Food production for juvenile seahorse culture

By

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Abstract

The aim of this project was to find a reliable, cost effective food for production of *Hippocampus* spp. juveniles. The first feeding preferences in terms of short term survival and growth were investigated. *H. guttulatus* juveniles had a significantly higher rate of survival of 55.5% when fed on *Tisbe battagliai* compared to *Artemia* or two other copepods to day seven. *H. kuda* juveniles had a significantly higher survival rate of 61% when fed on enriched rotifers compared to *Artemia* or *T. battagliai* to day seven. Experiments feeding *T. battagliai* to *H. reidi* gave conflicting results. A survival and growth experiment with *H. fuscus* juveniles resulted in no significant differences in survival to day 17 when fed on enriched *Artemia*, newly hatched *Artemia* or *Tisbe battagliai*. The weight of the juveniles fed on enriched *Artemia* and newly hatched *Artemia* was significantly higher than juveniles fed on *T. battagliai* however.

Due to the success of *T. battagliai* as a live food for some species of juvenile seahorses, investigations were carried out to find optimal culture conditions. In survival experiments, it was found that *T. battagliai* would survive and reproduce on a variety of diets such as yeast and SMA milk in both small and large culture systems. A loss of pigment in the copepods was associated with these diets however. In a life time study of the effects of SMA milk and yeast on *T. battagliai* it was found that algae as a food resulted in a longer life span, more fertile females and more brood sacs. However *T. battagliai* fed on SMA milk had a shorter development time and had more nauplii per brood sac than those fed on a mixture of algae. The maximum ingestion rate of a *T. battagliai* in a 24 hour period was investigated for the algae *Tetraselmis suecica*. The highest pellet production was 32 pellets female $^{-1}$ day $^{-1}$ at a concentration of 3.74×10^{5} cell ml^{-1}.

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Chapter One

Introduction

Species Systematics

Seahorses are part of the family Syngnathidae, defined by their tubular jaws and use of specialized pipette feeding, thick bony plates, absence of ribs and lobed gills (Lourie *et al.* 1999). "Syn" is derived from the Greek word "together" or "fused", while "gnathus" is translated as "jaws" (Lourie *et al.,* 1999). Relatives include pipe fish, pipe horses and seadragons. Seahorses are all listed under the genus "*Hippocampus*". McAllister (1990 cited in Lourie *et al.,* 1999) lists 106 species names, however, many are miss-identifications or have subsequently been reduced to synonyms (Lourie, 1999), with recent research suggesting there are only around 35 species (Vincent, 1996). Generally there is a low rate of dispersal among young seahorses which may lead to localized "races" occurring within a species (Woods, 1999). According to Lourie *et al.* (1999) formal seahorse taxonomy began with an entry in Linnaeus' Systema Naturae (1758), a catalogue of the natural world as it was known then.

Seahorse Morphology

Seahorses are conservative in morphology, lack certain key physical features (e.g. pelvic and caudal fins) often used in the morphometric analysis of other fish species, and

variation in the body proportions and meristics (used to determine species membership) often overlap among species (Lourie *et al.,* 1999). Seahorses generally all look superficially similar, a head at right angles to an upright body, a tubular snout, an armour -plated body and a long prehensile, finless tail (Lourie *et al.*, 1999). They range in size from less than 4 centimeters for the pygmy seahorse *H. bargibanti* Whitley 1970 to more than 30 centimeters for *H. ingens* Girard 1858 (Woods, 1999) and *H. abdominalis* Lesson 1827 (Lourie *et al.,* 1999).

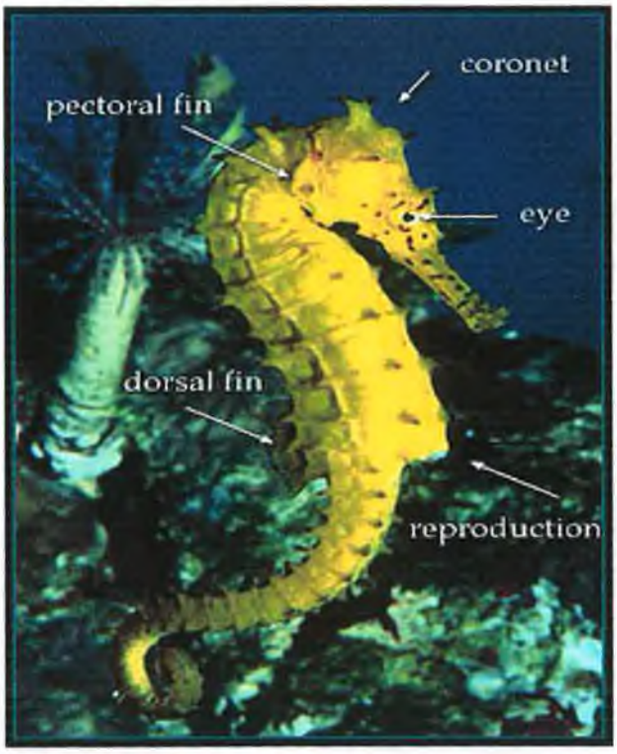

Figure 1:1 An adult *Hippocampus kuda* taken from url, 1

The seahorse swims in a vertical, head up position, using its pectoral fins to stabilize itself (Stoskopf, 1993). The pectoral fins are located on its head, below the gill openings (Lourie *et al.,* 1999). The seahorse swims exclusively by undulating its dorsal fin and

pectoral fins (Consi *et al.,* 2001). A pelvic fin is located next to the anus, but no value for locomotion has been found (Stoskopf, 1993). Their body armour makes it difficult to flex the body. Camouflage helps them in their role as ambush predators and to hide from predators (Lourie *et al.,* 1999). They are not believed to be the main food item for any species but have been found in the stomachs of red snappers and eldorado (dolphin fish) in the Caribbean, while other, largely undigested, seahorses have been found in the stomachs of tuna from Hawaii, and flatheads and striped anglerfish from Australia (Lourie *et al.,* 1999; Kuiter, 2000).

Seahorse Habitats

Seahorses mainly live among seagrasses, (e.g. the seahorse *H. whitei* Bleeker 1855) while others occur in flooded mangrove beds (e.g. the seahorse *H. kuda* Bleeker 1852), soft bottomed areas where sponges and sea squirts are abundant, or among corals in the tropics (Lourie *et al.*, 1999). They are fully marine, with the exception of *H. capensis* Boulenger 1900, which is tolerant of some fresh water influence as it lives in an estuary in South Africa (Lourie *et al.*, 1999). Seahorses are found in shallow water along the coasts of countries each side of the equator between 45° North and 35° South (Lourie *et al.*, 1999). Most species are found around the Indo Pacific region, with Australia having 10 species while Europe has two species found around its shores. The world wide distribution of seahorses is shown in Figure 1:2.

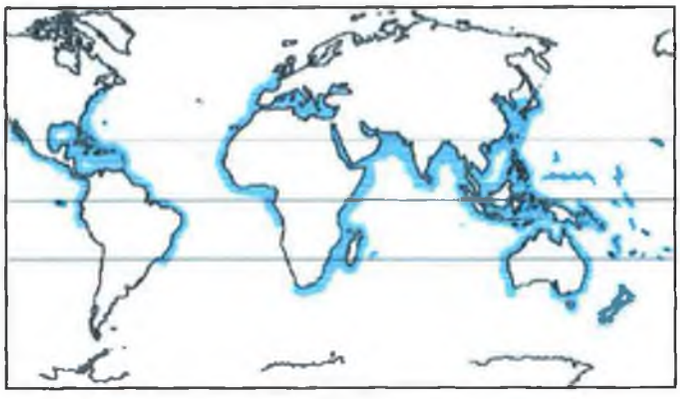

Figure 1:2
Map of world with seahorse distribution highlighted. Taken from Lourie *et al.*, (1999)

Seahorse Sightings in Ireland

Hippocampus hippocampus Linnaeus 1758 and *Hippocampus guttulatus* Cuvier 1929 (formerly known as *H. ramulosus*) are the two species of seahorse commonly found around European shores. Both have been observed frequently around the Southern coast of England (Garrick-Maidment, pers. comm.). Seahorses have been recorded on a number of occasions in Irish waters and records are listed in Table 1:3. Three taxonomic entities are reported. One, *H. brevirostris* Cuvier 1829, is not listed as a valid species by Lourie *et al.*, (1999) but is a synonym for *H. hippocampus.* A number of records are of *Hippocampus* spp. only. The other species identified is *H. guttulatus*. Both European species have therefore been found around the Irish coast.

Table 1:3. Recorded sightings of the seahorse *Hippocampus* spp. around Ireland.

Date	Location	Species & remarks	Collected/ Seen By	Cited
July, 1821	Beach at Red Bay, Co. Antrim	Dead specimen of *Hippocampus* (*Hippocampus brevirostris*, Cuvier)	William Ogilby, Esq.	(Fisher, 1926)
1834	Beach at Youghal	Dead specimen *Hippocampus* sp.	Dr. R. Ball	(Thompson, 1839)
July, 1837	Belfast Bay	*Hippocampus* sp. Live specimen	Richard Langtry	(Thompson, 1839)
September/ October 1837/38	Stomach of a codling caught above Carlise Bridge, Dublin	Dead specimen *Hippocampus* sp. (an inch long)	John Armstrong	(Thompson, 1839)
Summer, 1893	Giants Thumbstones, Whitehead Belfast Lough	*Hippocampus* sp. Live specimen seen from boat	George. C. Reilly	(Reilly, 1929)
July, 1921	Greenisland, Belfast Lough	*Hippocampus* sp. (an inch long) Live specimen seen from boat	Nora Fisher	(Fisher, 1926)
31 January, 1956	Cliffs of Moher, Co. Clare	Dead specimen Originally identified as *Hippocampus hippocampus* but later re- identified as *H. guttulatus* by M. Holmes National Museum Dublin	Denis McMahon of Ballysteen, Liscannon	(Roche, 1956)
1976, 1978, 1983	Zosterae beds between Muighinis and Feenish Islands Co. Galway.	*Hippocampus* sp. Live Specimen seen while snorkeling	Duncan Browne	Personal Communication
1976	Poll an Choire (Gherts Hole)	*Hippocampus* sp. Live specimen seen while snorkeling	Brian Ottway	Personal Communication

	Carna, Co. Galway			
18 August, 1989	Goleen inlet on Lough Hyne	*Hippocampus ramulosus* Leach (12 cm Total Length) Male Live specimen	Mark Holmes & E. J. Holmes	(Holmes, 1991)
1990s year unknown	Poll an Choire (Gherts Hole) Carna, Co. Galway	*Hippocampus* sp. Live specimen seen while diving	Ian Lawler	Personal Communication
1992	Berehaven Harbour, Millcove, Cork (intertidal area)	*Hippocampus* sp. (juvenile specimen) Live specimen seen while snorkeling	Gavin Power	Personal Communication

Only 12 recordings have been collected of this most unusual and recognizable fish in the last 180 years. The increase in recreational diving has not resulted in an increase of records. However, the ability of seahorses to blend into their surroundings makes them difficult to find. There are numerous beach seining (Allen, 2004) and plankton surveys carried out around the Irish coasts, but none of these have ever collected *Hippocampus* specimens to my knowledge. No pregnant males have ever been recorded and so it is not clear if any seahorses have reproduced around the Irish coastline. Of the 13 sightings around Ireland, five were dead specimens and only three were identified to species level. Some adults were seen and in two cases the seahorses were noted to be "an inch long" (2.54cm). No record has ever reported seeing more than one seahorse. A native species is described as "living naturally within a given area; indigenous/ naturally occurring or born in a place" (Cambridge Dictionary, 2004). No concrete evidence was found that would suggest *H. guttulatus* or *H. hippocampus* are native species.

Activity

Seahorses are usually more active during the day, making use of their independently moving eyes with excellent vision (Vincent, 1990), for ambushing their prey. Fishermen have reported, however, that *H. comes* could be seen and caught by day when seahorse fishing started in the late 1960s, but by 2002, *H. comes* had begun nocturnal hunting (Perante *et al.*, 2002). This is thought to be due to the heavy fishing pressure (Perante *et al.*, 2002).

The males of *H. whitei* are site faithful (Vincent & Sadler, 1995), moving infrequently and remaining in a home range of not more than $1m^2$ from their early morning location (Vincent, 1990). The female moves more frequently during the day in a 4-6m diameter area (Vincent, 1990). It is not known if all species have home ranges. Retaining the same home range and holdfast may facilitate crypsis, given that such seahorses adopt colours and camouflage quite specific to their location (Perante *et al.*, 2002). They are thought not to have any social hierarchies or schooling behaviour (Woods, 2003b).

Reproduction

Synganthids are sexually dimorphic. However the seahorse has an unusual reproductive pattern, with the male becoming the carrier of the fertilized eggs inside a specially designed pouch located on the ventral part of the tail and giving birth. The female

produces large, energy rich, immobile gametes (eggs) and the male produces smaller, energy-poor, mobile gametes (sperm) (Lourie, 1999). Eggs develop along a spiraling assembly line in each of two ovaries (Lourie *et al.*, 1999; Vincent, 1990). The eggs are hydrated shortly before copulation (Lourie *et al* 1999; Woods, 2003b). Sperm development continues throughout the year but the sperm themselves are only present in the breeding season, stimulated by courtship (Boisseau, 1967 cited in Lourie *et al.*, 1999). All syngnathids have long intricate courtships each morning with couples reaffirming their bond by swimming together and changing colour markings and patterns dramatically (Vincent, 1990; Pers. obs.). Courtship in seahorses commonly lasts a long time (Lourie *et al.*, 1999). Greeting rituals appear to facilitate reproductive synchrony of male and female so that the female has ripe eggs ready as soon as the male gives birth (Lourie *et al.*, 1999). Breaking up a pair bond may incur a substantial cost for the male in terms of travel distance and reduced reproductive rate while searching for a new mate (Kvarnemo, 2000). In existing pairs of *H. fuscus* Rüpell 1838, the hydration is synchronized with the brooding cycle of the male, but in newly formed pairs, this process takes about 2 days (Kvarnemo, 2000). The possibility remains that the males in recently formed pairs may still benefit from switching mates in terms of overall fitness, if the second female is more fecund or has some other preferred trait that might be naturally or sexually selected (Kvarnemo, 2000). A pairs capacity to produce offspring increases with the time elapsed since pair formation (Vincent, 1994; Pers. obs.).

The female will eventually align her ovipositor with the males pouch opening, they then lock together and a long sticky string of eggs is transferred (Lourie *et al.*, 1999). The

male will then settle the eggs in his pouch where they are fertilized. Each embryo will then embed in the epithelial tissue lining the pouch wall where capillaries will supply oxygen (Lourie *et al.*, 1999). The brood pouch becomes a honeycomb like structure with each egg occupying one compartment (Chang, 2000). The egg yolk provides the nutrients, while the male secretes a hormone, prolactin, that induces enzymatic breakdown of the outer part of the egg to produce a placental fluid (Lourie *et al.*, 1999; Chang, 2000). The male also provides calcium and controls the pouch environment, altering it from a body fluid environment to almost seawater as the pregnancy proceeds (Vincent, 1990; Lourie *et al.*, 1999; Woods, 2003b). The discovery of a series of genes related to brooding suggests that the brood pouch might not only provide nourishment, oxygen and osmoregulation, but also protect embryos from infection as does the uterus of female mammals (Zhang *et al.*, 2003).

Male seahorses brood the developing embryos for ten days to six weeks depending on species and temperature (Lourie *et al.*, 1999; Binsheng, 1992). Newborn seahorses are released by a pumping action of the pouch and body contortions. The number of young produced by the male is dependent on age/size, the number of previous successive spawnings (Woods pers. comm.; Garrick-Maidment, pers. comm.; Pers. obs.) and species (Vincent, 1994; Masonjones & Lewis, 1996; Lourie *et al.*, 1999). Fry are born as perfectly formed miniatures of the adults and are capable of feeding from the moment they are born (Garrick-Maidment, 1997; Chang, 2000; Pers. obs.). Newborns of most species measure 7-12mm with the length more dependent on latitude than on adult size, the nearer the equator, the smaller the young (Vincent, 1990; 1996). Number of fry born

can be as few as ten, or as many as 2000 (Pers. obs.). When the fry are born, they are ready to feed on rotifers, copepods and other small crustaceans in relatively large quantities (Binsheng, 1992).

Feeding

Seahorses are ambush predators relying on inconspicuousness, both visual and hydrodynamic, to avoid alerting their prey (Flynn & Ritz, 1999). When seahorses are in visual contact with and in the process of attacking their prey, it appears that they can suppress the release of kairomones that otherwise would reveal their presence (Cohen & Ritz, 2003). Kairomones are chemicals which are believed to be by-products of metabolism in fish (Cohen & Ritz, 2003). They are thought to be secreted through the urine or surface mucus of the skin (Cohen & Ritz, 2003). The seahorse is the only species in which the ability to suppress the release of this chemical has been reported. It makes intuitive sense because seahorses are well adapted ambush predators with a cryptic profile, excellent binocular vision and good maneuverability (Cohen & Ritz, 2003).

Seahorses naturally feed on a wide variety of benthic and pelagic organisms, mostly tiny zooplanktonic crustaceans and epibenthic isopods and worms, shrimps, and larval fish (Filleul, 1996). The gut contents of *H. abdominalis* sampled over a year period revealed copepods, amphipods including gammarids and caprellids, mysids, carids, and larval fish (Lovett, 1969 cited in Filleul, 1996). The diet of *H. guttulatus* consisted mainly of amphipods, decapods (euphasids, mysidaceans), isopods, ostracods, some gastropods and

harpacticoid copepods (d'Entremont, 2002). Plant material was also found in the guts by d'Entremont (2000), an item never previously reported as being part of the syngnathid's diet. Seahorses are likely to feed on most available zooplankton with selection primarily determined by gape size (Vincent, 1994). They have small, terminal mouths on a protruding tubiform snout and their jaws lack true teeth (Pers. obs.). Their mouths are not capable of distention. Consequently, food cannot be crushed and must be of an appropriately small size (Lovett, 1969 cited in Filleul, 1996).

Seahorses are mainly ambush predators (*H. abdominalis*: Flynn & Ritz, 1999: *H. zosterae* Jordan & Gilbert 1882: Tipton & Bell, 1988), but can also actively pursue prey (*H. erectus* Perry 1810: James & Heck, 1994). They visually locate their prey, and quickly thrust their head forward, which creates a vacuum, when the prey is close enough. They swallow the prey whole (*H. erectus*; James & Heck, 1994). Seahorses capture prey from the water column, on the surface of the plants and from the sediments.

The oesophagus of the seahorse leads to a stomach, which is merely a dorsal pouch (Stoskopf, 1993) at the crook of the neck. There is no muscle to separate the stomach from the intestines which are nearly 50% longer in males than in females (Stoskopf, 1993). Histological examinations by Leake (1990 cited in Filleul, 1996) found a dense concentration of mucin cells lining the oesophagus of all specimens. The anterior intestine was lined with a folded epithelium, which was taller and thrown into bigger folds than in the median and posterior intestine. The highest concentration of mucin cells were found in the posterior intestinal region. These features suggest the oesophagus and

anterior portion is mainly secretary and the posterior portion, which has a lower epithelium, is chiefly absorptive (Leake, 1990 cited in Filleul, 1996). The consistent alkaline pH readings taken from the digestive tracts of 5 different specimens of different age classes by Filleul (1996) suggest that digestion in seahorses occurs in an alkaline environment.

The intestine clearly elongates as the young seahorse develops. Stroband & Kroon (1981 cited in Filleul, 1996) stated that, although an ability to take up macromolecules is probably a general feature of the teleostean intestine, the physiological role of the posterior intestine might differ between larvae and adults. In larvae, the food reaches the caudal intestine very quickly and absorption of macromolecules might be of quantitative importance while in adults, including stomach-less fish, many hours pass before the food reaches the posterior gut, and digestion of protein may then be at an advanced stage (Filleul, 1996). The intestinal transit time in adult seahorses is still fairly rapid and fat stores tend to be rather minimal (Bull, 2002).

There is a gallbladder, a discrete organ at the most cranial portion of the liver, just below the crook of the neck (Stoskopf, 1993) and the kidneys are paired. The gonads can be quite variable, the testes appear as thin white lines running on the ventral surface of the kidneys, while the ovaries are a low paired orange structure (Stoskopf, 1993). The heart of a seahorse is a dark triangular organ in the apex of the neck just behind the gills and it surrounds the oesophagus with three major vessels on each side (Stoskopf, 1993). One vessel courses cranially to the gills and two run caudally, bringing returning blood from

the liver and kidneys (Stoskopf, 1993). The gills are distinct from those of other fishes due to their many individual gill projections making up a hemispherical organ (Stoskopf, 1993), giving them an appearance of a bunch of grapes or small chrysanthemum (Stoskopf, 1993). The gills are relatively inaccessible because the operculum has a nearly complete membranous attachment to the body (Stoskopf, 1993). The anatomy can be seen clearly in Figure 1:4.

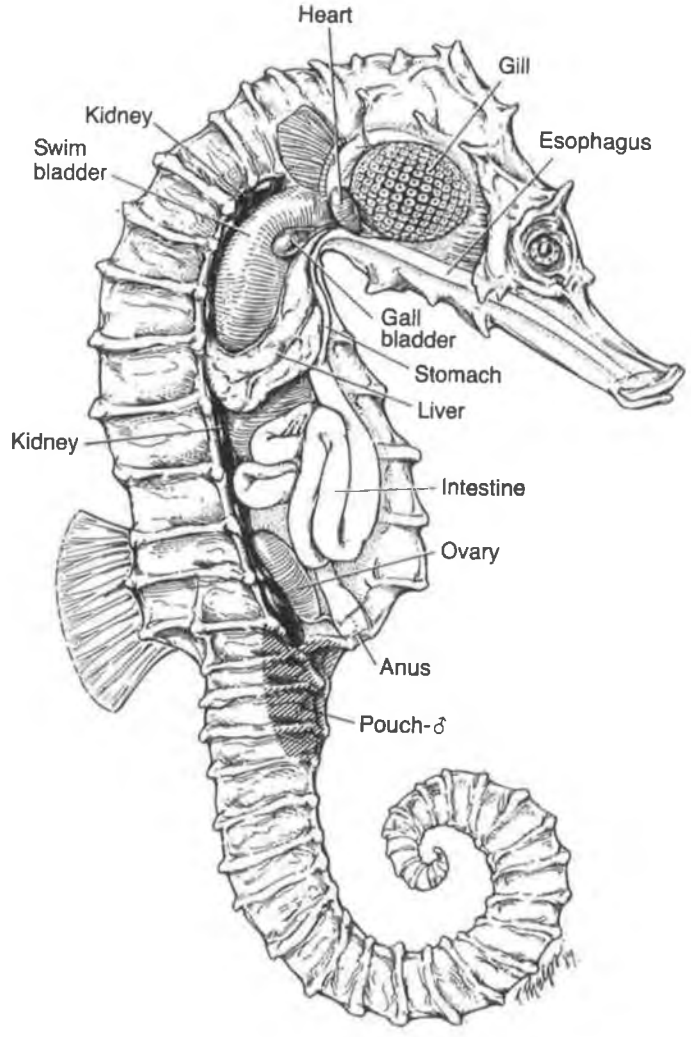

Figure 1:4
Anatomy of *Hippocampus* species. Taken from Stoskopf, (1993)

Markets

The aesthetic appeal of the seahorse has lead to them being widely used as ornamental aquarium fish and dried curios. Seahorses are also used as one of the most famous and expensive materials of Traditional Chinese medicine (TCM) (Zhang *et al.*, 2003). Most animals in the Syngnathidae family can be used as TCM material. Their uses were well documented by all versions of the China Pharmacopoeia even as early as the Liang Dynasty (A.D. 502-557) (Zhang *et al.,* 2003). According to the Ying Yang theory described in TCM, seahorses are a tonic for the kidney and activate the Yang. The former function is essentially related to the regulation of urinogenital, reproductive, nervous, endocrine and immune systems. Recent pharmacological studies suggested that seahorses not only have hormonal activities, boosting hematopoiesis function but also showed anti-tumor, anti-aging, anti-fatigue and Ca^{2+} channel blocking activities (Zhang *et al.*, 2003). They are also highly regarded as an aphrodisiac (Anon., 1998). The increasing demand for the seahorse as medicinal potion, or charm (in part due to the increase in Asian populations and their new found prosperity) has resulted in an increase in the trade of many species worldwide (Forteath, 1997).

Picture 1:5
Dried seahorses packaged and ready to be sold for Traditional Chinese Medicine

Demand for seahorses exceeds supply (Woods, 2003b; Anon., 1998), and prices quoted for the dried powdered product can range from NZ$400 (€206.72) to NZ$1200 (€620.17) a kilogram (Anon., 1998). Medium to large whole seahorses can fetch NZ$15- NZ$20 (€7.75 - €10.35) each (Anon., 1998). In 1992, it was reported in Austasia Aquaculture (Anon., 1998), that China consumed 20 tonnes of seahorses, which is thought to be around 6 million animals. In May 2004, Ocean Rider (a Hawaiian company selling seahorses) were pricing their seahorses for sale for the aquarium trade from €16.35 - €369.90 per pair. Wild caught seahorses will be cheaper than this, but their life expectancy is usually short due to the difficulty in training wild caught seahorses to eat frozen food.

Fisheries

At least 32 nations are actively involved in trading seahorses (Vincent, 1996). The seahorses are caught by subsistence fishers in places such as the Philippines and Thailand (Woods, 1999). Seahorse capture can comprise up to 80% of their annual income (Woods, 1999). They are also caught as accidental by-catch from shrimp trawling and other forms of net-fishing (Vincent, 1996). The intensity of effort dedicated to collecting seahorses depends on their availability relative to other fisheries resources. Virtually all seahorses sold for the aquarium and TCM markets are caught directly from the wild (Vincent, 1996).

Conservation Status

Seahorse characteristics of low fecundity, limited mobility, structured mating patterns and site fidelity, make them particularly vulnerable to heavy fishing pressure (Lourie *et al.*, 1999). They inhabit many ecologically sensitive aquatic habitats, including coral reefs, seagrasses, mangroves and estuaries, with most species in the Indo Pacific and western Atlantic regions (Sreepada, 2002). All 35 seahorse species have been added to the Appendix II list of CITES (The Convention on International Trade in Endangered Species

includes most of Indo-Pacific seahorse species, while the remaining species are judged as too data deficient to allow assessment. The Red List outlines the conservation concerns about species, but has no direct legislative or legal implications for trade (Sreepada, 2002).

Aquaculture Issues

Aquaculture is undergoing a major revolution with respect to marine fish species (Forteath, 1997). New technologies, advancing nutritional adjuncts, sophisticated breeding techniques and a highly skilled work force are ensuring species, previously considered difficult to rear in captivity, now may be mass cultured (Forteath, 1997). Such developments greatly enhance the conservation of a species by removing the threat to wild populations both directly and indirectly (Forteath, 1997).

There are impacts of ornamental fish farming, as there are with any farming. Among the more serious of these is the threat to wild populations and the release of non-indigenous species into the ecosystem (Tlusty, 2002).

Seahorse Culture Issues

When examining a new species for aquaculture, it is essential to investigate market demand, biology and life history of the target species (Chang, 2000). Recently, Western society has recognized the economic and social importance of the seahorse in Eastern

Aim of study

Lack of knowledge concerning the specific nutritional requirements of juvenile seahorses has been identified by Wong and Benzie (2003) and all seahorse aquaculturists I have contacted, as the largest gap in information required.

This project is concerned with finding a year round supply of high quality food items for the culture of several species of juvenile seahorses.

Chapter two will describe the general husbandry requirements needed for the culture of several species of seahorses in a commercial setting. It will also describe the protocols for the culture of micro-algae, rotifers, *Artemia* and copepods.

Chapter three will detail the juvenile experiments in which alternative diets will be tested to see which gives highest survival and growth for first feeding of several species of juveniles. The culture of all the diets used in chapter three will have been outlined in chapter two.

Chapter four will investigate methods of increasing production for *Tisbe battaggliai* cultures, as following the results of chapter 3, this was found to be a viable alternative feed to *Artemia* and rotifers for some seahorses.

Chapter five will be an overall discussion on the results obtained during this research.

species (Chang, 2000; Garrick-Maidment, pers. comm.; Pers. obs.). Their large size (adults may reach 30cm + in total length) requires that tanks in which successful egg transfer is to occur must be at least 90-100cm in height (Woods, 2000b; 2003b). Seahorses can adapt to a wide range of temperatures (Wong, 1982), but need well oxygenated water (Wong, 1982). Young seahorses are known to exhibit growth inflection points, as they switch between prey types (Boisseau, 1967 cited in Lourie *et al.*, 1999; Woods, pers. comm.). It only takes a few months for seahorses to reach sexual maturity (Wong, 1982) with male seahorses developing brood pouches and females developing rounder, more pronounced abdomens at less than 6 months old (Wong & Benzie, 2003). Courtship rituals are also initiated around this time (Pers. obs.). Environmental factors determine the growth rate (Wong, 1982). Research is currently being carried out in Portugal to determine the lifespan of seahorses, but it is thought to be at least three and possibly up to five years (Garrick-Maidment, pers. comm.; Woods, pers. comm.).

There are relatively few scientific studies published on the basic husbandry of seahorses (Wong & Benzie, 2003). The effects on juvenile growth of temperature (James & Woods, 2001), stocking density and gender segregation (Woods, 2003c), and general factors affecting breeding (Woods, 2000b) and juvenile survival (Woods, 2000a), have been reported for the pot bellied seahorse, *H. abdominalis*. The effect on growth and survival of different feeds has been examined in *H. subelongatus* (Payne & Rippinglae, 2000) and *H. erectus* (Correa *et al.*, 1989). The survival and growth of *Hippocampus* spp. juveniles fed on *Artemia* has been examined by Chang (2000).

