EVALUATION OF *FABREA SALINA* AND OTHER CILIATES AS

ALTERNATIVE LIVE FOODS FOR FIRST-FEEDING RED

SNAPPER, *LUTJANUS CAMPECHANUS*, LARVAE

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EVALUATION OF *FABREA SALINA* AND OTHER CILIATES AS ALTERNATIVE LIVE FOODS FOR FIRST-FEEDING RED SNAPPER, *LUTJANUS CAMPECHANUS*, LARVAE

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EVALUATION OF *FABREA SALINA* AND OTHER CILIATES AS ALTERNATIVE LIVE FOODS FOR FIRST-FEEDING RED SNAPPER, *LUTJANUS CAMPECHANUS*, LARVAE

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VITA

Melanie Anne Rhodes, daughter of Terry S. (Wood) Rhodes-Forsberg and Jay S. Forsberg, was born on November 16, 1979 in Phoenix, AZ. She graduated from Calvert Senior High School in Prince Frederick, MD in June 1997. In August 1997, she entered Long Island University-Southampton College in Southampton, NY. She graduated with a Bachelor of Science degree in Marine Science, Biology concentration in May 2001. While attending college, she gained work experience through internships at the Wisconsin Aquatic Technology and Environmental Research Institute in Milwaukee, WI and the Chesapeake Biological Laboratory in Solomons, MD. She participated in the SEAmester program in the Fall of 1999, sailing from Boston, MA to San Juan, PR, on the tall ship, *Harvey Gamage*. During the Winter 2001 term she participated in a Tropical Marine Biology program in the South Pacific islands of the Kingdom of Tonga. From March 2001 to September 2001, she was employed by the East Hampton Town Shellfish Hatchery in Montuak, NY as a Hatchery and Field Technician. Returning to Maryland, she was employed from January 2002 to March 2003 as an Aquatic Biologist by Wildlife International, Ltd. in Easton, MD. From April 2003 to June 2003 she lived on board the Schooner *A.J. Meerwald* working as a Deckhand and Environmental Educator. In September 2003, she entered the Master of Science Program at Auburn University.

THESIS ABSTRACT

EVALUATION OF *FABREA SALINA* AND OTHER CILIATES AS ALTERNATIVE LIVE FOODS FOR FIRST-FEEDING RED SNAPPER, *LUTJANUS CAMPECHANUS*, LARVAE

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One of the major challenges with the culture of red snapper *Lutjanus campechanus* is providing an appropriate food source at the onset of feeding. At first-feeding the larvae has limited endogenous reserves and within hours must find an appropriate size food organism to survive. Protozoans are important trophic links from bacteria and phytoplankton to metazoans. Ciliated protozoans may be more important for first-feeding fish larvae than other similar sized traditional live feeds because they naturally occur in higher densities and without lorica they can be more easily digested. Behavioral observations of first feeding snapper larvae were conducted with three

different ciliates as a food source. The three ciliates, *Fabrea salina, Strombidium sp*. and *Strombidinopsis sp*. are various sizes and have unique swimming speeds and patterns. Observing the foraging behavior of fish larvae when exposed to different prey allowed for the selection of a diet for the production of fish. There was a reduction in protozoan densities suggesting the protozoans were consumed by the fish larvae. Experiments were conducted to determine the optimum culturing conditions of one of the naked ciliates, *Fabrea salina* to be used as a food source. Growth conditions evaluated included ciliate stocking density, algae food species and density and photoperiod. The trials optimum results, 12 hours light/day, initially stocking 3 *F. salina*/mL and feeding 9.0×10^4 *Rhodomonas lens* cells/mL/day were then applied to 40 and 200 L mass culture conditions. Average densities of 66 to 91 ciliates/mL were common in 6 to 8 days. The mass cultures of *F. salina* provided approximately 16 million ciliates in 7 days. The *F. salina* was compared to copepod nauplii as well as a combination of *F. salina* and copepod nauplii as first-foods for larval red snapper in a large-scale experiment. The treatment provided with the combination of *F. salina* and copepod nauplii resulted in $2.39 \pm 2.75\%$ survival which was much higher than the $0.28 \pm 0.15\%$ survival when fed copepod nauplii alone. There was no fish survival in the treatment fed *F. salina* alone for first-feeding. The growth was significantly higher in the treatment fed *F. salina* and copepod nauplii than the treatment fed copepod nauplii alone. The results of the snapper rearing trial imply that *F. salina* and possibly other protozoans can be used to supplement the diet for first-feeding fish larvae to increase growth and survival.

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I. INTRODUCTION

Red snapper, *Lutjanus campechanus* is an economically important marine fish that is subject to intense commerical and recreational fishing pressure. Culture of this fish for food fish production and wild stock enhancement have been limited due to low larval survival in hatcheries. Development of suitable first-feeding regimens for mass rearing of larval marine fish is one of the major barriers to successful propagation. The small mouth size, uncompleted development of their perception organs and digestive system are limiting factors in proper food selection for the first-feeding period (Lavens and Sorgeloos 1996). At the onset of feeding the digestive tract only has a few functional enzyme systems. Thus, a proper first-food source should be at least partially and easily digestible, contain enzyme systems which allow autolysis and supply in abudanance of the essential nutrients required by the larval fish (Lavens and Sorgeloos 1996). Based on these criteria, ciliated protozoans are suitable as a first food for larval fish.

Ciliates often dominate the microzooplankton and are a major link in the marine microbial loop (Azam et al. 1983). There is limited data on the predation of naked ciliates by fish larvae since there are no indigestible hard parts that can be identified in the fish guts. There have been several lab studies that have detected ciliates ingested by fish larvae using various techniques including epifluorescent microscopy, fluorescent mircrospheres, immunofluorescent antibody probes and fluorescent rRNA probes (Ohman et al. 1991, Ohman 1993; Lim et al. 1993; Lessard et al. 1996;

Fukami et al. 1999). Ciliates have a variety of swimming speeds, swimming patterns, and escape responses (Buskey et al. 1993) that may stimulate larval fish response. Most culture techniques for protozoans have been developed at a small laboratory scale. Determining the optimum culture conditions for ciliates is necessary to produce mass quantities that can be used for larval fish culture.

