in lengths, weights, and survival between groups were determined using one-way ANOVA. Mean length comparison between treatments were assessed using Tukey's honestly significant difference (HSD) test (Appendix 3.3) and Tukey's least significant difference (LSD) was used to determine which treatments differed with respect to survival and weight (Appendix 3.6; 3.9). Correlations between all dependent variables and dietary *n*-3 fatty acid content, DHA/EPA ratio, and dietary DHA and EPA contents were tested using Pearson's correlation (one-tailed). All data were analyzed using SPSS 8.0 student version.

3.2.4 Fatty acid analysis

Frozen seahorse samples were freeze-dried. Three replicate samples from each treatment were combined prior to analysis. Combining replicate samples was necessary to obtain sufficient sample for analysis. Lipid was extracted from weighed samples of dried seahorse fry using chloroform/methanol (Folch *et al*., 1957) saponified and derivatised to fatty acid methyl esters (FAMEs) according to Van Wijngaarden (1967). Gas chromatography (GC) with flame ionization detection was used to quantify FAMEs. The GC was fitted with a DB-23 fused silica column (30 m

x 0.25 mm i.d and 25 μm coating) with ultra high purity helium used as the carrier gas at 1 mL min^{-1}. Oven temperature commenced at 140°C for 5 min and increased by 3°C min^{-1} to 210°C, and then maintained for a further 6 min. An internal standard (heneicosanoic acid methyl ester, Sigma Chemicals) was used for the comparison of retention times of the samples.

3.3 Results

3.3.1 Survival

The first mortalities were observed on day 8 of the experiment in the unenriched treatment (11 days post hatch) (Fig. 3-1). After 30 days, mean (± S.E.) survival of seahorses fed unenriched *Artemia* was 48.3 (± 6.0) %. This value did not differ significantly from survival of seahorses fed *Artemia* enriched with DC Selco (66.7 ± 3.3 %), but was significantly lower than those from treatments receiving nauplii enriched with either DC DHA Selco (71.7 ± 6.0 %) or DC Super Selco (78.3 ± 6.0 %) (Table 3-2). No significant differences in survival were observed between the three enriched treatments (Table 3-2).

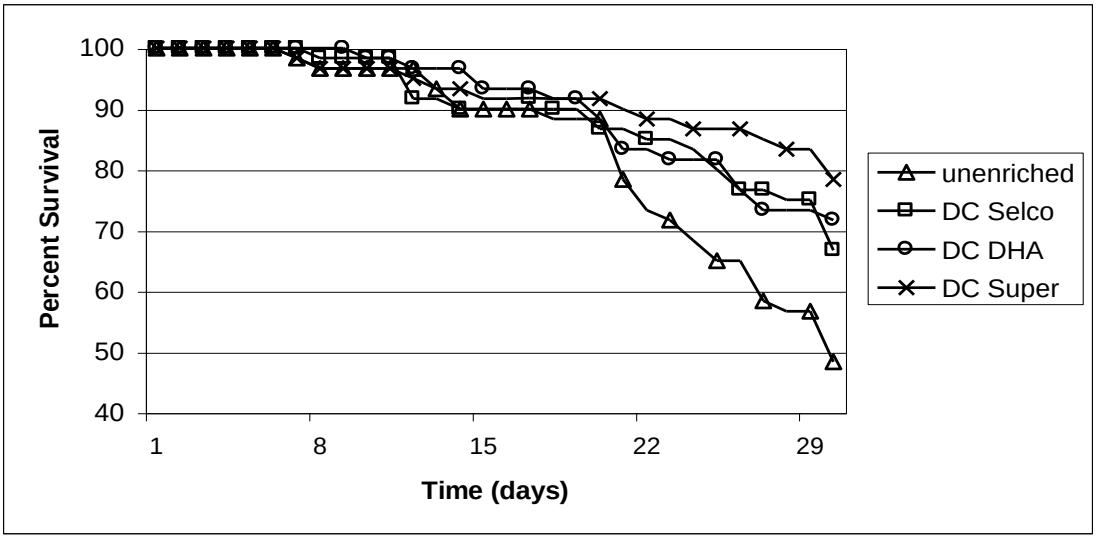


Figure 3-1: Percent survival of juvenile *Hippocampus* sp. fed unenriched *Artemia* or *Artemia* enriched with one of three enrichment preparations.

Table 3-2: Mean (± S.E., n=3) survival, wet weight and standard length of juvenile *Hippocampus* sp. at 30 days of a growth trial fed unenriched *Artemia* or *Artemia* enriched with DC Selco, DC DHA Selco or DC Super Selco[1]

Treatment	Survival (%)	Final weight (mg)	Final length (mm)
Control (no enrichment)	48.33 ± 6.01^{a}	16.43 ± 0.52	18.13 ± 0.33^{a}
DC Selco	66.67 ± 3.33^{ab}	16.93 ± 1.32	18.63 ± 0.31^{ab}
DC DHA Selco	71.67 ± 6.01^{b}	19.13 ± 0.37	20.20 ± 0.34^{c}
DC Super Selco	78.33 ± 6.01^{b}	18.30 ± 0.35	19.72 ± 0.34^{bc}

[1] Means within a column sharing a common superscript are not significantly different ($P \leq 0.05$)

Table 3-3: ANOVA of standard lengths of juvenile *Hippocampus* sp. fed unenriched *Artemia* or those enriched with three enrichment preparations

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.775E-02	3	1.592E-02	7.141	.000
Within Groups	.328	147	2.229E-03		
Total	.375	150			

3.3.2 Growth

Mean (± S.E.) standard length differed significantly between treatments (Table 3-2). There was no significant difference between the mean length of seahorses fed unenriched *Artemia* (18.1± 0.3 mm) and those fed *Artemia* enriched with DC Selco (18.6 ± 0.3 mm); however, seahorses in both these treatment were significantly smaller than those receiving *Artemia* enriched with DC Super Selco (19.7 ± 0.3 mm) or DC DHA Selco (20.2 ± 0.3 mm) (Table 3-2). No significant differences in weight were observed between the treatments (Table 3-4).

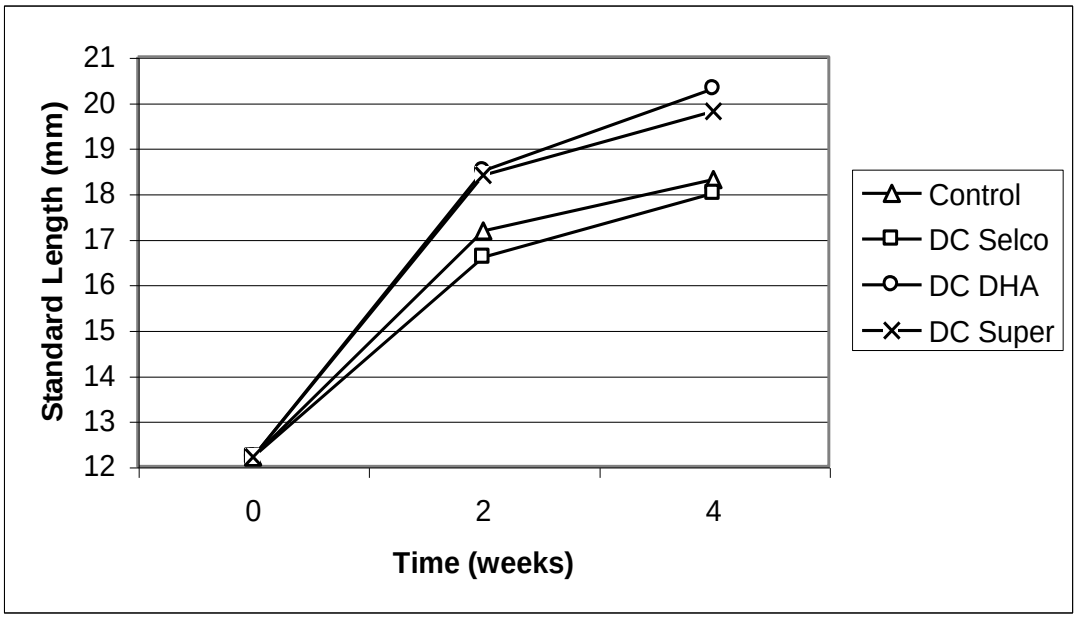


Figure 3-2: Changes in standard length of juvenile *Hippocampus* sp. fed unenriched *Artemia* or those fed on three enrichment preparations.

Table 3-4: ANOVA of wet weight of juvenile *Hippocampus* sp. fed unenriched *Artemia* or those enriched with three enrichment preparations

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8.613E-03	3	2.871E-03	2.667	.119
Within Groups	8.611E-03	8	1.076E-03		
Total	1.722E-02	11			

Table 3-5: Fatty acid composition (mg g^{-1} DW) of unenriched *Artemia* and *Artemia* enriched with DC Selco, DC DHA Selco or DC Super Selco and of seahorses (*Hippocampus* sp.) fed unenriched *Artemia* or *Artemia* enriched with DC Selco, DC DHA Selco or DC Super Selco

	Unenriched		DC Selco		DC DHA Selco		DC Super Selco	
Fatty acid	*Artemia*	seahorse	*Artemia*	seahorse	*Artemia*	seahorse	*Artemia*	seahorse
14	0.9	0.4	4.4	0.8	2.4	0.9	2.5	0.6
14:1*n*-5	0	0	0	0	0	0	0	0
15	0	0	0.5	0	0.6	0.2	0	0.2
16	13.9	5.4	23.1	7.6	23.6	8.8	25.4	8.2
16:1*n*-7	2.5	0.4	10.7	1.6	7.3	1.7	6.3	1
17	0.8	0.4	1	0.4	1.2	0.5	1.3	0.5
17:1*n*-8	0	0	0	0	0	0	0	0
18	7.5	5.4	8.4	5.9	9.7	6.3	12.3	6.4
18:1*n*-9	25.2	7.6	45.1	11.1	41.4	12.3	43.4	10.7
18:1*n*-7	7.7	2.3	10.8	3.6	10.4	3.9	12.2	3.4
18:2*n*-6	6.4	2.2	18.1	4	12.1	3.8	12.4	3
19	0.4	0	0.6	0	0.7	0.2	0	0.2
18:3*n*-3	35.5	3.7	41.3	5.8	44.7	7.2	52.6	5.7
18:4*n*-3	5.4	0.5	7	1	6.7	1.2	8.2	0.9
20	0	0	0.3	0	0	0.2	0	0.2
20:1*n*-11	0	0	0	0	0	0	0	0
20:1*n*-9	0.9	0	1.5	0.4	1.6	0.4	2.1	0.4
20:1*n*-7	0	0	0.2	0	0	0	0	0
20:2*n*-6	0.4	0	0.6	0	0.7	0.2	0.7	0.2
20:3*n*-6	0	0	0	0	0	0	0	0
20:4*n*-6	1.2	1.8	2.2	1.7	3	2.2	2.8	1.8
20:3*n*-3	1.4	0.4	1.3	0.5	1.6	0.5	1.9	0.4
20:4*n*-3	0	0	0	0	0	0	0	0
20:5*n*-3	3.3	1.5	19.5	4.2	14.1	3.7	30.9	5.5
22	0	0	0.2	0	0.4	0.2	0	0.2
22:1*n*-11	0	0	0.4	0	0	0	0.6	0
22:1*n*-9	0	0	0	0	0	0	0	0
22:1*n*-7	0	0	0	0	0	0	0	0
22:1*n*-6	0	0	0	0	0	0	0	0
22:1*n*-3	0	0	0	0	0	0	0	0
22:2*n*-6	0	0	0	0	0	0	0	0
22:3*n*-6	0	0	0	0	0	0	0	0
22:4*n*-6	0	0	0	0	0	0.2	0	0
22:3*n*-3	0	0	0	0	0	0	0	0
22:5*n*-6	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0
22:5*n*-3	0	0.5	2.1	1.3	1.5	0.9	3.1	1.5
22:6*n*-3	0	2	9.3	6.4	15.3	9.2	14.5	7.6
24:1*n*-9	0	0	0	0	0	0	0	0
total lipid	113.3	34.5	208.7	56.5	199.2	64.7	233.2	58.8
total *n*-3	3.3	4.4	32.2	12.4	32.5	14.3	50.4	15

3.3.3 Fatty acid content of *Artemia*

Total *n*-3 HUFA content of *Artemia* ranged from 3.3 mg g^{-1} dry weight (DW) (2.9% of total fatty acids) in unenriched *Artemia* to 50.4 mg g^{-1} DW (21.61% of total lipid) in *Artemia* enriched with the DC Super Selco (Table 3-5). The EPA content increased from 3.3 mg g^{-1} in unenriched *Artemia* to 19.5 mg g^{-1} DW, 14.1 mg g^{-1} DW and 30.9 mg g^{-1} DW in *Artemia* enriched with DC Selco, DC DHA Selco and DC Super Selco, respectively. Similarly DHA content increased from zero in unenriched *Artemia* to 9.3 mg g^{-1} DW, 15.3 mg g^{-1} DW and 14.5 mg g^{-1} DW in *Artemia* enriched with DC Selco, DC DHA Selco and DC Super Selco, respectively.

3.3.4 Fatty acid content of seahorses

The *n*-3 HUFA content of seahorses generally reflected that of the diet and increased from 4.4 mg g^{-1} DW in groups fed unenriched *Artemia* to 12.4 mg g^{-1} DW, 14.3 mg g^{-1} DW and 15 mg g^{-1} DW in seahorses fed *Artemia* enriched with DC Selco, DC DHA Selco and DC Super Selco, respectively (Table 3-5). Seahorses fed unenriched *Artemia* contained 1.5 mg EPA g^{-1} DW and 2.0 mg DHA g^{-1} DW. As would be expected, seahorses receiving enriched *Artemia* contained higher levels of EPA and DHA. EPA contents ranged from 4.2 mg g^{-1} DW to 5.5 mg g^{-1} DW in seahorses receiving *Artemia* enriched with DC Selco and DC Super Selco, respectively. The same enrichment treatments resulted in DHA contents of 6.4 mg g^{-1} DW and 7.6 mg g^{-1} DW, respectively. Seahorses receiving *Artemia* enriched with DC DHA Selco had a higher DHA content of 9.2 mg g^{-1} DW.

3.3.5 Correlations

There was a highly significant positive correlation between survival and dietary *n*-3 HUFA content ($r^2 = 0.97$, $p \leq 0.0125$); however, there were no significant correlations between dietary *n*-3 HUFA content and mean standard length or mean wet weight ($p > 0.05$). There were significant positive correlations between dietary EPA ($r^2 = 0.82$, $p \leq 0.05$) and dietary DHA ($r^2 = 0.92$, $p \leq 0.05$) contents and mean survival. There was also a significant relationship between dietary DHA content and mean standard length ($r^2 = 0.82$, $p \leq 0.05$); however, the relationship between EPA content and standard length was not significant ($r^2 = 0.16$, $p > 0.05$). Relationships between dietary DHA:EPA ratio and standard length and wet weight proved significant ($r^2 = 0.86$, $p \leq 0.05$ and $r^2 = 0.87$, $p \leq 0.05$, respectively); however the relationship between dietary DHA:EPA ratio and survival was not significant ($r^2 = 0.45$, $p > 0.05$).