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Chapter 2

Maintenance and husbandry of animals in culture

Introduction

The culture of marine fish today, particularly in the West, is mainly carried out in intensive systems under controlled conditions. Light, water temperature and feeding regimes are manipulated to ensure that production of fish is maximised but still cost-effective. The culture of species other than trout and salmon such as cod, red snapper, flame angelfish and seahorses has brought about the need for adapting existing culture techniques for these species. While salmon and trout will feed on dry diets as soon as they commence feeding, other cultured species which have very small larvae, require live feed of the correct size and nutritional composition at first feeding.

In this chapter, the husbandry of seahorses is dealt with as is the production of live feed required by the juveniles for first feeding.

Seahorse Husbandry

Adult husbandry

Adults of *Hippocampus* spp. were obtained from various locations by Seahorse Ireland and held in recirculation systems, one of which can be seen in Figure 2:1. The

seawater in the system was subjected to continuous biological filtration, ozone sterilization and cleaned using protein skimmers. Up to ten adults where held in 225 litre plastic barrels also shown in Figure 2:1. Each barrel had its own inlet and outlet pipe (which were covered in 300μm mesh). As far as possible, equal sex ratios of similar sized seahorses of a single species were kept in the tanks. During egg transfer, the female passes the eggs to the male while swimming upwards in the water column. The broodstock tanks need to be tall so that this can be carried out. The seahorses were subjected to a 12 hours light and 12 hours dark cycles at a constant temperature of 24.5°C. Tanks were oxygenated through the recirculation system. According to Wong & Benzie (2003) oxygen consumption in seahorse's increases particularly from 17°C to 26°C, so culturing seahorses at higher temperatures may increase their metabolic rate, as reported for other fish.

Figure 2:1
The tanks holding seahorse broodstock attached to a recirculation system.

Faeces and extra food were removed from the tanks by siphoning them off the bottom in the morning and in the evening. Daily water exchange in each tank was approximately 400%. Water quality is of major concern when live feeds are used since, particularly at high densities, they cause fouling of the water (Filleul, 1996).

Such conditions promote the growth of detrimental, opportunistic bacteria and ciliates such as *Uronema marinum* which has been described as a marine facultative parasite of seahorses (Filleul, 1996).

Standard food was provided three to four times a day in the form of frozen mysid shrimp (*Neomysis integer* (Leach 1814)) (obtained from Limestar Tropicals, Chester, and identified using Makings, 1977) which had been collected and frozen immediately into 5kg blocks. The mysids were defrosted by cutting the frozen mass into small blocks and soaking in saltwater for 30mins before feeding. Wild mysids were caught using a 5mm mesh net in tidal brackish lakes near the Seahorse Ireland facility in Carna, Connemara. *Praunus flexuous* (Müller 1776) and *Neomysis integer* where found at these sites (Geoff Oliver, pers. comm.; Pers. obs.). Wild caught seahorses have to be weaned onto frozen food. This is done by adding the frozen food as the first feed in the morning. Seahorses generally eat more in the first feed than in later feeds. Live mysids are added for the remaining feeds. When all seahorses are weaned onto frozen food the live mysids can be substituted with frozen mysids.

Seahorses are visual ambush predators that exhibit a hunger dependent foraging strategy. That is, hungry individuals will actively forage but they prefer to adopt a "sit and wait" feeding mode, attempting to conceal their presence by attaching to and mimicking the surrounding substrate and waiting for prey to come within striking distance (Woods, pers. comm.; Pers. obs.). Seahorses in culture conditions will become stressed and/or fatigued if they cannot hold onto something with their tails, consequently, some form of substrate is essential (Filleul, 1996). Garden hose pipe and polypropylene rope were used as substrate in each tank.

Quarantine Procedures

The seahorses should be quarantined for up to 2 months on arrival, in a closed recirculation system with all old water being treated before being disposed of. The seahorses should be checked daily for posture and buoyancy problems (gas entrapment in the brood pouch, subcutaneous emphysema of the tail, brood pouch gas accumulation), feeding response, and rate and pattern of breathing (rapid breathing and coughing could suggest gill disease). Most seahorses will only swim when in search of food, during courtship rituals or to attain a better position on the holdfasts. If a seahorse is frantically swimming in the tank, it strongly suggests that there is something wrong. Other problems to look out for are parasites both Protozoa and Metazoa (Bull, 2002). If a seahorse continues twisting and rubbing against the edge of the tank or tail flicking against its gills, a scraping from the area should be taken and viewed under the microscope. Analysis of faeces for intestinal worms etc should be carried out during the quarantine period especially if they are wild caught specimens. Bacterial diseases, most commonly *Vibrisosis*, are common, especially in stressed seahorses and mortality can be high (Bull, 2002; Geech, pers. comm.).

Juvenile husbandry

Juveniles were siphoned from the broodstock tanks the morning after they were born into grey 70 litre rectangle PVC tanks shown in Figure 2:2 & 2:3. The seawater feeding the PVC tanks was subjected to continuous biological filtration, ozone sterilization and cleaned using protein skimmers. Each tank had an inlet and outlet pipe, with the outlet pipe covered in 200μm filter. These can be seen in Figure 2:2. The juveniles were subjected to a 12L:12D cycle at a constant temperature of 24.5°C.

Figure 2:2
PVC tanks which hold the juvenile seahorses showing the inlet and outflow pipes

Figure 2:3
The juvenile tanks at Seahorse Ireland connected to the recirculation unit

Garden wire was used for juvenile attachment Most species however, have a planktonic stage for the first few weeks of life.

Newly born juveniles were fed on newly hatched *Artemia salina* (Linnaeus 1758) or rotifers (depending on seahorse species) 4-5 times a day. For larger juveniles, a mixture of newly hatched and on-grown *Artemia* was used. *Artemia* were grown on a mixture of algae until they reached an appropriate size and were then fed on commercial enrichments for 12 hours to increase their nutritional value. The larger juveniles were weaned onto frozen mysids as soon as possible and could then be moved into the adult systems.

The bottoms of the tanks were siphoned in the morning and in the evening to remove faeces and extra food. Daily water exchange in each tank was approximately 400%.

Algae Production

Micro-algae, like land plants, utilise photosynthesis to construct complex carbon molecules. Nutritionally, micro-algae are a source of macronutrients, vitamins and trace elements for aquatic communities. They also provide a source of protein, carbohydrates and most importantly essential fatty acids.

Suitable algal species for culture have been selected on the basis of their mass-culture potential, cell size and digestibility, and overall food value for the consumer. The species chosen for this project were *T-Isochrysis galbana* Parke 1949, *Rhinomonas reticulata* Novarino 1991 and *Dunaliella tertiolecta* Butcher 1959. This choice was based on findings of Miles *et al.*, (2001) for the culture of the copepod *Tisbe holothuriae*. The stock cultures were obtained from Brian Ottway, Department of Life Sciences, GMIT.

Conical flasks containing 300ml of sterilised water, nutrients and vitamins were inoculated with a single species of algae. The enrichment medium of nutrients and vitamins provides all requirements for rapid growth. After 10 days, the contents of the conical flask were up-scaled into a demi-john containing 3 litres of sterilised water, vitamins and nutrients. The conical flasks and demi-johns can be seen in Figure 2:4.

The saltwater was sterilised by autoclaving. Nutrients were stored in the fridge until needed. Light was provided in the form of two fluorescent tubes 24 hours a day. The temperature was approximately 19°C.

Continuous turbulence and aeration was provided with a glass pipette kept in place with cotton wool and tin-foil. As much as possible, all work was carried out quickly and aseptically with pipettes and funnels being sterilised with alcohol whenever used.

Figure 2:4
The conical and demi-johns containing the algae used for the culture of live food

When the cultures were at their near-maximum density, they were harvested and topped up with sterilised water and nutrients.

Growth Dynamics

There are five growth phases of micro-algae cultures, which can be manipulated if necessary (Coutteau, 1996). The growth phases are shown in Figure 2:5.

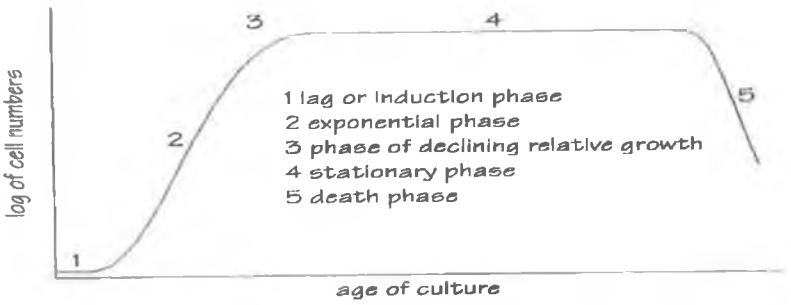


Figure 2:5
Graph of the five growth phases of micro-algae cultures taken from Hoff & Snell, 2004.

The lag or induction phase

- The lag phase is when the algae adapts to its new environment
- Doubling time is slow
- Requirement for threshold concentrations of specific compounds to be reached before exponential growth begins

The exponential phase

- Cell density increases rapidly

The slowing phase

- Slowing of cell division
- The nutrients or other factors begin to limit growth.

The stationary phase

- The limiting factor and growth factor are equal giving a balanced and relatively constant cell density.

The death phase

- This is when nutrients are exhausted and the water quality is unacceptable for continued survival. The culture crashes.

Cultures can be maintained in the exponential phase by adding sterilised water and nutrients after partial harvests. The quality of harvested cells will change according to the algal growth phase.

A weekly routine was set up that involved the up-scaling of the conical flasks, setting up new conical flasks and the harvesting of some algae from the demi-johns. The algae cultures were maintained for 10 months and reached a maximum density of 4.81 x 10^6 cells ml^{-1} (*T-Isochrysis galbana*), 6.58 x 10^5 cells ml^{-1} (*Rhinomonas reticulata*) and 1.76 x 10^6 cells ml^{-1} (*Dunaliella tertiolecta*).

When adequate supplies were not available in the Seahorse Ireland facility, algae was also obtained from the Martin Ryan Institute Marine Laboratory at Carna. Three algal cultures were obtained, when needed, with cultures having an average algae count of 8.77 x 10^6 cells $ml^{-1}$for *T-Isochrysis galbana*, 6.73 x 10^6 cells $ml^{-1}$for *Chaetoceros calcitrons* Takano 1968 and 3.74 x 10^6 cells $ml^{-1}$for *Tetraselmis suecica* Butcher 1959. The larger algal culture systems used in the Martin Ryan Institute Marine Laboratory can be seen in Figure 2:6.

Figure 2:6
Algae culture at the Martin Ryan Institute Marine Laboratory, Carna.

Counting Cell Density

Algal cell density was counted using a haemocytometer. A haemocytometer is a thick glass slide with a mirrored surface which has precisely etched grids defining a known volume. A cover slip is placed on top of the mirrored surface and a drop of the algae to be counted is added. The sample is drawn under the cover-slip by capillary action. All 25 squares are counted if possible (otherwise 5 squares are counted and then multiplied by 5) and then this number is multiplied by 10,000 to get the number of cells ml^{-1}.

Rotifer Production

Brachionus plicatilis Muller 1786, shown in Figure 2:7, tolerate a wide range of conditions, have high reproduction rates and can be reared at high densities of up to 3000 animals ml^{-1} (Reed, pers. comm.) in intensive systems.

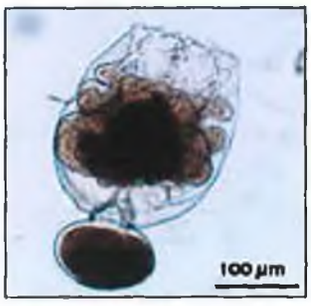


Figure 2:7 Picture of *Brachionus plicatilis* from url, 2

Brachionus plicatilis were obtained from Brian Ottway, Department of Life Sciences, GMIT and were kept in 10litre plastic water bottles at ambient temperatures. No aeration or heat was supplied to the cultures. The plastic bottles were stored in a cupboard without additional lighting. The water used for the cultures had been filtered to 1µm and sterilised with UV lights. Every week the rotifers were collected on a 50µm sieve and then poured back into a clean bottle with clean water. The cultures were then given an equal mix of *Chaetoceros calcitrons, T-Isochrysis galbana and Tetraselmis suecica* until the water became turbid (approximately 1.9 x 10^8 cells added to 1 ltr culture water (1.9 x10^6 cells ml^{-1})). The ability of rotifers to filter feed allows enrichment of the rotifers. When numbers increased sufficiently the cultures were split into more plastic bottles.

At 25°C, the estimated lifespan of rotifers is 3.4 to 4.4 days with the larvae reaching adulthood at 0.5 to 1.5 days (Dhert, 1996). Females start to lay eggs approximately every 4 hours, having up to 10 egg clutches before they die. The life cycle can be completed by two modes of reproduction as shown in Figure 2:8.

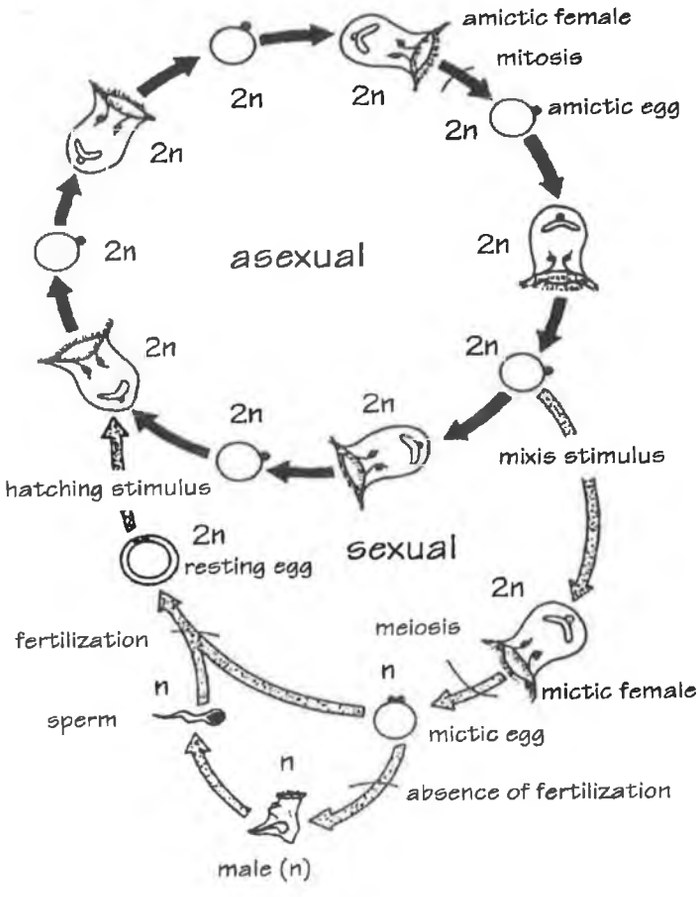


Figure 2:8
Life cycle of *Brachionus plicatilis* showing two modes of reproduction from Hoff & Snell (2004)

Males are smaller with no digestive tract and so are not capable of enrichment. A healthy rotifer population will contain few males while a large proportion of the females will have an egg sac. Rotifers can also be fed on bakers yeast and these are usually larger than those fed on live algae (Dhert, 1996).

Rotifers can be enriched with several products to increase their protein or vitamin C proportions depending on the need of the larval fish, by adding the enrichment to the rotifers for approximately 8 hours before harvest. The rotifers were enriched on algae or Culture Selco 3000 (INVE, Belgium) for the juvenile seahorse experiments in chapter three.

Rotifers were maintained successfully from 19.02.2003 to 01.12.2003 and maximum numbers reached 3.93 animals ml $^{-1}$.

Artemia Production

Artemia salina are brine shrimp which grow in salt lakes and in certain environmental conditions will produce cysts which can be collected and sold in tins. A newly hatched *Artemia* nauplius can be seen in Figure 2:9.

Figure 2:9
Picture of a newly hatched *Artemia salina* from url, 3

Artemia cysts were obtained from INVE Technologies, Dendermonde, Belgium and Argent Laboratories. On the morning prior to an experiment, a gram of cysts was weighed and hydrated in 700ml of freshwater with vigorous aeration for one hour. The cysts were then decapsulated by adding 300ml of hypochlorite to the freshwater. The cysts were monitored closely and when a colour change to light grey had occurred or 10 minutes had elapsed, they were poured into a sieve. These were then washed with freshwater for 5 minutes, or until the hypochlorite odour had been removed.

Decapsulation disinfects the cysts material and removes the outer shell. The nauplii energy content from decapsulated cysts will be higher than nauplii hatched from undecapsulated cysts as they do not have to break out of the hard shell.

The decapsulated cysts were then added to a 70 l blue PVC hopper which contained approximately 60 litres of sterilised and filtered saltwater. The water was heated to around 28°C with the aid of an aquarium heater in the hopper. Vigorous aeration from the bottom of the tanks kept the cysts in suspension while light was supplied 24 hours a day by two fluorescent tube lights overhead. Strong illumination triggers embryonic development. The nauplii hatch in approximately 12-16 hours depending on the quality of the cysts (Merchie, 1996).

To harvest the nauplii, firstly the hoppers are covered to exclude light and the air is turned off. The cysts float to the surface and the nauplii remain in suspension allowing them to be drained off into a sieve. They can then be rinsed quickly with freshwater. Newly hatched nauplii can be used directly for feeding, or they may be returned and enriched a number of ways.

Artemia were enriched by growing them to the required size with a mixture of algae, changing the water and then adding a commercial enrichment. Most enrichments required mixing with water and then being liquidised in a blender. The enrichment used for *Artemia* was A1-DHA Selco (INVE, Belgium) for the seahorse juvenile experiments in chapter three. The *Artemia* were fed to the seahorses after 12 hours of enrichment.

Newly hatched nauplii can be stored in the fridge with slight aeration for 24 hours and this will arrest their development without significant mortalities or a reduction of energy (Merchie, 1996).

Artemia production was set up a few days before the start of a juvenile seahorse feeding trial as most experiments outlined in chapter three required newly hatched *Artemia.*

Copepod Production

Standard methods were available for small scale production from Shannon Toxicology Laboratories and for large scale production from the Seafish Marine Farming Unit at Ardtoe.

Tisbe battagliai Production

Tisbe battagliai Volkmann-Rocco 1972 were obtained from the Shannon Toxicology Laboratory where they had been kept in culture for 2 years. They were maintained in 70 litre blue PVC hoppers. The hoppers were held in a wooden frame with the outflow being approximately 20cm from the floor to allow for draining and harvesting. Each hopper held up to 35 litres of seawater which had been sterilised and filtered to 1μm. No airlines were added. The hopper system can be seen in Figure 2:11. Extra substrate was introduced in the form of 15.25cm bio-media filters (media for biofiltration from Dryden Aqua Ltd Edinburgh Scotland) to each hopper (Figure 2:10)). This was to increase the surface area and bio-film which the adults can graze

on. This system and the media were adapted from a tank system used in the Seafish Marine Farming Unit, Ardtoe.

Figure 2:10
Media from Dryden Aqua ltd. which was used to increase the surface area for *Tisbe battagliai* to graze on in the hopper production system.

Figure 2:11
The hoppers used in the production of *Tisbe battagliai*

The room was illuminated by two fluorescent tube lights and three Perspex windows allowing natural light into the room. The lights were turned on for approximately 10 hours every day. In the winter time, a radiator heated the room and water was kept at ambient temperatures throughout the year. The temperature range of the seawater was 9-18°C and was checked on occasion with a thermometer.

Maintenance and Feeding

A water change was carried out once a week and the copepods were fed twice a week to satiation by monitoring the colour of the culture water. An equal mix of *Chaetoceros calcitrons, T-Isochrysis galbana* and *Tetraselmis suecica* was added until the water became murky (approximately 6.4 x 10^9 cells twice a week (2.1 x10^5 cells ml^{-1})). An initial stocking density of 10,000 adult *Tisbe battagliai* would double within two weeks.

Some hoppers were also fed on bakers yeast and a formulated milk diet. Chapter four gives further details on these experiments.

The *Tisbe* populations were maintained from 9.01.2003 until 30.04.2004 and achieved a maximum density in the hoppers of 1.5 animals ml^{-1}. The *Tisbe* used in the juvenile experiments in chapter three were all between the size ranges of 50–150μm.

Harvesting

Tisbe were removed from this system when required for experiments by draining the water through two filter bags with a pore size of 150 and 50μm, with the nauplii being caught in the 50μm bag while the adults and large copepodites were caught in the 150μm bag. The contents of each filter bag were then rinsed into a plastic container so that counts could be made. All filter bags and containers were rinsed between the harvesting of each hopper to reduce the risk of contamination (See contamination section below).

Tray Culture System

Some *Tisbe* were maintained in black PVC trays with a maximum volume of 20litres. This system was adapted from Miles *et al.*, (2001). The trays were stored in a wooden shelving unit as shown in the Figure 2:12. Six litres of seawater filtered to 1μm and sterilised with UV lights were added to the trays with the *Tisbe* cultures. They were fed twice a week with an equal mixture of *Chaetoceros calcitrons, T-Isochrysis galbana* and *Tetraselmis suecica* (approximately 1.9 x 10^9 cells twice a week (3.16 x10^5 cells ml^{-1})). The trays had high organic loadings and were difficult to harvest so culture was moved to the hopper system. The *Tisbe* achieved a higher population number, 2.6 animals ml^{-1} compared to the hoppers maximum of 1.5 animals ml^{-1}, probably because of the high surface area to volume ratio.

Figure 2:12: Tray system for the culturing of *Tisbe battagliai*

Contamination of cultures

A number of organisms other than copepods and algae were observed in the hopper and tray systems. Most of these were innocuous and small enough to be removed by sluicing water through the filter bags during harvesting. Both ciliates and nematode worms were often found in copepod cultures but the presence of these organisms did not appear to have any influence on production levels and were usually associated with high organic loadings.

Contamination of the copepod batch culture system by rotifers was a problem as they could overtake a copepod population due to the speed of their asexual reproduction. In order to prevent the spread of rotifers among the hoppers it is advisable to harvest the hopper as soon as possible and disinfect the hopper and filter bags and containers associated with the harvesting of that tank.

Culture of Other Species

Tigriopus brevicornis Muller and *Eurytemora velox* (Lilljeborg 1983) were obtained from salt water splash pools located close to Seahorse Irelands facility in Carna, Connemara. They were kept in glass aquaria and fed an equal mix of *Chaetoceros calcitrons, T-Isochrysis galbana* and *Tetraselmis suecica. Eurytemora velox* were collected in a splash pool with a salinity of 5 ‰. The salinity of the culture water was reduced to match that of their natural habitat by adding fresh water. The water was sterilised and filtered to 1μm and kept at room temperature. *Tigriopus* and *Eurytemora* were removed from the glass aquaria when required for experiments by draining the water through two sieves, the nauplii were caught on a 50μm sieve while

the adults and large copepodites were caught on a 150μm sieve. The water was changed every ten days or when required.

Tigriopus brevicornis was maintained in culture for five months. New stock was added to these populations every month with the numbers reaching a maximum of 0.08 animals ml $^{-1}$. The *Tigriopus* used in the juvenile experiments in chapter three were all between the size range of 150-200μm.

Eurytemora velox was maintained in culture for three months but only achieved a maximum of 0.04 animals ml $^{-1}$. The *Eurytemora* used in the juvenile experiments in chapter three were all between the size range of 80-200μm.

Tisbe holothuriae Humes 1957, a sister species of *Tisbe battagliai,* was obtained from Seafish Aquaculture Laboratory, Ardtoe and were maintained in small tanks and conical flasks for over a year. Production was not increased due to the limited space available. Population numbers reached a maximum of 0.26 animals ml $^{-1}$.

Acartia tonsa (Dana 1849), a calanoid copepod, was obtained from Orkney Laboratories in Scotland. The cultures were maintained from 7.11.2002 to 1.12.2003 and reached a maximum density of 0.53 animals ml $^{-1}$. The maintenance of these copepods was slightly different from the other species as they produced resting eggs which had to be collected when the culture water was changed. The culture water was poured through 150μm and 50 μm sieves to collect the adults and nauplii. The bottom of the culture vessel was then rinsed in freshwater and poured through a 50μm sieve to collect the resting eggs. They could then be backwashed into a petri dish with some

saltwater and a few drops of algae. When the cultures were checked the next week, the petri dished could be viewed under the microscope and if nauplii were present they could be added to a nauplii container. Adults were separated from the nauplii every week to reduce cannibalism. Only 30-40 % of the culture water was changed weekly as adding too much clean water is thought to shock these delicate species (Rhodes, pers. comm.). They were fed on a mixture of *Chaetoceros calcitrons, T-Isochrysis glabana* and *Tetraselmis suecica.* Over-addition of food resulted in high organic loadings and ammonia levels due to the small amount of clean water added weekly. When the *Acartia* were collected on sieves, they were kept out of water for as little time possible.

Counting of Cultures

Copepods were counted by draining the culture water through a sieve and then back washing the adults/nauplii (depending on size of sieve) into a 2 litre jug. The water was topped up to the 2litre mark and 6 samples were taken while the water from the jug was poured to and from another jug to keep the copepods in suspension. The number of copepods in each of the six samples was recorded and from this the approximate number of copepods in the population could be noted.

Other methods of counting were investigated and the results can be seen in chapter 4.

Discussion

Production of ornamental fish in a culture system is advantageous in many ways as it is more predictable in terms of supply and price and the fish are conditioned to

holding in captivity and so are of higher quality than wild harvested fish (Tlusty, 2002). The biology of the fish are also thoroughly investigated, with techniques learned in their rearing and feeding that can be transferred to other species (Dhert *et al.*, 1997; Tlusty, 2002).

The knowledge learnt in the commercial cultivation of seahorses in places such as Seahorse Ireland, can be transferred to the areas where they are caught commercially but stocks are declining. This would allow the natural populations to recover while maintaining employment in these normally disadvantaged areas. The farmers would also get higher prices for seahorses which are already trained onto frozen food, thus reducing deaths of wild caught specimens sold to aquarium markets.

Intensive culture of any live food can have high overhead costs and be labour intensive. The knowledge of life history parameters in relation to different temperature and food is helpful in achieving maximum numbers without an increase in labour and costs. Most of the life history parameters and production needs are well known for *Artemia* and rotifers due to the level of research that has been carried out on these organisms in the last 40 years (Hoff & Snell, 2004). Copepods have been used for many years in toxicology laboratories and their life history is well documented. Their use in marine fish culture is increasing as is the need for intensive culture (Shields, pers. comm.). The production outline given in this chapter has shown that sustained culture can be achieved relatively easily for *Tisbe battagliai.* However further work is needed to increase the amount of *Tisbe* that can be harvested daily and this will be discussed further in chapter 4.

Culture guidelines for live foods including copepods are provided in manuals such as "Plankton Culture Manual" by Hoff & Snell (2004) and "Manual on the production and use of live food for aquaculture' by Lavens & Sorgeloos (1996). Home aquarists web pages also discuss small scale production of copepods, rotifers and *Artemia.*

The use of copepods as live prey for larval marine fish has recently gathered momentum due to the increase in research and culture of difficult to grow marine fish. Small scale production systems for copepods are well established for Toxicology laboratories. Mass culture systems however are currently being developed. Stottrup and Norsker (1997) achieved a daily average yield of 300,000 nauplii in a tray system which had been stocked with 40,000 adult *Tisbe holothuriae* (predominantly ovigerous females). The hopper system described in this chapter had a much lower stocking density and was not harvested daily. Rippingale and Payne (2001) could produce 0.5 – 1x 10^6 nauplii daily for the copepod *Gladioferens imparipes* Thomson 1946 in static tanks with a stocking density of 0.5 copepods.ml^{-1}. The stocking density in the hopper system at Carna, was the same (0.5 copepods.ml^{-1}) as the one for *G. imparipes* but as no daily harvesting was carried out the number of nauplii produced cannot be compared. Miles et al., (2001) found that after stocking with 60,000 adult *Tisbe holothuriae* in a tray system, 300-600,000 nauplii could be harvested 10 days later. The tray system described in this chapter was based on the same methods but the temperature and lighting regime was stricter in Miles *et al.,* (2001). The stocking density was much lower in the hopper system at Carna, but results would have been similar at the end of ten days.

Chapter Three

Experiments on alternative diets for the first feeding of *Hippocampus spp.*

Introduction

Several key problems associated with sustainable culture of seahorses have been identified. These include lack of information concerning dietary requirements of juvenile seahorses and general water quality parameters to maximize growth and survival in captivity (Chang, 2000). In the previous chapter, the production methods at Seahorse Ireland for seahorse culture were outlined and in this chapter, the successfulness of several live feeds for juvenile seahorses are investigated.

Young seahorses (*Hippocampus* spp.) are born as well developed fry that begin feeding immediately. While extensive pre-natal parental care may alleviate some of the commonly encountered problems associated with larviculture, first feeding remains the period of greatest mortality (Filleul, 1996). The first food of seahorses must meet specific criteria in order to be suitable. They must be an appropriate size for the mouth gape and they must swim in a manner that elicits a feeding response (Payne, 2001). The nutritional profile is, however, the most critical. The first food must provide the nutrients that cannot be manufactured by the larvae, and are therefore essential dietary requirements. These essential nutrients include highly

unsaturated fatty acids (HUFAs) (Watanabe *et al.,* 1983), amino acids and vitamins (Merchie *et al.,* 1997).

Cultivation of most marine fish species depends on the provision of live prey during the larval stages (Howell, 1979; Watanabe *et al.*, 1983; Leger *et al.*, 1986). The success of mass culture techniques for rotifers and *Artemia* has resulted in their widespread use as food for fish larvae (Cutts, 2003). Both organisms have low levels of essential fatty acids such as docosahexaneoic acid (22:6n-3, DHA) and eicosapentaneoic acid (20:5n-3, EPA), which are required for normal development in several marine species (Sargent *et al.,* 1997). *Artemia a*nd rotifers are often too large for first feeding and may not elicit a feeding response (Marcus & Murray, 2001). Seahorse juveniles require fresh, live foods with the correct nutritional profile, size, and behaviour (Vincent, 1995).