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II. LITERATURE REVIEW

Marine Larval Fish Culture

Development of suitable feeding regimens for mass rearing of larval fish represents one of the primary barriers to successful propagation of a variety of marine species. Many of the first-feeding problems result from the relatively small mouth size of first-feeding larvae (Shirota 1970) and their limited yolk reserves. Fish larvae are visually oriented feeders. Foraging success is largely dependent on the availability of suitable prey items. If feeding is initiated too late, after the optimum period, the larvae may irreversibly lose their ability to digest and utilize food (Lasker et al. 1970; May 1974; Laurence 1974; Bailey and Stehr 1986). It may be important to introduce food even before the digestive system of the larvae is completely developed in order to simulate normal feeding behavior.

The microplankton assemblage (20-200 μm) represents particle-size ranges that are utilized by invertebrates and fish larvae as food. Within the microplankton are the phytoplankton, including phyto-flagellates and dinoflagellates and the zooplankton including copepod nauplii and ciliates. At the onset of feeding, most marine fish larvae select prey ranging from 50-100 μm in width (Hunter 1980; Houde and Scheketer 1980; Thielacker 1987). Depending on the mouth size of fish larvae, rotifers, copepod nauplii or brine shrimp have been traditionally provided to many first-feeding fish larvae in aquaculture.

In addition to the size of the prey organism, the swimming speed and pattern of prey may be important. The swimming patterns and speeds of the prey items presented may visually stimulate larvae in different manners and influence the ease of capture. Protozoans were found to have a mean speed of 0.154 ± 0.06 cm/s compared to a speed of copepod nauplii 1.65 ± 0.20 cm/s (Hunt von Herbing and Gallager 2000). Protozoan escape response was found to be poor, 0.27 ± 0.15 cm/s while nauplii had an escape speed of 2.07 ± 0.5 cm/s (Hunt von Herbing and Gallager 2000). Copepod nauplii also have a very high rate of change of direction ranging from 589 to 1,931 degrees/s compared to ciliates with a range of 88 to 502 degrees/s (Buskey et al. 1993). The slower rate of changing direction of a given live food may make its capture easier by a fish larvae.

Capture success is a critical factor determining whether first-feeding larvae succeed in consuming enough prey to survive. Bay anchovy search time decreased from 0.4 h at 100 prey/L to only 0.04 h at 1,000 prey/L with the difference in consumption being 1.2 prey/h compared to 12.5 prey/h (Houde and Schekter 1980). First-feeding bay anchovy and sea bream attacks were only successful 50% of the time (Houde and Schekter 1980). The encounter rate may not only be affected by density and swimming rate of prey but also turbulence and other environmental factors (Chesney 1989).

There are several ways to evaluate the successful feeding of fish larvae. The feeding response can be estimated by the disappearance of prey (clearance rate), calculating ingestion rates, digestion or gut evacuation rates, fish growth rates, survival and the condition of larvae by comparing physical attributes and biochemical properties,

and observing foraging behavior (Ivlev 1960; Ricker 1975; Houde 1978; Chesson 1983; Webster and Lovell 1990; Boujard and Leatherland 1992; Dou et al. 2000).

Final measures of growth and/or survival are the simplest and most direct ways to evaluate feeding success without sacrificing fish larvae. Growth can be measured by length, weight or other measures of condition. Estimates of production as defined by Ricker (1975) as the total elaboration of new body substance in some unit of time, regardless of whether it survives to the end of that time is calculated in many studies like that of Houde (1978).

Stomach contents can be useful to determine if feeding occurs, but doesn't necessarily imply growth and survival. Gut content analysis is often time consuming and difficult to quantify, estimating gut fullness (Omori and Ikeda 1992), presence or absence of prey (Webster and Lovell 1990) and feeding incidence defined as the proportion of larvae within a sample with food in the gut (Dou et al. 2000) are often used. The disappearance of prey can be determined by counting the concentration of prey remaining after some period of time. Prey disappearance can be used to estimate ingestion rate if prey growth rate is accounted for. There are several indices that can by used to measure prey selectivity (Ivlev 1960; Chesson 1983).

Understanding feeding behavior of first-feeding fish larvae is important to predict feeding rhythms, rates and food selectivity (Boujard and Leatherland 1992). Fish larvae are poor swimmers and many have a S-flex attack posture (Hunter and Kimbrell 1980). The occurrence of this attack posture and other Modal Action Patterns (Barlow 1977) which include search and capture actions can be used to estimate the feeding activity for a given period of time when presented with different prey choices and densities. The

number of strikes or attempted captures can be used to calculate capture success, which is the proportion of prey attacks that were successful. Search rates may also be useful to determine the optimum prey stocking density.

Artemia

Brine shrimp *Artemia* are in the phylum Arthropoda, class Crustacea. The life cycle begins by hatching from dormant cysts, containing encysted embryos that are metabolically inactive. When the cysts are placed in seawater, they re-hydrate and the embryos resume development (Persoone et al. 1980; Sorgeloos et al. 1986). After 15-20 hours at 25 C, the cyst bursts and the embryo exits the shell. The embryo hangs beneath the cyst shell for the first few hours enclosed in a hatching membrane (umbrella stage). Inside the hatching membrane the nauplius completes development, its appendages begin to move and it emerges free-swimming. The first larval stage (Instar I) is 400-500 μm in length. Newly hatched nauplii do not feed since their mouth and anus are not yet developed, they consume their own energy reserves (Benijts et al. 1976). At the high temperatures used during cyst incubation, freshly hatched *Artemia* nauplii develop into the second larval stage (Instar II metanauplii) within 6-8 hours. They are about 50% larger than first-instar, swim faster and they contain less free amino acids.