3.4 Discussion

Numerous studies have shown that the fatty acid composition of enriched *Artemia* resembles that of their food source (e.g. Witt *et al*., 1984; Izquierdo *et al*., 1992; Southgate and Kavanagh, 1999) and this was confirmed in the present study. This study also showed that the fatty acid composition of *Artemia* significantly affected growth and survival of seahorses and that fatty acid enrichment of *Artemia* may improve their nutritional value. This confirms the results of similar studies with fish larvae which have shown the beneficial effects of improved dietary *n*-3 HUFA larvae which have shown the beneficial effects of improved dietary *n*-3 HUFA content for commercially cultured (Izquierdo *et al*., 1992; Witt *et al*., 1984) and coral reef (Southgate & Kavanagh, 1999) species. The importance of *n*-3 HUFA for normal

growth and development of marine fish results from their limited capacity to bioconvert dietary C18 precursors to longer chain C20 and C22 fatty acids (Bell *et al*., 1986). The latter have important roles in the structure and functioning of biological membranes (Bell *et al*., 1986).

While there was a highly significant relationship between survival of juvenile seahorses and dietary *n*-3 HUFA content, the results of this study do not clearly indicate the relative physiological importance of EPA and DHA. Prior studies with finfish have suggested that the physiological roles of EPA and DHA are distinct (Kraul *et al*., 1993; Mourente *et al*., 1993; Watanabe, 1993). However, DHA is thought to have greater importance as an essential fatty acid for fish and has been shown to be of greater importance than EPA in reducing stress-related mortality in red sea bream, *Pagrus major*, larvae (Watanabe, 1993). The results of this study showed a significant relationship between survival of juvenile seahorses and dietary content of both EPA and DHA. There was also a significant relationship between dietary DHA content and mean standard length. Increased standard lengths were also correlated to increased DHA/EPA ratio; however, there were no similar relationships between dietary EPA content in standard length. This may indicate a more important role for dietary DHA as a factor influencing the growth of *Hippocampus* sp.

For example, mortality of yellowtail larvae, *Seriola quinqueradiata*, fed *Artemia* enriched with EPA but lacking DHA, was associated with an imbalance of EPA and DHA in membrane phospholipids similar to that shown by larvae fed diets deficient in both EPA and DHA (Watanabe, 1993). On this basis, it has been suggested that optimal functioning of biomembranes relies on the presence of EPA and DHA in the correct proportions (Watanabe, 1993). The positive correlation found

in this study between dietary DHA:EPA ratio and both standard length and wet weight in seahorses provides additional support to the importance of DHA:EPA ratio.

It is interesting to note that seahorses fed unenriched *Artemia* contained 2.0 mg DHA g^{-1} DW even though their diet lacked DHA. This may indicate that *Hippocampus* sp. are capable of limited biosynthesis of DHA from a dietary precursor, as reported for turbot larvae (Linares and Henderson, 1991). If this is the case, the improved growth rates of seahorses fed higher levels of DHA indicates that the rate of synthesis is suboptimal. The presence of DHA in these seahorses could also indicate the presence of yolk reserves; however, given the advanced age of the seahorses when analyzed (30 days after birth), this is unlikely. Mortality of seahorses in this study began 11 days after hatch; this is likely to indicate the point at which maternally-derived nutrient reserves are exhausted.

Final lengths, wet weights and survival greatly increased in seahorses fed the DC DHA Selco and DC Super Selco treatments where dietary DHA content was raised above 14.5 mg g^{-1} DW. However, there was no significant improvement in growth and survival of seahorses fed *Artemia* enriched with DC Selco (9.3 mg DHA g^{-1}DW) when compared to those fed unenriched *Artemia*. This indicates that the level of dietary DHA supporting optimal growth and survival of juvenile *Hippocampus* sp. is greater than 9.3 mg DHA g^{-1} DW. However, *Artemia* enriched with DC Selco had a higher EPA content than those enriched with DC DHA Selco. The observed improvement in growth of *Hippocampus* sp. fed the latter, indicates that dietary DHA content has a greater influence on the growth of *Hippocampus* sp. than dietary EPA content.

CHAPTER 4

IMPROVEMENT OF SHRIMP (*Acetes sibogae*) AS A BROODSTOCK DIET FOR *Hippocampus* SP. AND THE SUBSEQUENT EFFECTS ON LARVAL QUALITY AND FATTY ACID CONTENT

4.1 Introduction

4.1.1 Lipids in broodstock nutrition

Lipids in broodstock nutrition are considered to be one of the most important factors influencing egg quality and fecundity of many fish (Watanabe *et al*., 1984a; Watanabe *et al*., 1984b; Watanabe, 1985; Kjorsvik *et al*., 1990; Mourente and Odriozola, 1990; Harel *et al*., 1994; Rainuzzo *et al*., 1997). Lipid reserves in fish eggs are used by the developing larvae both as substrates for energy metabolism and as structural components in membrane biogenesis during the transition from endogenous to exogenous feeding (Sargent, 1995; Rainuzzo *et al*., 1997). In addition, a close relationship between the fatty acid content of broodstock diet and the fatty acid composition of resulting gametes has been reported (Mourente and Odriozola, 1990). These studies have shown that a deficiency of HUFAs in broodstock diets, especially those in the *n*-3 series, negatively affects fecundity, fertilization rate and hatching rate (Rainuzzo *et al*, 1997). Therefore, lipids are now considered to be the major nutritional constituent in broodstock diets determining food quality.

4.1.2 Diet of broodstock seahorses

The influence of dietary *n*-3 HUFAs on broodstock fecundity and subsequent quality of newborn seahorses has received little attention. Previous studies have identified broodstock nutrition of the seahorse, *H. trimaculatus,* to be a critical factor affecting the success of captive breeding efforts (Nan'er *et al.*, 1984). However, broodstock nutrition of seahorses still remains an unexplored area of research despite increasing commercial interests.

The diet of seahorses in captivity is generally limited to adult *Artemia* and wild-caught sources of live and frozen foods (Lourie *et al*., 1999). In preliminary studies, pelagic schooling shrimps *Acetes sibogae* (*australis*) were used successfully as a broodstock and "grow out" diet for the species examined in the current study. Shrimps of the genus *Acetes* occur in coastal and estuarine waters in tropical, subtropical, and temperature parts of the world (Xiao and Greenwood, 1993) and were seasonally abundant at the study site at the time of the experiment (Section 2.1). Although *Acetes* were found to be an acceptable diet, previous studies have indicated that seasonal variations in the nutritional quality of *Acetes* sp. may exist (Xiao and Greenwood, 1993). As such, the nutritional quality of *Acetes* as a broodstock diet and its subsequent effects on broodstock fecundity is not known.

4.1.3 Research aim

Understanding the nutritional requirements of seahorses prior to breeding will play a key role in developing successful large-scale seahorse culture techniques. Fecundity and egg/fry quality are a key component of this success and development of means to optimise these factors is very important. The aim of this study was to examine the

fatty acid composition and quality of newborn seahorses with respect to the nutritional quality of *Acetes* fed to broodstock *Hippocampus* sp. To achieve this, broodstock seahorses were fed either *n*-3 HUFA enriched *Acetes* or unenriched *Acetes*.

4.2 Materials and Methods

4.2.1 Live feed treatment of *Acetes* fed to broodstock *Hippocampus* sp.

Acetes were distributed equally in four separate 30-L rectangular glass aquaria at a density of 20 individuals L^{-1}. Aquaria were supplied with separate undergravel filtration and aeration with water conditions of 26^{o}C, pH 8, and salinity 32 ppt. Ten percent water changes and bottom siphoning were conducted twice weekly. Two replicate aquaria containing *Acetes* were allocated for each of the two treatments: (1) 'enriched' *Acetes* and; (2) 'unenriched' (control) *Acetes*`.

Results from the first experiment determined that the HUFA content of *Artemia* could be increased through dietary enrichment (Chapter 3). As *Acetes* readily consume *Artemia*, enriched *Artemia* were used as a food source for *Acetes* as a means of increasing their HUFA content. *Acetes* in each aquarium were fed approximately 40,000 *Artemia* nauplii, twice daily. 'Enriched' *Acetes* were fed a diet of DC DHA Selco enriched *Artemia* (Section 3.2.1), while control *Acetes* received unfed *Artemia* (Section 3.2.1). After 15 minutes of feeding, *Acetes* were fed to respective pairs of broodstock, *Hippocampus* sp., *ad libitum.* Three random samples of *Acetes* were separated from each treatment and analyzed for fatty acid content according to the methods outlined in Section 3.2.4.

4.2.2 Broodstock seahorse culture conditions

Adult seahorses were maintained in a 1200 L pond system using the methods described in Section 2.2.1. Six pairs (male/female) of seahorses were selected on the basis of uniformity of size and parental relation (all seahorses were from the same parentage). Pairs of seahorses were placed randomly into 6 separate 100 L glass aquaria supplied with aeration and constant recirculation. Seahorses were fed respective diets for one month prior to data collection. Twenty-five percent water changes were conducted once per week and water quality was maintained at 28^{o}C, 35 ppt and pH 8. Broodstock seahorses were fed either unenriched *Acetes* (control) or HUFA enriched *Acetes* (enriched) as detailed above. Resulting clutches of newborn seahorses were counted, measured for standard length (Section 2.3), weighed (wet weight) and analyzed for fatty acid content as described in Section 3.2.4.

4.2.3 Data collection and analysis

The experiment was set up as a randomized block design and lasted 45-days. Newborn seahorses were collected and counted on the day of birth and a subsample of 50 individuals were removed for measurements of mean standard length (Section 2.3) and mean wet weights. A 2-way nested ANOVA (General Linear Model, GLM ANOVA) was used to determine significant differences in lengths, weights and numbers of offspring produced between pairs (Appendix 4.2). Broodstock pairs were nested within dietary treatments to determine if any broodstock pair biases existed. Lavene's test was used to assess homogeneity of variance (Appendix 4.1). All statistics were computed using SPSS 8.0 student version.

4.3 Results

4.3.1 Reproductive performance of broodstock fed different diets

Five of the six pairs bred successfully during the course of the experiment. One pair did not breed in the unenriched treatment during the experiment. Broodstock pairs gave birth 2.6 ± 1.16 times during the 45-day period of the experiment with an average of 20 ± 1.61 days between births.

The mean standard length (± S.E.) of fry resulting from broodstock fed the two diets were 10.61 ± 0.11 mm in the control and 10.99 ± 0.12 mm in the enriched diet group (Table 4-1). No significant differences in length were observed between the two dietary treatments. The mean (± SE) weight of fry from the enriched group was significantly higher (5.4 ± 0.12 mg) than that of the unenriched group (5.04 ± 0.12 mg) (Table 4-1). Wet weight of newborn seahorses did not differ significantly within the nested dietary groups (Table 4-2). The mean number of young produced per clutch were very similar between the two treatments (190.63 ± 22.01 versus 192.2 ± 26.73 for unenriched and enriched treatments, respectively) and there was no significant difference between the two (Table 4-1).

Table 4-1: Mean (± S.E.) standard length, wet weight and number per clutch of newborn seahorses *Hippocampus* sp. hatched from broodstock fed unenriched *Acetes* or *Acetes* fed with DC DHA Selco enriched *Artemia*[1]

Acetes Diet	Mean Standard Length	Mean Wet Weight	Mean Newborns Per Clutch
Control	10.61 ± 0.11^{a}	5.04 ± 0.12^{a}	192.2 ± 26.73^{a}
Enriched	10.99 ± 0.12^{a}	5.4 ± 0.10^{b}	190.63 ± 22.01^{a}

[1] Means within a column sharing a common superscript are not significantly different ($P \leq 0.05$)

Table 4-2: Tests of within subject effects of newborn weight with broodstock pairs nested within diet

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
PAIR(DIET)	Hypothesis	.219	3	7.289E-02	1.346	.327
	Error	.433	8	5.417E-02		

4.3.2 Fatty acid content of *Acetes*

Acetes readily consumed enriched and unenriched 2-day old *Artemia.* The DHA content of *Acetes* increased from 3.9 mg g^{-1} DW in the unenriched treatment to 5.5 mg g^{-1} DW in the enriched treatment and EPA increased from 5.1 mg g^{-1} DW in the unenriched treatment to 6.0 mg g^{-1} DW in the enriched treatment (Table 4-3). Likewise, the DHA/EPA ratio in *Acetes* increased from 0.76 in the unenriched treatment to 0.92 in the enriched treatment. Total *n*-3 HUFA content in *Acetes* increased from 9.6 mg g^{-1} DW in the unenriched treatment to 13.6 mg g^{-1} DW in the enriched treatment. Total *n*-6 HUFA's were similar in both treatments and ranged from 2 mg g^{-1} DW in the unenriched treatment to 1.9 mg g^{-1} DW in the enriched treatment (Table 4-3). Unenriched *Acetes* contained no detectable levels of 22:5*n*-3; however, enriched *Acetes* contained 1.7 mg 22:5*n*-3 g^{-1} DW. All fatty acids in the 18: series were consistently lower in enriched *Acetes* when compared to unenriched *Acetes.* It was also interesting to note that 18:1*n*-9 decreased substantially from 7.9 to 5.8 mg g^{-1} DW in the control and enriched *Acetes* diets, respectively; however 18:1*n*-9 increased from 10.5 to 11.1 mg g^{-1} DW in newborn seahorses produced from broodstock fed the control and enriched diets, respectively.

4.3.3 Fatty acid content of newborn seahorses *Hippocampus* sp.

DHA content of newborn seahorse increased from 9.7 mg g^{-1} DW when fed unenriched *Acetes* to 11.1 mg g^{-1} DW when fed enriched *Acetes* (Table 4-3). EPA content in seahorse samples also increased from 3.9 mg g^{-1} DW when fed unenriched *Acetes* to 4.5 mg g^{-1} DW when fed the enriched *Acetes*. These corresponded to similar increases in the respective broodstock diets (Table 4-3). The content of 22:5*n*-3 increased from 0.4 to 2.1 mg g^{-1} DW in newborn seahorses produced from broodstock fed the control and enriched diets, respectively. This followed an increase in the content of 22:5*n*-3 in the broodstock diets.