Even with an adequate supply of correct food, juvenile survival success can still be low. Mortality can be attributed in some cases to imbalanced parental nutrition resulting in poor quality fry (Payne, 2001). Over-inflation of the swim bladder has been suggested by a number of authors as another major cause of mortality during the initial stages of rearing seahorses in captivity (Wilkerson, 1995 cited in Filleul, 1996). It is possible that this occurs more often under culture conditions as a consequence of using photopositive prey and overhead lighting. *Artemia* and rotifers tend to congregate at the air/water interface where young seahorses may swallow air while attempting to feed (Filleul, 1996).

Rotifers and brine shrimp are usually deficient in at least one of the essential fatty acid's (EFA's) and possibly other nutrients (Watanabe *et al.,* 1983) required by larval marine fish. Live feeds that are poor in EFA can be improved by enrichment. Enrichment is accomplished by feeding live feeds emulsion preparations which typically contain a large amount of fish oils. At instar I *Artemia* cannot take up food and thus consume their own energy reserves. Once they have moulted into the second larval stage (i.e. Instar II, approximately 8-24 hours following hatching) they begin non-selective feeding on particulate matter, making enrichment a convenient method of enhancing their nutritional value (Sorgeloos & Leger, 1992; Filleul, 1996).

Hippocampus zostera and *H. abdominalis* are capable of ingesting instar II *Artemia* at first feeding (Filleul, 1996). Feeding on instar II *Artemia* enriched with algae and Frippak, a commercial enrichment resulted in significantly improved growth rates for *H. abdominalis* juveniles compared to unenriched *Artemia* (Filleul, 1996).

Chang (2000) found poor survival and growth in juvenile seahorses when they were fed on *Artemia* that was not enriched. With the addition of enriched *Artemia* there were marked improvements in the survival of the juveniles. The fatty acid composition of the larval seahorses also showed enhanced levels of those fatty acids provided by the booster feeds given to *Artemia.* Wong and Benzie (2003) found that the growth rate of *H. whitei* was consistently higher in treatments that were fed with enriched *Artemia* diets. This is consistent with other studies, which report that enriching *Artemia* makes them more suitable for feeding to young fish (Wong & Benzie, 2003).

Some researchers have used copepods as a successful first food for seahorse juveniles. Payne (2001) used the calanoid copepod *Gladioferens imparipes* Thompson (1946), to successfully culture *H. sublegonatus* Castelnau 1973, juveniles. Job *et al.*, (2002) used wild zooplankton as a food source for the first ten days of culture of *H. kuda* Bleeker 1852 juveniles. Gardner (pers. comm.) also used wild caught zooplankton as a first food for *H. erectus* Perry 1810.

The aim of the experiments in this chapter was to find a supply of live food which was of a high quality and economically viable for the culture of several species of juvenile seahorses. The optimum food can be determined in a number of ways. Such response variables include; do they attack it, do they ingest it, do they digest it, do they have short term survival on it, do they have long term survival on it, do they grow on it and do they reproduce on it. The response variables for the experiments in this chapter are:

- Striking at food
- Ingestion of food
- Digestion of food
- Short term survival
- Growth in terms of weight

Materials and Methods

A number of different species of juvenile seahorses were used and these are discussed below.

Species Description

Hippocampus guttulatus Cuvier 1829 (long snouted seahorse) broodstock were obtained from Portugal and shipped to the facility at Seahorse Ireland where they were kept in quarantine and then moved into the main broodstock room. *H. guttulatus* is widespread in the Mediterranean Seas and the adjacent Eastern Atlantic, and is mainly associated with shallow inshore algae habitats (Kuiter, 2000). Their young are approximately 15 to 16mm long at birth and the adults can grow to around 14cm in total length (Lourie *et al.,* 1999).

Hippocampus kuda Bleeker 1852 (yellow seahorse) broodstock were obtained from the Tropical Marine Centre UK. These seahorses are found in estuaries, harbours and lower reaches of rivers entering brackish waters along the coasts of The Maldives, Sri Lanka, Andaman Sea, Singapore and western Indonesia to Ryukus, Japan (Kuiter, 2000). They are between 6-8mm at birth and the adults can grow up to 15cm in height (Lourie *et al.*, 1999).

Hippocampus reidi Ginsburg 1933 (slender seahorse) broodstock were obtained from the Tropical Marine Centre U.K. This species is widespread from Florida to the West Indies. It is extremely variable in colour, with both sexes having pale saddle-like

banding (Kuiter, 2000). The adults can grow up to 15 cm and the juveniles are between 10-11mm (Gomezjurado, pers. comm.) and pelagic when born.

Hippocampus fuscus Rüppell, 1838 (sea pony) broodstock were obtained from the Tropical Marine Centre U.K. These seahorses live in shallow protected waters on the edge of algal reefs or sea grass beds in 1-10m depth. The adults can grow up to 15cm and the juveniles are around 10mm when born. They are mainly found in the Red Sea and Arabian seas (Kuiter, 2000).

Live feed Culture

The methods for the production of *Artemia,* rotifers and copepods in culture are described in detail in the previous chapter. All *Artemia* cysts were grade AF from INVE Technologies Belgium (size ± 430μm), unless otherwise stated.

General Materials and Methods

Hippocampus spp. juveniles were collected in the morning, from a single birth during the previous night. The juveniles were siphoned out of the adult tanks and into buckets and then siphoned into a randomly selected tank where the experiments were carried out. This was repeated until all the tanks had an equal number of juveniles.

There were at least four replicates of each experimental diet, with the tanks being randomly assigned to a diet. The experimental tanks were kept at a constant temperature of 23 or 24°C by placing them in a water bath which contained two aquarium heaters. The salt water used was filtered to 1μm and sterilised with UV lights. Each tank had a separate airline which provided oxygen and broke the surface

tension. All work was carried out in a separate room from commercial production in the Seahorse Ireland facility.

The experimental tanks used were either 10 litre glass aquaria or 3 litre plastic lunch boxes. The tanks and water bath can be seen in Figure 3:1 and 3:2. It was assumed that after initial cleaning neither tank type leached contaminants.

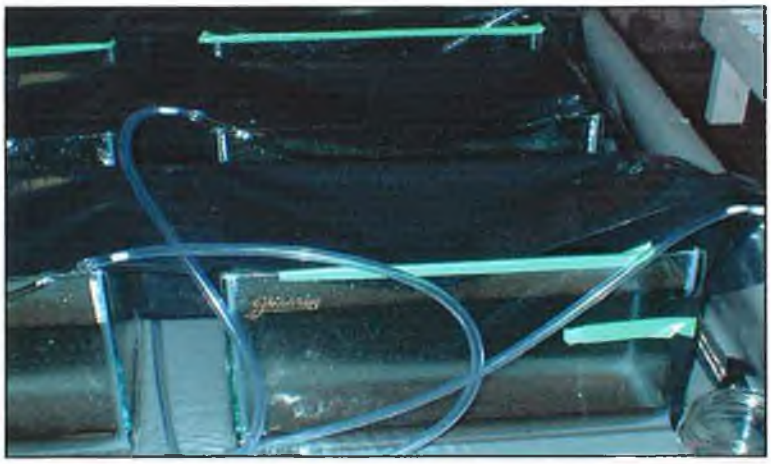


Figure 3:1
10 litre glass aquaria in a water bath used for the juvenile experiments

Figure 3:2
The 3 litre plastic lunch boxes used for the juvenile experiments in the water bath.

The juveniles were fed their specific diet in the morning and afternoons to excess. Feeding to excess was ensured by checking that there were still food items remaining by the next feeding session. A 10ml sample was taken from each experimental tank

and the number of food items remaining was observed. The amount of food added was recorded with all diets receiving similar quantities of food. This was so that juveniles had the same opportunity of attacking a food item. The live food was collected between two sieve ranges so that a definite size range of food was added (except for newly hatched *Artemia* which were expected to be the same throughout trials). The alternative diets tested and size ranges of diets are given in Table 3:3.

Table 3:3. Characteristics of the live food used in the survival experiments carried out on *Hippocampus* spp. juveniles. All diets are grown on algae unless otherwise stated.

***Hippocampus* species**	**Experimental Diet**	**Size range (μm)**
H. guttulatus	Newly hatched *Artemia*	430
	Tisbe battagliai	50-150
	Tigriopus brevicornis	150-200
	Eurytemora velox	80-200
H. kuda	*Tisbe battagliai* (grown on yeast)	50-100
	Tisbe battagliai	50-100
H. kuda	*Tisbe battagliai*	50-100
	Brachionus plicatilis	80-150
H. kuda	Newly hatched *Artemia*	430
	Newly hatched *Artemia* enriched with FishVits®	430
H. kuda	Newly hatched *Artemia*	450-525
	Brachionus plicatilis enriched with Culture Selco 3000	80-150
H. reidi	Newly hatched *Artemia*	430
	Tisbe battagliai	50-150
	Brachionus plicatilis	80-200
H. reidi	Newly hatched *Artemia*	430
	Tisbe battagliai	50-150
H. fuscus	Newly hatched *Artemia*	430
	Brachionus plicatilis	80-200
H. fuscus	Newly hatched *Artemia*	430
	Artemia enriched with A1-DHA-Selco®	80-200
	Tisbe battagliai	50-200

Siphoning was carried out every morning (before the first feed) to remove waste and excess food from the bottom of the tanks. Water was added when required.

The experiments were concluded at day 7 unless otherwise stated. Survival data was then recorded. If juveniles were observed feeding, this was recorded, as was evidence of faecal pellets.

Specific Materials and Methods

Hippocampus guttulatus

The *H. guttulatus* juveniles in this experiment were given a 0.5% formalin bath for one hour to remove potential parasites. This treatment was not carried out on the remaining experiments due to the stress it caused the juvenile seahorses. Stress was indicated by juveniles swimming in an erratic fashion and having no interest in food items when added.

Hippocampus kuda Experiment D

The newly hatched *Artemia* were collected and split into two 1litre containers. One of these containers had 15ml of FishVits® added. Both the containers were then placed in a fridge for at least three hours before being used.

Hippocampus reidi Experiment B

The *H. reidi* juveniles in this experiment were given a one minute freshwater bath before the start of the experiment. It was essential that this freshwater was the same temperature as the water from the broodstock tank. They were placed in freshwater to remove any external parasites. Although the juveniles were still stressed, this method lasted for one minute and appeared to minimise stress of the juveniles.

Hippocampus fuscus

Hippocampus fuscus juveniles attach onto a holdfast a few hours after birth. Garden pea climbing wire was used for juvenile attachment.

Hippocampus fuscus Experiment B

The *H. fuscus* juveniles were weighed on the afternoon of day 14 after being starved in the morning. Juveniles were collected according to tank number with an aquarium net which was placed onto paper towels to dry off the excess water, and then placed into a known quantity of water. The weight of the total number of juveniles from that tank was then divided by the actual number of juveniles in the tank to get the average weight of an individual. Juveniles were weighed again on day 17 and the experiment was then concluded.

Statistical Analysis

The survival data were checked for normality using a Ryan-Joiner normality test. If the data were normal it was analysed using a one way ANOVA. If there was a significant difference in the results, the data were analysed using Fisher's one way multiple comparisons. When viewing the Fishers test results, two negative values indicated the column level was significantly lower than the row level. If both values were positive, then the column level was significantly higher than the row level. If one value was negative and one was positive then the column and row levels were not significantly different.

If the data were not normally distributed they were analysed with the non-parametric Kruskal-Wallis test.

All statistical analysis was carried out by MINITAB version 13.31.

Results

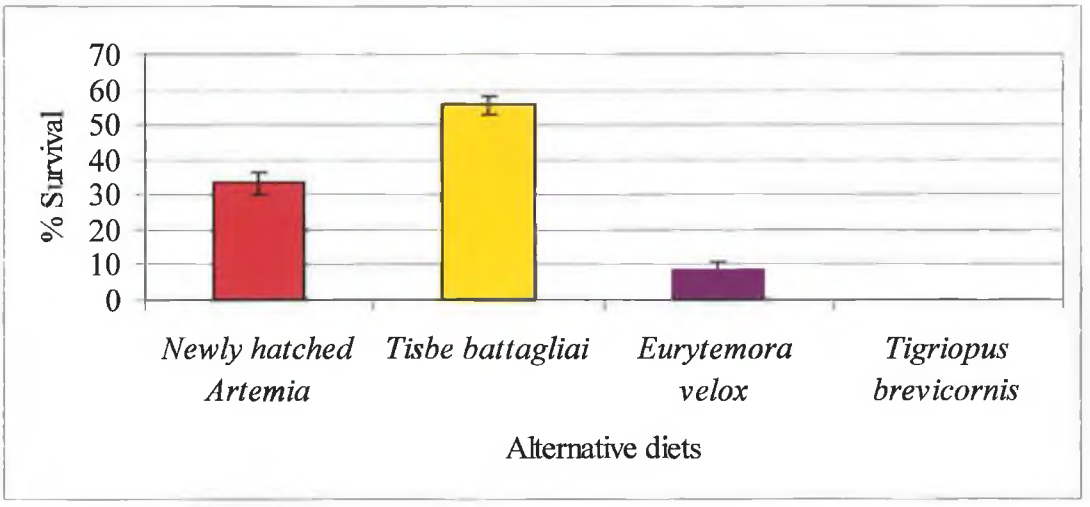
Hippocampus guttulatus

H. guttulatus juveniles were fed for seven days on newly hatched *Artemia, Tisbe battagliai, Tigriopus brevicornis* or *Eurytemora velox.* The results are shown in Table 3.4 and Figure 3.5.

Table 3:4. Results of a seven day feeding trial on *Hippocampus guttulatus* juveniles. Experimental diets were newly hatched *Artemia, Tisbe battagliai, Tigriopus brevicornis* or *Eurytemora velox.* (N = number of juveniles in each tank, NR = number of tank replicates and ED = experiment duration in days)

N	NR	Experimental Diet	Number to Survive	ED
9	5	Newly hatched *Artemia*	15 (33.3 ± 3.15%)	7
		Tisbe battagliai	25 (55.5 ± 2.75%)	
		Tigriopus brevicornis	0 (0 ± 0 %)	
		Eurytemora velox	4(8.8 ± 1.84%)	

Figure 3:5. Histogram showing the percentage survival to day seven of *Hippocampus guttulatus* juveniles when fed on newly hatched *Artemia, Tisbe battagliai, Eurytemora velox* and *Tigriopus brevicornis*. There was no survival to day seven when *H. guttulatus* were fed on *T. brevicornis*.



All *H. guttulatus* juveniles fed on *Tigriopus brevicornis* died before the end of the seven day experiment. The juveniles had low survival (8.8%) when fed on *Eurytemora velox*. Survival increased to 33.3% when fed on newly hatched *Artemia* and increased again to 55.5% when fed on *Tisbe battagliai*.

Data were found to be normally distributed and a one way ANOVA was carried out.

Hypotheses:

Ho: There is no significant difference in the survival of *Hippocampus guttulatus* when fed on newly hatched *Artemia*, *Tisbe battagliai* or *Eurytemora velox*.

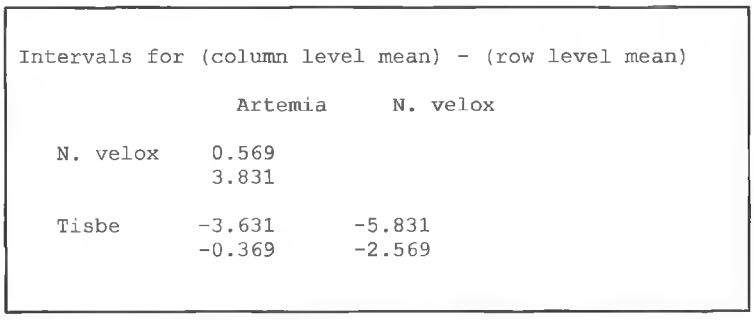
Ha: There is a significant difference in the survival of *Hippocampus guttulatus* when fed on newly hatched *Artemia*, *Tisbe battagliai* or *Eurytemora velox*.

Critical value (p=0.05; df=2,12)=3.89

Decision: Calculated F (15.76) is greater than critical F (3.49). Therefore reject Ho.

Conclusion: There is a significant difference in the survival of *Hippocampus guttulatus* when fed on newly hatched *Artemia*, *Tisbe battagliai* or *Eurytemora velox.*

The results of the Fisher's test showed that *H. guttulatus* fed *Tisbe battagliai* had significantly higher survival rates than those fed on newly hatched *Artemia.* Juveniles fed on newly hatched *Artemia* and *Tisbe battagliai* had significantly higher survival than those fed on *Eurytemora velox.* The results of the Fishers test can be seen in Figure 3:6.



```
Intervals for (column level mean) - (row level mean)

                Artemia      N. velox

   N. velox     0.569
                3.831

   Tisbe       -3.631       -5.831
               -0.369       -2.569
```

Figure: 3:6
Results of Fishers test on survival data from *Hippocampus guttulatus* juveniles fed on 4 diets

Hippocampus kuda

Experiment A

H. kuda juveniles were fed on *Tisbe battagliai* which had been grown on either a mix of algae or bakers yeast. The results can be seen in Table 3:7.

Table 3:7. Results of a seven day feeding trial on *Hippocampus kuda* juveniles. Experimental diets were *Tisbe battagliai* fed on yeast or *Tisbe battagliai* fed on a mixture of algae. (N = number of juveniles in each tank, NR = number of tank replicates and ED = experiment duration in days)

N	NR	Experimental Diet	Number to survive	ED
22	4	*Tisbe battagliai* (grown on yeast) *Tisbe battagliai* (grown on algae)	0 (0%) 0 (0%)	6

The tanks were siphoned to see if there were any faecal pellets, as no feeding had been observed. No faecal pellets were found however, and an unidentified worm was present in all the tanks. The same worm had been found in some of the broodstock tanks and was removed by a treatment with a drug called Panacur.

Experiment B

H. kuda juveniles were fed on *Tisbe battagliai* which had been grown on either a mix of algae or bakers yeast. The results can be seen in Table 3:8.

Table 3:8. Results of a seven day feeding trial on *Hippocampus kuda* juveniles. Experimental diets were *Tisbe battagliai* fed on yeast or *Tisbe battagliai* fed on a mixture of algae. (N = number of juveniles in each tank, NR = number of tank replicates and ED = experiment duration in days)

N	NR	Experimental Diet	Number to survive	ED
15	5	*Tisbe battagliai* (grown on yeast) *Tisbe battagliai* (grown on algae)	4 (5.3%) 0 (0%)	7

The majority of the juveniles died before day seven, with only 5.3% survival to day 7 when fed on *T. battagliai* grown on yeast. The tanks were siphoned daily and checked, but no worms were found in any of the tanks. Statistical analysis was not carried out due to the low number of seahorse juveniles to survive to day seven.

Experiment C

H. kuda juveniles were fed for seven days on *Tisbe battagliai* or *Brachionus plicatilis*. The results are shown in Table 3.9.

Table 3:9. Results of a seven day feeding trial on *Hippocampus kuda* juveniles. Experimental diets were *Tisbe battagliai* or *Brachionus plicatilis* (N = number of juveniles in each tank, NR = number of tank replicates and ED = experiment duration in days)

N	NR	Experimental Diet	Number to survive	ED
30	4	*Tisbe battagliai* *Brachionus plicatilis*	0 (0%) 4 (4.4%)	7

Only four *H. kuda* juveniles remained on day seven. These had all been fed on *Brachionus plicatilis* grown on a mixture of algae (see previous chapter). Statistical analysis was not carried out because of the low survival rate.

Experiment D

H. kuda juveniles were fed on newly hatched *Artemia* or newly hatched *Artemia* which had been enriched with FishVits®. The results are shown in Table 3.10.

Table 3:10. Results of a six day feeding trial on *Hippocampus kuda* juveniles. Experimental diets were newly hatched *Artemia* and enriched newly hatched *Artemia*. (N = number of juveniles in each tank, NR = number of tank replicates and ED = experiment duration in days)

N	NR	Experimental Diet	Number to survive	ED
40	4	Newly hatched *Artemia* Newly hatched *Artemia* enriched with FishVits®	0 (0%) 1 (0.6%)	6

Only one juvenile remained on day 6. This trial was carried out in larger tanks connected to a flow through system in the main Seahorse Ireland juvenile system (see chapter two). Some *Uronema marinum* were present on dead juveniles that were siphoned from the bottom of the tanks. Juveniles were observed striking and ingesting prey items. Some juveniles siphoned from the bottom of the tanks were observed under the microscope to have live *Artemia* present in their guts. Statistical analysis was not carried out.

Experiment E

H. kuda juveniles were fed for seven days on newly hatched *Artemia* or enriched *Brachionus plicatilis*. The results can be seen in Table 3.11.

Table 3:11. Results of a seven day feeding trial on *Hippocampus kuda* juveniles. The experimental diets were newly hatched *Artemia* or enriched *Brachionus plicatilis* (N = number of juveniles in each tank, NR = number of tank replicates and ED = experiment duration in days)

N	NR	Experimental Diet	Number to survive	ED
18	4	Newly hatched *Artemia* Enriched *Brachionus plicatilis*	0 (0%) 44 (61%)	7

No *H. kuda* juveniles grown on newly hatched *Artemia* (Brand Argentemia Grade III Bronze) survived to day seven whereas there was an average percentage survival of juveniles grown on *Brachionus plicatilis* of 61.1% (± 15.7). The *Artemia* used in this experiment were reduced quality (Brand Argentemia Grade III Bronze size 450-525μm) and no feeding was observed on these, while feeding was observed on the rotifers. The rotifers had been enriched on Culture Selco 3000 (INVE Brand). The mean survival rate of *H. kuda* fed rotifers in this experiment was the highest (61% ± 15.7) of all the juvenile experiments on this species.

Hippocampus reidi

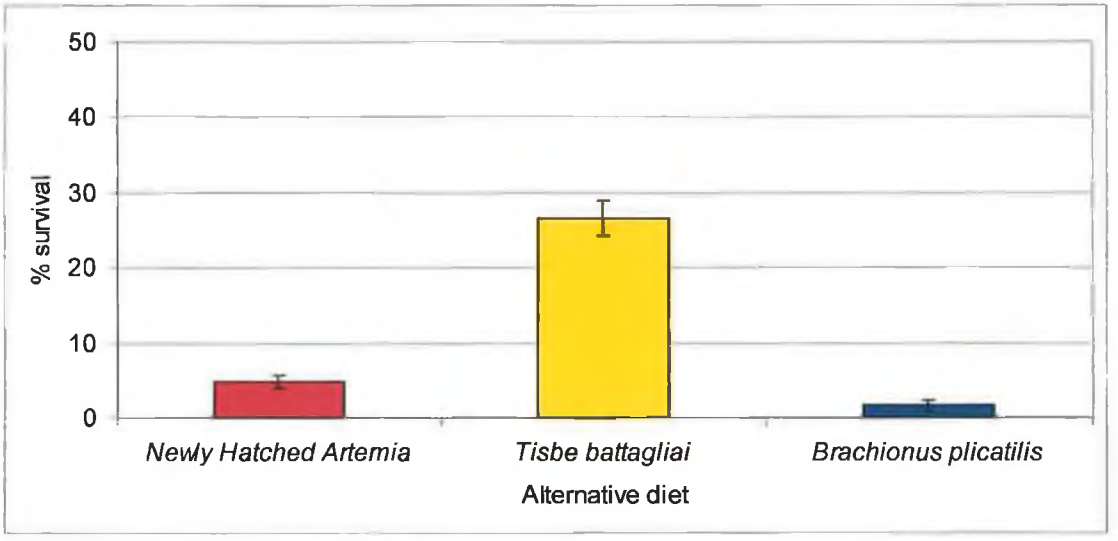
Experiment A

H. reidi juveniles were fed for seven days on newly hatched *Artemia, Tisbe battagliai* or *Brachionus plicatilis*. The results are shown in Table 3.12 and Figure 3.13.

Table 3:12. Results of a seven day feeding trial on *Hippocampus reidi* juveniles. Experimental diets were newly hatched *Artemia*, *Tisbe battagliai* or *Brachionus plicatilis*. (N = number of juveniles in each tank, NR = number of tank replicates and ED = experiment duration in days)

N	NR	Experimental Diet	Number to survive	ED
12	5	Newly hatched *Artemia* *Tisbe battagliai* *Brachionus plicatilis*	3 (4.9 ± 0.8%) 16 (26.6 ± 2.36%) 1 (1.6 ± 0.8 %)	7

Figure 3:13. Histogram showing the percentage survival of *Hippocampus reidi* to day seven when fed on newly hatched *Artemia*, *Tisbe battagliai* or *Brachionus plicatilis*.



The *H. reidi* juvenile survival to day seven was very low. Only 1.6% of juveniles fed on rotifers survived and this increased to 4.9% when they were fed on newly hatched *Artemia*. The highest overall survival (26.6%) of the *H. reidi* was when they were fed on *Tisbe battagliai*.

Data were found to be normally distributed and a one way ANOVA was carried out.

Hypotheses:

Ho: There is no significant difference in the survival of *Hippocampus reidi* when fed on newly hatched *Artemia*, *Tisbe battagliai* or *Brachionus plicatilis*.

Ha: There is a significant difference in the survival of *Hippocampus reidi* when fed on newly hatched *Artemia*, *Tisbe battagliai* or *Brachionus plicatilis*.

Critical value (P=0.05; df=2,9)=4.26

Decision: Calculated F (19.9) is greater than critical F (4.26). Therefore reject Ho.

Conclusion: There is a significant difference in the survival of *Hippocampus reidi* when fed on newly hatched *Artemia*, *Tisbe battagliai* or *Brachionus plicatilis*.

The results of the Fishers test showed that the juveniles fed *Tisbe battagliai* had significantly higher survival rates than those fed on newly hatched *Artemia* and *Brachionus plicatilis*. The juveniles fed on newly hatched *Artemia* did not have significantly higher survival rates than those fed on *Brachionus plicatilis*. The results of the Fishers test are shown in Figure 3: 14.

```
Intervals for (column level mean) - (row level mean)

             Artemia      Rotifer

 Rotifer     -0.9601
              1.9601

 Tisbe       -4.7101      -5.2101
             -1.7899      -2.2899
```

Figure: 3:14 Results from Fishers test on survival of *Hippocampus reidi* fed on three diets

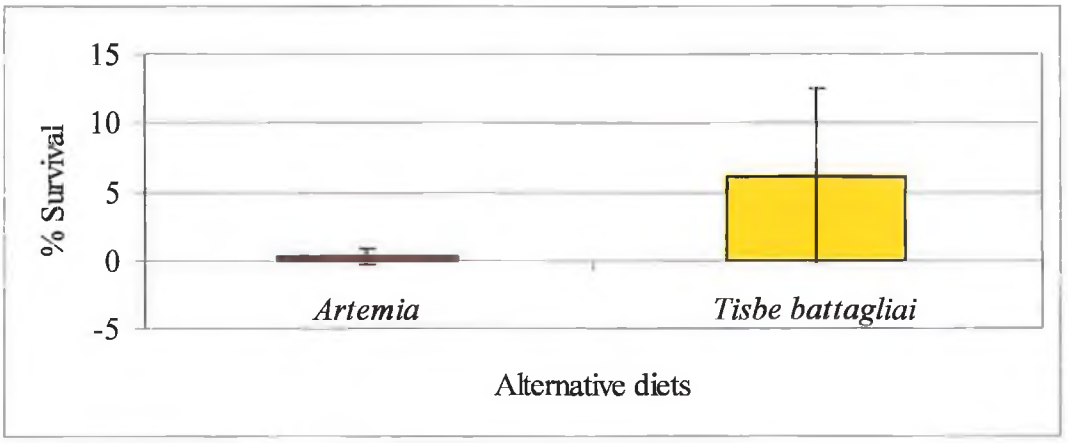
Experiment B

H. reidi juveniles were fed for ten days on newly hatched *Artemia* or *Tisbe battagliai*. The results can be seen in Table 3:15 and Figure 3:16.

Table 3:15. Results of a ten day feeding trial on *Hippocampus reidi* juveniles. Experimental diets were newly hatched *Artemia* or *Tisbe battagliai*. (N = number of juveniles in each tank, NR = number of tank replicates and ED = experiment duration in days)

N	NR	Experimental Diet	Number to survive	ED
85	4	Newly hatched *Artemia*	1 (0.3%)	10
		Tisbe battagliai	26 (7.6%)	

Figure 3:16. Histogram showing the mean percentage survival of *Hippocampus reidi* to day ten when fed on newly hatched *Artemia* or *Tisbe battagliai*.



Survival for the *H. reidi* juveniles was less than 8 % by day ten. Only one juvenile fed on newly hatched *Artemia* survived.

Data were found to be normally distributed and a one way ANOVA was carried out.

Hypotheses:

Ho: There is no significant difference in the survival of *Hippocampus reidi* when fed on newly hatched *Artemia* or *Tisbe battagliai.*

Ha: There is a significant difference in the survival of *Hippocampus reidi* when fed on newly hatched *Artemia* or *Tisbe battagliai.*

Critical value (p=0.05; df=1,8)=5.32

Decision: Calculated F (4.25) is less than critical F (5.32). Therefore accept Ho.

Conclusion: Survival was higher in *Tisbe* fed *H reidi* (7.6%), but it was not significantly higher than *H. reidi* fed on newly hatched *Artemia*. This is due to some of the replicated tanks fed on *Tisbe battagliai* having 0% survival.