Relative to small marine larvae, *Artemia* nauplii are normally supplied later in the larval fish rearing process at a density ranging from 2-20/mL. Hunter (1980) compared the relationship between prey size and larval length for 12 marine fishes. Only hake larvae, *Merluccius merluccius*, could adequately feed on *Artemia* nauplii at the onset of feeding. Since then, other species of fish larvae including striped bass, *Morone saxitilis* (Webster and Lovell 1990) have been fed *Artemia* nauplii as a first food. Although using

Artemia cysts appears to be simple, several factors are critical for hatching the large quantities needed for larval fish production. These include cyst disinfection or decapsulation prior to incubation, and hatching under the following optimal conditions: temperature of 25-28 C, 15-35 ppt salinity, minimum pH of 8.0, near saturated oxygen levels, maximum cyst densities of 2 g/L, and strong illumination of 2,000 lx (Lavens and Sorgeloos 1996). After hatching, prior to feeding to larvae, *Artemia* nauplii should be separated from the hatching wastes. It is essential and common practice to enrich these live prey with emulsions of marine oils because commercially available strains are relatively poor in docosahexaenoic acid (DHA), 22:6n-3, needed for fish development (Sorgeloos et al. 2001).

Rotifers

The rotifer *Brachionus plicatilis* is the major component of initial feed for a variety of marine fish larvae (Watanabe et al. 1978 a,b; Lubzens 1987). Rotifers are in the phylum Rotifera, a lower invertebrate phylum closely related to the nematodes (round worms). There are three classes of Rotifera: Seisionidea which are unusual marine forms, Bdelloidea which are a worm-like group that reproduce strictly asexually, and Monogononta which is the class containing *Brachionus plicatilis*, *B. calyciflorus* and *B. rubens* (Wallace and Snell 1991). Monogononts have a cyclically parthenogenetic life cycle that contains both asexual and sexual phases (Birky and Gilbert 1971; King and Snell 1977).

Rotifers possess several characteristics that make them attractive as live food in mariculture: (1) They are relatively small, ranging in size from 60-300 μm, depending on the zoogeographical strain and stage of development; (2) They are slow swimmers, yet

maintain their position in the water column; (3) They can be cultured at high densities; (4) They reproduce rapidly, making them available in large quantities in a relatively short period; and (5) They can be enriched with fatty acids or other nutrients which are required for growth and survival of larvae (Lubzens et al. 1989).

Rotifers were found to be a suitable first-feed for marine fish larvae in Japan in the late sixties and early seventies (Lubzens et al. 1989; Hagiwara et al. 2001). The introduction of rotifers marked the first regular successes in the mass larval rearing of several marine species such as grey mullet, *Mugil cephalus* (Nash et al. 1974), sole, *Solea solea* (Howell 1973; Girin 1974; Fuchs 1978, 1982; Dendrinos and Thorpe 1987), gilthead seabream, *Sparus aurata* (Person-Le Ruyet and Verillaud 1980, 1981; Tandler and Helps 1985), sea bass, *Dicentrarchus labrax* (Barnabé 1974; Girin 1974), turbot, *Scophthalmus maximus* (Kuhlmann et al. 1981; Olsen and Minck 1983; Witt et al. 1984), flounder, *Paralichthys olivaceus* (Fukusho et al. 1985) and milkfish, *Chanos chanos* (Liao et al. 1979; Juario et al. 1984). Larval red snapper have not successfully been reared with rotifers as the only food item.

The variation among strains of *B. plicatilis* has been documented in several papers (Fukusho and Okauchi 1982; Yufera 1982; Serra and Miracle 1983; Snell and Carrillo 1984; Snell and Winkler 1984). Fukusho (1983) categorized *B. plicatilis* into two major groups. These have commonly been called the S-type Hawaiian strain and L-type Tokyo strain. Hirayama (1990) compared the S- and L-types and reported that S-type strains tend to be round, ranging in length from 150-220 μm as compared to the 200-360 μm for oblong L-type strains. Genetic characterization of these strains has led to the conclusion that S- and L-types are different species (Fu et al. 1991 a,b). Recognition of these

differences has led to the naming of L-type as *Brachionus plicatilis* and S-type as *B. rotundiformis* (Segers 1995). Another type used less widely is the SS-type, *Brachionus sp*., ranging in length from 130-190 μm making it smaller than the S-type. Some other small size strains include Java, Tahiti, Spain, Eilat small, JS1 and JS2 and large size strains N.R.E. and JL (Lubzens et al. 1989).

Choosing the appropriate rotifer strains to be mass cultured depends on the environmental conditions of the hatchery and the size of the larval predator. Different fish species may also prefer different densities, 10 rotifers/mL is generally the most accepted level (Eda et al. 1990a, b; Ostrowski and Molnar 1998).

In initiating mass cultivation of rotifers, the following problems must be considered. 1) A rotifer strain must be selected which will be an appropriate size for the larvae and suitable for the culture conditions; 2) The food quality and quantity must be maintained at an adequate level; and 3) Water quality parameters in culture tanks (i.e. temperature, salinity, pH) must be controlled and waste products must be removed (Lubzens 1987). Rotifer feeding rates and optimum growth change with environmental conditions like temperature, salinity, as well as food quantity and type (Hirayama and Ogawa 1972; Scott and Baynes 1978).

Depending on the scale of production needed, there are various methods of culturing rotifers. They can be sorted into three basic methods, batch cultures, semi-continuous cultures, and continuous 'feedback' culture systems (Lubzens 1987). For batch cultures, rotifers are introduced at low densities 50/mL into dense algal cultures (green-water) and harvested once all the algae are consumed. The batch method has distinct advantages, production is more predictable, there is less chance of contaminants

overtaking the rotifer culture and it is easier to maintain rotifer populations in the log phase of growth.

Semi-continuous cultures are similar to batch cultures but once the algae are consumed, an external supply of algae is provided until the rotifers reach a density greater than 100/mL. Every 1-3 days, about 10-30% of the water is removed and replaced by seawater containing algae. After several harvests, the rotifer reproduction rates decrease and all the rotifers are harvested. The culture volumes vary greatly from tens of liters to more than ten thousand liter cultures. The 'feedback' system uses a stream unit with partitions that the culture is circulated through to remove feces and accumulated particulate matter (Hirata 1979). The collected feces and particulate matter is used to fertilize algae cultures, which are then used to feed the rotifer cultures. Harvesting begins once the rotifer density reaches 100-150 rotifers/mL and can reach greater than 500 rotifers/mL.