Table 4-3: Fatty acid composition (mg g^{-1} DW) of *Acetes* fed DC DHA Selco enriched *Artemia* or unenriched *Artemia* and the corresponding fatty acid composition (mg g^{-1} DW) of newborn seahorse *Hippocampus* sp.[1]

	Control		Enriched	
Fatty acid	*Acetes*	Seahorses	*Acetes*	seahorses
14	0.6	2	0.8	1.7
14:1*n*-5	0	0	0	0
15	0.2	0.7	0.3	0.5
16	8.8	17.8	8.5	18.5
16:1*n*-7	1.6	2.9	1.7	2.7
17	0.7	0.8	0.6	0.8
17:1*n*-8	0	0	0	0
18	4	9.2	3.5	9
18:1*n*-9	7.9	10.5	5.8	11.1
18:1*n*-7	3.3	2.3	2.3	2.5
18:2*n*-6	2.3	1.8	1.7	2.1
19	0	0	0	0
18:3*n*-3	7.8	1.9	4.5	2.1
18:4*n*-3	0.6	0	0.4	0
20	0	0	0	0
20:1*n*-11	0	0	0	0
20:1*n*-9	0.2	0	0.3	0
20:1*n*-7	0	0	0	0
20:2*n*-6	0.2	0	0.2	0
20:3*n*-6	0	0	0	0
20:4*n*-6	2	3.7	1.9	3.9
20:3*n*-3	0.3	0	0.2	0
20:4*n*-3	0	0	0	0
20:5*n*-3	5.1	3.9	6	4.5
22	0.3	0	0.3	0
22:1*n*-11	0	0	0	0
22:1*n*-9	0	0	0	0
22:1*n*-7	0	0	0	0
22:1*n*-6	0	0	0	0
22:1*n*-3	0	0	0	0
22:2*n*-6	0	0	0	0
22:3*n*-6	0	0	0	0
22:4*n*-6	0	0	0	0
22:3*n*-3	0	0	0	0
22:5*n*-6	0	0	0	0
24	0	0	0	0
22:5*n*-3	0	0.4	1.7	2.1
22:6*n*-3	3.9	9.7	5.5	11.1
24:1*n*-9	0	0	0	0

[1]Analyses were conducted on samples resulting from the combination of subsamples taken from replicate clutches.

4.4 Discussion

Little information exists on the lipid contents of *Acetes*; however, previous studies have determined total lipid content to be about 16.5% of dry body weight in *Acetes* spp. (Nair *et al.,* 1975); 27.9% (Gopalakrishnan *et al.,* 1977) and 12.37% (Madhupratap *et al.,* 1979) in *A. japonicus*; and 5% (Chen *et al.,* 1965), 4-6% (Chung and Lee, 1976), and 19.9-27.9% (Su and Xiao, 1989) in *A. chinensis.* These variations have been attributed to seasonal and latitudinal differences and differences in lipid determination method (Xiao and Greenwood, 1993). Proximate analysis was not performed in the current study. As such, comparisons could not be made between *A. sibogae* in the current study and results from previous studies.

An optimal level of (*n*-3) / (*n*-6) HUFA in broodstock diets and in marine fish eggs has been suggested to be 5:1-10:1 (Sargent, 1995). *Acetes* examined in this study contained a (*n*-3) / (*n*-6) HUFA ratio of approximately 7:1 in the enriched treatment and approximately 5:1 in the unenriched treatments. Based on the criteria of Sargent (1995) these data indicate that *Acetes* are a suitable broodstock diet from the viewpoint of fatty acid nutrition and that dietary quality of *Acetes* can be effectively increased through the method of diet enrichment used in the study.

Year-round breeding of seahorses is possible by increasing water temperature and photoperiod thereby simulating an extended breeding season (Nan'er *et al.,* 1984). Previous studies have shown that in seabream (*Sparus aurata*) females, 52% of muscular lipids and 45% of adipose tissue lipids are lost during long spawning seasons; as final ovarian development in fish requires major physiological and biochemical changes which, in turn, require substantial incorporation of lipids and proteins into growing oocytes (Zohar *et al.,* 1995). The extent of muscular and adipose tissue lipid loss during spawning of seahorses is presently unknown; however

the extent of parental tissue loss of seahorses during spawning and the effects of year-round spawning may have significant implications for the culture methods used and for broodstock management.

Previous studies have shown that egg quality and the chemical composition of eggs are influenced by the nutritional quality of diets fed to broodstock before and during spawning (Leray *et al*., 1985; Watanabe *et al*., 1991; Watanabe and Kiron, 1994). The duration of the period during which broodstock must be fed on a particular diet to affect egg and larval quality is still unclear. Batch spawners such as salmonoids, with up to six months of vitellogenesis, must be fed the high quality diet several months before spawning season (Hardy *et al*., 1990). However, continuous spawners with short vitellogenic periods can be affected by dietary lipid immediately prior to spawning and during spawning (Harel *et al*., 1994). Seahorses are continuous spawners with short vitellogenic periods (Nan'er *et al*, 1984) and, in the present study, fatty acid composition and weight of newborn seahorses were affected by broodstock diet fed for one month prior and during spawning. The optimum duration of pre-feeding an improved broodstock diet to maximize reproductive performance remains unclear and requires further research.

Because of the unusually high content of DHA in neural cell membranes; this fatty acid plays a key role in the formation of the brain and eyes in developing fish larvae, which constitute a major proportion of embryonic and larval body mass (Sargent, 1995). DHA/EPA ratios were effectively increased from 0.76 in the unenriched treatment to 0.92 in the enriched treatment. Likewise, DHA and EPA levels in the newborn seahorses increased as a result of broodstock diet enrichment, demonstrating that improved fatty acid content of the enriched *Acetes* were effectively transferred from broodstock diet to the newborn seahorses. Therefore, it is likely that

increased *n*-3 content of the enriched *Acetes* diet was the main factor contributing to observed significant increase in mean wet weight of newborns from broodstock pairs fed the enriched *Acetes.*

In conclusion, the problem of varying newborn fish quality is one of the most critical constraints on current and future development of seahorse culture. This study concluded that the dietary quality of *Acetes* was improved when using HUFA enriched *Artemia* as an enrichment source for *Acetes.* The wet weight of newborn seahorses (and presumably vigour/quality) were significantly improved by the methods tested, demonstrating the importance of HUFA's in broodstock *Hippocampus* sp. diet.

CHAPTER 5

EFFECT OF INCREASED AMBIENT CALCIUM LEVELS ON GROWTH AND SURVIVAL OF NEWBORN SEAHORSE *Hippocampus* SP.

5.1 Introduction

5.1.1 Calcium requirements of fish

In most fish, bones and scales derive their sturdiness from deposited calcium phosphate materials (Flik and Verbost, 1993) and during growth, fish must accumulate calcium in their body. Calcium uptake in fish occurs mostly through ion-transporting cells, or 'chloride cells', also known as ionocytes (Herrmann-Erlee and Flik, 1989). These ionocytes are primarily located in the gills and less extensively in skin areas covering the inner operculum. Other sources of calcium uptake may be through the intestine by drinking water and from food.

Research has demonstrated the beneficial effects of increased environmental calcium in alleviating mortality in striped bass and its white hybrids (Grizzle *et al*., 1985; Weirich *et al*., 1992). Environmental calcium also been shown to decrease the toxicity of nitrogenous compounds to sunshine bass (Weirich *et al*., 1993). The stress relieving benefits of environmental calcium has been demonstrated in these studies; however, other studies have indicated that increased environmental calcium has no effect on Sunshine bass reared under normal conditions (Seals *et al*., 1994).

5.1.2 Calcium requirements of seahorses

Ambient calcium levels may have an important role in the growth and development of newborn seahorses. Past research reported that the calcium concentration of the incubating pouch fluid in the male seahorse, *Hippocampus erectus,* decreased as incubation of the embryos progressed; radioactive calcium was also taken up by embryos in the pouch (Linton and Soloff, 1964). These results indicate that calcium may be an essential element for newborn seahorses; however, the role of calcium and its effect on growth and survival of newborn seahorses is poorly understood.

Due to the bony nature of the seahorse and the relatively high amount of calcareous material found in seahorse bodies (compared with most fish), it is possible that seahorses require increased uptake of calcium for optimal growth. This study examines the effects of increased ambient calcium levels on the growth and survival of juvenile seahorse *Hippocampus* sp.

5.2 Materials and Methods

5.2.1 Seahorse *Hippocampus* sp. culture conditions

Newborn seahorses were obtained from captive bred parental stock held in the system described in Section 2.2.1. Juvenile seahorses from a single clutch were reared for 7 days using techniques described in Section 2.2.2 and transferred to the James Cook University Aquaculture facility. Seahorses were acclimated to the new system and respective treatments over a 24-hour period. The system consisted of 12 plastic tanks (10 L each) supplied with air-lift sponge filtration containing 20 seahorses each. The bottom of each tank was siphoned daily to remove detritus and 25 % water changes were conducted once weekly with respective calcium treatments added to new water. Salinity and water temperature in aquaria were maintained at 35 ppt, 24-26^{o}C and pH

8. Mean oxygen saturation was maintained above 90%. Light intensity was not measured but was kept at a low level. Seahorses were fed DHA Selco (INVE Aquaculture, Belgium) enriched *Artemia* (Prime *Artemia,* Great Salt Lakes, USA).

5.2.2 Method for the determination of calcium in seawater

Calcium concentrations in seawater were measured using an ICP system consisting of the Liberty Series II ICPAES with SPS-5 autosampler and diluter, controlled by a Compaq 386/20e computer. A V-groove nebulizer was also used. Power, plasma and auxillary flows were optimized and programmed to obtain high intensity while minimizing salt build up in the torch. Standard one piece, low flow torch was used with the Sturman-Masters spray chamber fitted with a V-Groove nebulizer. Dynamic background correction was used for the analysis with sulfur being run under vacuum and purged conditions. Photomultiplier tube voltage was lowered for sensitive lines produced by Ca.

Standards were prepared from 1000 and 10000 mg L^{-1} ICP standards purchased from Spex and Plasma Chem. The chloride standard was made from dry, reagent grade sodium chloride. In order to optimize accuracy and precision for analysis, various dilutions were applied to the samples using a 1:10 dilution with ASTM Type I Water. It was necessary to dilute the samples for the analysis of the major elements to overcome problems of ionization found in the samples. All samples were performed in triplicate. Results are reported in means of determinations. ICP system parameters are listed in Appendix 1.

5.2.3 Preparation of calcium supplemented seawater

Raw seawater was collected from Pallarenda, Townsville on a high tide. Salinity of the water was 35 ppt and temperature was 28^{o}C at the time of collection. Water was stored in a 1000 L holding tank, which was moderately aerated. Calcium content of the raw seawater (Control treatment) was determined to be 432.67 ± 13.44 ppm. Three treatments (Low, Medium, and High Calcium) were prepared from the collected seawater through addition of reagent grade calcium chloride (Aquasonic, NSW). Calcium concentrations were measured as: 489 ± 15.43 ppm, 520.83 ± 11.62 ppm, and 583 ± 10.21 ppm in the Low, Medium, and High treatments, respectively. Alkalinity in all treatments was maintained at 140.66 ± 5.23 mg $CaCO_3$ L^{-1}.

5.2.4 Experimental design, data collection and analysis

The experiment was arranged in a randomized block design with three independent replicate tanks assigned to each calcium treatment. After a period of 30 days, remaining seahorses in all treatments were counted (mean % survival ± SE) and weighed (mean dry weight 0.001 g accuracy). Tanks were considered as independent observations (n=3 for each treatment). Levene's test was used to assess equality of variances of survival and dry weight (Appendix 5.1, 5.4). If variances were equal, one-way ANOVA (95% confidence interval) was used to determine overall differences in weight and survival (Zar, 1984). All data were analyzed using SPSS 8.0 student version.

5.3 Results

During the 30 day period of exposure to elevated calcium levels, final mean (±SE) dry weights of juvenile seahorses in the treatments ranged from 10.67 ± 0.53 to 11.09 ± 0.82 mg (Table 5-1). ANOVA could not be used as dry weight variances between the groups were unequal (Appendix 5.3). Final mean (± SE) survival ranged from 56.67 ± 12.03 to 66.67 ± 12.03 % (Table 5-1). No significant differences in survival were observed between treatments (Appendix 5.2).

Table 5-1: Final mean dry weight (mg) and mean (± SE) survival (%) of juvenile seahorse *Hippocampus* sp. subjected to various levels of environmental calcium.[1]

Calcium Treatment (ppm)	Survival (%)	Dry Weight
433	66.67 ± 3.34	11.09 ± 0.82
489	56.67 ± 12.03	11.03 ± 1.74
521	66.67 ± 12.03	10.91 ± 0.39
583	60 ± 20.84	10.67 ± 0.53

[1] Means within a column sharing a common superscript are not significantly different ($P \leq 0.05$)

5.4 Discussion

No significant differences in growth or survival of 30 day old juvenile *Hippocampus* sp. were observed due to increased ambient calcium levels. This result indicates that all treatments used in the current study were above the required level and demonstrated that the lowest calcium treatment of 433 ppm was adequate for normal growth and survival of *Hippocampus* sp. in culture. Studies on sunshine bass (female white bass *Morone chrysops* x male striped bass *M. saxatilis*) have shown in non-stressful conditions, environmental calcium does not affect growth, food conversion, condition factor, hematocrit, plasma osmolality, or plasma calcium concentrations

(Seals *et al*., 1994). This study concluded that elevated calcium levels offered no advantages during non-stressful periods, agreeing with previous studies on sunshine bass (Seals *et al*., 1994).

Environmental calcium has been shown to help alleviate stress induced mortality in striped bass and its white hybrids (Grizzle *et al*., 1985, 1995; Weirich *et al*., 1993) and has been shown to decrease the toxicity of nitrogenous compounds to sunshine bass (Weirich *et al*., 1993). In addition, increased calcium concentration has been shown to benefit survival of juvenile channel catfish (*Ictalurus punctatus*) exposed to toxic concentrations of copper sulfate (Wurts and Perschbacher, 1994). Juvenile seahorses in the current experiment were reared under 'non-stressful' conditions using rearing protocols successful in previous studies (see Chapter 3). Further research may demonstrate the benefits of elevated calcium levels on the growth and survival of juvenile seahorses reared in stressful conditions such as elevated nitrogenous compound levels. The results of this further research may have direct implications on low-technology culture of seahorses in countries such as the Philippines and Vietnam where high-capital filtration devices, required to maintain high water quality, are not available. Future studies may focus on the effects of elevated calcium levels on the toxicity of ammonia and nitrite.

Previous studies have shown for the Atlantic cod, *Gadus morhua*, that intestinal calcium absorption increases significantly in pre-spawning fish (Sundell and Bjornsson, 1988), which require vast amounts of calcium for the development of the gonads. However, the relative importance of ambient calcium, the role of dietary calcium and the mechanisms and factors influencing absorption are poorly understood for many cultured species of fish, including seahorses. Further studies may use calcium isotopes as radio labels to track the preferential utilization of calcium.