Hippocampus fuscus

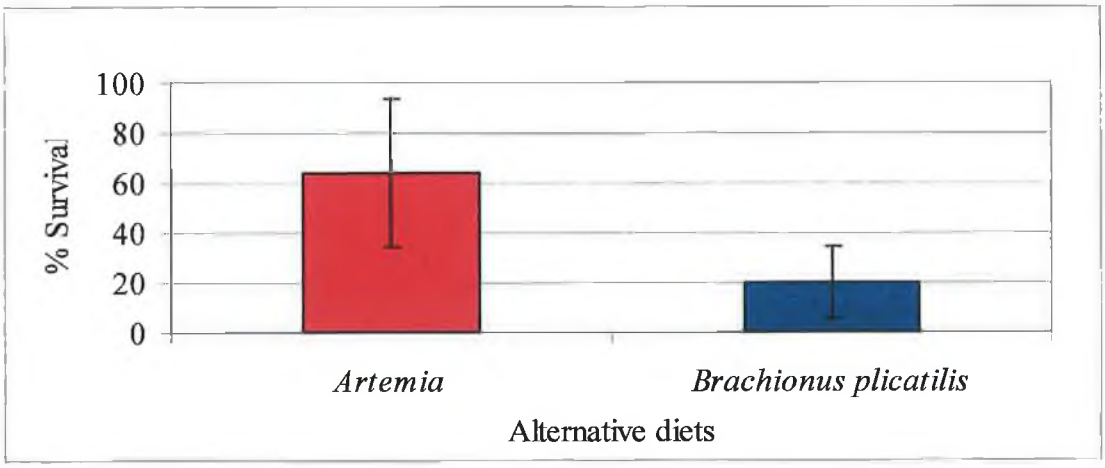
Experiment A

H. fuscus juveniles were fed on newly hatched *Artemia* or *Brachionus plicatilis* for seven days. These results are shown in Table 3:17 and Figure 3:18.

Table 3:17. The results of a seven day feeding trial on *Hippocampus fuscus* juveniles. Experimental diets were newly hatched *Artemia* or *Brachionus plicatilis*. (N = number of juveniles in each tank, NR = number of tank replicates and ED = experiment duration in days)

N	NR	Experimental Diet	Number to survive	ED
5	5	Newly hatched *Artemia* *Brachionus plicatilis*	16 (64 ± 5.93 %) 5 (20 ± 2.83 %)	7

Figure 3:18. Histogram showing the percentage survival of *Hippocampus fuscus* juveniles to day seven when fed on newly hatched *Artemia* or *Brachionus plicatilis*.



The survival of *Hippocampus fuscus* juveniles for seven days was 20% for those fed on rotifers and 64% for those fed on newly hatched *Artemia*. The results were

analysed to see if the survival rates were significantly different. Data were found to be normally distributed and a one way ANOVA was carried out.

Hypotheses:

Ho: There is no significant difference in the survival of *Hippocampus fuscus* when fed on newly hatched *Artemia* or *Brachionus plicatilis*.

Ha: There is a significant difference in the survival of *Hippocampus fuscus* when fed on newly hatched *Artemia* or *Brachionus plicatilis*.

Critical value (p=0.05; df=1,8)=5.32

Decision: Calculated F (8.96) is greater than critical F (3.49). Therefore reject Ho.

Conclusion: There is a significant difference in the survival of *Hippocampus fuscus* when fed on newly hatched *Artemia* or *Brachionus plicatilis.*

The results of the Fishers test tell us that *H. fuscus* juveniles fed on newly hatched *Artemia* had significantly higher survival rates than those fed on *Brachionus plicatilis.* The results of the Fishers test are shown in Figure 3:19.

```
Intervals for (column level mean) - (row level mean)

             Artemia

Rotifers       0.505
               3.895
```

Figure: 3:19. The results from a Fishers test on the survival rates of *Hippocampus reidi* juveniles fed on two diets

Hippocampus fuscus

Experiment B

This experiment was set up to test the success of newly hatched *Artemia*, enriched *Artemia* and *Tisbe battagliai* over 17 days. Survival was assessed at three points (Table 3:20, 3:21 & 3:22) and growth, in terms of weight was assessed at two points (Table 3:23 & 3:26).

Survival to Day Seven

H. fuscus juveniles were fed on newly hatched *Artemia*, enriched *Artemia* and *Tisbe battagliai*. The survival was checked at day seven and the results are shown in Table 3:20.

Table 3:20. Survival rates of *Hippocampus fuscus* juveniles at day seven when fed on enriched *Artemia*, newly hatched *Artemia* or *Tisbe battagliai*.

Diet	**Survival (%) to day 7**
Enriched *Artemia*	95.8 ± 2.1
Newly hatched *Artemia*	95.8 ± 2.1
Tisbe battagliai	100 ± 0

Survival of the *H. fuscus* juveniles was 97.23% to day seven.

Data were not normally distributed and a Kruskal-Wallis test was carried out.

Hypotheses:

Ho: There is no significant difference in the survival of *Hippocampus fuscus* when fed on newly hatched *Artemia*, *Tisbe battagliai* or enriched *Artemia*.

Ha: There is a significant difference in the survival of *Hippocampus fuscus* when fed on newly hatched *Artemia, Tisbe battagliai* or enriched *Artemia.*

Critical value (df= 2; p=0.05)=5.99

Decision: Calculated H value (1.10) is less than critical value (5.99). Therefore accept Ho.

Conclusion: There is no significant difference in the survival of *Hippocampus fuscus* when fed on newly hatched *Artemia, Tisbe battagliai* or enriched *Artemia* and so the experiment was kept running.

All diets elicited a feeding response when added to the tanks and faeces were found in all tanks in varying amounts.

Survival to Day Fourteen

The *H. fuscus* juvenile survival rate was measured again at day fourteen and the results are shown in Table 3:21.

Table 3:21. Survival rates of *Hippocampus fuscus* juveniles at day fourteen when fed on enriched *Artemia*, newly hatched *Artemia* or *Tisbe battagliai.*

Diet	**Survival (%) to day 14**
Enriched *Artemia*	79.17 ± 2.065
Newly hatched *Artemia*	91.66 ± 2.425
Tisbe battagliai	87.5 ± 6

Data were found to be normally distributed and a one way ANOVA was carried out.

Hypotheses:

Ho: There is no significant difference in the survival of *Hippocampus fuscus* when fed on newly hatched *Artemia*, *Tisbe battagliai* or enriched *Artemia*.

Ha: There is a significant difference in the survival of *Hippocampus fuscus* when fed on newly hatched *Artemia*, *Tisbe battagliai* or enriched *Artemia*.

Critical value (p=0.05; df=2,9)=4.26

Decision: Calculated F (0.62) is less than critical F (4.26). Therefore accept Ho.

Conclusion: There is no significant difference in the survival of *Hippocampus fuscus* when fed on newly hatched *Artemia*, *Tisbe battagliai* or enriched *Artemia* to day 14.

Survival to Day Seventeen

The survival rates of the *H. fuscus* juveniles to day seventeen when fed on newly hatched *Artemia*, enriched *Artemia* or *Tisbe battagliai* are shown in Table 3:22.

Table 3:22. Results of a seventeen day feeding trial on *Hippocampus fuscus* juveniles. Experimental diets were enriched *Artemia*, newly hatched *Artemia* or *Tisbe battagliai*. (N = number of juveniles in each tank, NR = number of tank replicates and ED = experiment duration in days)

N	NR	Experimental Diet	Number to survive	ED
6	4	Newly hatched *Artemia*	22 (91.6 ± 2.41 %)	17
		Enriched *Artemia* with A1-DHA Selco	19 (79.2 ± 2.09 %)	
		Tisbe battagliai	16 (66.6 ± 11.79 %)	

Survival to day seventeen was high on all diets. Juveniles had a mean survival rate of 91.6% when fed on newly hatched *Artemia*, 79.2% when fed on enriched *Artemia* and 66.6% when fed on *Tisbe battagliai*.

Data were found to be normally distributed and a one way ANOVA was carried out.

Hypotheses:

Ho: There is no significant difference in the survival of *Hippocampus fuscus* when fed on newly hatched *Artemia, Tisbe battagliai* or enriched *Artemia.*

Ha: There is a significant difference in the survival of *Hippocampus fuscus* when fed on newly hatched *Artemia, Tisbe battagliai* or enriched *Artemia.*

Critical value (p=0.05; df=2,9)=4.26

Decision: Calculated F (0.79) is less than critical F (4.26). Therefore accept Ho.

Conclusion: There were still no significant differences in survival rates at day seventeen of *Hippocampus fuscus* juveniles fed on newly hatched *Artemia*, enriched *Artemia* or *Tisbe battagliai.*

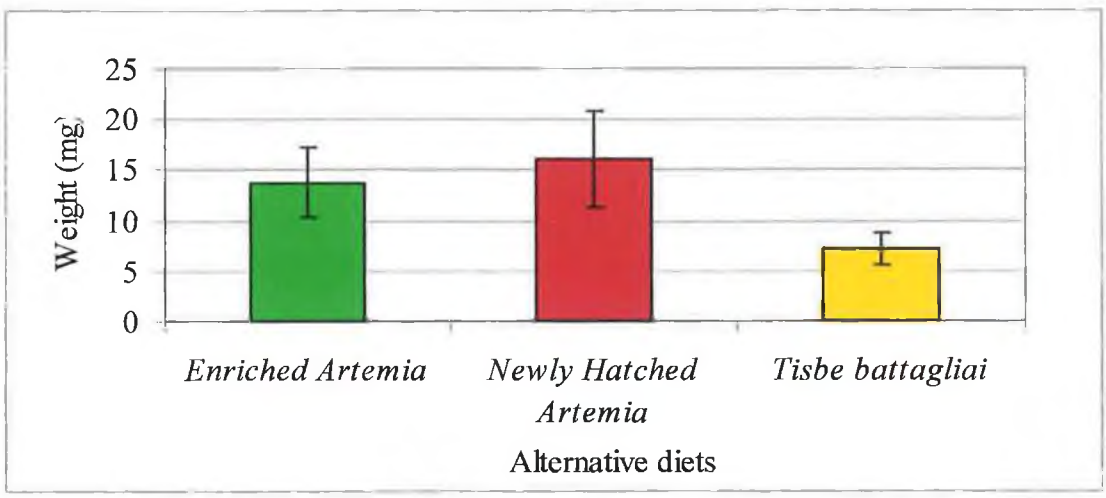
Weight at day 14

H. kuda juveniles fed on newly hatched *Artemia,* enriched *Artemia* and *Tisbe battagliai* were weighed at day 14 and the results are shown in Table 3:23 and Figure 3:24.

Table 3:23. Average weight at day fourteen of juvenile *Hippocampus fuscus* when fed on newly hatched *Artemia*, enriched *Artemia* or *Tisbe battagliai.*

Diet	**Weight (mg) at day 14**
Enriched *Artemia*	13.75 ± 3.5
Newly hatched *Artemia*	16.05 ± 4.75
Tisbe battagliai	7.05 ± 1.58

Figure 3:24. Histogram showing the average weight (mg) of individual *Hippocampus fuscus* juveniles at day fourteen when fed on enriched *Artemia*, newly hatched *Artemia* or *Tisbe battagliai*.



The juveniles fed on newly hatched *Artemia* weighed an average of 16.05mg. This was greater than the juveniles fed on enriched *Artemia* who weighed an average of 13.75mg, and the juveniles fed on *Tisbe* who weighed an average of 7.05mg.

Data were found to be normally distributed and a one way ANOVA was carried out.

Hypotheses:

Ho: There is no significant difference in the weight of *Hippocampus fuscus* when fed on newly hatched *Artemia*, *Tisbe battagliai* or enriched *Artemia*.

Ha: There is a significant difference in the weight of *Hippocampus fuscus* when fed on newly hatched *Artemia*, *Tisbe battagliai* or enriched *Artemia*.

Critical value ($p=0.05$; $df=2,9$)=4.26

Decision: Calculated F (7.03) is greater than critical F (4.26). Therefore reject Ho.

Conclusion: There is a significant difference in the weight of *Hippocampus fuscus* when fed on newly hatched *Artemia*, *Tisbe battagliai* or enriched *Artemia* to day 14.

From the Fishers test results, we can say that juveniles fed on newly hatched *Artemia* and enriched *Artemia* at day 14, had a significantly heavier weight than juveniles fed on *Tisbe battagliai*. There was no significant difference in the weight of juveniles fed on newly hatched *Artemia* or enriched *Artemia*. The results of the Fishers test are shown in Figure 3:25.

```
Intervals for (column level mean) - (row level mean)

                    Enriched        Newly hatched
                    Artemia         Artemia

Newly hatched       -0.007942
Artemia             0.003342

Tisbe               0.001058    0.003358
                    0.012342    0.014642
```

Figure: 3:25 Results of Fishers test on weight of juvenile *Hippocampus fuscus* when fed on three diets.

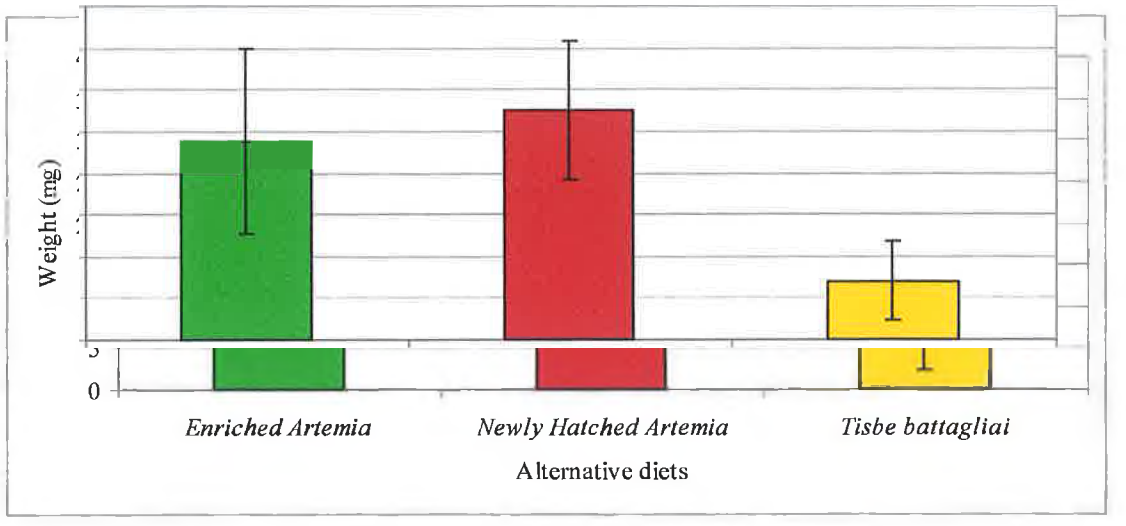
Weight Day 17

H. kuda juveniles fed on newly hatched *Artemia,* enriched *Artemia* and *Tisbe battagliai* were weighed at day 14 and the results are shown in Table 3:26 and Figure 3:27.

Table 3:26. Results of *Hippocampus fuscus* juvenile growth to day seventeen when fed on enriched *Artemia*, newly hatched *Artemia* or *Tisbe battagliai*.

Diet	**Weight (mg) at day 17**
Enriched *Artemia*	23.87 ± 11.14
Newly hatched *Artemia*	27.50 ± 8.23
Tisbe battagliai	7.00 ± 4.76

Figure 3:27. Histogram showing the average weight (mg) of individual *Hippocampus fuscus* juveniles at day seventeen when fed on enriched *Artemia*, newly hatched *Artemia* or *Tisbe battagliai*.



The *Hippocampus fuscus* juveniles fed on newly hatched *Artemia* still had a greater weight (27.5mg) than the juveniles fed on enriched *Artemia* (23.87mg) and *Tisbe battagliai* (7.0mg). The juveniles fed on *Tisbe battagliai* had not increased in weight from day 14.

Data were found to be normally distributed and a one way ANOVA was carried out.

Hypotheses:

Ho: There is no significant difference in the weight of *Hippocampus fuscus* when fed on newly hatched *Artemia*, *Tisbe battagliai* or enriched *Artemia*.

Ha: There is a significant difference in the weight of *Hippocampus fuscus* when fed on newly hatched *Artemia*, *Tisbe battagliai* or enriched *Artemia*.

Critical value ($p=0.05$; $df=2,9$)=4.26

Decision: Calculated F (6.70) is greater than critical F (4.26). Therefore reject Ho.

Conclusion: There is a significant difference in the weight of *Hippocampus fuscus* when fed on newly hatched *Artemia*, *Tisbe battagliai* or enriched *Artemia* to day 17.

From the results of the Fishers test we can say that juveniles fed on newly hatched *Artemia* and enriched *Artemia* at day 17, still had a significantly heavier weight than juveniles fed on *Tisbe battagliai*. There was still no significant difference in the weight of juveniles fed on newly hatched *Artemia* or enriched *Artemia*. The results of the Fishers test can be seen in Figure 3:28.

Intervals for (column level mean) - (row level mean)

	Enriched *Artemia*	Newly hatched *Artemia*
Newly hatched *Artemia*	-0.017147 0.009897	
Tisbe	0.003353 0.030397	0.006978 0.034022

Figure: 3:28. Results of Fishers test on growth of *Hippocampus fuscus* juveniles fed on three diets to day 17.

Summary of feeding trials

In the experiments above, *H. fuscus* is the only species to have a high mean percentage survival (91.6% to day 17) when fed on newly hatched *Artemia* as a first food. Mean survivals decreased to 33% for *H. guttulatus* juveniles, 4.9 & 0.3% for *H. reidi* juveniles and 0.6% (experiment D) for *H. kuda* when fed on newly hatched *Artemia*. *H. guttulatus* had a significantly higher mean survival (55%) when fed on *Tisbe* than newly hatched *Artemia* (33%) or two other copepods (0 & 8.8%). *H. reidi* also had higher mean survival on *Tisbe* (26.6 & 7.6 %) than newly hatched *Artemia* (4.9 & 0.3 %) or rotifers (1.6%). This difference was not significant in the second experiment however. *H. kuda* had a higher mean survival (61%) when fed on enriched rotifers than newly hatched *Artemia* (1%), *Tisbe* (5.3%) or rotifers fed on algae (4.4%). A summary of all the results is given in Table 3:29.

Table 3:29. Summary of feeding trials carried out on *Hippocampus* spp. juveniles. NE is the number of experiments

***Hippocampus* species**	**NE**	**Treatments**	**Response Variable**	**Preferred Food with average survival**
H. guttulatus	1	Newly hatched *Artemia*, *Tisbe battagliai*, *Tigriopus brevicornis* and *Eurytemora velox*	Survival to day seven	*Tisbe battagliai* (55.5%)
H. kuda	5	*Tisbe battagliai*, newly hatched *Artemia* and *Brachionus plicatilis*	Survival to day seven	*Brachionus plicatilis* (61%) (enriched on Selco 2000)
H. reidi	1	*Tisbe battagliai*, newly hatched *Artemia* and *Brachionus plicatilis*	Survival to day seven and day ten	*Tisbe battagliai* (26.6)
H. reidi	1	*Tisbe battagliai* and newly hatched *Artemia*	Survival to day ten	*Tisbe battagliai* (7.6%) (not significantly different)
H. fuscus	2	*Tisbe battagliai*, newly hatched *Artemia* and *Brachionus plicatilis*	Survival and growth to day 17	Newly hatched *Artemia* (91.6%) Enriched *Artemia* (79.2%)

Discussion

For commercial success, the culture of any marine fish requires a reliable and cheap food source. The aquaculture industry has relied primarily on brine shrimp and rotifers to provide the necessary nutrition for rearing the early life stages of fish (Marcus & Murphy, 2001). The survival of juvenile seahorses is one of the most critical aspects of seahorse aquaculture as they need a steady supply of fresh, live foods with the correct nutritional profile, size, and behaviour. The initial food must stimulate the seahorse to feed, be palatable and contain all the nutrients the growing juveniles need. Seahorses require relatively large amounts of live feed, up to 15% of

their body weight per day (Forteath, 1996; Giwojna & Giwojna, 1999). *Artemia* are used widely to feed seahorses, because they are easily cultured and are commercially available, but are not a natural prey item for seahorses. Also, it has been reported that seahorse species such as *H. subelongatus* have difficulty in assimilating this feed (Payne & Rippingale, 2000).

The copepods *Tigriopus brevicornis* and *Eurytemora velox* were found not to be suitable for *H. guttulatus*. *Tigriopus brevicornis* are harpacticoid copepods found in splash pools along the European coast line and can survive a range of salinities and temperature. They are also bright red in colour so should be visible to the juvenile seahorses. However, their thick exoskeleton and the size range given in the current project may make them difficult to digest even though juveniles were seen striking at *Tigriopus* in the experiment. Other authors have found that *Tigriopus* are successful for larval culture. *Tigriopus japonicus* were used to grow the giant croaker *Nibea japonica* by Ide *et al.,* (1998) and the grouper *Epinephelus-septemfasciatus* by Kitajima *et al.,* (1991). *Eurytemora velox* are calanoid copepods and they are also found in splash pools. They are mainly pelagic and can withstand a range of salinities and temperatures. *E. velox* were found to give significantly higher survival than enriched *Artemia* during the first 45 feeding days of halibut larvae *Hippoglossus hippoglossus* by Shields et al., (1999). There was some survival (8.8%) with *E. velox* to day seven as a diet for juvenile *H. guttulatus*, but it was significantly lower than juveniles fed on newly hatched *Artemia* or *Tisbe*.

The introduction of *Tisbe holothuriae* nauplii seemed to have an appetite-stimulatory effect in trials with turbot larvae (Cutts, 2003) and with their zig-zag movement they

are easily spotted by the seahorses. Harpacticoids copepods are also rich in essential fatty acids (EFA) such as docosahexaneoic acid (22:6n-3, DHA), eicosapentaneoic acid (20:5n-3, EPA) and arachidonic acid (20:4n-6, ARA), which are required for normal development in several marine species (Cutts, 2003). As mentioned in the introduction *Artemia* have low levels of docosahexaneoic acid (22:6n-3, DHA) and eicosapentaneoic acid (20:5n-3, EPA).

Many species of copepods have been cultured for toxicology laboratories and recently for their use as a live feed in larviculture. Payne (2001) cultured a calanoid copepod *Gladioferens imparipes* for the rearing of *H. subelongatus*. He found that *G. imparipes* as a first food gave significantly better length, wet weight and survival than Super Selco enriched *Artemia*.

H. kuda were found to have a 97% survival by Job *et al.,* (2002) when fed on wild caught plankton for the first ten days of sizes 210 to 500μm. The juvenile *H. kuda* used in the experiments in the current project had very low survival on *Tisbe* nauplii and copepodites (5.3%) and newly hatched *Artemia* (1%). They had a high mean survival rate when fed on enriched *Brachionus plicatilis* which is much smaller (80-150μm) than the plankton used by Job *et al.,* (2002). A reason for the differences in preference could be that *H. kuda* covers a wide geographical area and each small geographical population could have adapted to specific food items. Also the enrichment of the rotifers with Selco 2000 may have increased the levels of EFAs available to the juvenile seahorses, thus increasing their survival.

FishVits® (Zoolife, London UK) is a commercial enrichment used by Seahorse Ireland to increase the nutritional value of newly hatched *Artemia*. Newly hatched *Artemia* are unable to ingest food but FishVits® is thought to be absorbed by the *Artemia* as they respire (Geech, pers. comm.). *H. kuda* juveniles in the current project ingested the newly hatched *Artemia* but still did not survive to day seven. *H. kuda* juveniles were observed to have newly hatched *Artemia* still alive in their guts when some were siphoned from the bottom of the tank. *H. kuda* had a mean survival of 0.6% but this increased to 61% when fed on enriched rotifers. The response variable of ingestion is not as helpful in understanding the needs of a juvenile seahorse as regards survival rates. Even if the prey is the correct size and shape, it has to be digestible and nutritious for the seahorse juveniles to survive.

Hippocampus reidi are very large and extremely colourful, thus creating a huge market demand for them. The male of this species can have over 2000 juveniles (Authors obs.) every month, but they are very small and few researchers have been able to grow them in captivity. In the *H. reidi* experiment A in this chapter, *Tisbe battagliai* gives significantly higher mean survival (26.6%) than newly hatched *Artemia* (4.9%) or *Brachionus plicatilis* (1.6%). In experiment B, *Tisbe* still gave higher mean survival (7.6%) than newly hatched *Artemia* (0.3%) but it was not significantly higher. It is not known why there was a difference in survival rates between the two experiments. Other factors such as tank size, amount of light, turbulence or air flow may have been involved. Some of the juveniles from these experiments survived until week 6 on a mixture of *T. battagliai* and newly hatched *Artemia*, and then died, possibly due to a bacterial infection or another crucial change in feeding preferences.

In the experiments conducted in this chapter, *H. fuscus* juveniles were the only species to have significantly higher survival rates when grown on newly hatched *Artemia* compared to other alternative diets. In the same experiment, *H. fuscus* were fed on enriched *Artemia* (A1-DHA Selco), but there was no significant difference in survival or weight gain. This is in contrast to other authors and could be due to the levels of EFAs in this enrichment.

Survival may be inadequate as a measure of success in longer term experiments. This was shown with juvenile *H. fuscus* when growth was also used as a response variable. Although there was no significant difference in survival of the juveniles fed on three diets, there was a significant difference in the weight of the juveniles at day 14 and 17. The juveniles fed on *Tisbe battagliai* did not appear to grow between day 14 and 17.

The results show that no single feed has been found to be suitable for rearing all of the seahorse species: a range of species were found to be required to provide feed of different sizes and attractiveness. The differences in food preference could be due to several factors including size, visibility, speed and the way they swim. The striking and ingestion of a food item still does not guarantee that the juvenile seahorse will survive. The food items need to be easily digestible and nutritious.

In this investigation the results for *H. guttulatus* and *H. reidi* suggest that *Tisbe battagliai* is a viable alternative and is more successful in the commercial cultivation of these species than newly hatched *Artemia* for the first feeding stage. Many factors contribute to survival of the juveniles including light intensity, air flow and water

movement. Only when all these factors are investigated will mass cultivation of *Hippocampus* spp. commence for this most difficult to rear species. Mortalities associated with the commencement of first feeding continue to be a major obstacle facing the potential commercial culture of seahorses (Filleul, 1996). With an increase in research of different species, it will be possible to create a list of diets and their success for first feeding seahorse juveniles.

Chapter 4

Experiments on the culture of the copepod,

Tisbe battagliai

Section 1

Introduction

Copepoda are a class of animals within a larger group, the Phylum Crustacea. Copepods occur in most bodies of marine and fresh water. Many are parasitic, some swim freely as part of the plankton while others are benthic (bottom dwelling). There are three major groups of copepods; the Calanoida, mainly free swimming planktonic animals, the Cyclopida, which may be planktonic or demersal and the Harpacticoida, which are almost entirely benthic (Rippingale & Payne, 2001).

Copepods pass through very distinct life history stages. The female of species such as *Tisbe battagliai* carries the eggs in an egg sac, from which the nauplius emerges. After 6 nauplius stages, with growth between each stage, the body shape changes and a series of usually 6 copepodid stages follow (Miliou *et al.,* 1992). The last of these is the adult stage in which the separate sexes can be identified. Reproduction is sexual. Following sexual maturity, females mate once, and produce up to 12 broods (depending on species) over their lifetime (Hoff & Snell, 2004).

Copepods are the most dominant group in the zooplankton (Alcaraz, 1997). In the sea, the potential food items most likely to be encountered by fish larvae are the nauplius stages of calanoid copepods. For many older fish, adult copepods are very important diet items. Copepods have probably been important in the diet of many marine fish during their evolution and effective predation strategies have evolved for their capture (Rippingale & Payne, 2001).They have a nutritional composition that matches the nutritional requirements of marine finfish larvae, particularly with regard to fatty acids (McKinnon *et al.,* 2003).

The advantages of copepods for culture are high reproductive potential, short turnover time (from egg to egg) and fast individual and population growth rates. Other advantages are that they will have good growth on a variety of food sources and tolerate a wide range of environmental factors such as temperature and salinity (Cutts, 2003).

Copepods are routinely grown on a mixture of algae (Hoff & Snell, 2004) and previous researchers have looked at the ingestion, fecundity, growth rates and culture of copepods on algae in the laboratory (Lee *et al.,* 1985; Miliou & Moraitou-Apostolopoulou, 1991; Lee & Yan, 1994; Abu-rezq *et al.,* 1997; Lacoste *et al.*, 2001; Pinto *et al.,* 2001; Murray & Marcus, 2002; Thor *et al.,* 2002). Data on the amount of food required by copepods for maximum production in culture systems are generally lacking. The use of faecal pellet production over 24 hours by a single copepod can be used to determine maximum ingestion rates of algae. The maximum ingestion rates can then be related to optimum algae density in a culture system. Lacoste *et al.,* (2001) found, in experiments with the calanoid copepod *Calanus helgolandicus* Claus

1863, that daily production of faecal pellets varied with the concentration of the diet. Lacoste *et al.,* (2001) set out to eliminate the problem related to food limitation of copepods by feeding the females ad libitum. They then compared the effect of single and mixed diets using starved females as controls. The dinoflagellate *Prorocentrum minimum* was well utilised by these copepods, as indicated by a mean faecal production above 45 pellets female $^{-1}$ day^{-1}. The diatom *Phaeodactylum tricornutum*, the chlorophyte *Dunaliella tertiolecta* and the prymnesiophytes *Isochrysis glabana* and *Pavlova lutherii* gave pellet productions of $\leq$ 8 pellets female -1 day $^{-1}$ at 10^4 cells ml^{-1}. A mixture of these diets gave >40 pellets female $^{-1}$ day $^{-1}$. From these experiments Lacoste *et al.,* (2001) assumed that 10^4-10^5 cells ml^{-1} was a satisfactory concentration of cells, allowing females to reach food saturation.

Tisbe battagliai Volkmann-Rocco, 1972 are harpacticoid copepods. They possess a nauplius larva, which, after embryonic development, hatches from the egg sac directly onto the substratum inhabited by the adults (Hicks & Coull, 1983). *Tisbe* spp. demonstrate high rates of juvenile survival and development, possess relatively short generation times and sustain high levels of fecundity (Williams, 1997). *Tisbe battagliai* can be grown successfully in large cultures and *Tisbe* spp. have been used successfully in the culture of halibut larvae (McEvoy *et al.,* 1998), haddock larvae (Nanton & Castell, 1998) and larval Dover sole (Heath & Moore, 1997 cited in Cutts, 2003).

The aim of this chapter was to culture *Tisbe battagliai* successfully in a cheap, efficient and convenient way. The response variables to determine success in the culture experiments were:

- Survival to day seven
- Number of fertile females
- Development rate
- Number of nauplii in the first brood
- Mean number of broods
- Mean number of nauplii produced by each female over life time
- Mean number of nauplii in each brood
- Life expectancy
- Faecal pellet production in 24 hours

Success would also require that the copepod would be nutritionally adequate for the seahorse diet.