Brachionus plicatilis is an omnivorous filter feeder, feeding on a variety of food types including, algae, yeast, bacteria or inert food such as microcapsules. Food quantity and quality play an important role in the growth of rotifers and may change the biochemical composition. The marine alga, introduced as marine *Chlorella sp.* by the Japanese but now recognized as *Nannochlorpsis sp.* (Maruyama et al. 1986) is considered one of the best. Other microalgae suitable for rotifer culture include: *Chaetoceros sp., Dunaliella sp., Pyramimonas sp.*, *Isochrysis sp*., and *Tetraselmis sp.* Okauchi and Fukusho (1984) presented evidence that *Tetraselmis tetrathele*, a motile microalga, may even be better than *Nannochloropsis sp.*

An extensive analysis of the biochemical composition of *B. plicatilis* was completed by Watanabe et al. (1983b). *Nannochloropsis* fed rotifers were composed of 75% protein, 22% lipid and 3% ash by dry weight, similar to yeast fed rotifers of 71%, 17%, and 12%, respectively. Other studies have examined the biochemical composition of rotifers fed different diets (Scott and Baynes 1978; Ben-Amotz et al. 1987; Frolov et al. 1991; Nagata and Whyte 1992; Tamaru et al. 1993). The dry weight of a single rotifer depends upon its nutritional state and body size, but ranges from 0.12 to 0.36 µg per female, excluding eggs (Doohan 1973; Theilaker and Kimball 1984; Lubzens et al. 1989). The ash-free caloric content of rotifer biomass ranges from 4.8 to 6.7 cal/mg (Lubzens et al. 1989).

Mixed Zooplankton/Copepods

Wild net-collected plankton may be a more beneficial food source for marine fish larvae than the easily cultivated *Brachionus sp.* and *Artemia*. Studies where an *Artemia* diet has been supplemented with wild net-collected plankton have often led to better growth and survival of turbot, *Scophthalmus maximus* larvae (Witt et al. 1984; Nellen 1985). Such results raise doubts concerning the nutritional suitability of *Artemia* for fish larvae. Evidence that copepods may be preferable to *Artemia* as a food source for marine fish larvae also comes from the work of Pederson (1984) who examined the digestion in first-feeding herring, *Clupea hargenus* larvae hatched in the laboratory and found that copepods from wild zooplankton samples were passed through the gut and were digested more thoroughly than *Artemia*. Natural plankton collected with a 35 µm net was sorted by size and most potential food organisms offered to larvae were less than 150 µm wide during the initial phase of rearing. The scaled sardine, *Harengula pensacolae* and the bay

anchovy *Anchoa mitchilli*, both preferred copepod nauplii, copepodites, and copepods as food (Detwyler and Houde 1970) when the density of potential food organisms averaged 1,600-1,800/L. Size selection of food by larvae is thought to be regulated through visual attraction and limitations caused by size of mouth and gullet. The use of wild plankton has disadvantages: samples are expensive and time-consuming to collect and the available species and quantities are seasonally dependent.

Copepods belong to the Class Crustacea further placed in the Subclass Copepoda which is divided into eight Orders: Calanoida, Cyclopoida, Harpacticoida, Monstrilloida, Notodelphyoida, Caligoida, Lernaeopodoida, and Arguloida. Of the three free-living, non-parasitic orders, the calanoids are largely planktonic, the harpacticoids are largely benthic, and the cylopoids contain both planktonic and benthic species (Barnes 1963). Copepods sexually reproduce, depending on the species, it can take a month or more to complete their reproductive cycle. This makes it difficult to maintain a culture that can produce sufficient offspring to sustain a large population of rapidly growing nauplii. Newly hatched nauplii go through four to six naupliar stages and then become copepodites. After five copepodite stages they metamorphosis into an adult stage and molting stops. Depending on the species, the entire development may take less than one week or up to a year (Omori and Ikeda 1992).

Based on visual observations of larval fish guts collected in the field, copepod nauplii (generally > 100 μm) are considered to be the primary food of first-feeding pollock, *Theragra chalcogramma* (Nishiyama and Hirano 1985; Kendall et al. 1987; Nakatani 1988; Canino et al. 1991). Several attempts have been made to culture species such as, *Acartia spp.*, *Tigriopus japonicus*, *Oithona spp.*, *Paracalanus spp.*, and

Eurytemora spp. for feeding early larval stages in intensive systems (Støttrup et al. 1986; reviews by Nellen 1981 and Foscarini 1988).

The calanoid copepod, *Acartia tonsa,* is more commonly cultured than any other species (Zillioux 1969; Paffenhofer and Harris 1979; Ogle 1979, 2000; Turk et al. 1982). *A. tonsa* has been cultivated and used as a food organism for herring, *Clupea harengus*, turbot, *Sophthalmus maximus* L. and plaice, *Pleuronectes platessa* larvae (Munk and Kiørboe 1985). Turk et al. (1982) reported maximum densities of *A. tonsa* ranging from 870 to 1,680 nauplii/L, taking 6 to 33 days to reach this density.

Mass production of the calanoid *A. tsuensis* was reported successful by Støttrup et al. (1986) and Ohno and Okamura (1988). Lavens and Sorgeloos (1996) reported maximum densities of *A. tsuensis* of 1,300 nauplii, 590 copepodites with a maximum egg production of 350 eggs/L/day. They used a simplified synchronized method of placing filtered natural seawater in a tank and allowing it to progress through various stages of development from diatoms to nanoflagellates and dinoflagellates followed by blooms of ciliates, rotifers and eventually copepods.