Previous studies have also suggested that the absence of an observed effect of environmental calcium on growth or survival may be attributed to fish diets containing adequate levels of calcium (Seals *et al*., 1994). Weirich *et al*. (1992; 1993) suggested that when dietary calcium is adequate, ambient calcium plays an ameliorative role during stress. Several studies have also suggested that the major role of environmental calcium, in cases of sufficient dietary calcium, is to reduce flux across the gill membranes and not to provide calcium by the active transport system present in the gills (Flik *et al*., 1985; Ishihara and Mugiya, 1987; Flik and Perry, 1989). It is also possible that lack of response to varying ambient calcium levels by juvenile seahorses in this study resulted from an inappropriate range of calcium levels tested (ie at or above 'normal' seawater levels). In conclusion, this study indicated that there are no apparent growth or survival advantages in increasing calcium for rearing juvenile seahorses *Hippocampus* sp. Future studies may investigate the effects of decreased ambient calcium levels on growth and survival of seahorses to find minimum requirements.

CHAPTER 6

COMBINED EFFECTS OF TEMPERATURE AND SALINITY ON SURVIVAL AND GROWTH OF JUVENILE SEAHORSES, *Hippocampus* SP.

6.1 Introduction

6.1.1 The physiological effects of temperature and salinity on fish

Temperature and salinity are two of the most pervasive physical factors affecting the life of marine fish (Kinne, 1960; Reynolds and Casterlin, 1980; Watanabe *et al*., 1993). Previous studies have demonstrated that temperature and salinity should be considered together when measuring their biological effects, as temperature modifies the effects of salinity by enlarging, narrowing, or shifting the salinity tolerance range of an organism, just as salinity acts in a similar manner affecting temperature tolerance (Kinne, 1960; Kinne, 1963; Watanabe *et al*., 1993).

Fishes are poikilotherms, and therefore exhibit body temperatures similar to their surroundings. This decisively affects rates of metabolism, activity and therefore, growth and reproduction. Low temperatures may cause insufficient integration of nervous and metabolic process, insufficient rates of energy liberation and changes in water and mineral balance. Extreme high temperatures cause insufficient supply of oxygen, failure in process integration, water loss by evaporation, enzyme and lipid changes, and increased permeability of membranes.

6.1.2 Background of current species and previous studies

Hippocampus sp. are found inhabiting inshore waters, estuaries and mangrove areas in Townsville, Queensland. Due to the tidal nature of these areas, highly variable salinities may be encountered during heavy monsoonal rainfall where salinity may drop to 25 ppt (Hopley, 1982). It is therefore difficult to determine optimal temperature and salinity from information relating to natural habitats. Subsequently, little is known about the temperature nor salinity tolerance of this species despite the importance of these factors. Water temperature has been found to be an important factor affecting the reproductive function of seahorse, *H. trimaculatus*, broodstock (Nan'er, 1984) and can affect newborn seahorse *H. capensis*, length at birth (Lockyear *et al*., 1997). However, little is known about the effects of temperature and salinity on growth and survival of juvenile seahorses. This represents a large gap in fundamental information required for successful aquaculture of seahorses.

6.1.3 Experimental aim

Understanding the biological effects of salinity and temperature is fundamental to the development of more appropriate culture techniques for seahorses. In this study, the combined effects of temperature and salinity on growth and survival of juvenile *Hippocampus* sp. were investigated. To achieve this, the experiment was set up as a 4 x 4 factorial design with the aim of determining an optimal temperature and salinity range to maximize growth and survival of juvenile *Hippocampus* sp. in culture.

6.2 Materials and Methods

6.2.1 Broodstock spawning and newborn rearing conditions

Adult broodstock seahorses were maintained using the system and conditions described in section 2.2.1. Upon pregnancy, male seahorses, *Hippocampus* sp., were transferred to a 100 L circular hatching tank, supplied with airlift sponge filtration, to give birth. Water quality in the hatching tank was identical to water quality in the broodstock system described in section 2.2.1. Daily bottom siphoning to remove detritus and 10% weekly water changes were conducted. Enriched *Acetes* (Chapter 4) were fed to gravid males in the incubation tank and later removed (with the males) after birth. Incubation time was 17 days. Newborn seahorses were reared in the hatching tank for three days prior to the start of the experiment following methods described in Section 2.2.2.

6.2.2 Experimental design and materials used

A 4 x 4 factorial experimental design was used to test the affect of four temperatures (20^{o}C, 23^{o}C, 25^{o}C and 29^{o}C) and four salinities of (20 ppt, 25 ppt, 30 ppt and 35 ppt) to determine their effects on growth and survival of juvenile *Hippocampus* sp. Four temperature baths were used, which were regulated by heating against lower ambient air temperature. Salinity was regulated in the experiment by mixing synthetic sea salt (Instant Ocean, Aquasonic) with filtered fresh water to achieve the desired salinities. Evaporation in buckets was controlled by topping up evaporated water with de-chlorinated fresh water on a daily basis. Three replicate tanks for each salinity were placed randomly within the 4 temperature baths, creating the 4 x 4 factorial design with 3 replicate tanks allocated to each treatment.

Seahorses from two clutches were mixed and randomly assigned to the 16 temperature/salinity treatments and allowed to acclimate for seven days to their respective treatments prior to data collection. Four seahorses were placed in each of the replicate buckets. Twenty-five percent water changes and bottom siphoning were conducted every 3 days on each tank. Water was mixed to the correct salinity and heated one day prior. Seahorses were fed *Artemia* enriched with DC DHA Selco twice per day, *ad libitum*. Mortalities were recorded every 3 days when water changes were conducted. Mean standard lengths (± SE) and mean dry weights (± SE) were measured at 28-days post-birth.

6.2.3 Statistical methods

The factorial design of this experiment allows for greater precision in investigating interactions between different factors and also allows interpolation of interactions at intermediate levels of the factors being tested (Lough and Gonor, 1973). The effects of both temperature and salinity on growth and survival were compared by a general linear model (GLM) 2-way ANOVA (Appendices 6.2; 6.5; 6.8). Response surface estimation of survival and standard length were plotted. Data normality was assessed using residual plots (Appendices 6.4; 6.7). Assumption of equal variances was tested using Levene's test (Appendices 6.1; 6.3; 6.6). If variances were not homogenous, confidence limits were set to 99% for all ANOVAs. Multiple comparisons among means were made using Tukey's HSD test at the 99% confidence interval.

6.3 Results

6.3.1 Survival of juvenile seahorses *Hippocampus* sp., reared under different temperature/salinity regimes

Seahorses in the 20 ^{o}C treatment experienced 100% mortality within one week and were excluded from the analysis. In the remaining three temperature treatments, percent survival ranged from 33.33 ± 8.31 to 91.67 ± 8.31%. Survival was generally lower in the two higher salinity treatments (30 ppt and 35 ppt) for all temperatures tested (Fig. 6-1) and highest in the 20 ppt and 25 ppt salinity treatments. Highly significant effects of salinity on survival were detected ($P < 0.001$) while the interaction of temperature and salinity did not significantly effect survival ($P > 0.01$). Survival and standard length were slightly influenced by the range of temperatures used in the experiment ($P = 0.04$).

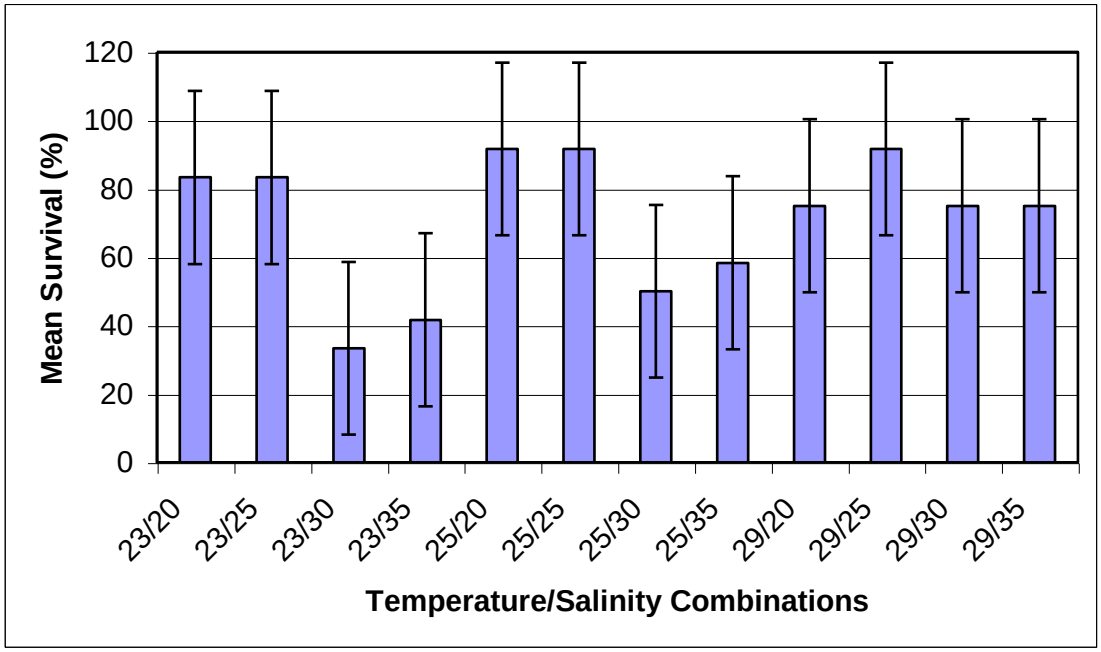


Figure 6-1: Mean (± SE) percent survival of juvenile seahorse *Hippocampus* sp. reared for 28 days under different temperature and salinity combinations.

Response surface estimation of survival (Fig 6-2) showed that survival was highest at the lowest salinity (20 ppt) and middle temperature ranges tested (25-26^{o}C). Optimal temperature salinity / temperature combination shown in surface contour diagrams for maximum survival appears to be in salinities below 20 ppt, however this range of salinity was not tested.

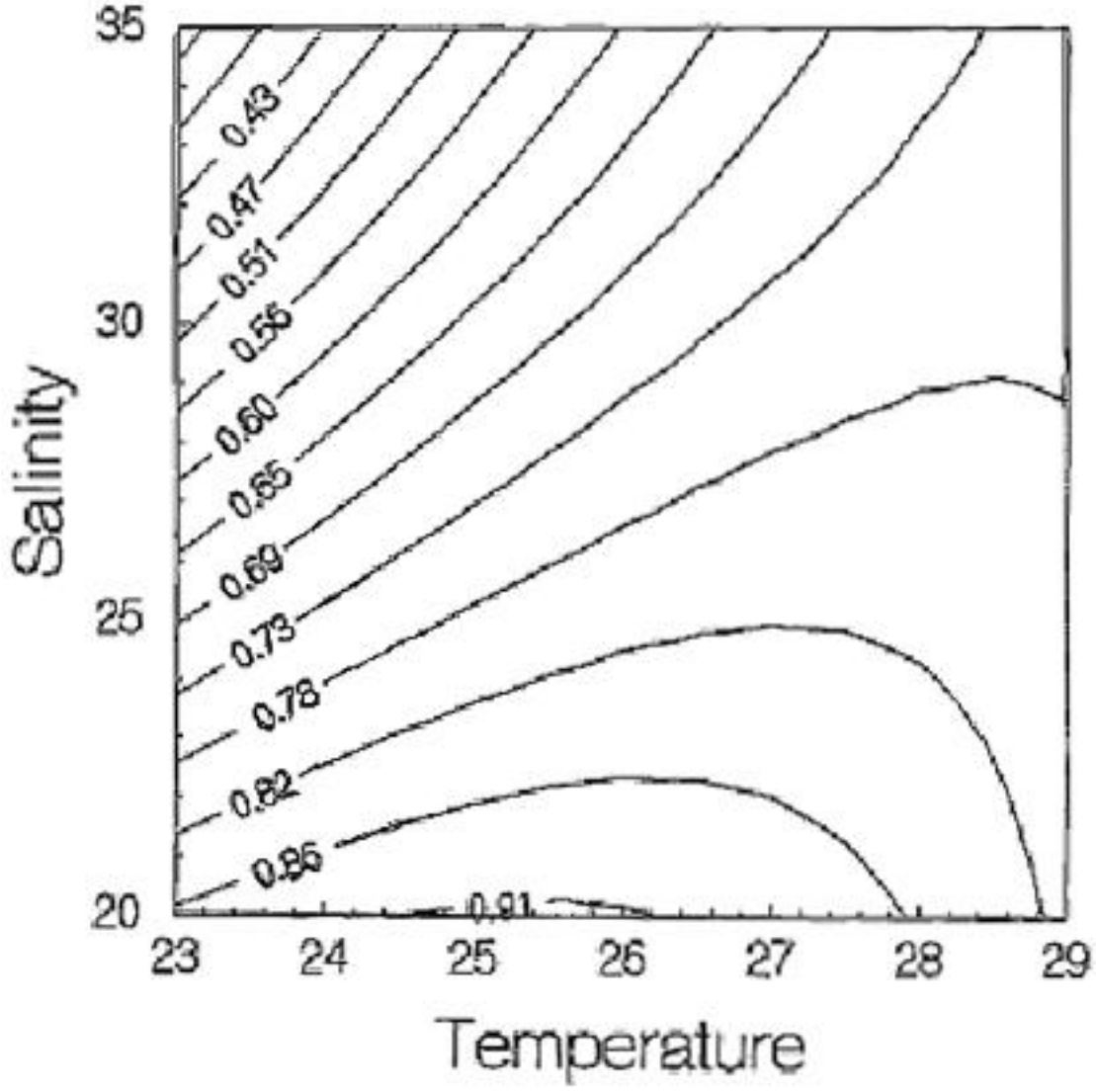


Figure 6-2: Response surface estimation of survival (0-1.0) of *Hippocampus* sp. juveniles reared for 28 days under different combinations of temperature and salinity.

6.3.2 Growth of juvenile seahorse *Hippocampus* sp. reared under different temperature/salinity regimes

Final mean dry weight ranged from 5.3 ± 0.76 mg to 15 ± 0.39 mg among treatments (Fig. 6-3) and were noticeably lower in all 23^{o}C treatments. Highly significant effects of temperature ($P < 0.001$) on dry weight were observed, with a general trend of increasing DW with increasing water temperature. Significant interactions were also observed between temperature/salinity combinations and dry weight ($P = 0.012$); however, salinity did not significantly influence dry weight ($P > 0.05$).