Materials and Methods

Tisbe battagliai

The *Tisbe* used in this study were sourced from Shannon Toxicology laboratory which in turn sourced them from Guernsey in January 2001.

Analysis of Results

Data were checked for normality using a Ryan-Joiner normality test. If the data were normal then analysis was carried out using a one way ANOVA. Some results in section 7 required the use of a two-way ANOVA.

If there was a significant difference in the results, the data were analysed using Fisher's one way multiple comparisons. When viewing the Fishers test results, two negative values indicated the column level was significantly lower than the row level. If both values were positive, then the column level was significantly higher than the row level. If one value was negative and one was positive then the column and row levels were not significantly different.

If the data were not normally distributed the data were analysed with the non-parametric Kruskal-Wallis test.

All statistical analysis was carried out by MINITAB version 13.31.

Section 2

Counting Copepods

Adult Copepod Counting

For small experiments it was acceptable to count the number of individuals with a glass pipette, but for counting larger populations a fast and reliable method of sub-sampling was required.

Materials and Methods

The adult copepods to be counted were concentrated onto a 100μm sieve and then washed into a beaker with filtered seawater. The beaker was topped up with saltwater and the amount of water recorded. A number of counting methods were tried and the one adopted. The adult copepods in the known quantity of water were poured between two containers so that the adults were evenly distributed throughout the water column. A 5ml plastic pipette with the top removed was used to collect a 2ml sample and this was emptied into a counting tray. The counting tray was viewed under a microscope and the copepods counted. The counting trays were obtained from Lennox laboratories (12 well tissue culture plates). The actual counts were made by collecting and counting all of the copepods individually with a glass pipette.

Results

The precision of the counting method is variable as the results show in Table 4:1; three sample counts were each compared with a total count. The estimated totals of counts B and C came within the 95% confidence levels estimated from the sub-sampling method. Count C, had an estimated total which was only 2.5 copepods outside of the 95% confidence levels. This method was adopted for the counting of all future experiments.

Table 4:1. Counting method for adult *Tisbe battagliai* showing 95 % confidence levels. S1-S6 are the sub-sample numbers.

Count	S1	S2	S3	S4	S5	S6	Estimated Total ± 95% confidence levels	Actual Total
A	20	23	20	25	22	19	5375 ± 1106.5	6483
B	1	3	4	2	3	1	116.6 ± 118.75	101
C	2	4	8	5	1	8	233.3 ± 288.5	375

Nauplii Counting

Nauplii were difficult to count. For some experiments, nauplii were individually collected with a glass pipette. For other experiments, the number of nauplii produced by female copepods was required. Lugols solution (an iodine solution for staining) was then added to the water in a round counting chamber or in a petri dish, which killed the nauplii, making them easier to identify and count.

Population Counts from Hoppers

Tisbe battagliai were cultured in hoppers in Seahorse Irelands facility for use in juvenile first feeding experiments as discussed in Chapter 3. Every week, each hopper was emptied through the bottom valve and the population was counted. It was noticed that if all nauplii were drained from a hopper and only copepods above 150μm were retained, copepodites and large nauplii were found the next day. An investigation was carried out to see if all *Tisbe battagliai* were harvested by emptying the hoppers.

Materials and Methods

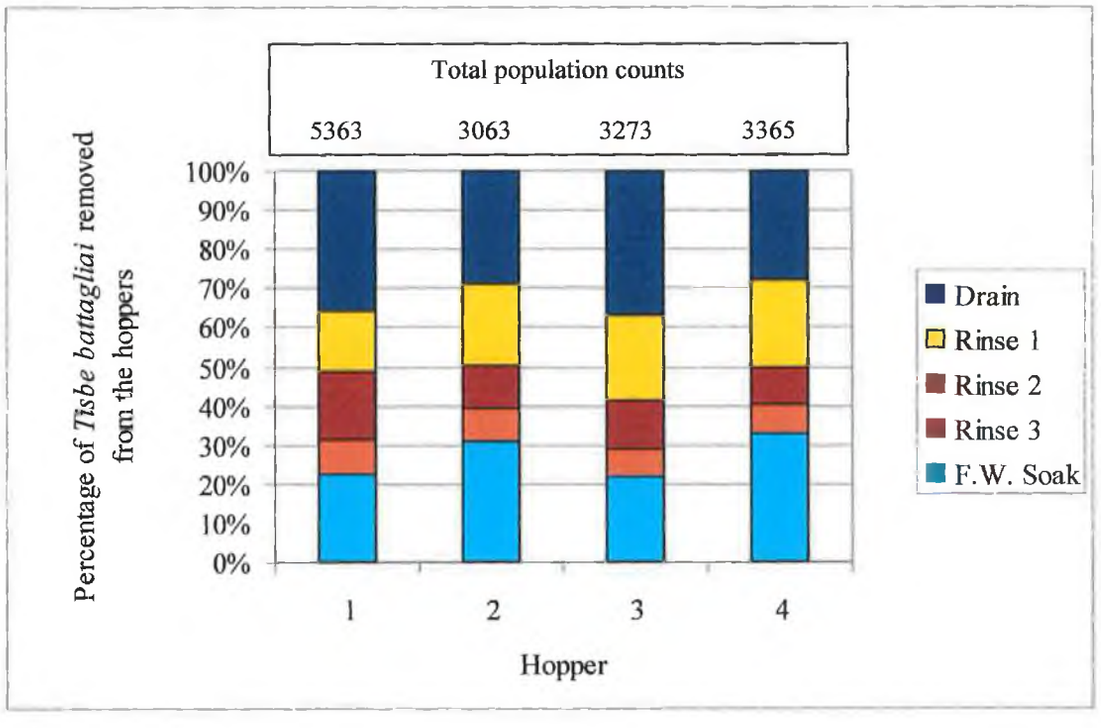
The hoppers were emptied and counted according to the procedures outlined in chapter 2. Following this first count, three further rinses with saltwater, each followed by a count, were carried out. Then each hopper was filled and rinsed with freshwater, and the remaining adults counted. For each hopper a total count was estimated by adding up the sum of the five counts.

It was assumed that by adding the freshwater to the hoppers, all the copepods were killed and removed.

Results

The results of the five counts expressed as a percentage, are given in Figure 4:2.

Figure 4:2. Numbers of the copepod *Tisbe battagliai* (expressed as a percentage of the total population) which are removed when the hopper is emptied, rinsed three times and given a freshwater rinse. The total counts are also shown, above each column.



An average of 32.33 ± 4.02 % of the population was removed when the hoppers were initially emptied. An average of 27.03 ± 4.9 % of the total population still remained in the hoppers after they were emptied and rinsed three times with saltwater. This is a large percentage of the population and will have to be taken into consideration when interpreting experimental results. *Tisbe* are benthic in nature and feed on the bio film which develops on the side of the tanks and on any additional substrate placed in the tanks. They also cling onto the surface of any substrate (pers. obs.).

Section 3

Short term survival of *Tisbe battagliai* on alternative diets

Aim of Investigation

The aim of this investigation was to find out what alternative diets would give best short term survival for *Tisbe battagliai*.

Materials and Methods

A number of alternative diets were identified as potential feeds for production of *Tisbe battagliai*. Formulated diets such as SMA Gold milk, chicken feed, bakers yeast, kelp tablets and *Spirulina* tablets, would be advantageous in the production of copepods as it would reduce the labour costs and problems of culture contamination and collapses associated with algal culture.

A novel formulated feed (named NFF from here on) was made from a mixture of 240ml tomato juice, 10g enriched brewers yeast, 5ml flax seed oil, 2ml liquid vitamin complex, 1ml liquid vitamin C and 1l of saltwater (kind permission of A. Rhodes). 0.5ml of this feed was used when needed in the experiments and the rest was frozen.

The SMA Gold Milk® (called SMA milk from here on) was designed for first feeding of infants. It is widely available, quality assured and relatively inexpensive. A suspension was made up by adding 0.5g of the SMA milk to 100ml of saltwater. Then 0.5ml / 0.2ml of this suspension was added as food to the experiments when required.

Yestamin® brewers yeast (known as yeast from now on) is readily available in health food stores. A suspension was made up by adding 0.5g of the yeast to 100ml of saltwater. Then 0.2 ml of this suspension was added as food to the experiments when required.

Chicken feed was obtained from a chicken farm. It is widely available in large quantities. A suspension was made up by adding 0.5g of the chicken feed to 100ml of saltwater. Then 1ml of this suspension was added as food to the experiments when required.

Kelp tablets are widely available in health food stores. A suspension was made up by crushing the tablet and adding it to 100ml of saltwater. Then 0.3 ml / 0.2ml of this suspension was added as food to the experiments when required.

Spirulina tablets are widely available in health food stores. A suspension was made up by crushing the tablet and adding it to 100ml of saltwater. Then 0.2 ml / 0.1ml of this suspension was added as food to the experiments when required.

Methods, Preliminary food selection trails:

Nauplii born within the previous 24 hours were counted and placed in coded petri dishes. Water filtered to 1μm and sterilised with UV lights was added. Then the appropriate diet was added to the coded petri dishes. At the end of the experiment, the remaining copepods were collected individually with a glass pipette and the numbers recorded. These preliminary experiments ran for 7-10 days.

Results

The results of two ten day, survival experiments when *Tisbe battagliai* were fed on a NFF, SMA milk or a mixture of algae are shown in the Table 4:3.

Table 4:3. Results from ten day survival trials when *Tisbe battagliai* are grown on alternative diets or a mixture of algae. N is the number of nauplii in each petri dish and NR are the number of replicates of each treatment.

N	NR	Experimental Diet	Number of nauplii to survive
20	1	NFF (0.5ml)	14 (70%)
		SMA Milk (0.5ml) (0.5g in 100ml seawater)	15 (75%)
10	1	NFF (0.5ml)	7 (70%)
	1	SMA Milk (0.5ml) (0.5g in 100ml seawater)	7 (70%)
	2	Algae mix (1ml) (approx 6.41 x 10^6 cells)	8 (40 %)

The mean percentage survival was 70% when fed on the NFF for the two experiments and 70 & 75% respectively when grown on SMA milk. The *Tisbe* grown on a mixture of algae had mean percentage survival of 40 & 85% respectively, in the two experiments. Statistical analysis was carried out to see if there were significant differences between the survival rates. For analysis, the results from the second experiment were multiplied by two and the results of the two trials were combined.

The data were not normally distributed and were therefore analysed using a Kruskal-Wallis test for non-parametric data.

Hypotheses:

Ho: There is no significant difference in short term survival of *Tisbe battagliai* fed on a NFF, SMA milk or a mixture of algae.

HA: There is a significant difference in short term survival of *Tisbe battagliai* fed on a NFF, SMA milk or a mixture of algae.

The critical value is taken from chi-squared tables.

Critical value (df=2; p=0.05) =5.99

Decision: Calculated H value (4.19) is less than critical value (5.99). Therefore accept Ho.

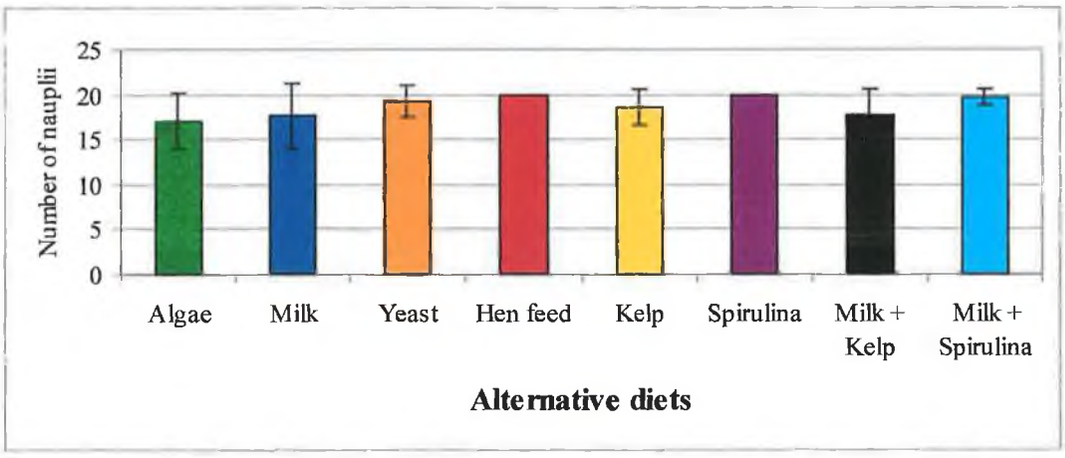
Conclusion: The results of the Kruskal-Wallis show that there is no significant difference in survival to day ten when *Tisbe battagliai* nauplii are fed on a NFF, SMA milk or a mixture of algae.

The results of a seven day survival trial on *Tisbe battagliai* nauplii when fed on SMA milk, yeast, chicken feed, kelp, *Spirulina*, kelp + *Spirulina*, SMA milk + *Spirulina* or a mixture of algae are shown in Table 4:4 and Figure 4:5.

Table 4:4. Results from short term survival trials when *Tisbe battagliai* are grown on alternative diets or a mixture of algae. N is the number of nauplii added to each petri dish and NR is the number of replicates of each treatment.

N	NR	Experimental Diet	Survival
20	5	SMA Milk (0.2ml) (0.5g in 100ml seawater)	88 (88%)
		Yeast (0.2ml) (0.5g in 100ml seawater)	96 (96%)
		Chicken feed (1ml) (0.5g in 100ml seawater)	100 (100%)
		Algae mix (1ml) (approx 6.41 x 10^6 cells)	85 (85%)
		Crushed kelp tablet in 100ml seawater (0.3ml)	93 (93%)
		Crushed *Spirulina* tablet in 100ml (0.2ml)	100 (100%)
		Kelp (as above) (0.2ml) & *Spirulina* (as above) (0.1ml)	88 (88%)
		SMA Milk (as above) (0.2ml) & *Spirulina* (as above) (0.2ml)	98 (98%)

Figure 4:5. Mean number of twenty *Tisbe battagliai* nauplii to survive to day seven when fed on a number of alternative diets with standard deviation lines. Standard deviation was zero for chicken feed and *Spirulina*.



The survival to day seven when *Tisbe battagliai* nauplii were fed on SMA milk, yeast, chicken feed, kelp, *Spirulina*, kelp + *Spirulina*, SMA milk + *Spirulina* or a mixture of algae results in an overall mean survival of 93.5%. *Tisbe battagliai* nauplii fed on chicken feed or crushed *Spirulina* achieved 100% survival to day seven. Survival rates dropped to 98% for *Tisbe* fed on a mixture of SMA milk and crushed *Spirulina*, 96% for a yeast diet and 93% for crushed kelp tablet diet. SMA milk on its own as a diet achieved an 88% survival rate and a mixture of kelp and *Spirulina* gave a survival to 88%. The lowest mean survival percentage was 85% for *Tisbe battagliai* fed on a mixture of algae.

The data were not normally distributed and a Kruskal-Wallis test was carried out.

Hypotheses:

Ho: There is no significant difference in short term survival of *Tisbe battagliai* fed on SMA milk, yeast, chicken feed, kelp, *Spirulina*, kelp + *Spirulina*, SMA milk + *Spirulina* or a mixture of algae.

Ha: There is a significant difference in short term survival of *Tisbe battagliai* fed on SMA milk, yeast, chicken feed, kelp, *Spirulina*, kelp + *Spirulina*, SMA milk + *Spirulina* or a mixture of algae.

The critical value is taken from chi-squared tables.

Critical value (df=7; p=0.05) =14.07

Decision: Calculated (10.46) is less than critical value (14.07). Therefore accept Ho.

Conclusion: The results of the Kruskal-Wallis show that there is no significant difference in survival to day seven when *Tisbe battagliai* nauplii are fed on SMA

milk, yeast, chicken feed, kelp, *Spirulina*, kelp + *Spirulina*, SMA milk + *Spirulina* or a mixture of algae.

Summary of results of short term experiments

The results above show that the use of a wide range of diets will allow short term survival of *Tisbe battagliai.* They can survive on SMA milk, bakers yeast, chicken feed, crushed kelp tablets, crushed *Spirulina* tablets as well as on a mixture of algae. A longer investigation would be required to find out if they can grow and reproduce through several generations on these diets.

Section 4

Long term survival experiments on alternative diets:

Introduction

Tisbe and other copepods are normally cultured on micro algae. Five diets were selected for long term survival experiments: an algal mix of *Chaetoceros calcitrons, T-Isochrysis galbana, Tetraselmis suecica,* NFF, chicken feed, SMA milk and brewers yeast. NFF (as above) was made from tomato juice, enriched brewers yeast, flax seed oil, liquid vitamin complex and liquid vitamin C. It was frozen into plastic bottles and defrosted when required. These diets were chosen because of their success in the short term survival experiments. The formulated diets are also quality controlled, very convenient, widely available and relatively inexpensive. Kelp and *Spirulina* were not used in these experiments as they are more expensive.

Aim of Experiment

The aim of the experiment was to determine whether *Tisbe battagliai* could reproduce through several generations successfully on diets other than micro-algae.

Materials and Methods

Twenty nauplii, born within the previous 24 hours, were placed in 25 water bottles each with 100 ml of seawater filtered to 1μm. The water bottles had been soaked overnight with a mild concentration of bleach and were then rinsed and left to dry. They were then randomly allocated to a diet with each diet having five replicates. The quantities of food in each diet are shown in Table 4:6.

Table 4:6. The quantities of food added into each bottle for the long term survival experiment.

Diet	Made up of	Amount given to each bottle
Algae	0.5mls of three algae	1.5ml (9.6×10^4 cells ml^{-1})
Milk	0.5g in 100ml in seawater	1ml
Yeast	0.5g in 100ml in seawater	1ml
Chicken Feed	0.5g in 100ml of seawater	1ml
NFF	Defrosted from original stock	0.5ml

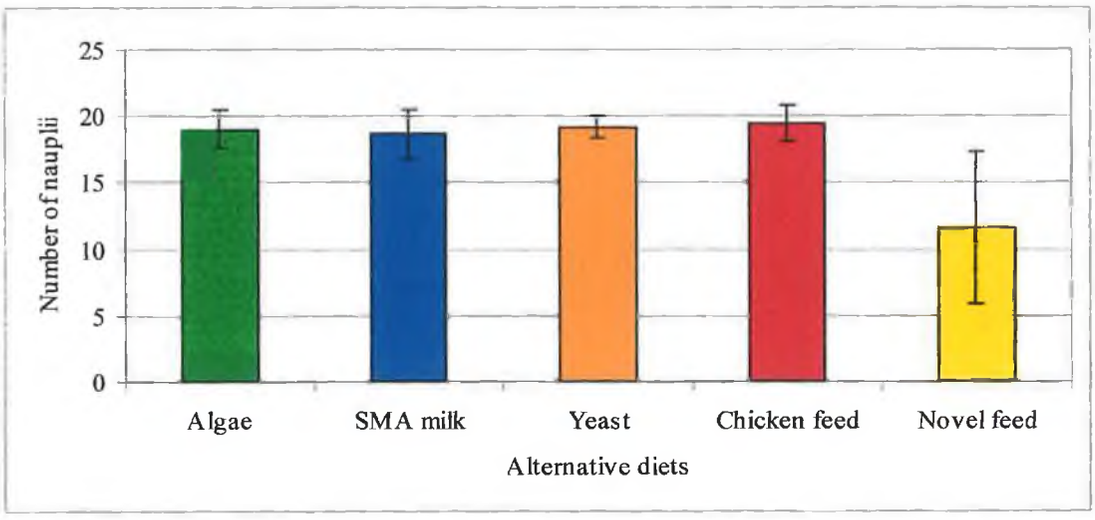
The populations were counted at day four to check short term survival. All treatments were fed, checked and their water replaced every week. Every second week the bottles were checked for egg bearing females and newly released nauplii. Once a large population of newly released nauplii were found, the five replicates from one treatment were concentrated onto two sieves of 50 and 150μm. The nauplii which

were retained on the 50μm sieve were then backwashed into a petri dish. 30 nauplii were then removed and placed into each of the five replicate bottles. This was repeated for all treatments. Codes were used to ensure that the replicates were fed the same food throughout the 10 generations in the experiment.

Results

The results of the survival to day four of *T. battagliai* fed on an algal mix, SMA milk, yeast, chicken feed, and NFF can be seen in Figure 4:7.

Figure 4:7. Short term survival of *Tisbe battagliai* fed on a mixture of algae, SMA milk, yeast, chicken feed or a NFF to day four showing standard deviation lines.



Mean survivals were all similar apart from those fed on NFF. Data were checked to see if this drop in survival of nauplii on NFF was significantly different from the survival of nauplii on other diets.

Data were found to be normally distributed and a one way ANOVA was carried out.

Hypotheses:

Ho: There is no significant difference in the short term survival of *Tisbe battagliai* when fed on a mixture of algae, SMA milk, yeast, chicken feed or a NFF.

Ha: There is a significant difference in the short term survival of *Tisbe battagliai* when fed on a mixture of algae, SMA milk, yeast, chicken feed or a NFF.

Critical value (df=2; 20; p=0.05) =3.49

Decision: Calculated F (6.89) is greater than critical F (3.49). Therefore reject Ho.

Conclusion: There is a significant difference in the short term survival of *Tisbe battagliai* when fed on a mixture of algae, SMA milk, yeast, chicken feed or a NFF.

Form the results of the Fishers test, we can say that *Tisbe battagliai* fed on the NFF have a significantly lower survival rate than any of the other treatments to day four. This is in contrast to the earlier short term survival tests that found there was no significant difference in these diets to day seven.

Discussion

After 5 months, all treatments had reached generation 10 and so the experiment ended. The results show that the original nauplii survived, moulted successfully to adults, reproduced and produced eggs. The next generations then hatched, survived, moulted, reproduced and had eggs successfully. A complete life history was achieved on all diets. All diets caused *T. battagliai* to lose its colour apart from those fed on a mixture of algae. The implications of this colour loss are discussed later. It would have been interesting to note if their life history characteristics had changed after being fed on these diets for so many generations, however, due to time constraints this was not possible. The reason for the low survival in the NFF to day four in the present

experiment contrasts with the results from the previous one and may be due to the freezing of the diet from the original stock which was used in the short term survival experiments.

Summary

Tisbe battagliai were able to reach generation F10 on a range of diets other than a mixture of algae. This proves that *T. battagliai* can survive and reproduce in a small culture, over a long period, on varied diets.

Section 5

Medium Scale Cultures

Aim of Experiment

The aim of this experiment was to investigate if SMA milk, one of the diets tested in section 3 & 4 above, could be used as successfully as a mixture of algae in medium scale systems (5ltr) for growing *Tisbe battagliai.*

Materials and Methods

Tisbe battagliai were harvested from the tray culture system in Seahorse Ireland (see chapter 2). The adult population above 200μm were concentrated onto petri dishes and viewed under the microscope. Egg bearing females were collected from the petri dishes with a glass pipette. 30 were then added to each of six five litre water bottles.

Each five litre container had three litres of seawater added. This had been filtered to 1μm with the temperature of the seawater being approximately 18°C. The containers were then randomly assigned to a diet, three being assigned to the mixture of algae already being used to grow the copepods in the main system, *Chaetoceros calcitrons, T-Isochrysis galbana* and *Tetraselmis suecica,* and three being assigned to the alternative diet of SMA milk.

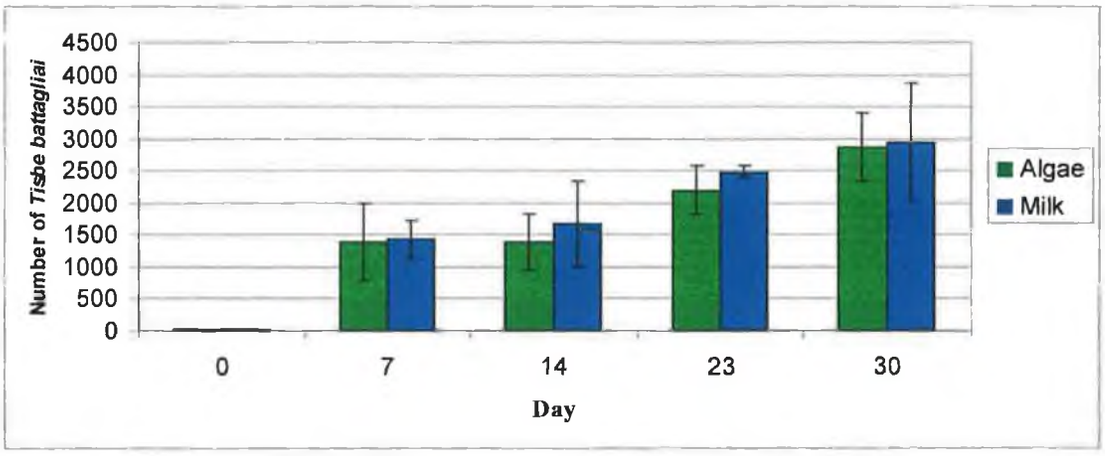
The containers selected to be grown on algae were given 50mls of an equal mix of *Chaetoceros calcitrons, T-Isochrysis glabana* and *Tetraselmis suecica* (approximately 3.2×10^8 cells container^{-1} [1.06×10^5 cells ml^{-1}]). The containers selected to be grown on SMA milk were given 10mls of a suspension of the SMA milk. This suspension was made up by adding 0.5g of SMA milk to 100mls of seawater which had been filtered to 1μm.

The experiments had a water change once a week and were fed twice a week. During the water change, the populations were counted. The experiment ran for 30 days. Survival was assessed at day 30.

Results

The population counts of *Tisbe battagliai* when fed on SMA milk or a mixture of algae over 30 days are shown in Figure 4:8.

Figure 4:8. The mean population counts of three replicate cultures of *Tisbe battagliai* fed on a mixture of algae and three replicate populations of *Tisbe battagliai* fed on SMA milk over 30 days.



The growth rates of the *Tisbe* fed on a mixture of algae and SMA milk were very similar over the 30 days. The populations of *Tisbe* reached on average 2867 ± 536 when fed on a mixture of algae and 2955 ± 915 when fed on SMA milk after 30 days.

The five litre bottles which were fed SMA milk were difficult to harvest as the excess milk clogged the 50μm sieve which collected the nauplii when the water was changed.

The data for survival to day 30 were analysed to see if there was a significant difference in the populations when fed on a mixture of algae or SMA milk.

Data were found to be normally distributed and a one way ANOVA was carried out.

Hypotheses:

Ho: There is no significant difference in the population numbers to day thirty when fed on SMA milk or a mixture of algae.

Ha: There is a significant difference in the population numbers to day thirty when fed on SMA milk or a mixture of algae.

Critical value (p=0.05; df=1, 4) =7.71

Decision: Calculated F (0.02) is less than critical F (7.71). Therefore accept Ho.

Conclusion: There is no significant difference in the population numbers of *Tisbe battagliai* to day thirty when fed on SMA milk or a mixture of algae in medium scale culture.

Discussion

When *Tisbe battagliai* are grown in 5 ltr jars there are no significant differences in the population numbers at day thirty when fed on a mixture of algae or SMA milk. The growth rate is also similar when the *Tisbe* are fed on the two diets as can be seen in Figure 4:8. In the thirty days, the original egg bearing females could have had up to six broods (see life studies in section 7). The nauplii from these broods could also have had a number of broods within the thirty days. This would explain the 100% increase in the population. SMA milk was just as successful in medium scale culture as a mixture of algae.

SMA milk is very easily obtained, quality controlled and readily available. In this medium scale (5ltr) production, milk would therefore be a cheaper, more convenient and more reliable food source for *Tisbe battagliai* than a mixture of algae. The copepods do however lose their colour when grown on milk. The implications of such colour loss are discussed later.

In section 4, SMA milk was shown qualitatively to allow *T. battagliai* to reproduce to generation ten. In this medium scale culture experiment, it is shown quantitatively that *T. battagliai* grown on SMA milk can pass through a number of generations and produce the same population sizes as those grown on a mixture of algae.

Section 6

Large scale cultures

Aim of Experiment One

The aims of this experiment were to establish if SMA milk and yeast could be used instead of a mixture of algae in the culture of *Tisbe battagliai* in a hopper system (30 ltr).

Materials and Methods

Tisbe battagliai were cultured as described in chapter two. There were nine hoppers so three were assigned to SMA milk, three were assigned to a yeast and three were assigned to a mixture of algae.

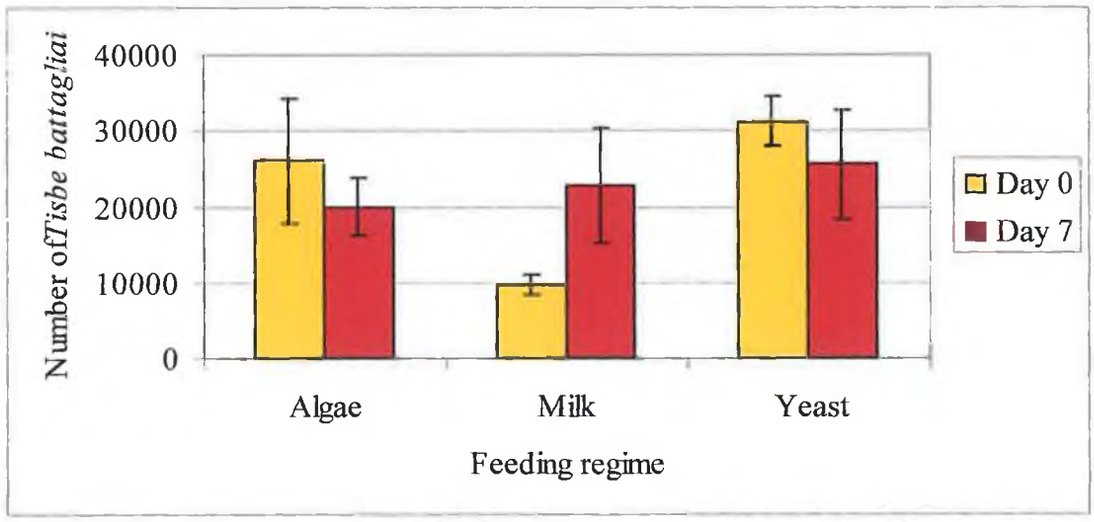
All hoppers had previously been fed on an equal mix of *Chaoetceros calcitrons, T-Isochrysis glabana* and *Tetraselmis suecica.* The hoppers were harvested and counted by the method described in chapter 2 at the beginning and end of the experiment.