Tisbe holothuriae is a benthic species, copepodites remain primarily on the vessel wall and unavailable to pelagic larval fish. Their naupliar stages are planktonic but their small size limits the applicability to the first week of turbot culture (Støttrup et al. 1993). The harpacticoid *T. holothuriae* fed *Rhodomonas baltica* was chosen as a model species for this work but other harpacticoids like *Tigriopus sp.* have been part of commercial rearing practices (Fukusho 1980). Hagiwara et al. (1995b) had success in culturing the harpacticoid *Tigriopus japonicus* as feed for larviculture. Methods for its mass culture were described by Fukusho (1980) and Kahan et al. (1981) and its physiological

requirements by Lee and Hu (1980). Attempts have been made to rear turbot in intensive systems on wild copepods or with supplement of copepods (Witt et al. 1984) and a few attempts at rearing copepods or other zooplankton in the laboratory (review by Nellen 1985; Støttrup et al. 1986). In laboratory feeding studies with copepods, the use of microalgae is standard. As alternatives to microalgae, rice brans and artificial foods have been utilized (Turk et al. 1982).

Outdoor tanks fertilized with cottonseed meal and pelleted hog feed were seeded with copepods producing nauplii densities > 1,000/L (Bootes, 1998). Red snapper eggs were added to these tanks and larvae survived in 36% of the tanks. Copepod nauplii harvested from outdoor brackish-water ponds as a first-food for red snapper has yielded variable results, often without survival (Lan, 2000; Lindley, 2004).

Protozoans

The kingdom Protista contains the subkingdom Protozoa, defined as single-celled eukaryotic organisms. The protozoans are numerically an important component of the microplankton assemblage (included in both the phytoplankton and zooplankton) that can be used as alternative live feeds. Lorica of tintinnid ciliates, skeletal material of radiolarians and shells of planktonic foramniferans are reported in the gut contents of most taxa of planktonic invertebrates and fish larvae (reviewed by Conover 1982). Tintinnids and dinoflagellates were found frequently in the guts of northern anchovy, *Engraulis encrasicholus* (Berner 1959) and the *Engraulis anchoita* (Vinas and Ramirez 1996) collected at sea. However, the majority of protozoa have no hard parts and thus the role in diets of zooplankton and fish larvae have been ignored (Stoecker and Govoni 1984). Protozoans may also be a useful food for first-feeding larvae in culture since they

are high in omega-3 fatty acids and may be easier for first-feeding larvae to capture and digest than other organisms, thus potentially increasing larval survival (Gallager et al. 1996a).

Flagellates/Dinoflagellates

There have been several attempts to feed larval fish with members of the Phylum Sarcomastigophora, which includes the flagellates and autotrophic dinoflagellates. Fabre-Domergue and Bietrix (1905) successfully fed cultured *Dunaliella salina*, an algal flagellate, to newly-hatched sole, *Solea vulgaris* larvae, while Kasahara et al. (1960) reared black porgy, *Mylio macrocephalus* by supplying first-feeding larvae with *Oxyrrhis sp*., an unarmored heterotrophic dinoflagellate. Stoecker and Govoni (1984) showed that young larval gulf menhaden, *Brevoortia patronus* fed on the tintinnid *Favella sp*.and the dinoflagellate *Prorocentrum sp*. Two armored dinoflagellates, *Prorocentrum micans* and *Fragilidium heterolobum*, did not sustain life in first-feeding anchovy larvae (Lasker et al. 1970). Both were eaten by the larvae, but the day of maximum mortality coincided with that of the starvation controls. The unarmored dinoflagellate, *Gymnodinium splendens* was eaten immediately and the mortality rate was sharply curtailed. The survival of anchovy larvae fed on *G. splendens* alone was compared to that of *G. splendens* and one of each of the 3 veligers (*Haminoea vesicular*, *Navanax inermis*, *Bulla gouldiana*) and compared to specimens fed only *B. gouldiana*, and a starvation control. When larvae were fed *G. splendens* alone or in combination with veligers, mortality was slowed considerably. The combination of *G. splendens* and veligers supported growth to a greater degree than did *G. splendens* alone (Lasker et al. 1970).

A technique was developed for the semi-mass culture of the autotrophic dinoflagellate, *G. splendens* yielding a maximum density of 4,600 to 6,800 cells/mL in 8 to 11 days of culture (Rodriguez and Hirayama 1997). An initial feeding trial showed red spotted grouper, *Epinephelus akaara* preferred *G. splendens* fed 200 cells/mL with 44% survival. However, the Japanese stripe knife jaw, *Opelgnathus fasciatus* and red sea bream, *Pagrus major*, did not respond well to the feeding *G. splendens* (Rodriguez and Hirayama 1997).

Ciliates

The Phylum Ciliophora is the largest group within the Protozoa. Marine ciliates are conventionally divided into loricate (tintinnid) and aloricate (naked) forms and comprise a large proportion of total microzooplankton abundance and/or biomass (Sanders 1987; Kamiyama 1994; Uye et al. 1996). Reviews by Sherr et al. (1986) and Pierce and Turner (1992) suggest that ciliates represent a significant trophic link between the microbial food web and metazoans, although mainly via ciliate-copepod links. There is little information on the predation on protozoa by fish larvae, except for the loricate tintinnids (Last 1978a, b, Govoni et al., 1983, Stoecker and Govoni 1984, Nishiyama and Hirano 1985; Ikewaki and Tanaka 1993).

Naked ciliates may be more important food for fish larvae than tintinnids, because naked ciliates occur in considerably larger numbers than tintinnids (Pierce and Turner 1992). Fukami et al. (1999) investigated predation on naked protozoa by fish larvae collected in the field. The gut contents of fish larvae belonging to 52 different taxonomical groups were examined using epifluorescence microscopy. Protists were found in the guts of 42 fish taxa.

Van der Meeren and Naess (1993) found that mesocosm-reared first-feeding larval cod maintained normal growth and survival by feeding mostly on ciliates, when ciliates were abundant (<7,130/L) and copepod nauplii were scarce (<5/L). This provides evidence that ciliates may enhance survival of first-feeding larvae in natural environments when copepods are scarce.

There are only a few available reports, based on laboratory experiments, which determined the effect of dietary ciliates on larval survival. Investigations of predation on protists by metazoans have been largely relied on the indirect method of following the disappearance of prey in long-term incubations (Stoecker and Govoni 1984; Gifford and Dagg 1988; Tiselius 1989). Korniyenko (1971) noted a few authors who stated protozoa was present in the food of larvae and adult freshwater fish and found consumption of ciliates by first-feeding larvae of freshwater fishes enhanced their survival. Larval sea bream, *Lithognathus mormyrus*, appeared to grow faster with lower mortality when microzooplankton including ciliates were available as prey (Kentouri and Divanach 1983). However, Howell (1972) reported the ciliate, *Euplotes vannus* was not accepted by lemon sole larvae. Feeding on ciliates by larval fish at first-feeding may prevent initial starvation and prolong the time to irreversible starvation of fish larvae.