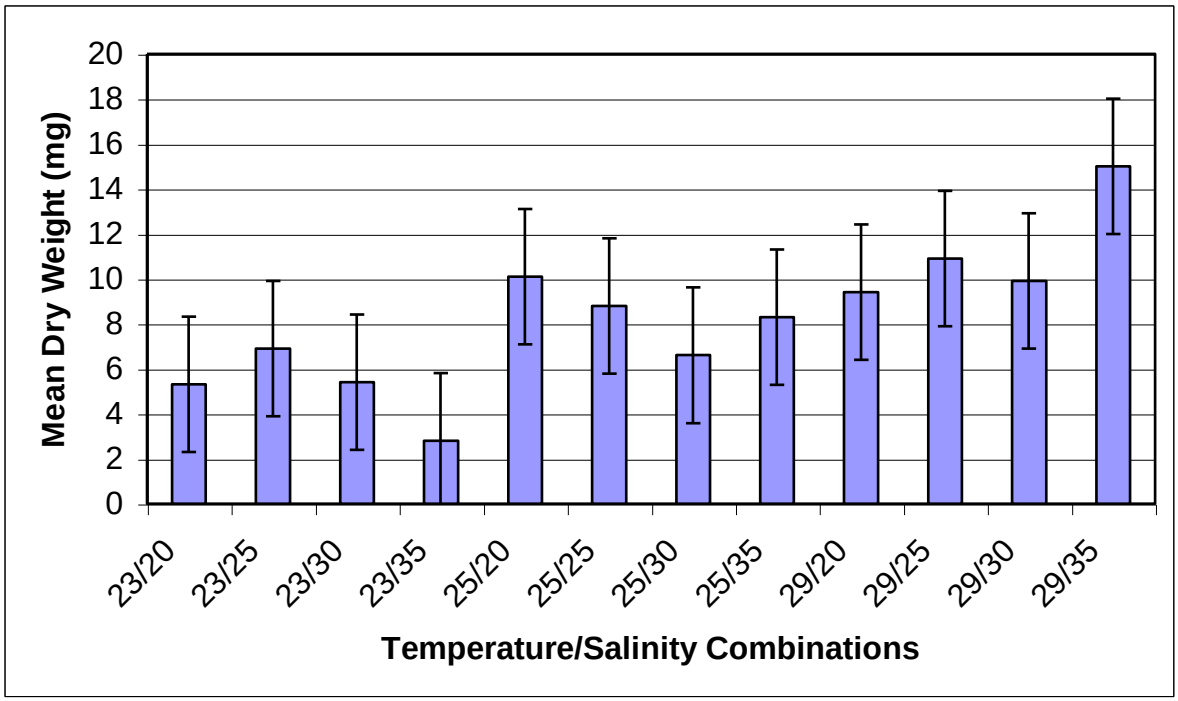


Figure 6-3: Final mean (±SE) dry weights (mg g^{-1} DW) of seahorse *Hippocampus* sp. juveniles reared for 28 days under different temperature and salinity combinations.

Final mean standard lengths ranged from 18.36mm ± 0.68 to 31.67mm ± 0.33 among treatments and were generally highest at 29 ^{o}C (Fig. 6-4). No significant effects of salinity on standard length ($P > 0.05$) were observed; however highly significant effects of temperature ($P < 0.001$) and the interaction of temperature and salinity ($P < 0.01$) on standard length were observed.

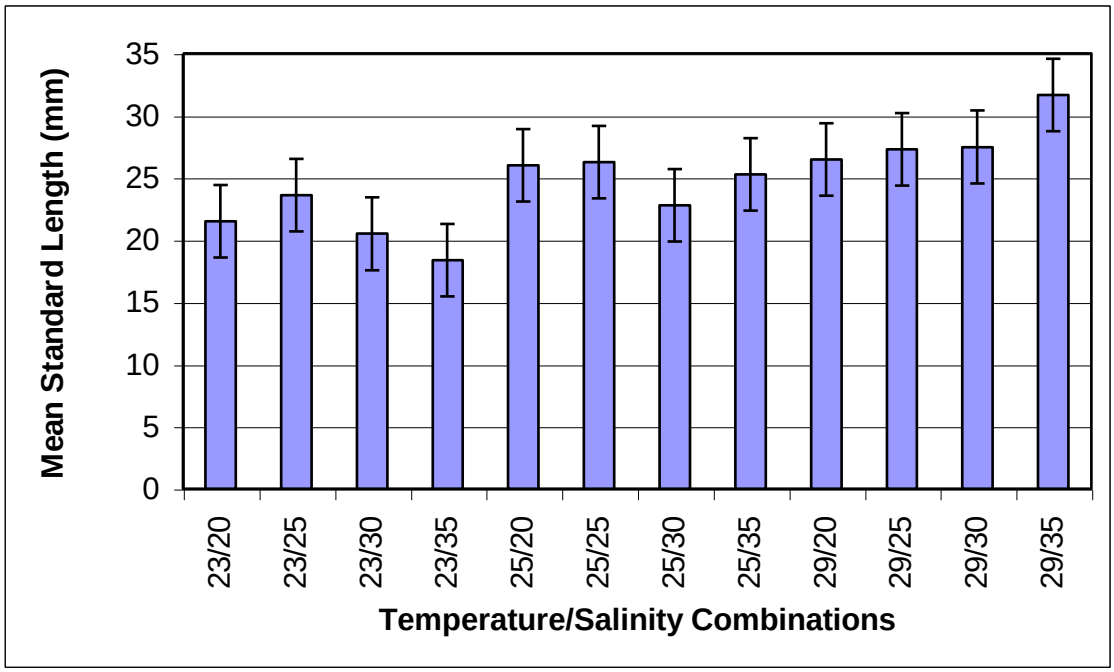


Figure 6-4: Mean (±SE) standard length (mm) and of seahorse *Hippocampus* sp. juveniles reared for 28 days under different temperature and salinity combinations.

Response surface estimation of standard lengths (Fig. 6-5) showed that mean lengths were highest at the upper temperature/salinity ranges used in the experiment.

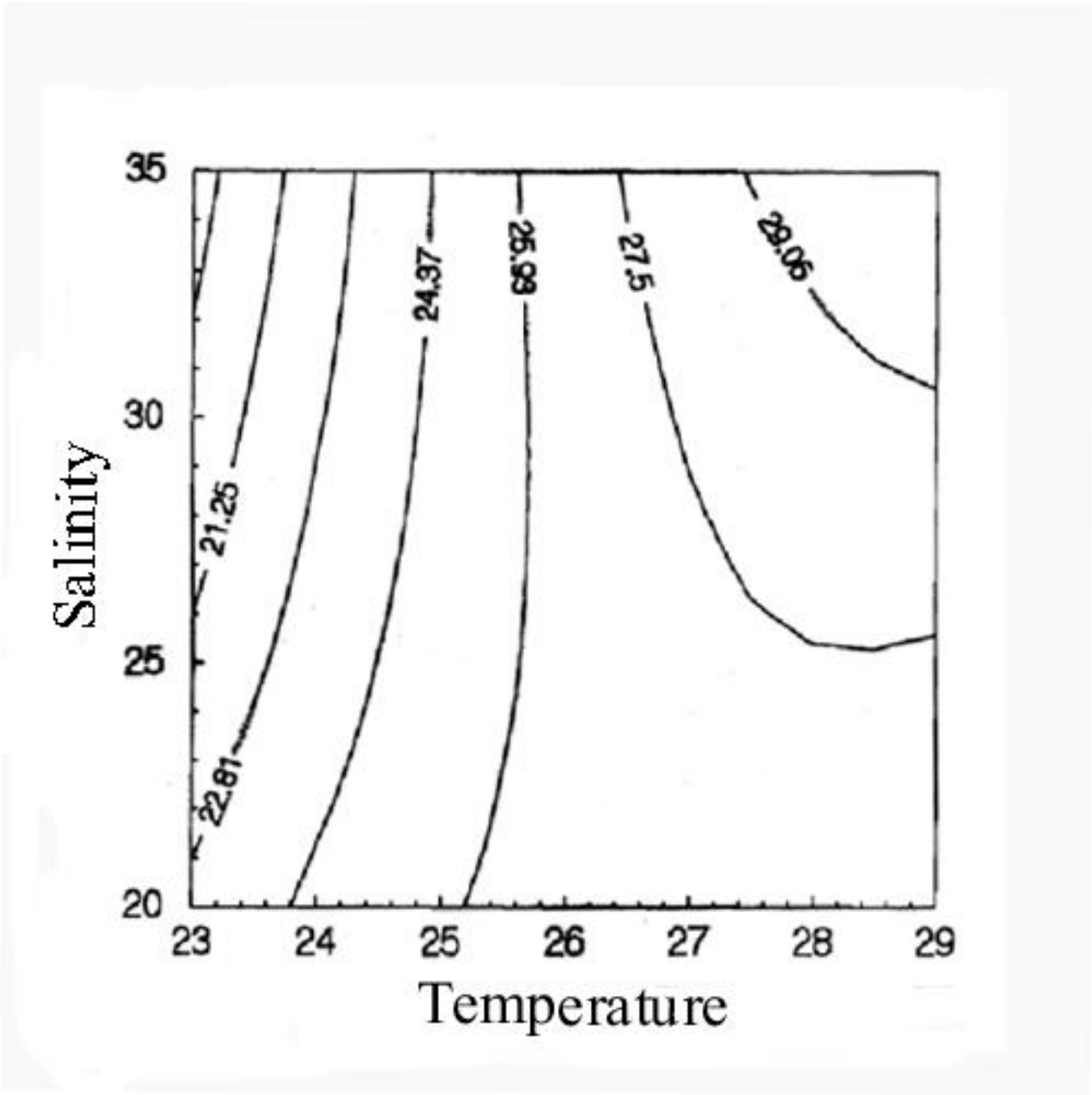


Figure 6-5: Response surface estimation of standard length (mm) of *Hippocampus* sp. juveniles reared for 28 days under different temperature and salinity combinations.

6.4 Discussion

Seahorses in the 20 ^{o}C treatment experienced near 100% mortality within the first week of the experiment indicating a lower temperature threshold in the range of 20-23 ^{o}C for juvenile *Hippocampus* sp. A maximum temperature threshold was not

determined within the temperature ranges tested, as no reduction in survival or growth were observed at the highest temperature tested of 29^{o}C. In general, mean standard length and dry weight of seahorses after 28 days increased with increasing temperature. This may be attributed to a number of physiological factors affected by increasing temperature in aquatic environments. Temperature has been found to influence the metabolism and activity of fish through factors such as: (1) affecting enzyme rates of reaction (Somerno, 1978); (2) changes in respiratory function (Crawshaw, 1979); (3) changes in blood pH correlated with maintenance of cellular metabolism (Crawshaw, 1977); (4) affecting various structural proteins (Vitvitskii, 1977) and lipid-membrane structures (Cossins and Prosser, 1978); and (5) changes in metabolic rate and gas exchange (Reynolds, 1980). Although increased growth at high temperatures for *Hippocampus* sp. could not be correlated with any specific physiological factor, it is likely that most of these factors were related to the observed increase in final standard length and dry weight of juvenile seahorses.

The optimum temperature for growth of fish has also been found to decrease if feed is restricted due to the subsequent increase in metabolic energy demands which therefore require higher feeding rates (Brett *et al.,* 1982; Reynolds and Casterlin, 1980; Kinne, 1963). In the present study, seahorses were fed *ad libitum* and the relationship between feeding rates and temperature was not measured. It was assumed that final standard length and dry weight were not influenced by food availability. However, the relation between optimum temperature and food availability requires further study as commercial-scale operations may have logistical or economical problems with feeding animals *ad libitum*.

Previous studies on the desert pup-fish, *Cyprinodon macularius,* have shown salinity to affect metabolic rate during early ontogeny (Kinne, 1964). This study

found that the narrowest range of salinity tolerance was confined to the period of embryonic development and that hatched young were able to tolerate wider salinity ranges and more pronounced salinity fluctuations than adults. Early ontogeny and development in seahorses occurs in the broodpouch of the male, which has been shown to have a significant role in osmoregulation of developing young (Linton & Soloff, 1964). The broodpouch fluid has been reported to be isomotic to the blood of the parent, however hyposmotic to environmental salinity. The conclusion was drawn that the broodpouch fluid served as an osmotic buffer for developing eggs and embryos, during the stage at which fish are most vulnerable to changes in salinity. Therefore, it was concluded that live bearing or viviparous fish are better suited to estuarine conditions for this reason (Quast and Howe, 1980).

In the current study, salinity did not significantly affect growth and presumably metabolic rate of *Hippocampus* sp. during the 28-day period of experimentation. However, juvenile seahorses showed significantly higher survival at lower salinities of 20 ppt and 25 ppt, when compared to the higher salinities of 30 ppt and 35 ppt. Lower survival in high salinity treatments may have been influenced by a decrease in temperature tolerance as previous studies have shown temperature tolerance in fish may be modified by manipulating salinity in culture (Peters and Boyd, 1972) due to the interactive effects in osmoregulation (Tilney and Hocutt, 1987).

Previous studies have found that, in general, the range of salinity tolerated is usually greater in animals living in estuarine areas (Kinne, 1964). The study of Kinne (1964) also found salinity tolerance in fish may be different at different ages, life cycle stages and between sexes (Kinne, 1964). Although significant effects of salinity on survival of *Hippocampus* sp. were observed in the current study, the physiological

role of salinity in growth, survival and reproduction of this species is presently unclear and requires further research.

This study found a general trend of high temperature and high salinity and low temperature and low salinity to be the most beneficial for growth. Previous studies on other juvenile euryhaline and eurythermal fish such as *C. macularius* (Kinne, 1960) and Florida red Tilapia (Watanabe et al., 1993) have supported this observation with similar results: at lower temperatures, juvenile growth was fastest in fresh water, but at higher temperatures, growth of juveniles was fastest in the high salinity treatments. The reasons for this observed trend have been attributed to the interactive effects of osmoregulation and temperature (Tilney and Hocutt, 1987).

The temperature/salinity combination of 29 ^{o}C and 35 ppt resulted in the highest overall growth of *Hippocampus* sp. in the present study. From these results it may be recommended that this was the optimum combination tested during the experiment; however, previous studies on *C. macularius* (Kinne, 1960) and the guppy, *Lebistes reticulatus* (Gibson and Hirst, 1955), demonstrated that differences in growth rate established in young fish do not necessarily persist through life. Initially, slow growing fish receiving colder water treatments were found to surpass initially fast growing fish receiving warmer water treatments and reached a significantly greater final length and age. These studies concluded that optimum temperature shifts downward with age due to a high metabolic rate and lowered efficiency of food conversion at high temperatures. Although high temperature significantly increased growth for *Hippocampus* sp., further long-term research is required to determine if temperature/salinity for maximum growth rates in *Hippocampus* sp. varies with age with as previous studies have suggested for other fish.