One litre of a mixture of algae (6.4×10^9 cells or 2.1×10^5 cells ml^{-1}), 1g of SMA milk and 1 g of yeast were added to the allocated hoppers on day 0. On day 4, the hoppers were fed again with 1ltr of algae mix, 0.5g of milk and 0.5 g of yeast. On day 7 the hoppers were emptied to allow for counting.

Results

The populations in the nine hoppers were different at the start of the experiment (see Figure 4:9).

Figure 4:9. The mean population counts of *Tisbe battagliai* in a hopper system over seven days when fed on SMA milk, yeast or a mixture of algae, showing standard deviation lines.



The mean population of hoppers were 26,055 ± 8260 when allocated to a mixture of algae, 9,639 ± 1305 when allocated to SMA milk and 31,222 ± 3289 when allocated to yeast.

Data were found to be normally distributed and a one way ANOVA was carried out.

Hypotheses:

Ho: There is no significant difference in the population of *Tisbe battagliai* in the hoppers at day 0 of the experiment.

Ha: There is a significant difference in the population of *Tisbe battagliai* in the hoppers at day 0 of the experiment.

Critical value (P=0.05; DF= 2, 6) = 5.14

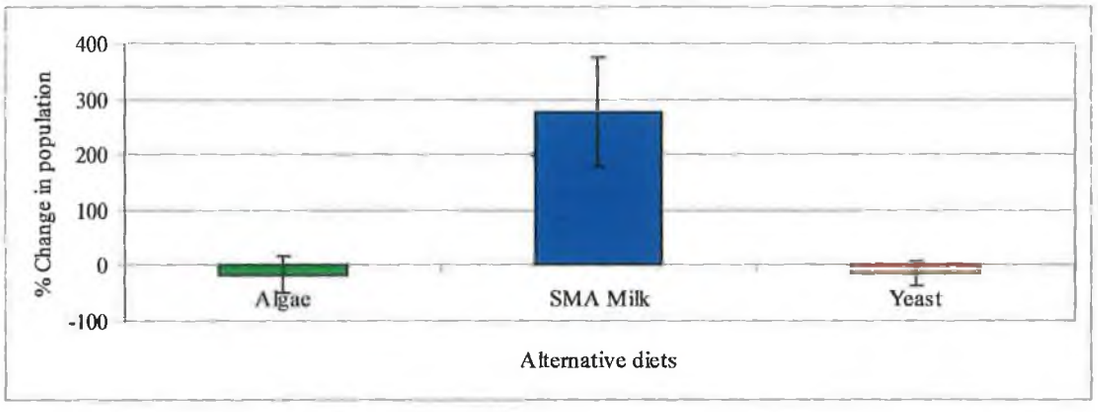
Decision: F calculated (14.16) is greater than F critical (5.14). Therefore reject Ho.

Conclusion: There is a significant difference in the population numbers at the beginning of the trial. The Fishers test tells us there is a significant difference between the population numbers in the SMA milk hoppers and the rest of the hoppers. There is no significant difference in the population numbers of the hoppers allocated to a mixture of algae or yeast. The difference between the hopper populations at the start of the experiment occurred despite the fact that hoppers were randomly assigned to a treatment. This is an unexpected phenomenon and the reason for it is unknown.

Day Seven

The copepod populations in the algae and yeast fed hoppers decreased at a very similar rate over seven days by 16.47 ± 34.19 % and 15.08 ± 21.65 % respectively. However, the copepod populations fed on SMA milk increased by on average 276.98 ± 97.26 % over the seven days. These results can be seen in Figure 4:10.

Figure 4:10. Mean percentage of change in population numbers of *Tisbe battagliai* in a hopper system when fed on SMA milk, yeast or a mixture of algae showing standard deviation lines.



No statistical analysis was carried out with the end results due to the significant difference in population counts at day 0. The numbers of *T. battagliai* in the hoppers at the start of the experiment were low for the hoppers assigned to milk and this allowed them the opportunity to increase. The numbers of *T. battagliai* in the hoppers at day 0 were high for the hoppers assigned to algae and yeast and they may not have had the opportunity to increase. This hopper experiment should be repeated.

Despite the fact that no statistical analysis was carried out, it can be clearly seen that SMA milk results in a growth of *T. battagliai* population in large scale cultures.

Although there was a slight smell from the hoppers which had formulated milk added, the harvesting was not restricted as in the small and medium scale experiments.

Experiment Two

Aim of Experiment

In this experiment, two comparisons were made. The production of *T. battagliai* grown on yeast was compared to those grown on a mixture of algae. Also, growth of a population of *T. battagliai* fed on a mixture of algae was compared to the growth of a population fed on a mixture of algae but with extra substrate added to the hoppers.

Materials and Methods

On day 0, the hoppers were drained through a 150μm sieve and then given a freshwater bath, killing the remaining *T. battagliai* in the hoppers. The nauplii and dead *T. battagliai* were disposed of. The adult *T. battagliai* retained on the sieve, were counted and divided equally among the nine hoppers. The hoppers were then randomly assigned to a diet of yeast, algae or algae with extra substrate. The extra substrate was 15cm bio-media filters from Dryden Aqua. Ltd. as described in chapter 2. All hoppers had previously been fed on an equal mix of *Chaetoceros calcitrons, T-Isochrysis glabana* and *Tetraselmis suecica.*

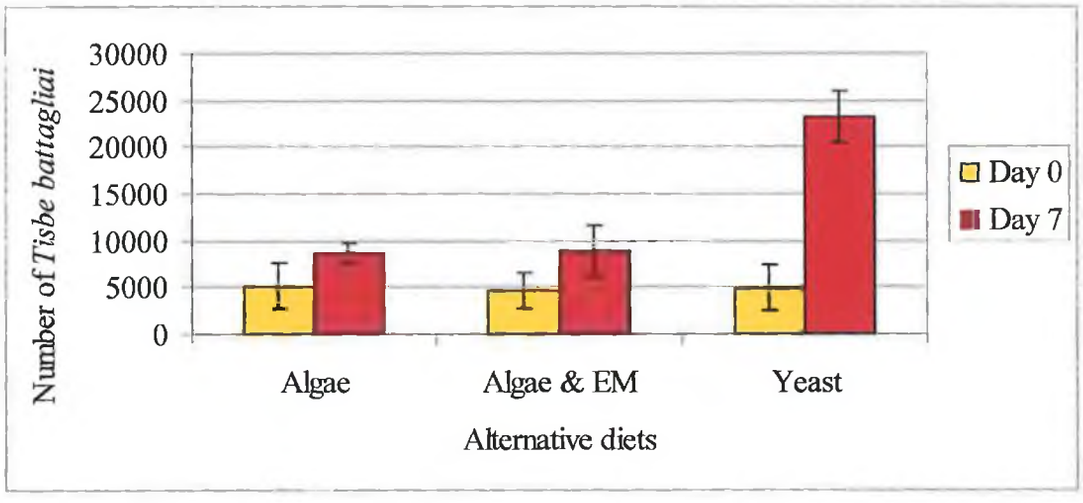
The control and extra substrate hoppers were fed 400ml (8.6×10^4 cells ml^{-1}) of the algae mix on day 0, 200ml (4.3×10^4 cells ml^{-1}) on day 2, 50ml (10.7×10^3 cells ml^{-1}) on day 3 and 300ml (6.4×10^4 cells ml^{-1}) on day 6 of the experiment. The yeast hoppers were fed 1.5g of yeast on day 0 of the experiment.

The hoppers were drained and rinsed with freshwater on day 7 so that all *T. battagliai* in the hoppers were counted.

Results

The mean population counts of the hoppers on day seven when fed on yeast, algae or algae with extra substrate are shown with standard deviation lines in Figure 4:11.

Figure 4:11. The mean population counts of *Tisbe battagliai* in a hopper system when fed on algae, algae with added substrate or yeast over seven days showing standard deviation lines. EM stands for extra substrate.



The population numbers were similar at the start of the experiment in all nine hoppers. The hoppers increased by 3562 ± 991 when fed on algae, 4334 ± 2813 when fed on algae and with extra substrate and 18,328 ± 2715 when fed on yeast. Yeast fed hoppers had the highest growth with the other treatments having similar growth.

The population numbers of *Tisbe battagliai* in the hopper system at day 0 were analysed to see if they were significantly different.

Data were found to be normally distributed and a one way ANOVA was carried out.

Hypotheses:

Ho: There is no significant difference in the population numbers of *Tisbe battagliai* in the hoppers at day 0.

Ho: There is a significant difference in the population numbers of *Tisbe battagliai* in the hoppers at day 0.

Critical value (P=0.05; DF=2, 6) = 5.14

Decision: F calculated (0.04) is less than critical F value (5.14). Therefore accept Ho.

Conclusion: There was no significant difference in the population numbers of *Tisbe battagliai* in the hopper system at day 0.

The population numbers of *Tisbe battagliai* in the hopper system at day 7 were analysed to see if they were significantly different.

Data were found to be normally distributed and a one way ANOVA was carried out.

Hypotheses:

Ho: There is no significant difference in the population numbers to day seven when fed on algae, algae with extra substrate or yeast.

Ha: There is a significant difference in the population numbers to day seven when fed on algae, algae with extra substrate or yeast.

Critical value (p=0.05; df =2, 6) = 5.14

Decision: F calculated (38.91) is greater than critical F (5.14). Therefore reject Ho.

Conclusion: There are significant differences in the population numbers of *Tisbe battagliai* in the hopper production system when fed on two feeding regimes and extra substrate. From the Fishers test results we can say the *Tisbe* fed on yeast have

significantly higher population counts than the *Tisbe* fed on a mixture of algae and the *Tisbe* fed on a mixture of algae with extra substrate present in the hoppers.

Summary of Large Scale Experiments

In the first experiment, there was an increase of 276.99 ± 97.26 *T. battagliai* when the hoppers had SMA added as a food. This demonstrates that SMA milk can achieve growth in these large scale cultures. In the second experiment, all populations increased. The populations that were fed with yeast increased at a significantly higher rate than those fed on algae. This shows that yeast also has potential as a food for large scale cultures. The hoppers which were fed algae and were given extra substrate had a similar population growth to the hoppers fed on algae with no extra substrate. Therefore, extra substrate does not increase the population numbers significantly.

Section 7

Life time studies

In the present research, the successful use of yeast and SMA milk as a diet in large scale production systems has been demonstrated. To estimate the production rates under culture, however, requires data on longevity, fertility, number of nauplii and broods. The experiments below were set up in order to estimate these response variables when *T. battagliai* are grown on yeast, SMA milk or a mixture of algae.

Experiment 1

50 day study of *Tisbe battagliai* grown on yeast and a mixture of algae;

Aim of Investigation

An investigation was carried out to compare various population parameters for *T. battagliai* when fed on yeast or a mixture of algae.

The response variables which were investigated are:

- Survival to day seven
- Number of fertile females
- Mean number of broods
- Mean number of nauplii in each brood

The survival rates of the nauplii to day seven can be compared with the short survival experiments in section 3.

Materials and Methods

Twenty nauplii of *Tisbe battagliai* born within the previous 24 hours were placed into each of eight petri dishes. As the nauplii had been collected from a population of 3000 adults the selection of the nauplii was believed to be random. It was assumed that on average each petri dish received an equal ratio of male to females. The eight petri dishes were then randomly assigned to the yeast or algae treatment using a random numbers table so that there were four petri dishes of each treatment. The petri dishes had saltwater added which had been filtered to 1μm and sterilised with Ultra Violet. The diet was then added. 3mls of an equal mixture of *Chaetoceros calcitrons, T-*

Isochrysis galbana and Tetraselmis suecica (19.235 x 10^6 cells container^{-1} ($7.69x10^5$ cell ml^{-1})) was added to the algae petri dishes. 0.5g of the yeast was added to 100ml of saltwater and mixed, and then 0.5ml of this diluted suspension was added to the yeast petri dishes.

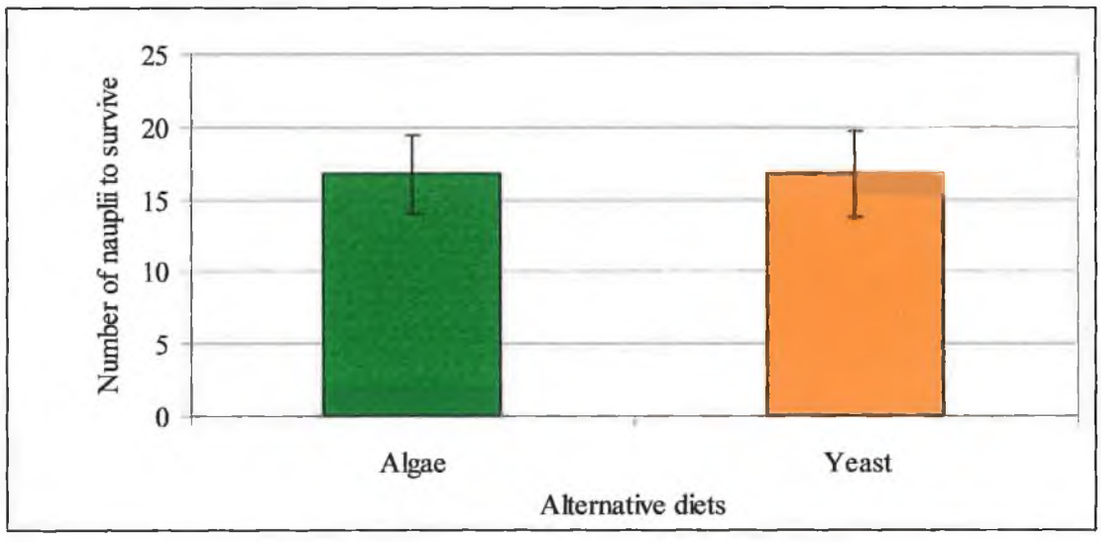
The petri dishes were then numbered and placed on a bench in the laboratory. Every few days, they were checked and when an egg bearing female was found, it was placed in a new petri dish, coded and given yeast or micro-algae as appropriate. The petri dishes containing these egg bearing females were then checked regularly for nauplii. When nauplii were discovered, the female was removed and placed in a clean petri dish with her original code plus its appropriate food and water. Lugols solution was added to the petri dish containing the nauplii. The nauplii were then counted. The petri dishes containing the egg bearing females were checked and any further nauplii released were counted as above. The experiment was concluded at day 50.

Results

Survival to day 7

Survival to day seven for the *Tisbe battagliai* when grown on a mixture of algae and when grown on yeast are shown in Figure 4:12.

Figure 4:12. The number of *Tisbe battagliai* nauplii to survive to day seven when fed on a mixture of algae or yeast showing standard deviation lines.



Survival to day seven for the *Tisbe battagliai* was 16.75 ± 2.75 when grown on a mixture of algae and 16.75 ± 2.99 when grown on yeast. The number of *T. battagliai* nauplii to survive was 80% for both treatments.

Data were normally distributed and so a one way ANOVA was carried out.

Hypotheses:

Ho: There is no significant difference in survival to day 7 when *Tisbe battagliai* are fed on a mixture of algae or bakers yeast.

Ha: There is a significant difference to day 7 when *Tisbe battagliai* are fed on a mixture of algae or bakers yeast.

Critical value ($p=0.05$; $df = 1, 6$) = 5.99

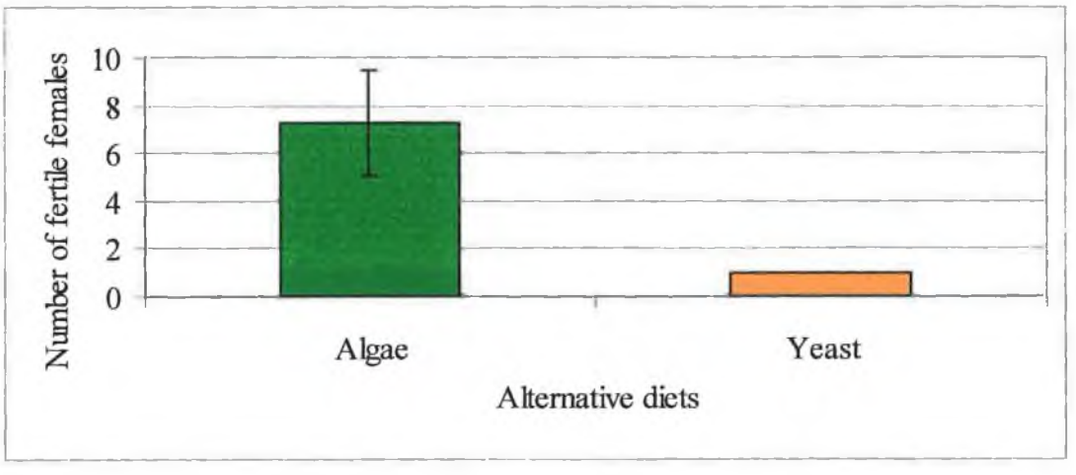
Decision: F calculated (0.00) is less than F critical (5.99). Therefore accept Ho.

Conclusion: There is no significant difference in the survival to day seven when *Tisbe battagliai* are fed on a mixture of algae or yeast. This is in agreement with the results of the short term experiments discussed earlier in the chapter.

Number of fertile females

The number of fertile females was higher when *T. battagliai* were fed on a mixture of algae than SMA milk. These results can be seen in Figure 4:13.

Figure 4:13. Mean number of fertile female *Tisbe battagliai* when fed on a mixture of algae or yeast showing standard deviation lines. Standard deviation was zero for yeast.



The average number of fertile females from the four replicates was 7.25 for the algae fed *Tisbe* and 1 for the yeast fed *Tisbe*. The data were checked to see if this was a significant difference.

Data were not normally distributed so a Kruskal-Wallis test was carried out.

Hypotheses:

Ho: There is no significant difference in the number of fertile female *Tisbe battagliai* when fed on yeast or a mixture of algae.

Ha: There is a significant difference in the number of fertile female *Tisbe battagliai* when fed on yeast or a mixture of algae.

Critical value: (df= 1, p=0.05) = 3.84

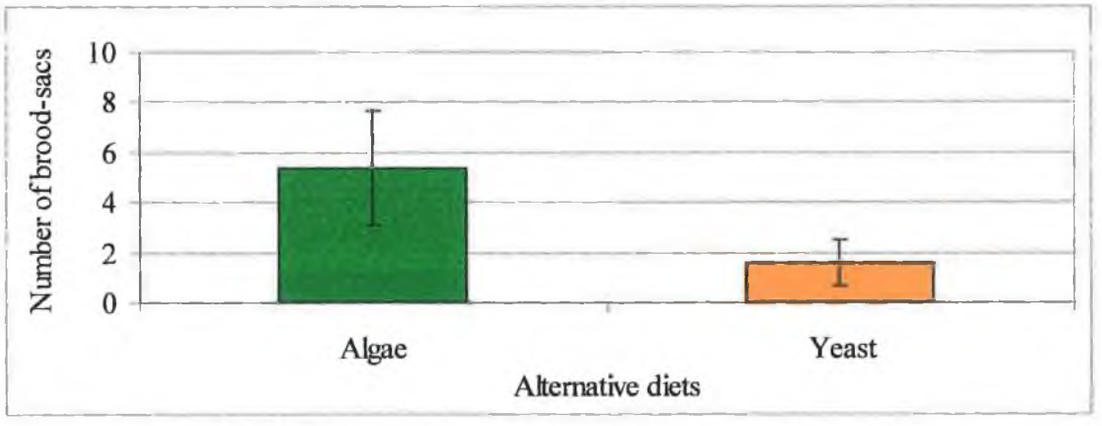
Decision: Calculated H value (6.05) is greater than critical value (3.84). Therefore reject Ho.

Conclusion: There is a significant difference in the number of fertile female *Tisbe battagliai* produced when grown on a mixture of algae or yeast. *Tisbe battagliai* grown on a mixture of algae produce significantly more fertile females than *Tisbe battagliai* grown on yeast.

The number of brood-sacs produced by *Tisbe battagliai* over 50 days when fed on a mixture of algae or yeast.

The number of brood-sacs produced by *T. battagliai* when fed on a mixture of algae is higher than when fed on yeast. These results are shown in Figure 4:14.

Figure 4:14. The mean number of brood-sacs produced by *Tisbe battagliai* when fed on a mixture of algae or yeast showing standard deviation lines.



The mean number of brood-sacs produced by *Tisbe battagliai* was 5.38 when fed on a mixture of algae and 1.6 when fed on yeast in the 50 day period. The minimum number of broods for *T. battagliai* was 1 when fed on a mixture of algae and yeast.

The maximum number of broods for *T. battagliai* was 11 when fed on a mixture of algae and 3 when fed on yeast. The data were analysed to find out if this difference was significant.

Data were normally distributed so a one way ANOVA was carried out

Hypotheses:

Ho: There is no significant difference in number of brood-sacs produced by *Tisbe battagliai* when fed on a mixture of algae or yeast.

Ha: There is a significant difference in number of brood-sacs produced by *Tisbe battagliai* when fed on a mixture of algae or yeast.

Critical value: (df= 1, 32; p=0.05) =4.17

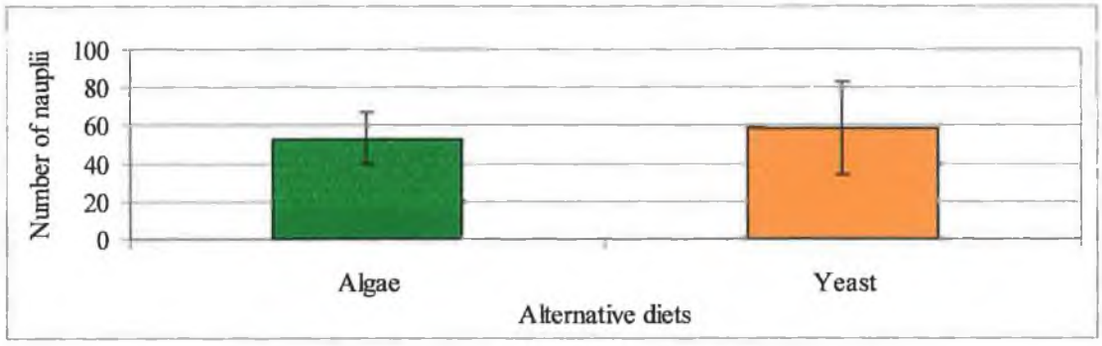
Decision: F calculated (0.44) is greater than F critical (4.17). Therefore reject Ho.

Conclusion: There is a significant difference in the number of brood-sacs produced by *Tisbe battagliai* when fed on a mixture of algae or yeast.

Mean number of nauplii produced in each brood-sac by female *Tisbe battagliai* during the 50 day trial when fed on a mix of algae or yeast

The numbers of nauplii produced in each brood-sac by *T. battagliai* appear to be similar. Figure 4:15 shows these results.

Figure 4:15. Mean number of nauplii produced in each brood-sac by female *Tisbe battagliai* when fed on a mixture of algae or yeast.



The mean number of nauplii per brood-sac was 53.08 for female *T. battagliai* when fed on a mixture of algae and 58.38 when fed on yeast. The data were analysed to see if there was a significant difference.

Data were normally distributed so a one way ANOVA was carried out

Hypotheses:

Ho: There is no significant difference in number of nauplii produced in each brood sac by *Tisbe battagliai* when fed on a mixture of algae or yeast.

Ha: There is a significant difference in number of nauplii produced in each brood sac by *Tisbe battagliai* when fed on a mixture of algae or yeast.

Critical value: (df= 1, 31; p=0.05) =4.17

Decision: F calculated (0.44) is less than F critical (4.17). Therefore accept Ho.

Conclusion: There is no significant difference in the number of nauplii produced in each brood sac when fed on either a mixture of algae or yeast. However, because female *T. battagliai* have significantly more brood-sacs when fed on a mixture of

algae, they will produce significantly more nauplii in their lifetime than females fed on yeast.

Summary

There was no significant difference in survival to day seven when *Tisbe battagliai* were fed on a mixture of algae or yeast. This is consistent with earlier results. The number of reproductive females and number of nauplii produced was significantly higher when *T. battagliai* were fed on a mixture of algae. Yeast could be used to some degree but it is not as good a diet as a mixture of algae and therefore production numbers would be significantly lower than when fed on algae. This is in contrast to the results in section 6 when yeast was used as a food in a large scale hopper system. In experiment 1, the yeast fed populations decreased in a similar way to algae fed populations over seven days but in experiment 2, yeast gave significantly higher population numbers than when hoppers were fed with a mixture of algae.

Experiment 2

Life time studies on *Tisbe battagliai* fed on SMA milk or a mixture of algae;

Aim of Investigation

An investigation was carried out to compare various population parameters for *T. battagliai* when it was grown on SMA milk or a mixture of algae.

The response variables which were measured are:

- Survival to day seven
- Number of fertile females
- Development rate
- Number of nauplii in the first brood
- Mean number of broods
- Mean number of nauplii produced by each female overall
- Mean number of nauplii in each brood
- Life expectancy

Materials and Methods

The method is the same as for the life time study of yeast versus algae.

The experiment lasted, however, until all of the original 20 nauplii were dead and there were five replicates rather than four.

2mls of an equal mixture of *Chaetoceros calcitrons, T-Isochrysis galbana and Tetraselmis suecica* (approximately 12.85 x 10^6 cells/ container ($5.14x10^5$ cell ml^{-1}))

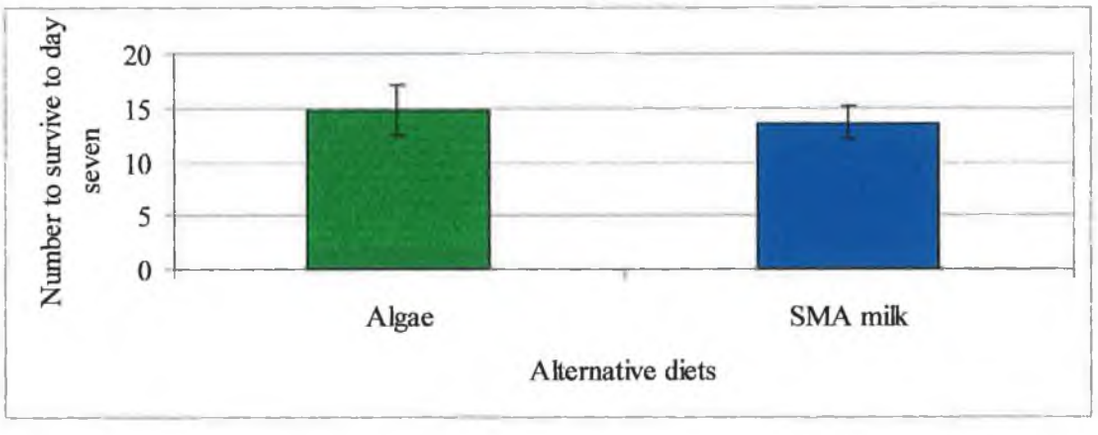
were added to the algae petri dishes. 0.5g of SMA milk was added to 100ml of saltwater and mixed, and then 0.5ml of this diluted suspension was added to the milk petri dishes.

Results

Survival to day 7

The survival of *T. battagliai* to day seven was very similar when fed on a mixture of algae or SMA milk. These results can be seen in Figure 4:16.

Figure 4:16. Mean survival to day seven of *Tisbe battagliai* when fed on algae or SMA milk showing standard deviation lines



Mean survival to day seven was 74% when *T. battagliai* nauplii were fed on a mix of algae and 68% when fed on SMA milk. The data were analysed to see if there was a significant difference.

Data were normally distributed so a one way ANOVA was carried out

Hypotheses:

Ho: There is no significant difference in survival to day 7 when *Tisbe battagliai* are fed on a mixture of algae or a SMA milk diet.

Ha: There is a significant difference in survival to day 7 when *Tisbe battagliai* are fed on a mixture of algae or a SMA milk diet.

Critical value: (df= 1, 8; p=0.05) = 5.32

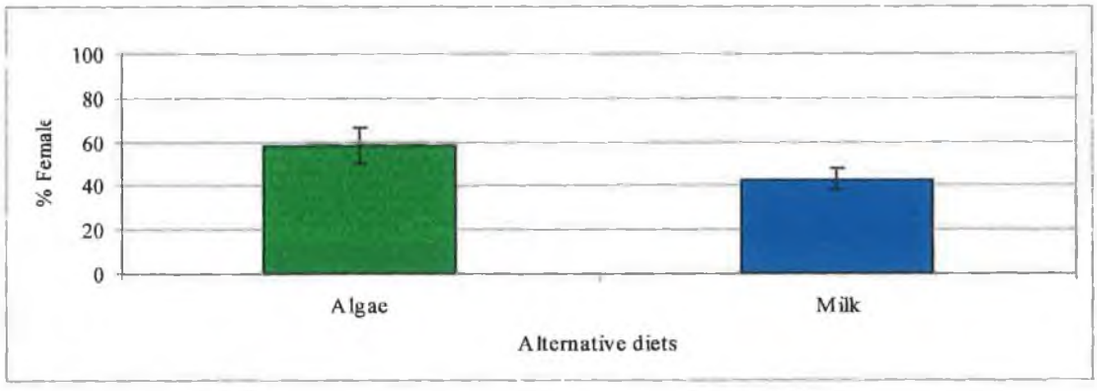
Decision: F calculated (0.96) is less than F critical (5.32). Therefore accept Ho.

Conclusion: There is no significant difference in survival to day seven when *Tisbe battagliai* are fed on SMA milk or a mixture of algae. This is consistent with the short term survival experiments in section 3.