There are laboratory reports where naked ciliates, including *Balanion sp*., *Euplotes sp.* and *Strombidium sp.* have been preyed upon by marine fish larvae (Ohman et al. 1991; Lessard et al. 1996; Fukami et al. 1999; Hunt von Herbing and Gallager 2000; Nagano et al. 2000a, b). Techniques for directly detecting ingestion of heterotrophic protists include the use of protist-specific immunofluorescent antibody probes (Ohman et al. 1991; Ohman 1993) and fluorescent rRNA probes (Lim et al. 1993).

These methods still require substantial development, analysis time and specialized equipment. An ideal labeling technique would 1) not effect prey behavior or chemical signals used by predator to find prey, 2) allow for easy detection of marked prey after ingestion and 3) allow marked prey to be readily distinguished from unmarked prey (Lessard et al. 1996).

Fabrea salina

Attempts have been made to culture the marine heterotrichous ciliate, *Fabrea salina* for aquaculture (De Winter and Persoone 1975; De Winter et al. 1976; Kahan 1976; Uhlig 1981; Rattan et al. 1999; Park and Hur 2001; Pandey and Yeragi 2004). The advantages of *Fabrea salina* as an alternative for rotifers or brine shrimp larvae were summarized by De Winter and Persoone (1975) as follows: It is one of the few truly "pelagic" heterotrich ciliates, it has the appropriate dimensions as a live food (50-500μm), the smooth cell wall and the absence of appendages facilitate its uptake by the predators, the generation time is very short, as a particle feeder it can be cultured on live algae as well as inert foods, according to the literature data its nutritional value for fish larvae seems to be excellent, it forms a tough cyst membrane when submitted to unfavorable environmental conditions and the cysts can be kept viable for a certain period of time without losing their hatchability.

De Winter et al. (1976) varied several abiotic factors to determine the effect on growth rate of *Fabrea salina*. Small-scale experiments were conducted in petri dishes containing 5 mL of yeast solution as sole food source to eliminate the bias that living food may have varied nutritional value due to changing abiotic conditions. Salinity experiments were conducted at 27 C, at 30, 50, 70 and 90 ppt. Salinities of 30 and 90 ppt

yielded the highest densities. Temperature experiments were carried out from 18-42 C at 70 ppt. It was shown that *F. salina* prefers high temperatures with best growth at >34 C However, due to the high metabolic rate at high temperatures, the appropriate temperature for mass culturing should be chosen between 27 and 34 C, in order to avoid a sudden collapse of the population. The results of Uhlig (1981) showed that *F. salina* had a generation time of about 2 days at 18-20 C.

In order to determine the optimum culturing condition with regard to temperature and salinity, a factorial experiment was conducted by De Winter and Persoone (1975). The ciliates were cultured in sixteen different temperature-salinity combinations of 18, 27, 36, and 45 C and 25, 35, 45 and 55 ppt salinity. The ciliates were counted after 2 days and analyzed by a computerized factorial program. The calculated optimum lies at 32 C and 46 ppt with a generation time of 8.7 h. Cultures were not affected by the presence of light. Rattan et al. (1999), recommends *F. salina* to be cultured at 28 C and a range of 50 to 70 ppt, which is similar to the finding by De Winter et al. (1976).

De Winter et al. (1976) also conducted experiments with two different food sources, the nanoplanktonic alga *Dunaliella viridis* and dry yeast. To test the influence of algal concentration, three different concentrations were offered, 2, 4, and 8 x 10^6 cells/mL. The lowest algal density gave the fastest reproduction rate. After 5 days, the food was depleted and the culture declined. Feeding lower algal concentrations several times was attempted. The best results were obtained with intermittent feeding with 2-4 x 10^6 cells/mL added 2 or 4 times throughout the trial as food was depleted.

De Winter et al. (1976) attempted culturing *F. salina* in 30 L cylinders using the previously determined optimum conditions. The ciliate density increased from 1.3

ciliates/mL to 130 ciliates/mL within 4 days. Similar results were obtained by Uhlig (1981), who found the density in 45 L bags increased from 0.5 ciliates/mL to 100 ciliates/mL within two weeks.

Rattan et al. (1999) attempted mass culture of *F. salina* using 40 L jars with fermented rice bran instead of live algal food. Cultures fed rice bran yielded higher densities than cultures fed with commercial yeast or fermented wheat bran. The salinity and temperatures were maintained at 50 ppt and 29 ± 1 C, respectively. The highest concentration obtained was 58 ciliates/mL in 5 days with an initial concentration of 3 ciliates/mL. The mass cultures that used inert food had lower overall densities than those fed algae. They concluded that more research was needed to improve the methodological and technical aspects of the mass culturing of *F. salina*.

Densities higher than 100 ciliates/mL were obtained in smaller volumes. De Winter et al. (1976), with a 7 L aquarium "raceway" system, achieved growth from 1 ciliate/mL to 187 ciliates/mL in 4 days. Uhlig (1981) experimented with a series of four aquaria each with 2 L of water and a continuous flow of a constant food suspension. After 2 ½ weeks, the population reached more than 1000 ciliates/mL in the first aquarium in the series.

There are several reports of feeding *F. salina* to larval fish (Renè 1974; Barnabè 1974; Park and Hur 2001). Renè (1974) used *F. salina* as a successful substitute for young *B. plicatilis* to feed larval gilthead, *Sparus aurata*, 3-7 days after hatching. Barnabè (1974) reported similar results with feeding larval sea bass, *Dicentrachus labrax*. There were no other reports mentioning the use of *F. salina* as a first food until Park and Hur (2001) found higher mortality of larval ayu, *Plecoglossus altivelis* when fed

F. salina when compared *Brachinous plicatilis*. This was probably because *F. salina* is much smaller than *B. plicatilis*. *F. salina* may be better suited for larval fish requiring a smaller first feed. Considering the results of other researchers, it seems probable that *Fabrea salina* can be mass cultured for practical applications in mariculture.