CHAPTER 7

CONCLUSIONS

Growing interest in the aquaculture of seahorses to supply the medicinal and aquarium trades has prompted research towards the improvement of culture techniques to increase survival, growth and reproductive performance. Through the study of dietary and water quality requirements of *Hippocampus* sp., this thesis presents important steps towards improvement of culture techniques and viability of *Hippocampus* sp. as an aquaculture species. Prior to this study, very little scientifically rigerous information existed on culture requirements of seahorses. As such, this study has significantly improved knowledge of the culture requirements of seahorses. Specifically, it addressed two of the major areas requiring development: (1) broodstock husbandry/nutrition; and (2) culture requirements of juveniles.

Chapters 3 and 4 illustrated the various uses of enriched *Artemia* nauplii as a food source for newborn-juvenile *Hippocampus* sp. and as a means of enriching live feeds (*Acetes*) for broodstock. These chapters demonstrated the importance of dietary fatty acid content in broodstock and newborn/juvenile seahorse diets. For these purposes, DC DHA Selco proved to be an effective enrichment medium and, from the results of this study, it may be recommended that *Artemia* used in seahorse culture be enriched with DC DHA Selco.

Recent declines in wild harvests of *Artemia* cysts, presumably caused by global weather changes and overharvesting, have increased prices and decreased the quality of available *Artemia* cyts (Lavens and Sorgeloos, 2000). It is a future

possibility that *Artemia* may not be economically viable for use in large-scale seahorse culture operations. Future availability, pricing, the quality of *Artemia* cysts and the development of alternative food sources for juvenile seahorses, such as copepods, will determine the role of *Artemia* in seahorse aquaculture.

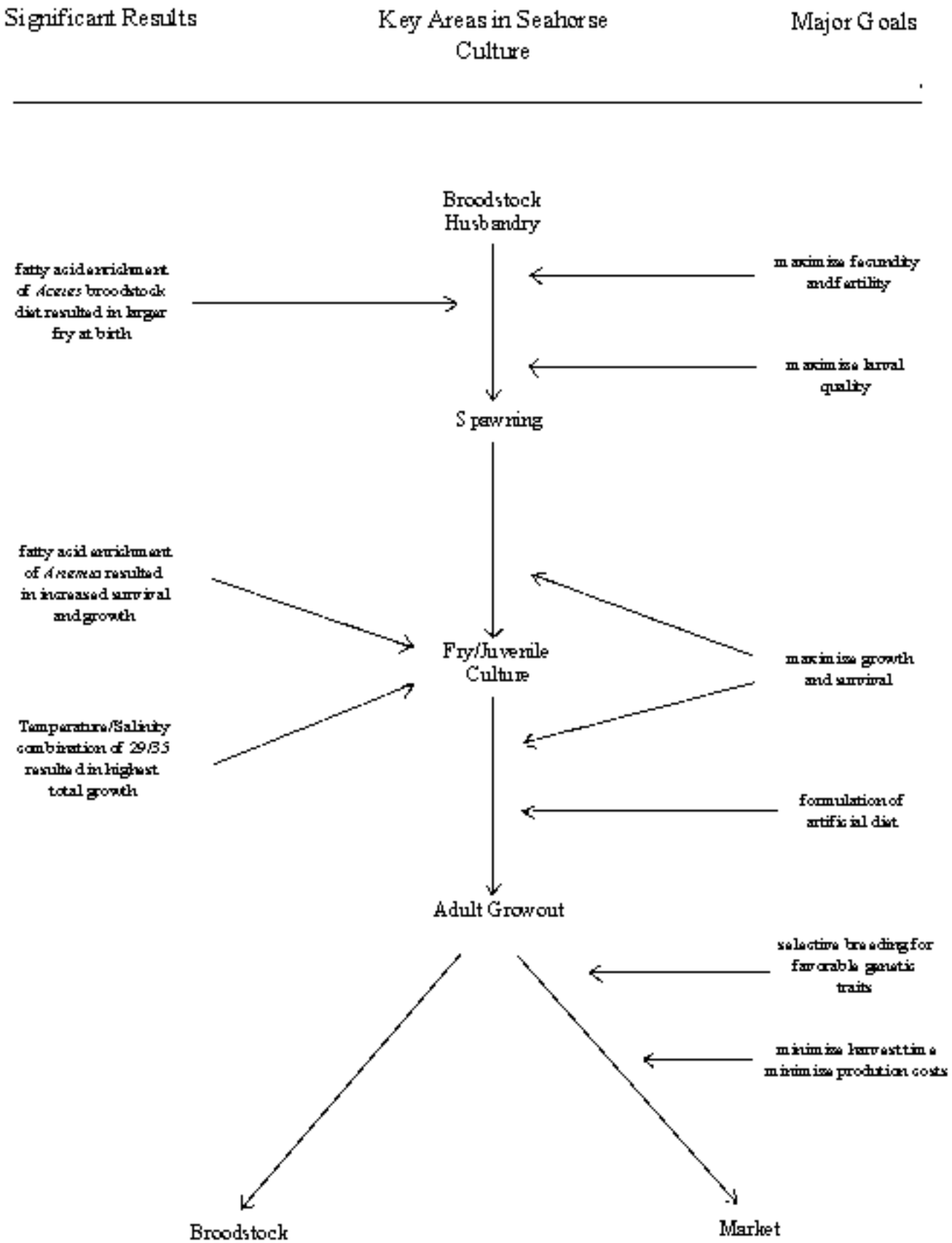
The formulation of an artificial diet for seahorses will reduce much of the labor and capital intensive live feed technology, currently required for seahorse aquaculture. The results of this study have direct implications for the formulation of an artificial diet for seahorses. From the results of the experiments presented, it may be suggested that *n*-3 HUFAs are one of the primary factors to be considered when assessing the quality of diets used for spawning and rearing seahorses. It may be proposed that a higher proportion *n*-3 to *n*-6 HUFAs and a higher proportion of DHA to EPA, be used when formulating a diet for *Hippocampus* sp. juveniles and broodstock. Successful (high growth and survival) HUFA profiles determined in this study may be used as a fatty acid model for future aritificial diet formulations for juvenile and broodstock *Hippocampus* sp.

The current study has also demonstrated the important role of temperature and salinity as two interacting environmental factors affecting growth and survival of juvenile seahorses. Previous studies have indicated a direct relationship between dietary requirements and water temperature (Watanabe, 1993; Kinne, 1964). Prior studies have shown that, in general, metabolism increases with increasing temperature and that the efficiency of metabolism is also affected. In addition, attainment of a larger final size at low temperatures was correlated with reduced metabolic rates and postponement of sexual maturity (Kinne, 1963). From these studies, it appears that age and diet are two of the most influential biological factors which affect the water temperature and salinity requirements of marine organisms and that these

requirements change as a function of age and diet. Future research should investigate these relationships and how they affect growth and survival of seahorses; the results will have a significant implication on improving culture techniques for seahorse culture.

In conclusion, the current research has provided a benchmark for future research on environmental and dietary requirements of seahorses. The current study has identified several key factors, which may improve culture techniques for this species and others like it (Fig 7-1). It is apparent that future studies must investigate combined biological and physical factors, which have an influence on each other as biological and environmental effects are seldom singular. Continued research may indicate the most critical of these factors and how they affect growth and reproduction of seahorses. The data generated in this study will allow further improvement of culture techniques and increased success and sustainability of seahorse culture.

Fig 7-1: Significant results of this study and how they contribute to the major goals and key areas of seahorse culture



List of References:

Bardach, J.E., Rhyter, J.H. and McLarney, 1972. *Aquaculture: the farming and husbandry of freshwater and marine animals*. Wiley-Interscience, New York, USA,868pp.

Barnabe, G. (ed.), 1994. Aquaculture: biology and ecology of cultured species. Ellis Horwood Ltd., Hertfordshire.

Bell, M.V., Henderson, R.J. and Sargent, J.R., 1986. The role of polyunsaturated fatty acids in fish. *Comp. Biochem. Physiol*. B 83, 711-719

Bensky, D. and Gamble, A. (eds.), 1993. *Chinese Herbal Medicine: Materia Medica* (*revised edition*). Eastland Press Inc., Seattle.

Binsheng, L., 1992. Research into the culture of *Hippocampus*. J. Ocean. Univ. Quingdao, Quingdao Haiyang Daoxoe Xoebao 22, 39-44

Brett, J.R., Clarke, W.C., and Shelbourne, J.E., 1982. Experiments on thermal requirements for growth and food conversion efficiency of juvenile Chinook salmon, *Oncorhynchus tshawytscha.* Can. Tech. Rep. Fish. Aquat. Sci. No. 1127, 29pp

Chen, S., Wang, M. and Wang, Y., 1965. The chemical composition of some marine zooplanktonic Crustacea off Amoy and its vicinity. Shui-chan Xue-bao 2, 75-80, in Chinese with English abstract.

Chung, S. and Lee, E., 1976. The taste compounds of fermented *Acetes chinensis.* Bulletin of the Korean Fisheries Society 9, 79-110, in Korean with English abstract.

Cossins, A. and Prosser, C., 1978. Evolutionary adaptation of membranes to temperature. Proc. Natl. Acad. Sci. U.S.A. 75, 2040-2043

Crawshaw, L., 1977. Physiological and behavioral reactions of fishes to temperature change. J. Fish. Res. Board Can. 34, 730-734

Crawshaw, L., 1979. Effect of rapid temperature change on mean body temperature and gill ventilation in carp. Am. J. Zool. 231, 837-841

Dhert, P., Divanach, P., Kentouri, M. and Sorgeloos, P., 1998. Rearing techniques for difficult marine fish larvae. World Aquaculture, March 1998, 49-55

Flik, G. and Perry, S., 1989. Cortisol stimulates whole body calcium uptake and the branchial calcium pump in freshwater rainbow trout. Journal of Endocrinology 120, 75-82

Flik, G., Van Rijs, J. and Wendelaar Bonga, S., 1985. Evidence for high-affinity calcium ATP-ase activity and ATP driven calcium-transport in membrane preparations of the gill epithelium of the cichlid fish *Oreochromis mossambicus.* Journal of experimental biology 119, 335-347

Flik, G. and Verbost, P., 1993. Calcium transport in fish gills and intestine. J. Exp. Biol. 184, 17-29

Folch, J., Lees, H. and Standard lengthoan-Stanley G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J.Biol. Chem. 726, 497-509

Gibson, M. and Hirst, B., 1955. The effect of salinity and temperature on the pre-adult growth of guppies. Copeia 3, 241-43

Gopalakrishnan, T., Nair, K., Peter, M., Sankaranarayanan, V. and Rao, T., 1977. Major chemical constituents of some faunal components of the Cochin backwaters. Indian Journal of Marine Sciences 6, 151-153

Grizzle, M., Mauldin II, A., Young, D., and Henderson, E., 1985. Survival of juvenile striped bass (*Morone saxatilis*) and *Morone* hybrid bass (*M. chrysops x M. saxatilis*) increased by addition of calcium to soft water. Aquaculture 46, 167-171

Grizzle, J. and Mauldin, A., 1995. Effect of calcium on acute toxicity of sodium chloride to larval and newly transformed juvenile striped bass. Journal of Aquatic Animal Health 7, 298-303

Hardy, R., Matsumoto, T., Faigrieve, W. and Stickney, R., 1990. The effects of dietary lipid sources on muscle and egg fatty acid composition and reproductive performance of coho salmon (*Oncorhyncus kisutch*). In: Takeda, M., Watanabe, T. (Eds.), The current status of fish nutrition in aquaculture. Japan, Tokyo, pp 347-356

Harel, M., Tandler, A. and Kissel, G.W., 1994. The kinetics of nutrient incorporation into body tissues of gilthead seabream (*Sparus aurata*) females and the subsequent effects on egg composition and egg quality. Br. J. Nutrition 72, 45-58

Herrmann-Erlee, M. and Flik, G., 1989. Bone: Comparative studies on endocrine involvement in bone metabolism. In Vertebrate Endocrinology: Fundamentals and Biomedical Implications, Vol. 3 (ed. P.K.T. Pang and M.P. Schreibman), pp. 211-243. New York: Academic Press.

Hoang, D.H., T.S. Ky and H.T. Hoa., 1996. Feeding behavior and food of seahorses in Vietnam.

Hopley, D., 1982. The Geomorphology of the Great Barrier Reef. Quarterly Development of the Coral Reefs. John Wiley & Sons, New York.

Hubbs, C.L., 1943. Terminology of early stages of fishes. Copeia 1943 (4), 260

Ishihara, A. and Mugiya, Y., 1987. Ultrastructural evidence of calcium uptake by chloride cells in the gills of goldfish, Carassius auratus. Journal of Experimental Zoology 242, 121-129

Izquierdo, M.S., Arakawa, T., Takeuchi, T., Haroun, R. and Watanabe, T., 1992. Effect of *n*-3 HUFA levels in *Artemia* on growth of larval Japanese flounder (*Paralichthys ovilacues*). Aquaculture 105, 73-82.

Kinne, O., 1960. Growth, food intake, and food conversion in a euryplastic fish exposed to different temperatures and salinities. Physiol. Zool. 33, 288-317

Kinne, O., 1963. The effects of temperature and salinity on marine and brackish water animals. Part I- Temperature. Oceanogr. Mar. Biol. Ann. Rev. 1, 301-340

Kinne, O., 1964. The effects of temperature and salinity on marine and brackish water animals. Part II-Salinity and temperature-salinity combinations. Oceanogr. Mar. Biol. Ann. Rev. 2, 281-339

Kjorsvik, E., Mangor-Jensen, A.T., and Holmfjord, I., 1990. Egg quality in fishes. Advances in Marine Biology 26, 71-113

Kraul, S., Ako H., Brittain, K., Cantrell, R. and Nagao, T., 1993. Nutritional factors affecting stress resistance in larval mahimahi, *Coryphaena hippurus*, J. World Aquac. Soc. 24, 186-193

Lavens, P. and Sorgeloos, P., 2000. The history, present status and prospects of the availability of *Artemia* cysts for aquaculture. Aquaculture 181, 397-403

Leger, P., Bengtson, D.A., Simpson, K.L. and Sorgeloos, P., 1986. The use and nutritional value of *Artemia* as a food source, Oceanogr. Mar. Biol. Ann. Rev. 24, 521-623

Lemm, C.A. and Lemarie, D.P., 1991. Survival and growth of larval striped bass (*Morone saxatilis*) fed *Artemia* enriched with highly unsaturated fatty acids (HUFA). Aquaculture 99, 117-126

Leray, C., Nonnotte, G., Rouband, P. and Leger, C., 1985. Incidence of (*n*-3) essential fatty acid deficiency on trout reproduction progress. Reprod. Nutr. Develop. 25, 567-581

Linares, F. and Henderson, R., 1991. Incorporation of ^{14}C-labeled polyunsaturated fatty acids by juvenile turbot, *Scophthalmus maximus* (L.) in vivo. Journal of Fish Biology 38, 335- 347.