Number of fertile female *Tisbe battagliai* produced when fed on SMA milk or a mixture of algae

The number of fertile females was higher when *T. battagliai* were fed on a mixture of algae than when fed on SMA milk. These results can be seen in Figure 4:17.

Figure 4:17. Mean percentage of *Tisbe battagliai* which are fertile females when fed on algae or SMA milk showing standard deviation lines.



The percentage of the population which were fertile females when fed on algae was 58.3% and this reduced to 42.7% when fed on SMA milk. The results were analysed to see if this difference was significantly higher.

Data were normally distributed so a one way ANOVA was carried out

Hypotheses:

Ho: There is no significant difference in the number of fertile females *Tisbe battagliai* when fed on a mixture of algae or a SMA milk diet.

Ha: There is a significant difference in the number of fertile females *Tisbe battagliai* when fed on a mixture of algae or a SMA milk diet.

Critical value: ($p=0.05$; $df=1, 8$) $=5.32$

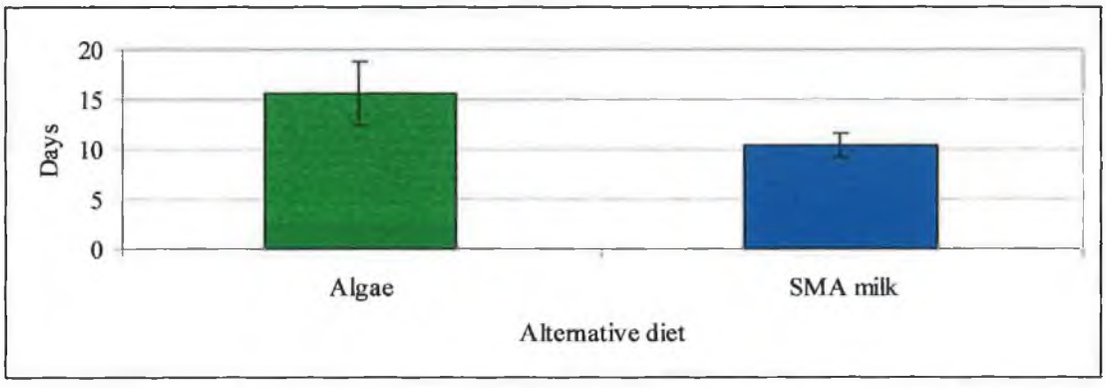
Decision: F calculated (12.5) is greater than critical F value. Therefore reject Ho.

Conclusion: There is a significant difference between the two treatments in numbers of fertile females produced. The *Tisbe battagliai* fed on a mixture of algae have significantly more fertile females than *T. battagliai* fed on SMA milk.

Development rate

The development rate was measured as the number of days, from the nauplius stage, it took for the female *T. battagliai* to produce their first brood sac. The development rate of *T. battagliai* was shorter when fed on SMA milk than when fed on a mixture of algae. These results are shown in Figure 4:18.

Figure 4:18. Mean development rate of female *Tisbe battagliai* in days when fed on a mixture of algae or SMA milk showing standard deviation lines.



The mean number of days was 15.58 for *T. battagliai* fed on a mixture of algae and 10.48 days for those fed on SMA milk. The data were analysed to see if there was a significant difference.

Data were not normally distributed so a Kruskal-Wallis was carried out

Hypotheses:

Ho: There is no significant difference in the number of days for female *Tisbe battagliai* to produce egg sacs when fed on a mix of algae or SMA milk.

Ha: There is a significant difference in the average number of days for female *Tisbe battagliai* to produce egg sacs when fed on a mix of algae or SMA milk.

Critical value: (df=1; p=0.05) =3.84

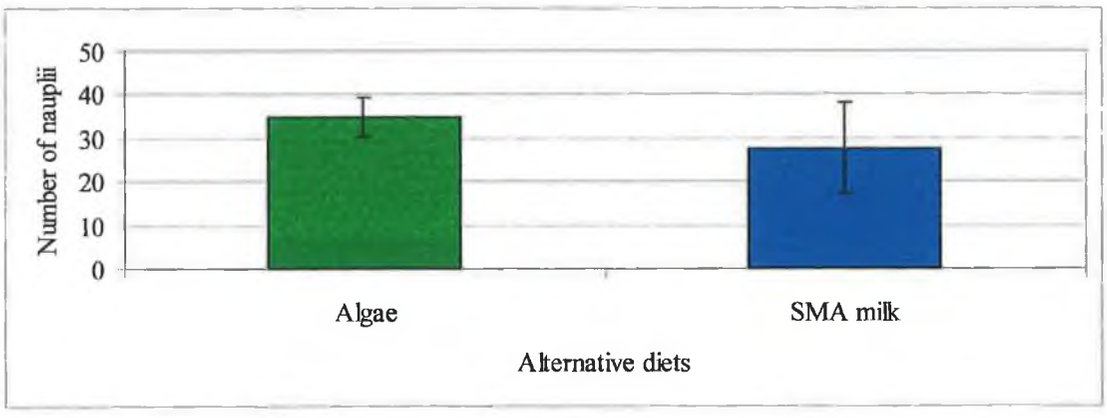
Decision: Calculated H (46.54) is greater than critical H value (3.84). Therefore reject Ho.

Conclusion: There is a significant difference in the development rate of *T. battagliai* when fed on a mixture of algae or SMA milk. The *T. battagliai* fed on SMA milk produced egg sacs in significantly fewer days than those fed on a mixture of algae.

Number of nauplii in the first brood

The number of nauplii produced by *T. battagliai* in the first brood-sac is higher when fed on a mixture of algae than when fed on SMA milk. The mean results of brood sac size for each replicate can be seen in Figure 4:19.

Figure 4:19. Mean number of nauplii produced by female *Tisbe battagliai* in their first brood when fed on a mix of algae or SMA milk showing standard deviation lines



The mean number of nauplii in the first brood was 35.0 for *T. battagliai* fed on a mix of algae and 27.6 for *T. battagliai* fed on SMA milk. The data were analysed to see if this was a significant difference.

Data were normally distributed so a one way ANOVA was carried out

Hypotheses:

Ho: There is no significant difference in number of nauplii produced in the first brood sac of *Tisbe battagliai* when fed on a mix of algae or SMA milk.

Ha: There is a significant difference in number of nauplii produced in the first brood sac of *Tisbe battagliai* when fed on a mix of algae or SMA milk.

Critical value ($p=0.05$; $df=1, 69$) = 4.00

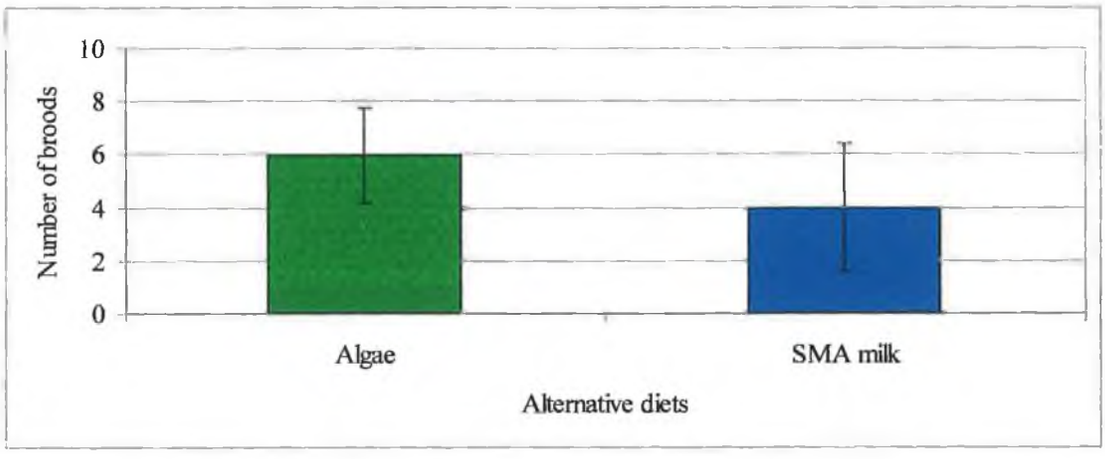
Decision: F calculated (2.96) is less than critical F value (4.00). Therefore accept Ho.

Conclusion: The number of nauplii produced in the first pregnancy, when fed a mixture of algae, is not significantly different from the number of nauplii produced in the first pregnancy when fed a SMA milk diet.

Number of brood-sacs produced by *Tisbe battagliai*

The number of broods produced by *T. battagliai* when fed on a mixture of algae is higher than when fed on SMA milk. These results can be seen with standard deviation lines in Figure 4:20.

Figure 4:20. Mean number of broods produced by female *Tisbe battagliai* when fed on a mix of algae or SMA milk showing standard deviation lines.



The average number of broods was 5.98 for *T. battagliai* fed on a mix of algae and 4.00 for *T. battagliai* fed on SMA milk. The minimum and maximum number of brood sacs for *T. battagliai* was one and nine respectively for both diets. The data were analysed to see if this was a significant difference.

Data were normally distributed so a one way ANOVA was carried out

Hypotheses:

Ho: There is no significant difference in the number of broods from *Tisbe battagliai* when fed on a mixture of algae or SMA milk.

Ha: There is a significant difference in the number of broods from *Tisbe battagliai* when fed on a mixture of algae or SMA milk.

Critical value ($p=0.05$ $df=1, 69$) = 3.92

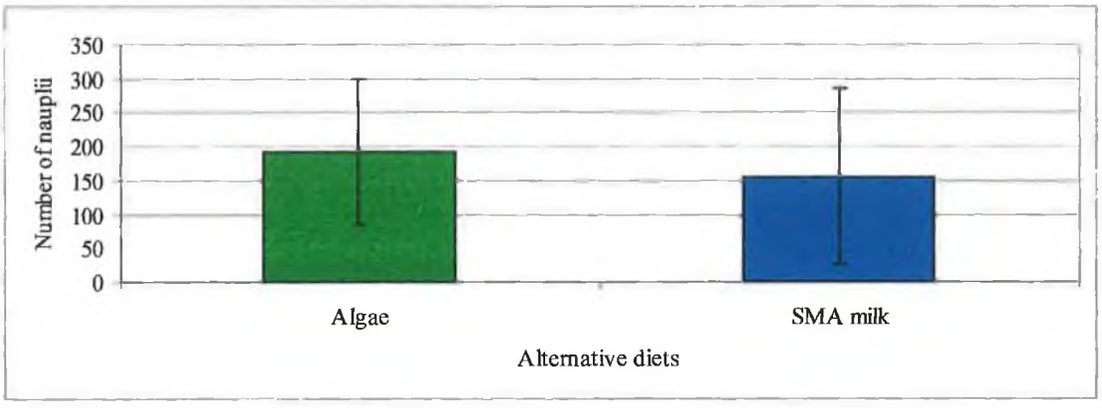
Decision: F calculated (12.36) is greater than critical F (3.92) value. Therefore reject Ho.

Conclusion: There was a significant difference in the number of broods produced when *Tisbe battagliai* were fed on a mixture of algae or a formulated milk diet. The *Tisbe battagliai* fed on a mixture of algae have significantly more broods of nauplii than those fed on SMA milk.

Total number of nauplii produced per female lifetime

The number of nauplii produced by each female *T. battagliai* is higher when fed on a mixture of algae than SMA milk. These results can be seen in Figure 4:21.

Figure 4:21. Mean number of nauplii produced by each female *Tisbe battagliai* when fed on a mix of algae or SMA milk showing standard deviation lines



The average number of nauplii produced by each female from the five replicates was 192.95 ± 107.03 when fed on a mixture of algae and 155.47 ± 129.08 when fed on SMA milk. The data were analysed to see if there was a significant difference between the treatments.

Data were normally distributed so a one way ANOVA was carried out

Hypotheses:

Ho: There is no significant difference in the total number of nauplii produced by each female of *Tisbe battagliai* when fed on a mix of algae or SMA milk.

Ha: There is a difference in the total number of nauplii produced by each female of *Tisbe battagliai* when fed on a mix of algae or SMA milk

Critical value (p=0.05; df= 1, 8) =5.32

Decision: F calculated (8.48) is greater than critical F value (5.32). Therefore reject Ho.

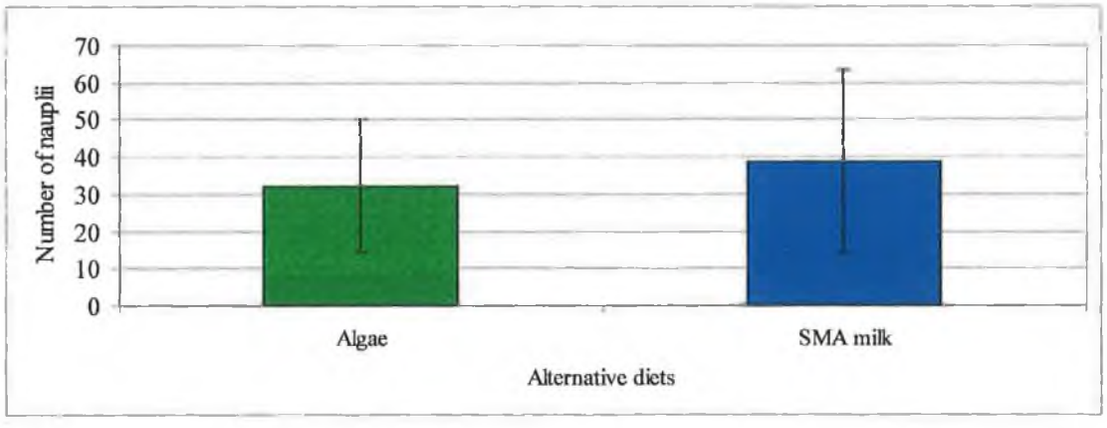
Conclusion: There was a significant difference in the number of nauplii produced by *Tisbe battagliai* when fed on a mixture of algae or a SMA milk diet. The average

number of nauplii produced by each female over her lifetime was higher when the *T. battagliai* were fed on a mixture of algae rather than those fed on SMA milk.

Average number of nauplii produced in each brood

The mean number of nauplii produced in each brood by female *T. battagliai* when fed on SMA milk was higher than when fed on a mixture of algae. These results are shown in Figure 4:22.

Figure 4:22. Mean number of nauplii produced in each brood by each female *Tisbe battagliai* when fed on a mix of algae or SMA milk showing standard deviation lines.



The average number of nauplii in a brood sac is 32.28 ± 17.8 when *Tisbe battagliai* are fed on a mix of algae and 38.90 ± 24.3 when fed on SMA milk. The minimum number of nauplii produced in a brood by *T. battagliai* was 2 when fed on a mixture of algae and 3 when fed on SMA milk. The maximum number of nauplii produced in a brood by *T. battagliai* was 74 when fed on a mixture of algae and 90 when fed on SMA milk. Failure to produce a brood is not recorded in this analysis. Data were analysed to see if this difference was significant.

Data were not normally distributed so a Kruskal-Wallis test was carried out

Hypotheses:

Ho: There is no significant difference in the average number of nauplii in each brood from *Tisbe battagliai* when fed on a mix of algae or SMA milk.

Ha: There is a significant difference in the average number of nauplii in each brood from *Tisbe battagliai* when fed on a mix of algae or SMA milk.

Critical value: (df=1, p=0.05) =3.84

Decision: Calculated H (4.64) is greater than critical H value (3.84). Therefore reject Ho.

Conclusion: There is a significant difference in the mean number of nauplii produced per brood by each female when fed on a mixture of algae or a SMA milk diet. From Figure 4:22 we can say that *T. battagliai* fed on SMA milk have a significantly higher number of nauplii per brood sac than when fed on a mixture of algae.

The results of the mean number of nauplii produced in subsequent brood sacs are shown in Table 4:24 and Figure 4:23 for *T. battagliai* when grown on SMA milk or a mixture of algae.

Figure 4:23. Mean number produced in every brood sac by female *Tisbe battagliai* when fed on algae or SMA milk.

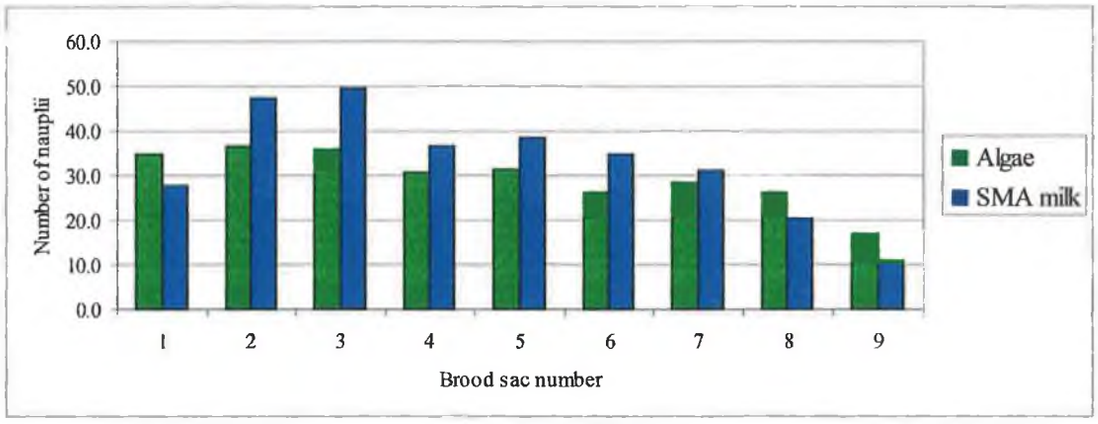


Table 4:24. The mean number of nauplii from each brood sac from female *Tisbe battagliai* when fed on SMA milk or a mixture of algae.

Replicate & Treatment		Brood-sac number								
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Algae	**1**	33.1	40.5	34.5	33.3	31.6	35	14.8	2	0
	2	28.6	28.2	30	35.1	29.3	27.5	30	8	0
	3	37.7	39.7	31.9	33.3	30.3	26.3	51.3	49.5	26
	4	39.4	35.4	33.6	22.3	24.7	12.7	9.5	0	0
	5	36.2	39.8	43.3	35.5	35.4	28.2	40.3	23.5	17
	Mean	35	36.7	35.9	30.9	31.3	26.3	28.9	26.3	17
SMA milk	**1**	13.6	51.4	54	40	50	19	0	0	0
	2	32	55.2	51.3	31	13.5	5	0	0	0
	3	29.9	49.5	34	42.8	60	38	38	0	0
	4	21.4	35.5	45	22	33	74	34	6	11
	5	41.3	45.3	64.8	47.8	36.8	38.3	21.7	35	0
	Mean	27.6	47.4	49.8	36.7	38.7	34.9	31.2	20.5	11

The mean number of nauplii from each brood sac produced by female *T. battagliai* when fed on SMA milk or a mixture of algae over her lifetime were analysed to see if there were significant differences in the mean brood^{-1} numbers of nauplii produced over the lifetime of the female. Brood failure was included in the analysis.

Data were normally distributed and a two way balanced ANOVA was carried out.

Where an interaction occurs, no conclusions can be drawn on the two main hypotheses (Underwood, 1997).

Null Hypotheses:

1: There is no significant difference in mean number of nauplii brood^{-1} over the lifetime of the female when fed on SMA milk or a mixture of algae.

2: There is no significant difference in sequential broods

3: There is no interaction between the mean number in various broods and the diet.

Conclusion:

There is no interaction between the mean number in various broods and the diet.

There is no significant difference in the number of nauplii in the various broods when fed on a mixture of algae or SMA milk.

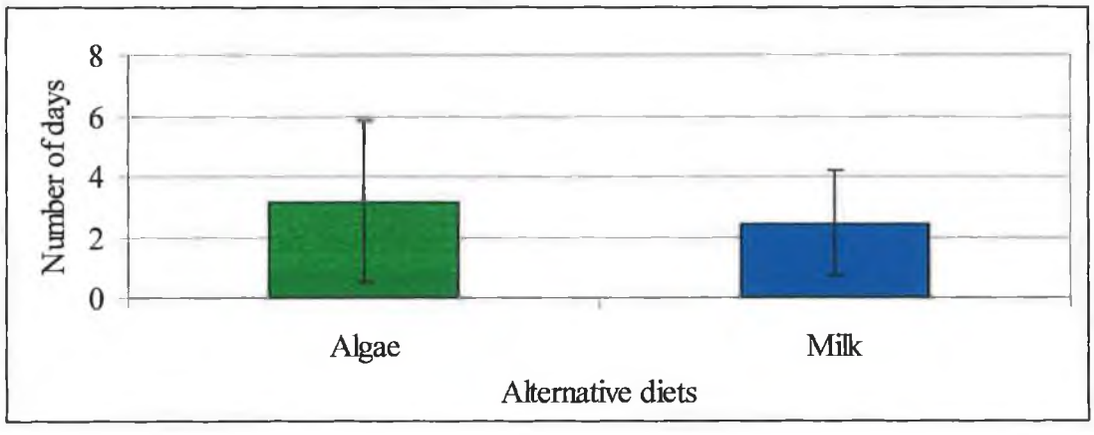
There is a significant difference in the number of nauplii in the sequential broods.

This latter result is not investigated further here as it is not a significant outcome for the culture of *Tisbe battagliai* on SMA milk or a mixture of algae.

Mean number of days between brood hatching dates

The mean number of days between each batch of nauplii hatching was longer for *T. battagliai* fed on a mixture of algae than on SMA milk. These results can be seen in Figure 4:25.

Figure 4:25. The mean number of days between brood hatching dates for *Tisbe battagliai* when fed on a mixture of algae or SMA milk, with standard deviation lines.



The mean number of days between each batch of nauplii hatching in *T. battagliai* was 3.17 days when fed on a mixture of algae and 2.45 days when fed on SMA milk. The data were analysed to see if this was a significant difference.

Data were not normally distributed so a Kruskal-Wallis test was carried out

Hypotheses:

Ho: There is no significant difference in the number of days between each batch of nauplii hatching for *Tisbe battagliai* when fed on a mix of algae or SMA milk.

Ha: There is a significant difference in the number of days between each batch of nauplii hatching for *Tisbe battagliai* when fed on a mix of algae or SMA milk.

Critical value: (df=1; p=0.05) =3.84

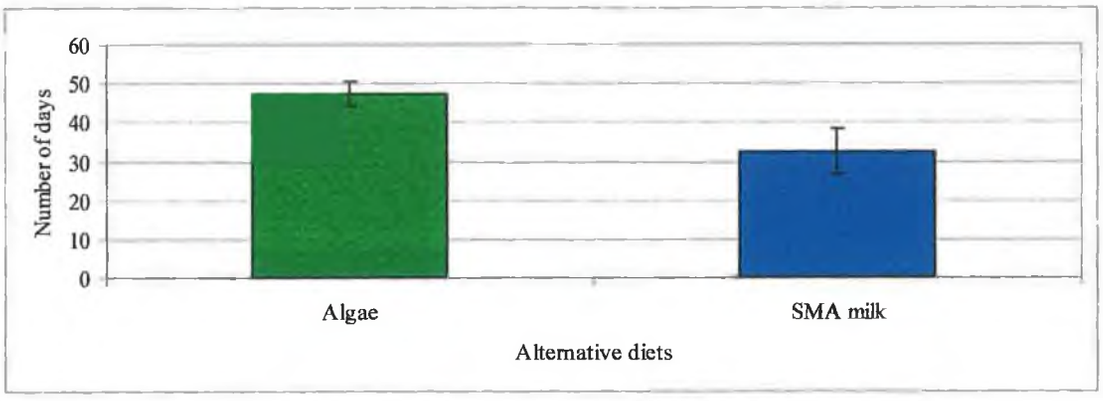
Decision: Calculated H (15.25) is greater than critical H value (3.84). Therefore reject Ho.

Conclusion: There is a significant difference in the number of days between nauplii hatching when *T. battagliai* are fed on a mixture of algae or SMA milk. The *T. battagliai* fed on SMA milk have a significantly shorter period between each batch of nauplii hatching.

Life Expectancy

The original twenty nauplii were followed from day of birth through to the day they died. Therefore, an estimate of mean life expectancy for *Tisbe battagliai* can be achieved. The mean life expectancy is higher when *T. battagliai* are fed on a mixture of algae and than on SMA milk. These results can be seen in Figure 4:26.

Figure 4:26. Mean life expectancy of *Tisbe battagliai* when fed on algae or SMA milk showing standard deviation lines.



Mean life expectancy was 47.48 ± 5.75 days for *Tisbe* fed on a mix of algae and 32.5 ± 3.27 days for *Tisbe* fed on SMA milk. The data were analysed to see if this difference was significant.

Data were normally distributed so a one way ANOVA was carried out

Hypotheses:

Ho: There is no significant difference in the life expectancy of *Tisbe battagliai* when fed on a mixture of algae or SMA milk.

Ha: There is a significant difference in the life expectancy of *Tisbe battagliai* when fed on a mixture of algae or SMA milk.

Critical value: (p=0.05; df=1, 8) =5.32

Decision: F calculated (25.63) is greater than F critical (5.32). Therefore reject Ho.

Conclusion: The life expectancy of *Tisbe battagliai* fed on a mixture of algae is significantly different from *Tisbe battagliai* fed on SMA milk. The *Tisbe* fed on a mixture of algae have a significantly longer life expectancy.

Summary

Tisbe battagliai grown on algae have a significantly longer life expectancy than *T. battagliai* fed on SMA milk. They also have significantly more fertile females, with each fertile female having significantly more broods and therefore significantly more nauplii over their life time than SMA milk fed *T. battagliai*. There is no significant difference however, in the short term survival of *T. battagliai* when fed on the two diets. The development rate of *T. battagliai* is significantly shorter when fed on SMA milk and the fertile females have a significantly shorter brood development time.

The current price of SMA milk is €0.015/g, while algae costs approximately €0.735/g for small scale or €0.363/g for large scale production (Green & McGrath, 2003). The

cost of production of *Tisbe battagliai* could therefore be reduced greatly by using SMA milk as the food source.

Production Value Predictions

From the results in the experiments 1 and 2 above, the production values of *Tisbe* when fed on a mixture of algae and SMA milk can be predicted. The *Tisbe* are produced in hoppers described in chapter 2 and used in section 6 for large scale experiment. The short term survival values are used in the calculations.

***Tisbe battagliai* production predictions when fed on a mixture of algae**

Stock 50,000 nauplii

At 74% survival = 37000

At 58.3% female ratio = 21460

Development time = 15.58 days

Mean number of nauplii per brood 32.28

Brood sac every 3.17 days

32.28 / 3.17 = production per day = 10.18 nauplii per day female $^{-1}$

Number of brood-sacs = 5.98 female $^{-1}$

Reproductive period= number of broods x days between broods

5.98 x 3.17 = 18.96

After 15.58 days 21,460 females will have 10.18 nauplii every day for 18.96 days (218,462.8 nauplii)

Over a lifetime of 34.51 days, they will have 4,142,054.6 nauplii.

***Tisbe battagliai* production predictions when fed on SMA milk**

Stock 50,000 nauplii

At 68% survival = 34000

At 42.7% female ratio = 14280

Development time = 10.48 days

Mean number of nauplii per brood 38.9

Brood sac every 2.45 days

38.9 / 2.45 = production per day = 15.88 nauplii per day female $^{-1}$

Number of brood-sacs = 4 female $^{-1}$

Reproductive period= number of broods x days between broods

4 x 2.45 = 9.8

After 10.48 days 14,280 females will have 15.88 nauplii every day for 9.8 days (226,766.4 nauplii)

Over a lifetime of 20.28 days they will have 2,222,310.7 nauplii.

Cost of production when *Tisbe battagliai* are grown on a mixture of algae.

As in section 6, 1ltr of a mixture of algae would be added to the hoppers, every 3 days.

The production lasted for 34.51 days and therefore they will require feeding on 11.5 occasions.

Grubb (1996) estimated commercial scale batch culture systems at:

0.08694 cent litre^{-1} for a large culture system

11.5 feeds x 0.08694 = €1.00055 to produce 4,142,054.6 nauplii

The cost per million nauplii = € 0.244

Cost of production when *Tisbe battagliai* are grown on SMA milk.

As in section 6, 1g of SMA milk would be added to the hoppers, every 3 days.

The production lasted for 20.28 days therefore they will require feeding on 6.76 occasions.

1g of SMA milk costs €0.015.

6.76 x 0.015 = €0.1014 to produce 2,222,310.7 nauplii.

The cost per million nauplii = € 0.046

Summary

Tisbe battagliai fed on a mixture of algae could produce nearly twice as many nauplii as *T. battagliai* fed on SMA milk. However, the production time is two weeks longer for those fed on a mixture of algae and the cost of production per million is five times higher than those fed on SMA milk.

Section 8

Maximum Consumption Rate of *Tisbe battagliai* on *Tetraselmis suecica*

In the above experiments, it has been shown that a mixture of algae is a significantly better diet than the alternative diets tested although SMA milk does have potential. It was unclear however how much algae an adult copepod needed for maximum growth. To estimate the algae concentration for maximum growth, the maximum consumption

rate of algae in terms of faecal pellet production was analysed following the method of Lacoste *et al.,* (2001).

Aim of Experiment

This experiment was carried out to study the maximum consumption rate of *Tetraselmis suecica* for an adult female *Tisbe battagliai* copepod in a 24 hour period in terms of faecal pellet production.

Materials and Methods

42 plastic petri dishes had *Tetraselmis suecica* added at seven increasing concentrations of algae. Each concentration of *Tetraselmis* had six replicates. Each of the replicates had an egg bearing female added to the dish. As a steady state consumption rate was to be investigated the females were not starved before the start of the trial.

The *Tetraselmis suecica* was harvested at the start of the experiment and cell density was counted.

Cell density was found to be 467000cells ml^{-1} for *Tetraselmis suecica.*

Treatment and Concentrations

(1) 0.25ml algae	5.8×10^3 cells ml^{-1}	0.58 cells μl^{-1}
(2) 0.5ml algae	11.7×10^3 cells ml^{-1}	1.17 cells μl^{-1}
(3) 1ml algae	23.4×10^3 cells ml^{-1}	2.34 cells μl^{-1}
(4) 2ml algae	46.7×10^3 cells ml^{-1}	4.67 cells μl^{-1}

(5) 4ml algae	93.4 x 10^3 cells ml $^{-1}$	9.34 cells μl^{-1}
(6) 8ml algae	187 x 10^3 cells ml $^{-1}$	18.68 cells μl^{-1}
(7) 16ml algae	374 x 10^3 cells ml $^{-1}$	37.36 cells μl^{-1}

Control: Five dishes had an egg bearing female added with no food as a control.