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III. CULTURE CONDITIONS FOR THE MARINE HETEROTRICHOUS CILIATE, *FABREA SALINA* HENNEGUY FOR AQUACULTURE PRODUCTION

Abstract

Ciliated protozoans are abundant in marine waters but their significance as a first food for fish larvae is poorly understood. Many have no lorica to facilitate their identification in the gut of a larval fish. *Fabrea salina* is a potential candidate for as a first food for many marine fish larvae. It is an appropriate size, 140 x 100 μm for many first-feeding marine fish larvae and has high levels of essential fatty acids. Culture experiments altering stocking densities of ciliates and algae, algal food type and photoperiod were conducted. Optimum conditions for growth were found to be 12 hours light day^{-1}, an initial stocking rate of 3 *Fabrea* mL^{-1} and 9.0 x 10^4 *Rhodomonas lens* cells mL^{-1} day^{-1}. Temperate and salinity were not controlled but were in a range of 25-30 C and 32-36 g L^{-1} respectively. Optimum conditions were applied to 200 L solar tubes resulting in a consistent batch culture of approximately 16 million ciliates in 7 days.

Introduction

Protozoans are important trophic links from bacteria and phytoplankton to the metazoans (Azam, Fenchel, Field, Gray, Meyer-Riel & Thingstad 1983). Ciliated protozoans may be important natural first foods for fish larvae because they often dominate coastal waters, are more abundant than copepod nauplii and rotifers (Kamiyama 1994) and most of the ciliates in the plankton are of a similar or smaller size than copepod nauplii

(Taniguchi 1978). There is some information on the predation of protozoans by fish larvae, especially for the loricate tintinnids, which have indigestible hard parts that can be identified in the guts (review by Pierce & Turner1992). Korniyenko (1971) found that larvae of three fish species (silver carp, bighead and grass carp) consumed mixed freshwater protist enrichments. Gut content analysis showed protists accounted for 92-96% of the ingested material and the protists satisfied food requirements of the fish larvae. Grouper larvae (Nagano, Iwatsuki, Kamiyama, Shimzu & Nakata 2000a) and surgeonfish larvae (Nagano, Iwatsuki, Kamiyama & Nakata 2000b) were reported to be able to feed on the aloricate ciliate, *Euplotes sp*. immediately after yolk resorption and survived better than the starved controls. Nagano et al (2000a, b) detected the ciliate in the larval guts by feeding fluoresent microspheres to the ciliate. Ohman, Theilacker & Kaupp (1991) detected the aloricate choreotrich *Strombidium sp*. with immunochemical methods in the guts of larval anchovy when ciliates were at densities as low as 0.8 ciliates mL^{-1} and determined that 5-8 cells mL^{-1} are needed to meet metabolic requirements of the larvae. Fukami, Watanabe, Fujita, Yamoka & Nishijima (1999) investigated predation on aloricate protozoa by 52 different taxonomical groups of fish larvae collected in the field. They found fishes in the superorder Acanthopterygii (spiny rayed fishes) consumed the greatest quantity of protozoans (>30 protists $individual^{-1}$).

Fabrea salina Henneguy is a heterotrich ciliate in the family Climacostomidae (Repak 1972). *F. salina* naturally occur in hypersaline areas (salt marshes, hypersaline lakes and salterns) in subtropical and tropical environments (Kirby 1934; Ellis 1937; Post, Borowitzka, Borowitzka, Mackay & Moulton 1983 and Rattan, Ansari, & Sreepada 1994). *F. salina* is a relatively large protozoan, ranging from 120-220 μm long by

67-125 μm wide (Kirby 1934). The size is variable and dependent on culture conditions. It is smallest when starved and largest when cannibalistic feeding occurs (Pandey & Yeragi 2004). Microalgae, bacteria or yeast have been routinely used to culture this ciliate (De Winter & Persoone 1975; Uhlig 1981; Rattan, Ansari & Chatterji 1999; Park & Hur 2001; Pandey & Yeragi, 2004).

In addition to being the appropriate size range for first-feeding fish larvae, *F. salina* has no appendages other than cilia or hard parts, which will facilitate its uptake and digestion by the predators. Another characteristic of *F. salina* making it a good candidate for aquaculture is that it forms a tough dessicant resistant cyst when faced with unfavorable environmental conditions. The cysts can be stored and retain viability (De Winter & Persoone 1975). Such cysts could allow for quick start up cultures when needed without the neccessity of maintaining cultures similar to the practice of using brine shrimp *Artemia*. In addition to a lack of data on the suitability of ciliates as a first food in aquaculture setting, the general conditions for mass culture are not available. The objective of this study was to better understand the ciliate inoculation, algal feeding and lighting requirements of *Fabrea salina* in mass culture settings.

Materials and Methods

Four trials were conducted to evaluate the response of *Fabrea salina* to the initial *F. salina* culture density, algae species, algae density and photoperiod. The *Fabrea salina* culture was obtained from the laboratory of E. Buskey, University of Texas, Marine Science Institute, Port Aransas, TX, USA. Stock cultures were maintained at the Claude Peteet Mariculture Center (CPMC), Gulf Shores, AL, USA. Algae species *Isochrysis sp.* (T-ISO strain) and *Rhodomonas lens* obtained from CCMP, Bigelow Laboratory for

Ocean Sciences, Maine, USA, were cultured in Gulliard's F/2 media at the CPMC laboratory. Based on the results of De Winter & Persoone 1975; Uhlig 1981; Rattan *et al.* 1999; Pandey & Yeragi 2004; and preliminary observations conducted at CPMC, a series of studies were conducted.

Stocking Density Trial

Trial one was designed to determine an appropriate *F. salina* inoculation density. For each treatment, (3, 6 or 9 *F. salina* mL^{-1}) three replicate 40 L plastic bags initially containing 25 L of filtered (5 μm) seawater were utilized. Algae densities of 1.2×10^5 *R. lens* cells mL^{-1} were added initially then every other day of the trial. Aeration was provided at a rate of 2.5 mL min^{-1} to maintain algal cells in suspension.