Linton, J.R. and Soloff, B.L., 1964. The physiology of the broodpouch of the male seahorse *Hippocampus erectus*. Bulletin of Marine Science of the Gulf and Carribbean 14(1), 45-61

Lockyear, J., Kaiser, H. and Hecht, T., 1997. Studies on the captive breeding of the Knysna seahorse, *Hippocampus capensis.* Aquarium Sciences and Conservation 1, 129-136

Lough R.G. and Gonor, J.J., 1973. A response-surface approach to the combined effects of temperature and salinity on survival and growth of bivalve larvae using response surface techniques. Fishery Bulletin 73, 86-94

Lourie, S.A., Vincent, A.J. and Hall, S.A., 1999. Seahorses: An Identification Guide to the World's Species and their conservation. Project Seahorse. London, UK, pp.24-25.

Luizi, F., Gara, B., Shields, R. and Bromage, N., 1999. Further description of the development of the digestive organs in Atlantic halibut (*Hippoglossus hippoglossus*) larvae, with notes on differential absorption of copepod and *Artemia* prey. Aquaculture 176, 101-116

Madhupratap, M., Venugopal, P., and Heridas, P., 1979. Biochemical studies on some tropical estuarine zooplankton species. Indian Journal of Marine Sciences 8, 155-158

Masonjones, H.D. and Lewis, S.M., 1996. Courtship behaviour in the dwarf seahorse, *Hippocampus zosterae*. Copeia 1996(3), 634-640

Mourente, G., Rodriquez A., Tocher D.R. and Sargent J.R, 1993. Effects of dietary docosahexaenoic acid (DHA: 22:6n-3) on lipid and fatty acid compositions and growth in gilt-head sea bream (*Sparus aurata* L.) larvae during first feeding. Aquaculture 112, 79-98

Mourente, G., Odriozola, J.M., 1990. Effect of broodstock diets on lipid classes and their fatty acid composition in eggs of gilthead sea bream (*Sparus aurata* L.). Fish Physiol. Biochem. 8, 93-101

Nair, V., Saraswathy, M.,, Saraladevi, K. and Gopalakrishnan, T., 1975. Biochemical composition of *Sagitta bedoti* and other zooplankton organisms from an estuarine area. Mahasagar-Bulletin of the National Institute of Oceanography 8, 109-111

Nan'er, C., Quanhan, X., Fucai, Y., and Xianhan, W., 1984. Studies on the reproduction of the seahorse *Hippocampus trimaculatus*. Studia Marina Sinica 23, 83-92

Ozkizilcik, S. and Chu, F.E., 1994. Evaluation of omega-3 fatty acid enrichment of *Artemia* nauplii as food for striped bass *Morone saxatilis* Walbaum larvae. J. World Aquac. Soc. 25, 147-154

Peters, D., and Boyd, M., 1972. The effect of temperature, salinity and availability of food on the feeding and growth of the hogchoker, *Trinectes maculatus* (Bloch and Schneider). J. Exp. Mar. Biol. Ecol. 7, 201-207

Quast, W.D. and Howe, N.R., 1980. The osmotic role of the brood pouch in the pipefish *Syngnathus scovelli.* Comp. Biochem. Physiol. 67A, 675-678

Rainuzzo, J., Reitan, K., and Olsen, Y., 1997. The significance of lipids at early stages of marine fish: a review. Aquaculture 155, 103-115

Ratafia, M., 1995. Aquaculture today, a worldwide status report. World Aquaculture 26(2), 18-24

Reynolds, W. and Casterlin, M., 1980. The role of temperature in the environmental physiology of fishes. Environmental Physiology of Fishes. Plenum Press New York, NY, pp. 497-518

Rimmer, M.A., Reed, A.W., Levitt, M.S. and Listandard Lengthe, A.T., 1994. Effects of nutritional enhancement of live food organisms on growth and survival of barramundi/sea bass *Lates calcarifer* (Block) larvae, Aquac. Fish. Manag. 25, 143-156.

Sargent, J., 1995. Origins and functions of eggs lipids: nutritional implications. In: Bromage, N. and Roberts, R. (Eds.), Broodstock Management and Egg and Larval Quality. Blackwell Science, London, pp. 353-372.

Seals, C., Kempton, C., and Tomasso, J., 1994. Environmental calcium does not affect production or selected blood plasma characteristics of Sunshine bass reared under normal culture conditions. The Progressive Fish Culturist 56, 269-272

Somerno, G., 1978. Temperature adaptation of enzymes: biological optimization through structure-function compromises. Ann. Rev. Ecol. Syst. 9, 1-29

Sorgeloos, P., Lavens, P., Leger, P., Tackaert, W. and Versichele, D., 1986. Manual for the culture and use of *Artemia* in aquaculture. State University of Ghent, Belgium-Faculty of Agriculture. pp. 319

Southgate, P.C. and Kavanagh, K., 1999. The effect of dietary *n*-3 highly unsaturated fatty acids on growth, survival and biochemical composition of the coral reef damselfish *Acanthochromis polyacanthus*. Aquat. Living Resour. 12, 31-36

Strawn, K. 1958. Life history of the pigmy seahorse, *Hippocampus zosterae* Jordan and Gilbert, at Cedar Key, Florida. Copeia 1958, 16-22

Su, Y. and Xiao, J., 1989. Biochemical composition of some marine planktonic crustaceans. Journal of Oceanography in Taiwan Strait 8, 132-139, in Chinese with English abstract.

Sundell, K. and Bjornsson, B., 1988. Kinetics of calcium fluxes across the intestinal mucosa of the marine teleost, *Gadus morhua*, measured using an in vitro perfusion method. J. Exp. Biol. 140, 170-186

Tilney, R. and Hocutt, C., 1987. Changes in gill epithelia of *Oreochromis mossambicus* subjected to cold shock. Environ. Biol. Fish. 19(1), 35-44

Tipton, K. and S.S. Bell., 1988. Foraging patterns of two sygnathid fishes: importance of harpacticoid copepods. *Mar. Ecol. Prog. Ser.* 47, 31-43

Van Wijagaarden, D., 1967. Modified rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. Anal. Chem. 39(7), 848-849.

Vincent, A., 1994. Operational sex ratios in seahorses. Behaviour 128(1-2), 153-167

Vincent, A., 1995. A role for daily greetings in maintaining seahorse pairbonds. Animal Behaviour 49, 258-260

Vincent, A., 1996. The international trade in seahorses. TRAFFIC International, Cambridge.

Watanabe, T., 1985. Importance of the study of broodstock nutrition for further development of aquaculture. In "Nutrition and Feeding in Fish" (Cowey, C.B., Mackie, A.M. and Bell, S.G. eds), 395-414. Academic Press, London

Watanabe, T., 1993. Importance of docosahexaenoic acid in marine larval fish. Journal of the World Aquaculture Society 24:2, 152-161

Watanabe, T., Kitajima, C. and Fujita, S., 1983. Nutritional values of live organisms used in Japan for mass propagation of fish: a review. Aquaculture 34, 115-143

Watanabe, T., Arakawa, T., Kitajima, C. and Fujita, S., 1984a. Effect of nutritional quality of broodstock diets on reproduction of red seabream. Nippon Suisan Gakkaishi 59, 1023-1028

Watanabe, T., Arakawa, T., Kitajima, C. and Fujita, S., 1984b. Effect of nutritional quality of diets given to broodstock on the verge of spawning on reproduction of red sea bream. Bull. Jap. Soc. Fish. 50, 1207-1215

Watanabe, T., Lee, M., Mizutani, J. Yamada, T., Satoh, S., Takeuchi, T., Yoshida, N., Kitada, T. and Arakawa, T., 1991. Effective components in cuttlefish meal and raw krill for improvement of quality of red seabream (*Pagrus major*) eggs. Nippon Suisan Gakkaishi 57, 681-694

Watanabe, T., Kiron, V., 1994. Broodstock management and nutritional approaches for quality offsprings in the red seabream. In: Proceedings, Broodstock Management and Egg and Larval Quality, 23-27 June 1992. Stirling University, Scotland, UK

Watanabe, W., Ernst, D., Chasar, M., Wicklund, R., and Bori, O., 1993. The effects of temperature and salinity on growth and feed utilization of juvenile, sex-reversed male Florida tilapia cultured in a recirculating system. Aquaculture, 112, 309-320

Webster, C.D. and Lovell, R.T., 1990. Responses of striped bass larvae fed brine shrimp from different fatty acid compositions. Aquaculture 90, 49-61

Weirich, C., Tomasso, J., and Smith, T., 1992. Confinement and transport induced stress in white bass *Morone chrysops* x striped bass *M. saxatilis* hybrids: effect of calcium and salinity. Journal of the World Aquaculture Society 23, 49-57

Weirich, C., Tomasso, J., and Smith, T., 1993. Toxicity of ammonia and nitrite to sunshine bass in selected environments. Journal of Aquatic Animal Health 5, 64-72

Wilson, M.J. and Vincent, A.C.J., 2000. Preliminary success in closing the life cycle of exploited seahorse species, *Hippocampus* spp., in captivity. Aquarium Sciences and conservation 2, 179-196

Witt U., Quantz G. and Kulhmann, D., 1984. Survival growth of turbot larvae *Scophthalmus maximus* larvae reared on different food organisms with special regard to long chain polyunsaturated fatty acids. Aquacultural Engineering., 177-190

Xiao, Y. and Greenwood, J., 1993. The biology of *Acetes* (Crustacea; *Sergestidae*). Oceanogr. Mar. Biol. Annu. Rev. 31, 259-444

Zar H., 1984. Biostatistical analysis, Prentice-Hall International Inc., London, 718 p.

Zohar, Y., Harel, M., Hassin, S. and Tandler, A., 1995. Gilt-Head Seabream (*Sparus aurata*). In: Bromage, N. and Roberts, R. (Eds.), Broodstock Management and Egg and Larval Quality. Blackwell Science, London, pp. 94-117.

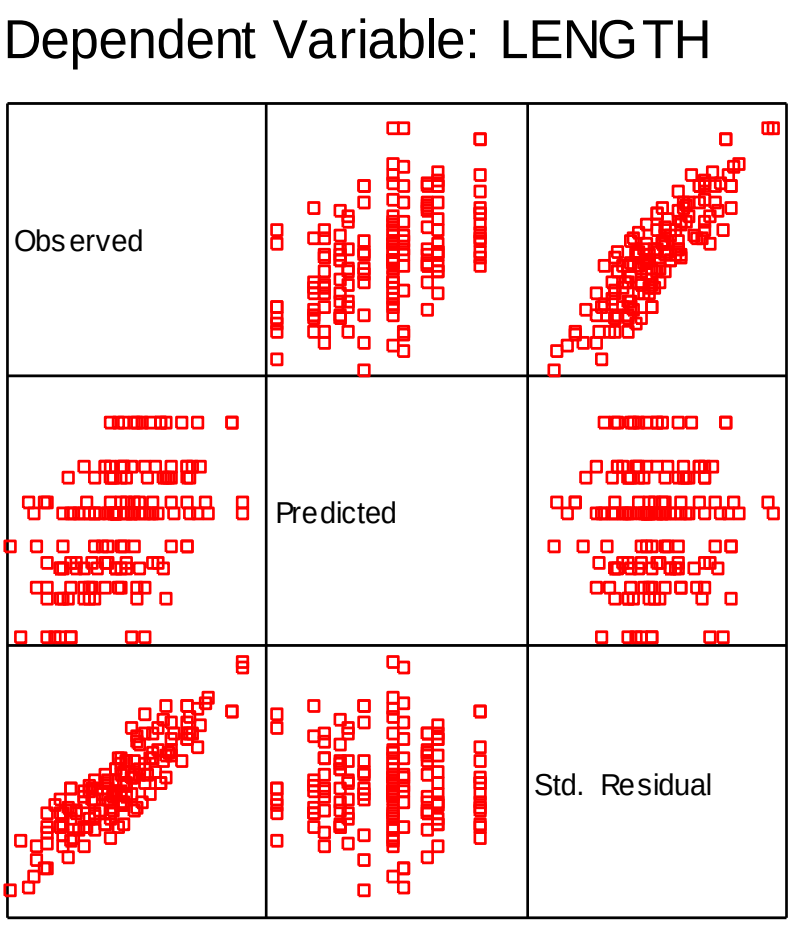
APPENDIX

3.1 Levene's test of equality of variances for standard lengths of seahorses fed various diets

Levene Statistic	df1	df2	Sig.
.311	3	147	.818

[1] computed using log values

3.2 Residual plot of standard lengths of seahorses fed various diets



3.3 Tukeys HSD multiple comparison of standard lengths of seahorse fry fed various diets

		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
(I) DIET	(J) DIET				Lower Bound	Upper Bound
1.00	2.00	-.5053	.509	.754	-1.8136	.8030
	3.00	-2.0671	.492	.000	-3.3313	-.8029
	4.00	-1.6021	.481	.005	-2.8375	-.3667
2.00	1.00	.5053	.509	.754	-.8030	1.8136
	3.00	-1.5618	.467	.005	-2.7609	-.3628
	4.00	-1.0968	.455	.075	-2.2655	7.185E-02
3.00	1.00	2.0671	.492	.000	.8029	3.3313
	2.00	1.5618	.467	.005	.3628	2.7609
	4.00	.4650	.436	.709	-.6540	1.5840
4.00	1.00	1.6021	.481	.005	.3667	2.8375
	2.00	1.0968	.455	.075	-7.1851E-02	2.2655
	3.00	-.4650	.436	.709	-1.5840	.6540