The control dishes had 20mls of saltwater added that had been filtered to 1μm. In each of the remaining petri dishes, for every ml of algae added, the same amount of saltwater was reduced from 20mls of saltwater.

The vessels were incubated for 24 hours. At the end of the incubation period the vessel contents were preserved in 1ml of 5% formalin for further analysis.

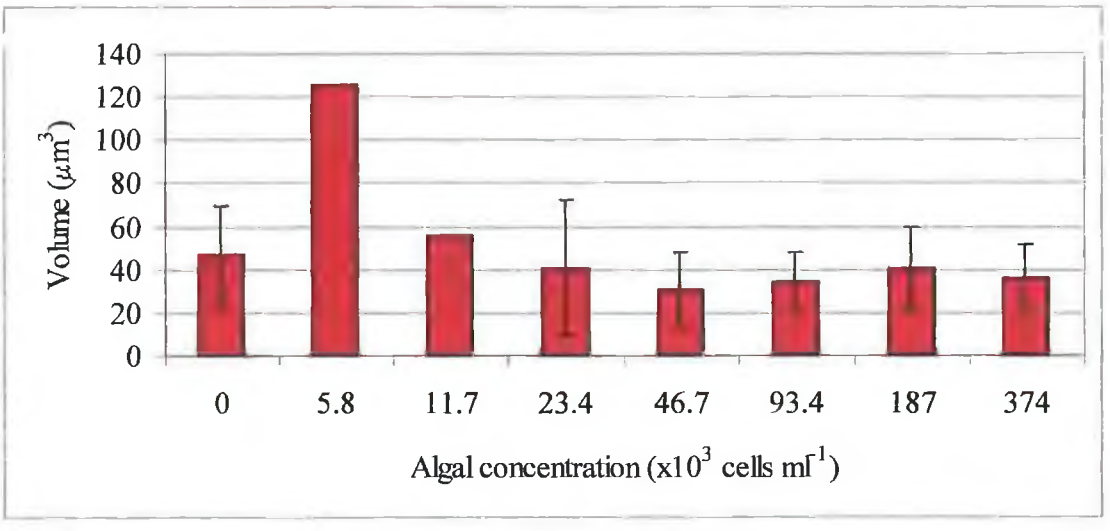
The petri dishes were viewed under a microscope and any faecal pellets discovered were counted and placed on a glass slide. The length and width of each faecal pellet was then measured with an eye piece graticule at zoom lens x 40.

Results

Volume of faecal pellet

An estimate of volume was obtained by multiplying the length by the width of each pellet following Pinto *et al.,* (2001). These results can be seen clearly in Figure 4:27.

Figure 4:27. Mean faecal pellet volume produced by *Tisbe battagliai* when fed increasing concentrations of *Tetraselmis suecica* showing standard deviation lines. (Where no standard deviation lines are present, only one faecal pellet was present)



The mean volume of faecal pellets was 51.47μm^3 for the seven treatments and the control. The largest faecal pellet was 126 μm^3 from a *Tisbe battagliai* which was not fed (part of the control). The smallest faecal pellet was 8 μm^3 from a *T. battagliai* in group 4 (46.7 x 10^3 cells ml $^{-1}$).

Data were normally distributed and a one way ANOVA was carried out.

Hypotheses:

Ho: There is no significant difference in the mean volume of faecal pellets produced by *Tisbe battagliai* when fed on the increasing *Tetraselmis suecica* concentrations.

Ha: There is a significant difference in the mean volume of faecal pellets produced by *Tisbe battagliai* when fed on the increasing *Tetraselmis suecica* concentrations.

Critical value (p=0.05; df =7, 38) =2.25

Decision: Calculated F (1.08) is less than critical F value (2.25). Therefore accept Ho.

Conclusion: There is no significant difference in the mean volume of faecal pellets produced by *Tisbe battagliai* when fed on increasing *Tetraselmis suecica* concentrations.

Number of faecal pellets produced with increasing *Tetraselmis suecica* concentration

Table 4:28 shows the number of faecal pellets produced by each *Tisbe battagliai* in the 24 hour period.

Table 4:28. The number of faecal pellets produced by each *Tisbe battagliai* with treatments receiving increasing amounts of *Tetraselmis suecica*.

	Replicates						
Treatment (cells ml^{-1})	A	B	C	D	E	F	Mean
0×10^3	1	2	0	0	-	-	0.75
5.8×10^3	0	0	1	0	0	0	0.167
11.7×10^3	1	0	0	0	0	0	0.167
23.4×10^3	3	1	1	2	1	2	1.667
46.7×10^3	8	5	4	4	4	4	4.834
93.4×10^3	6	5	8	9	3	3	5.667
187×10^3	18	8	13	17	15	17	14.667
374×10^3	18	20	13	26	32	1	18.334

As the concentration of algae was increased, the number of faecal pellets that were produced by the copepods generally increased. Some of the controls, which were not fed during the trial, still produced some faecal pellets.

Data were normally distributed so a one way ANOVA was carried out

Hypotheses:

Ho: There is no significant difference in the total number of faecal pellets individual^{-1} produced by *Tisbe battagliai* when fed on the increasing *Tetraselmis suecica* concentrations.

Ha: There is a significant difference in the total number of faecal pellets individual^{-1} produced by *Tisbe battagliai* when fed on the increasing *Tetraselmis suecica* concentrations.

Critical value (p=0.05; df=7, 38) =2.25

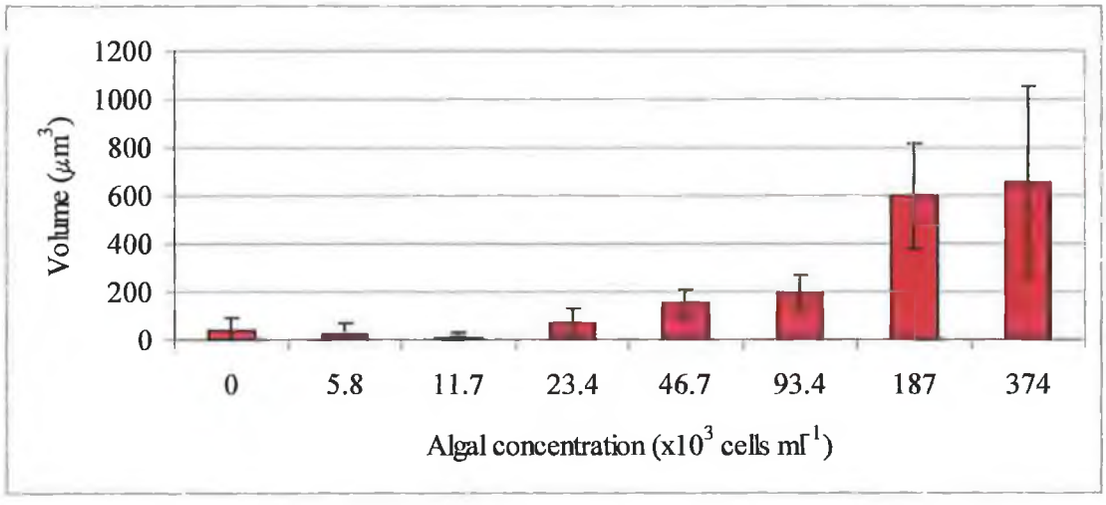
Decision: Calculated F (15.57) is greater than critical F value. Therefore reject Ho.

Conclusion: There is a significant difference in the number of faecal pellets produced when *Tisbe battagliai* are fed increasing concentrations of *Tetraselmis suecica.* From the Fishers test results we can say the control is not significantly different from treatment 1, 2, 3, 4 & 5. Treatment 5 is significantly different from treatment 1 & 2. Treatment 6 & 7 are not significantly different from each other but are significantly different from all other treatments.

<u>The total volume of faecal pellet production by *Tisbe battagliai* when fed on increasing *Tetraselmis suecica* concentrations.</u>

The total volume of faecal pellets was measured by adding together the volume of all the faecal pellets from a single *Tisbe battagliai* in a 24 hour period. The results can be seen in Figure 4:29.

Figure 4:29. The mean faecal pellet production volumes for *Tisbe battagliai* over a 24 hour period when fed on increasing *Tetraselmis suecica* concentrations with standard deviation lines.



The highest mean volume (655.5 μm^3) was in treatment seven (37.36 cells μl^{-1}) and the lowest mean volume (9.3 μm^3) was in treatment two (1.17 cells μl^{-1}). Data were normally distributed so a one way ANOVA was carried out

Hypotheses:

Ho: There is no significant difference in the total volume of faecal pellets produced by *Tisbe battagliai* when fed on the increasing *Tetraselmis suecica* concentrations.

Ha: There is a significant difference in the total volume of faecal pellets produced by *Tisbe battagliai* when fed on the increasing *Tetraselmis suecica* concentrations.

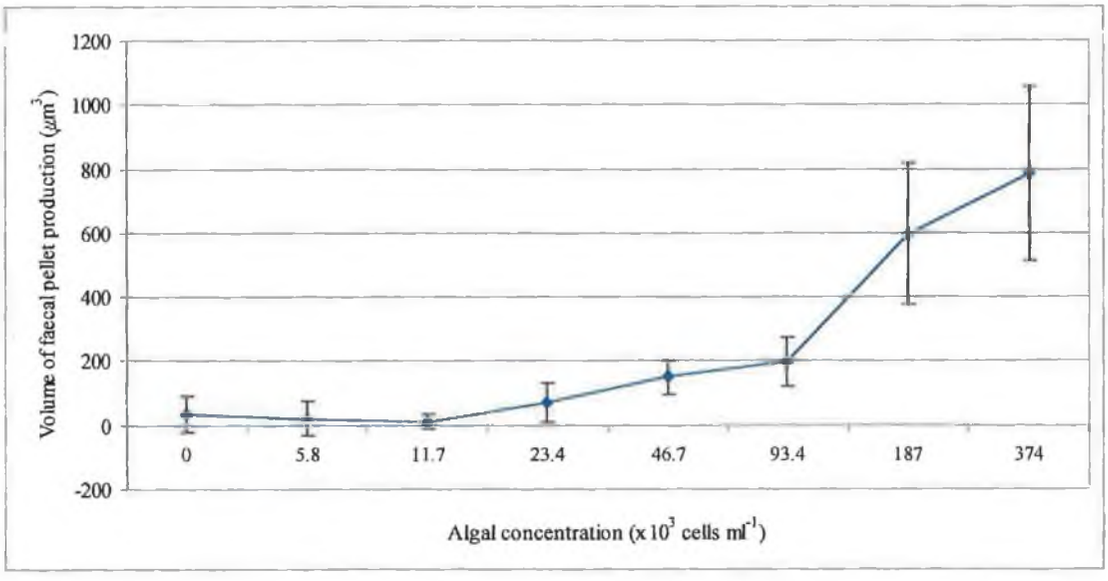
Critical value (p=0.05; df=7, 38) =2.25

Decision: Calculated F (13.57) is greater than critical F. Therefore reject Ho.

Conclusion: There is a significant difference in the sum of faecal pellets produced by each copepod when fed on different amounts of *Tetraselmis suecica.* From the Fishers test results we can say that the control, treatment 1, 2, 3, 4 & 5 are not significantly different from one another but are significantly different from treatment 6 & 7.
6 & 7 are not significantly different from one another, therefore, from the results it would appear that concentrations at this level of *Tetraselmis suecica* are close to the maximum ingestion rate.

One of the results of the total volume of faecal pellet production in treatment seven was very low, possibly due to the death of the individual. As can be seen in Table 4:24, this individual only produced one faecal pellet. It was removed from the results and a one way ANOVA was carried out on the remaining replicates.

Figure 4:30. The mean faecal pellet production volumes for *Tisbe battagliai* over a 24 hour period when fed increasing concentrations of *Tetraselmis suecica* showing standard deviation lines. One abnormal result has been removed from treatment seven.



When this one result was removed, there was a significant difference in the sum of faecal pellets produced by treatment 6 & 7. This difference in the mean production rates when this one individual is removed can be seen clearly if we compare Figures 4:29 and 4:30. While there is a significant difference between 6 & 7, there is evidence from the slope that ingestion rates are close to maximum.

Summary

The volumes of the faecal pellets produced by *Tisbe battagliai* did not increase significantly when they were fed increasing concentrations of *Tetraselmis suecica.* The increasing *Tetraselmis suecica* concentrations fed to *Tisbe battagliai* did result in a significant increase in number and volume of faecal pellets produced. The slope of the curve in Graph 4:30, shows evidence that ingestion rates are close to maximum at 374 x 10^3 cells ml^{-1}.

Section 9

Discussion

In this chapter, experiments were carried out to see if successful production of *Tisbe battagliai* could be carried out without the use of a mixture of algae.

For ease of production, a simple cost-effective diet is needed to grow *Tisbe battagliai,* which can be a quality food item for some species of larval/juvenile fish such as *H. guttulatus*. The short term survival experiments, in section 3 showed that *T. battagliai* were able to grow on a variety of food. Longer term experiments to see if they would

successfully grow and reproduce on these experimental foods in section 4 were successful, with the copepods all reaching generation 10 in five months. The growth rate of *T. battagliai* grown on a mixture of algae and SMA milk were also shown to be similar in section 5. Production of *T. battagliai* in large scale systems when fed on SMA milk or yeast was demonstrated in section 6. All of the diets with the exception of those grown on a mixture of algae, lost their colour, but this had no obvious affect on life history. In experiments by Miliou & Moraitou-Apostolopoulou (1991), a total loss of pigmentation was also observed when *Tisbe holothuriae* Humes was fed on any one food exclusively, with the exception however, of *Ulva.* Tanaka *et al.,* (1976 cited in Miliou & Moraitou-Apostolopoulou, 1991) reported that the total carotenoid content in crustaceans varies according to food type.

Copepods rely on the availability of carotenoids such as astaxanthin through the consumption of precursors from their diet (Andersson *et al.,* 2003). Carotenoids are believed to be important for copepods in photo-protection and other antioxidant activities during the day (Andersson *et al.,* 2003). The alternative diets provided to the copepods in this experiment must be lacking in the precursors needed for carotenoid production. The loss of carotenoids is significant as dietary based carotenoids are thought to be important in the establishment of colour in seahorses (Wyles, 2002). The eggs of seahorses are also bright red and carotenoids may be important in egg production (pers. obs.).

Providing live food in the form of copepods, which do not have all the dietary requirements that the seahorses require, may cause long term problems to arise. A way of adding the carotenoids or precursors at a critical level to the copepods before

feeding to the fish will be required as prey characteristics such as colour can affect feeding success of juvenile seahorses (Woods, 2003a).

Mixed algal diets seem to fulfil the nutritional requirements for high *Tisbe* productivity better than other diets, probably due to a richer supply of trace elements and vitamins (Miliou & Moraitou-Apostolopoulou, 1991). A study by Miliou & Moraitou-Apostolopoulou (1991) found that all life cycle parameters were affected to a greater or lesser degree by the type of food. In section 7, the life history parameters for *Tisbe* fed on yeast and SMA milk are compared with *Tisbe* fed on algae. The productivity of *Tisbe* populations is directly correlated with the following life cycle parameters: (a) survival during development, (b) sex ratio, (c) number of egg sacs per female, (d) number of offspring per egg sac, (e) development rate. One type of food might favourably affect one particular parameter.

The results of the lifetime studies in section 7 showed no significant difference in survival to day seven of *T. battagliai* when fed on a mixture of algae, SMA milk or yeast, thus agreeing with results in section 3 and 4. The development rate of *T. battagliai* grown on SMA milk was significantly shorter than when *T. battagliai* were grown on a mixture of algae. The average life span of *Tisbe battagliai* fed on algae was found to be 47.48 days compared to 32.5 days when *Tisbe* were fed on SMA milk. Therefore a diet of SMA milk will increase development rate but reduce life expectancy. The average number of brood-sacs produced by *T. battagliai* was 5.98 when they were fed on a mixture of algae, 4 when fed on SMA milk and 1 when fed on yeast. Fertility is therefore a response variable affected strongly by food type.

Survival experiments in section 3 and 4 showed that the addition of yeast would enable *T. battagliai* to survive and reproduce over several generations. Yeast is used as a diet for intensive rotifer culture (Hoff & Snell, 2004) but no survival to maturity was observed in *G. imparipes,* a calanoid copepod, by Payne (2001), when they were fed yeast. Cereal-based diets, such as rice bran and cereal flour, have been used to culture calanoid copepods with mixed success. Like yeast, these inert diets may contribute indirectly to copepod nutrition by providing a food source for heterotrophic flagellates which are then consumed by copepods (Payne, 2001). These copepods have a larger proportion of fatty acids in their lipid stores which have been synthesised by bacteria rather than phytoplankton. These are less valuable in the diet of fishes (Rippingale & Payne, 2001). The number of fertile females and number of brood sacs produced by *T. battagliai* were significantly affected when they were fed on yeast and so this is not recommended as a diet for the culture of copepods. SMA milk however is a potential diet replacement for *T. battagliai* culture.

The results of the maximum consumption experiment in section 8 found that the volume of faecal pellet production at the highest *Tetraselmis suecica* concentration of 3.74×10^5 cells ml^{-1} was still increasing. Frost (1972 cited in Abu-Rezq *et al.,* 1997) reported that the ingestion of *Calanus* increased proportionately with the increase in concentration of food up to a saturation point. He suggested that above this, ingestion rate may be determined by the passage of food through the alimentary canal or the mechanical efficiency of the filtering process. In the experiment above a stabilizing of faecal pellet production was not seen. The slope of the cure when plotted in a graph, showed that the saturation point was near as shown in Figure 4:30.

Abu-Rezq *et al.*, (1997) found that *Tisbe furcata* (Wilson 1932) initiated feeding on *Rhinomonas reticulata* and *Skeletonema costatum* at low densities of less than 10 cells μl^{-1} but it did not initiate feeding unless the density exceeded 25 cells μl^{-1} for *Pavlova lutheri*. Frost (1972 cited in Abu-Rezq *et al.*, 1997) highlighted similar observations as the ingestion of the alga *Thalassiosira* by the copepod *Calanus* ceased at low densities. He suggested that a critical density of algae might be required before feeding would take place because organisms would not expend energy in feeding when the energetic return from the food was too low. Urry (1965 cited in Abu-Rezq *et al.*, 1997) showed that a concentration of 5 cells μl^{-1} of *Isochrysis galbana* was insufficient to satisfy the nutritional requirements of the copepod *Pseudocalanus elongates*. In the maximum ingestion experiment, faecal pellet production started for all *T. battagliai* individuals at concentration level three (2.34 cells μl^{-1}) as shown in Figure 4:25 and Table 4:24. This may be the critical density of *Tetraselmis suecica* needed for *Tisbe battagliai* to initiate feeding.

A mixture of algae increased the faecal pellet production of *Calanus helgolandicus* as seen by Lacoste *et al.*, (2001) when 10^5 cells ml^{-1} gave >40 pellets female $^{-1}$ day $^{-1}$. The highest pellet production in the experiment described in the present work was 32 pellets female $^{-1}$ day $^{-1}$ at a concentration of 3.74 x 10^5 cell ml^{-1} or 37.36 cells μl^{-1}. Therefore, the maximum ingestion rate for *Tetraselmis suecica* is greater than 3.74 x 10^5 cells ml $^{-1}$ which is of similar concentrations as the cell density reported by Lacoste *et al.*, (2001).

The life time studies of *T. battagliai* grown on a mixture of algae or SMA milk in section 7, had an algal concentration of 5.14 x10^5 cells ml^{-1}. This concentration was above the highest concentration tested in experiments described here. Therefore, all *T.*

battagliai in the life time studies in section 7, had a concentration of algae close to or exceeding the maximum ingestion rate. It is possible, that during medium scale (5ltr) cultures in section 5, the concentration of algae may have been too low for maximum production rates. It is important to provide enough algae for all stages of the copepod populations to survive, grow and reproduce. In the small scale culture experiments, three types of algae were added, increasing the chance of their availability to the different copepod stages. If too much algae is added to a static copepod culture system, the algae cells not eaten will die; increasing the bacterial load while reducing oxygen. Therefore it is important to provide just enough algal cells for the copepods to grow and reproduce on.

Population prediction rates were worked out based on the life history parameters determined for *T. battagliai* grown on algae or on SMA milk. Production of a batch culture of *T. battagliai* may be estimated as numbers of nauplii produced l^{-1} for SMA milk as follows: as a maximum density achieved at Carna, of adult *T. battagliai* of 1000 adults l^{-1}, with 43% fertile female population, 16 nauplii d^{-1} produced in a fertile period of 15 days gives 103,200 l^{-1}. This is the same as Miles *et al.,* (2001) who achieved 100,000 nauplii 1^{-1} in a small volume culture of the Harpacticoid *Tisbe holothuriae.* This production rate along with the reduced cost and ease of production when *T. battagliai* are grown on SMA milk, makes it an attractive food possibility for copepod culture systems.

The production rates of copepods even in intensive systems are well below the production rates for *Artemia* and rotifer cultures. The life history parameters of these live feeds, have been investigated thoroughly however. The use of copepods in the

larval culture of difficult to rear species is increasing (Shields, pers. comm.) as is the need for higher density cultures of small prey items suitable for high-value finfish (McKinnon *et al.,* 2003). The cost of production of *Tisbe battagliai* could be reduced greatly by using SMA milk as an alternative food source to algae. The loss of pigment in the copepods would need to be addressed however.

Conclusion

In this chapter we have found that *Tisbe battagliai* will reproduce on alternative diets, but that a mixture of algae is still significantly better in terms of number of nauplii produced. However the ease of use and the reduced costs of SMA milk as an alternative diet would make up for the reduced performance of the populations. It is not known however what affect SMA milk would have on larval fish survival.

Chapter 5

General Discussion

The aim of this research was to find a reliable food suitable for the first feeding of *Hippocampus* spp. juveniles. Currently, most seahorse aquaculture ventures grow juvenile seahorses on *Artemia*. The price of *Artemia* (€190 for 500g) and the low survival of some species resulted in a need for an alternative food source. Juvenile survival is still one of the main bottlenecks facing successful seahorse culture (Woods, 2003a).

A separate and important issue in an Irish context was to establish if there is a resident population of native seahorses in Ireland. This is because, if seahorses are deemed native, it will have implications for the aquaculture of seahorses in Ireland. As with the aquaculture of any fish species, there is a possibility of disease transfer and genetic weakening of the wild stock by escapees (Tlustry, 2002). Escapees could breed with the native populations or out-compete them reducing the biodiversity. The effluent water from a farm, if not sterilised correctly, can transfer pathogens into the surrounding coastal waters. Salmon farming is a typical example of how aquaculture can affect the wild populations. A report published by Morton *et al.,* (2004) found sea lice infestation rates of 90% on juvenile chum and pink salmon near salmon farms, compared to rates of near zero in areas without salmon farms.

Although most research on seahorses will be carried out in recirculation systems at higher temperatures than the surrounding coastal water, it is still necessary to

investigate the possible side effects from culture practises. From the limited sightings in Ireland however, and as discussed in chapter one, there is no concrete evidence of a native population of seahorses.

In the juvenile seahorse experiments in chapter three, the success of a diet for several *Hippocampus* spp. juveniles was investigated by monitoring ingestion of the diet and survival and growth of the juveniles. The striking and ingestion of food by the juvenile seahorses still did not ultimately lead to survival in all cases. This indicates survival as a more accurate response variable than ingestion. For example, the *H. kuda* juveniles ingested newly hatched *Artemia* but still did not survive to day seven. There was also a difference in growth rate of the juvenile seahorses fed on alternative diets even when survival was not significantly different. This was shown in the *H. fuscus* experiment, when survival was not significantly different to day 14 and 17, but weight was significantly different between newly hatched *Artemia,* enriched *Artemia* and *Tisbe battagliai.* This indicates that for longer juvenile trials, growth is a more accurate response variable. It was found that the success of a diet depended on the species of seahorse. In table 5:1 a list of recommended first feeding diets is given for 4 species of seahorse.

Table 5:1 List of recommended first feeding diets for four species of *Hippocampus* juveniles.

***Hippocampus* Species**	**Food type that gave highest survival rates for first feeding**
H. guttulatus	*Tisbe battagliai* nauplii
H. reidi	*Tisbe battagliai* nauplii
H. fuscus	Newly hatched *Artemia*
H. kuda	*Brachionus plicatilis* (enriched with Selco 2000)

The juvenile seahorses' survival success was high for *H. guttulatus* and *H. reidi* when fed on the copepod *Tisbe battagliai*. Other species of seahorse had high survival success on rotifers and newly hatched *Artemia*. There are already well established protocols for rotifer and *Artemia* production. The advantage of using newly hatched *Artemia* is that they are hatched from cysts bought in a tin and do not require food. To on-grow the *Artemia* and to enrich the *Artemia* will increase the cost of production. This is why newly hatched *Artemia* are recommended in Table 5:1 as there was no significant difference in survival or weight of *H. fuscus* juveniles when fed on enriched or newly hatched *Artemia.*

Provision of copepod nauplii as food increases larval survival in many fish species that are difficult to rear using standard procedures. For many fish species, hatchery production is uneconomical or impossible using either rotifers or *Artemia* as larval diets. This is mostly a result of poor survival at the first feeding stage (Payne & Rippingale, 2000). Increased larval survival when fed on copepod nauplii has been demonstrated for red snapper (Doi *et al.*, 1997a) and some of the groupers (Doi *et al.*,

1997b). Also, the provision of copepods in the early larval diet reduces malpigmentation in halibut (McEvoy *et al.,* 1998) and increases stress resistance in mahi-mahi (Kraul *et al.,* 1993, cited in Payne, 2001). These improvements are mostly attributed to increased feeding by fish on copepods and the high nutritional content of copepods (Payne & Rippingale, 2000).

Copepod culture must demonstrate reliable and prolific production to be accepted as part of commercial operations. Both calanoid and harpacticoid copepods have been cultured for use in fish larviculture (Payne & Rippingale, 2000). The doubling time of populations is 5 – 10 times longer for copepods than it is for rotifers (Tuckers, 1992 cited in Filleul, 1996). However the densities needed in fish-rearing tanks are 5 – 10 times lower than those usually used for rotifers (Hernández Molejón & Alvarez-Lajonchère, 2003).

Copepod cultures that produce highly nutritious nauplii are well suited for use with ornamentals where the value per unit of biomass is much higher than for table fish (Payne, 2001). Payne (2001) found that feeding copepod nauplii for the first 12 days of life maximised the survival of *H. subelongatus* through the first-feeding stage. *Artemia* were then digested by the later juvenile stage. Payne *et al.,* (1998) found that juveniles of pipefish recorded very high survival when provided with cultured copepods in captivity (Payne *et al.,* 1998).

The desirable characteristics of *Tisbe* species for mass production are (Cutts, 2003):

- high reproductive potential
- short turnover time (from egg to egg)

- fast individual growth rates
- fast population growth rates
- good growth on a variety of food sources
- a tolerance of a wide range of environmental factors such as temperature and salinity

Copepods offer a great variety of sizes, species and qualities, and have high levels of protein, highly unsaturated fatty acids (HUFA), carotenoids and other essential compounds (Hernández Molejón & Alvarez-Lajonchère, 2003). The introduction of *Tisbe* nauplii can have an appetite-stimulatory effect (Stottrup & Norsker, 1997). Nauplii that are not eaten would be able to find nourishment in fish rearing tanks by feeding on detritus, the biofilm and bacteria, maintaining their nutritional value as well as keeping the tank clean (Stottrup & Norsker, 1997).

Copepods are routinely grown on a mixture of algae. The process of growing algae is an added expense and cultures can crash (See algae culture section chapter 2). In chapter 4, a cheap, reliable alternative diet to algae was investigated. The different response variables in the diet experiments included initial survival, development through to copepodite stage, maturation, reproduction and reproduction through several generations. A variety of alternative diets were tested and the up-scaling of these experiments into larger systems was investigated. Overall, the addition of SMA milk was the most promising. Although *Tisbe* had fewer brood sacs when fed on milk, the development time was significantly reduced and the number of nauplii per brood-sac was significantly higher than when fed on a mixture of algae. The cost per million of *T. battagliai* nauplii when grown on milk was €0.046, approximately five times cheaper than a million nauplii produced on a mixture of algae (€0.244).

The addition of the SMA milk resulted in the loss of colour from the copepod. This is important as it is thought to be due to a reduction in the carotenoid content. This will need to be investigated in future work as it may have implications for the visibility of the copepods as prey to larval fish. The availability of carotenoids to the growing larval or juvenile fish will be limited and this could have long term consequences on their growth and colour. Copepods rely on the availability of carotenoids such as astaxanthin through the consumption of precursors from their diet (Andersson *et al.*, 2003). Carotenoids are believed to be important for copepods in photo-protection and other antioxidant activities during the day (Andersson et al., 2003). The SMA milk must be lacking in the precursors needed for carotenoid production. Providing live food in the form of copepods, which do not have all the dietary requirements that the seahorses require, may cause long term problems to arise. A way of adding the carotenoids or precursors at a critical level to the copepods before feeding to the fish will be required and this requires further investigation.

In this study, the need for different live food species for the first feeding of several species of seahorses has been demonstrated. This should help to improve the initial survival and the culture techniques for seahorse aquaculture. The successful survival and reproduction of *T. battagliai* on diets other than a mixture of algae was also shown. The significant reduction in price and overall convenience of the SMA milk as a food for *T. battagliai* makes it a viable alternative for culture.

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url 2: www.dobis.ori.u-tokyo.oc.jp/watabe-lab.html laboratory for rotifer culture

url 3: www.angelfire.com/wa/AquariaWeb/artemia.html newly hatched *Artemia*