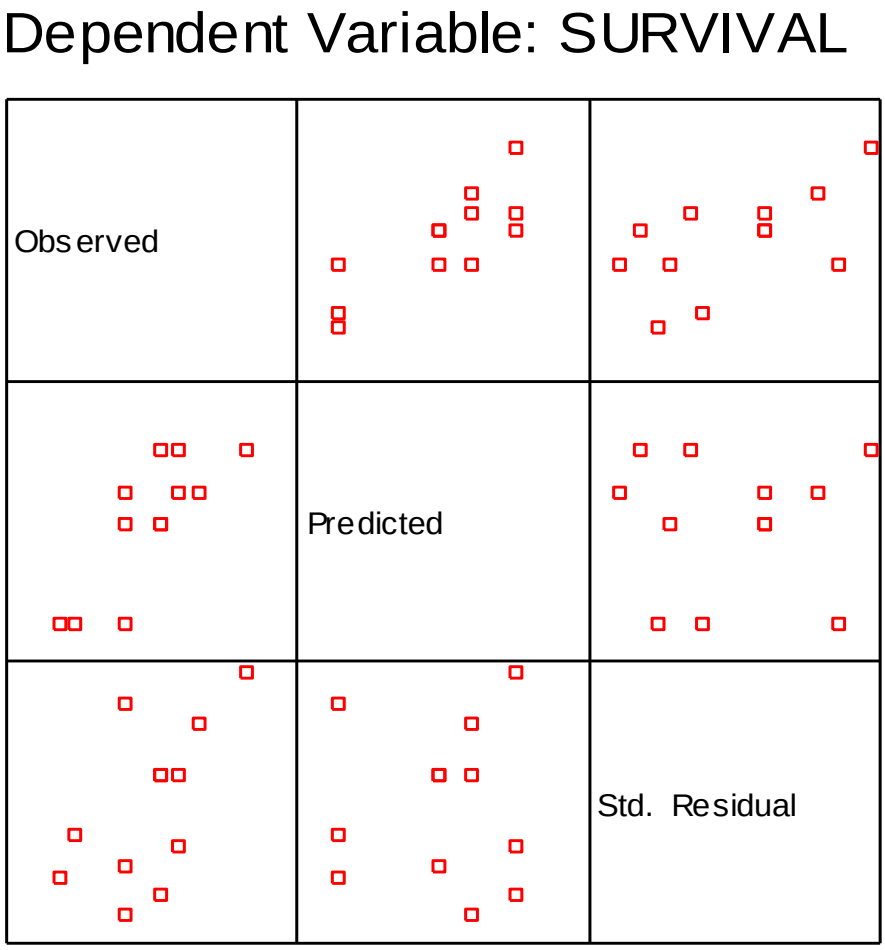
[1] calculated using log values

3.4 Levene's test of equality of variances for survival of seahorses fed various diets

Levene Statistic	df1	df2	Sig.
.898	3	8	.483

[1] calculated using arcsin values

3.5 Residual plot of survival of seahorses fed various diets



3.6 Tukeys LSD multiple comparison of survival of seahorse fry fed various diets

		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
(I) DIET	(J) DIET				Lower Bound	Upper Bound
1.00	2.00	-10.7400	5.024	.065	-22.3247	.8447
	3.00	-14.0233	5.024	.024	-25.6081	-2.4386
	4.00	-18.7433	5.024	.006	-30.3281	-7.1586
2.00	1.00	10.7400	5.024	.065	-.8447	22.3247
	3.00	-3.2833	5.024	.532	-14.8681	8.3014
	4.00	-8.0033	5.024	.150	-19.5881	3.5814
3.00	1.00	14.0233	5.024	.024	2.4386	25.6081
	2.00	3.2833	5.024	.532	-8.3014	14.8681
	4.00	-4.7200	5.024	.375	-16.3047	6.8647
4.00	1.00	18.7433	5.024	.006	7.1586	30.3281
	2.00	8.0033	5.024	.150	-3.5814	19.5881
	3.00	4.7200	5.024	.375	-6.8647	16.3047

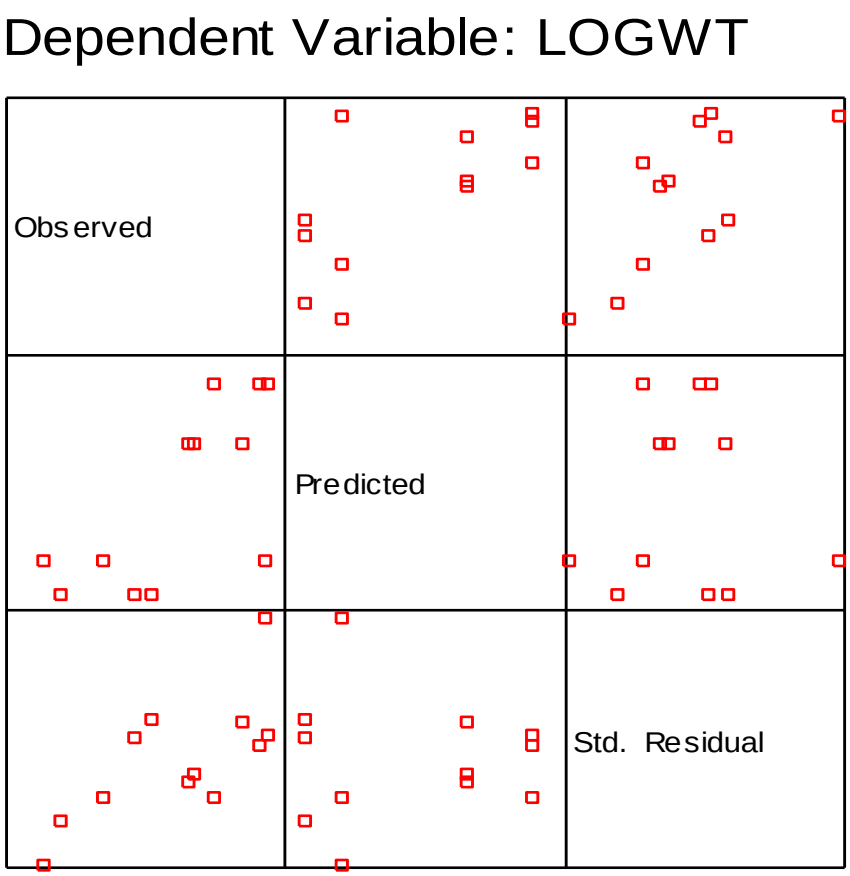
[1] calculated using arcsin value

3.7 Levene's test of equality of variances for wet weight of seahorses fed various diets

Levene Statistic	df1	df2	Sig.
3.793	3	8	.058

[1] calculated using log values

3.8 Residual plot of wet weight of seahorses fed various diets



3.9 Tukey's LSD multiple comparison test of wet weight of seahorses fed various diets

		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
(I) DIET	(J) DIET				Lower Bound	Upper Bound
1.00	2.00	-.5000	1.0685	.652	-2.9639	1.9639
	3.00	-2.7000	1.0685	.035	-5.1639	-.2361
	4.00	-1.8667	1.0685	.119	-4.3306	.5973
2.00	1.00	.5000	1.0685	.652	-1.9639	2.9639
	3.00	-2.2000	1.0685	.073	-4.6639	.2639
	4.00	-1.3667	1.0685	.237	-3.8306	1.0973
3.00	1.00	2.7000	1.0685	.035	.2361	5.1639
	2.00	2.2000	1.0685	.073	-.2639	4.6639
	4.00	.8333	1.0685	.458	-1.6306	3.2973
4.00	1.00	1.8667	1.0685	.119	-.5973	4.3306
	2.00	1.3667	1.0685	.237	-1.0973	3.8306
	3.00	-.8333	1.0685	.458	-3.2973	1.6306

4.1 Levene's test of equality of variances for standard length, weight and number of newborn seahorses *Hippocampus sp.* hatched from broodstock fed *Acetes* unenriched or enriched with DC DHA Selco *Artemia*

	F	df1	df2	Sig.
LENGTH	.867	1	11	.372
WEIGHT	.129	1	11	.726
NUMBER	.469	1	11	.507

4.2 ANOVA of standard length, weight and number of young produced by broodstock seahorses fed either enriched or unenriched *Acetes*

Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Noncent. Parameter	Observed Power
LENGTH	.447	1	.447	4.618	.055	4.618	.500
WEIGHT	.399	1	.399	6.728	.025	6.728	.657
NUMBER	7.633	1	7.633	.002	.965	.002	.050

5.1 Levene's test of equality of variances for survival of juvenile seahorses reared in varying ambient calcium levels

F	df1	df2	Sig.
2.614	3	8	.123

5.2 ANOVA of survival of seahorses reared in varying ambient calcium levels

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	225.000	3	75.000	.136	.936
Intercept	46875.000	1	46875.000	85.227	.000
TREAT	225.000	3	75.000	.136	.936
Error	4400.000	8	550.000		
Total	51500.000	12			
Corrected Total	4625.000	11			

5.3 Levene's test of equality of error variances for dry weight of seahorses reared in varying ambient calcium levels

F	df1	df2	Sig.
4.670	3	8	.036

6.1 Levene's test of equality of error variances for survival of seahorses reared under varying temperature and salinity combinations

F	df1	df2	Sig.
2.107	11	24	.062

6.2 ANOVA of survival of seahorses reared under different temperature and salinity combinations

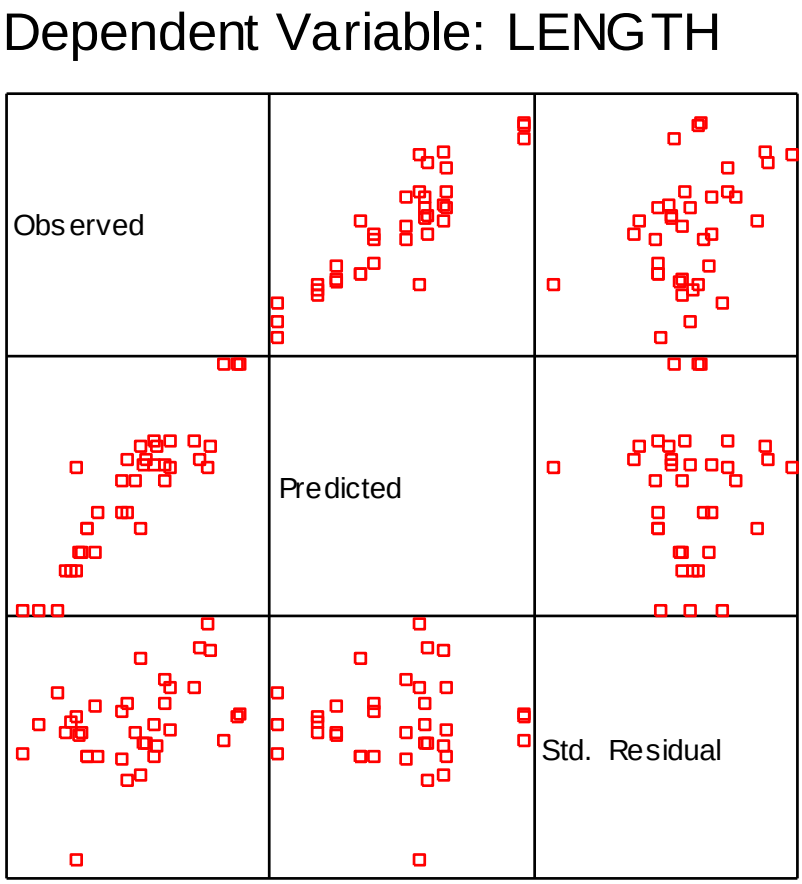
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	13541.667	11	1231.061	4.171	.002
Intercept	180625.000	1	180625.000	612.000	.000
TEMP	2187.500	2	1093.750	3.706	.040
SAL	8680.556	3	2893.519	9.804	.000
TEMP * SAL	2673.611	6	445.602	1.510	.217
Error	7083.333	24	295.139		
Total	201250.000	36			
Corrected Total	20625.000	35			

a R Squared = .657 (Adjusted R Squared = .499)

6.3 Levene's test of equality of error variances for standard length of seahorses reared under different temperature and salinity combinations

F	df1	df2	Sig.
3.663	11	24	.004

6.4 Residual plot of standard lengths of seahorses reared under different temperature and salinity combinations



6.5 ANOVA of standard length of seahorses reared under different temperatures and salinities

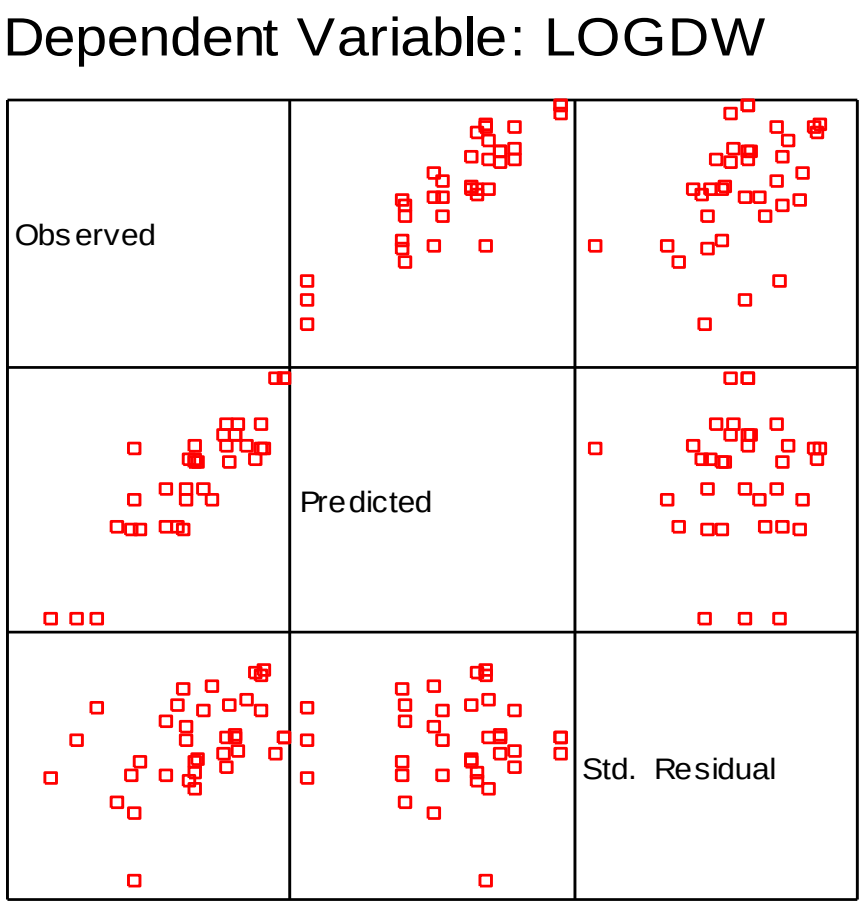
Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Noncent. Parameter	Observed Power
Model	22511.335	12	1875.945	478.675	.000	5744.096	1.000
TEMP	315.436	2	157.718	40.244	.000	80.488	1.000
SAL	21.603	3	7.201	1.837	.167	5.512	.416
TEMP * SAL	92.831	6	15.472	3.948	.007	23.687	.917
Error	94.057	24	3.919				
Total	22605.392	36					

a Computed using alpha = .05
b R Squared = .996 (Adjusted R Squared = .994)

6.6 Levene's test of equality of error variances for dry weight of seahorses reared under different temperatures and salinities

F	df1	df2	Sig.
3.682	11	24	.004

6.7 Residual plot of dry weight of seahorses reared under different temperatures and salinities



Model: Intercept + SAL + TEMP + SAL*TEMP

6.8 ANOVA of dry weights of seahorses reared under different temperatures and salinities

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.176	11	.107	7.975	.000
Intercept	27.557	1	27.557	2056.226	.000
SAL	4.943E-02	3	1.648E-02	1.230	.321
TEMP	.809	2	.405	30.194	.000
SAL * TEMP	.317	6	5.282E-02	3.941	.007
Error	.322	24	1.340E-02		
Total	29.055	36			
Corrected Total	1.497	35			

a R Squared = .785 (Adjusted R Squared = .687)