Algae Species, Algae Density and Photoperiod Trials

The remaining three trials were carried out for 7 days in 2 L Erlenmeyer flasks initially containing 1 L of chlorinated then dechlorinated, 1 μm filtered seawater (32-34 g L^{-1}). Each treatment contained three replicate flasks, which were not aerated. The flasks were gently swirled before sampling to evenly distribute the ciliates and resuspend algal cells. The temperature ranged between 25-30 °C.

A trial to determine best algae species compared *F. salina* growth when fed a daily density 9.0×10^4 *Isochrysis sp.* or *R. lens* cells mL^{-1}. The next trial compared feeding *R. lens* density of 9.0×10^4 and 1.35×10^5 cells mL^{-1} day^{-1}. Any algae remaining in the flasks was not quantified. The experimental densities of algae were added daily, not adjusted to a specific density. The algae trials were evaluated stocking *F. salina* at 3 mL^{-1} and provided with 12 hours light day^{-1}.

Three light:dark cycles (6L:18D, 12L:12D and 18L:6D) were evaluated, using *F. salina* stocked at 3 mL^{-1} and *R. lens* at 9.0 x 10^4 cells mL^{-1} day^{-1}. The lighting was provided with a combination of 75 W Gro-Lux WS and 60 W Cool White fluorescent bulbs for a wide spectrum of wavelengths (SYLVANIA Westfield, IN, USA). The light intensity for all trials was measured using a light meter (Fisherbrand Traceable®, Fisher Scientific, Pittsburgh PA USA) and ranged from 1,240 to 1,680 lux.

In all four trials, daily samples were removed from each replicate, preserved and stained using Lugol's iodine solution for seven days. Triplicate counts of *F. salina* in each sample were accomplished using Sedgwick-Rafter slides on a compound microscope with 40x magnification. The algae density in the culture flasks was not enumerated daily, additions of algae were provided based on a daily rate determined for the trial. Water quality including temperature, salinity, dissolved oxygen and pH were measured daily in each replicate with a YSI 556 MPS meter (YSI Incorporated, Yellow Springs, CO, USA) sterilized before monitoring each treatment. Samples for ammonia were collected daily with a sterile glass pipette and analyzed using the Nesslerization method (Clesceri, Greenberg & Trussell 1989).

Intrinsic growth rate was determined for the day of peak density of each of the treatments using the equation given by Preisser & Spittler (1979) as follows: $r= 2.3026 (\log N_t - \log N_0) t^{-1}$ where r=intrinsic growth rate, N_t=number of *F. salina* mL^{-1} at peak growth, N_0=initial number of *F. salina* mL^{-1} and t=day of peak growth. The doubling time (T_D) of the culture in days from the initial density was calculated as follows: $T_D= 0.6932/r$.

All statistical analyses were performed using SAS Version 8.02 (SAS Institute, Cary, North Carolina, USA). One-way ANOVA was used to compare the *F. salina* densities and water quality parameters for the trials with three treatments. Trials containing only two treatments were compared using the two-sample *t*-test. If the variances were found to be unequal by Folded F method, Satterthwaite's approximation was used to compare the two means as an alternative to the *t*-test. Simple linear regression models were used to investigate the relationships between *F. salina* densities and water quality parameters.

The best treatment was selected from each of the four trials and applied to mass cultures of *F. salina* in 40 L plastic bags or flat bottom 200 L (152 cm x 46 cm diameter) cylindrical transparent polymer fiberglass culture tubes (Solar Components Corporation, Manchester, New Hampshire, USA).

Results

The following results are based on *F. salina* culture trials where *F. salina* stocking density, algal species and density, and photoperiod were evaluated. The day of peak density, peak density, intrinsic growth rate (r), and doubling time (T_D) for each trial are listed in Table 1. Mean water quality measurements for each treatment in the four trials are presented in Table 2.

Stocking density trial

On days 5, 6 and 7 of the stocking density trial, there were no significant differences ($p=0.50$, $p=0.35$, and $p=0.56$, respectively) in the growth when stocked at 3, 6 or 9 *F. salina* mL^{-1}. The mean maximum density for all flasks reached 97 ± 9 mL^{-1} after

Table 1. Intrinsic growth rate (r), doubling time (T_D) and mean peak density ± SE for each treatment of each trial.

Trial	Treatment	r	T_D	Peak day	Density (*F. salina* mL^{-1})
F. salina	3 mL^{-1}	0.47	1.46	7	89 ± 14
stocking	6 mL^{-1}	0.52	1.34	6	104 ± 9
	9 mL^{-1}	0.50	1.40	5	110 ± 7
Algae Species	*R. lens*	1.04	0.66	4	216 ± 20
	Isochrysis sp.	0.72	0.97	5	76 ± 5
Algae Density	9.0×10^4	0.46	1.50	7	70 ± 6
	1.35×10^5	0.46	1.50	7	51 ± 8
Photoperiod	6L:18D	0.49	1.41	7	45 ± 2
	12L:12D	0.52	1.34	7	58 ± 22
	18L:6D	0.60	1.15	7	84 ± 5
40 L Bags	Indoor	0.40	1.73	8	67 ± 5
200 L	Indoor	0.58	1.19	6	91 ± 10
Culture Tubes	Outdoor	0.47	1.47	7	66 ± 21

Table 2. Mean ± SD of water quality parameters for each trial over the 7-day culture period.

Trial	Treatment	Temp (°C)	Salinity (g L^{-1})	DO (mg L^{-1})	pH	TAN (mg L^{-1})
F. salina	3 mL^{-1}	30.4 ± 1.8	32.0 ± 0.3	4.6 ± 1.0	7.6 ± 0.3	0.50 ± 0.56
stocking	6 mL^{-1}	30.7 ± 1.9	32.1 ± 0.3	4.4 ± 0.8	7.6 ± 0.3	0.74 ± 0.65
	9 mL^{-1}	31.1 ± 1.9	32.1 ± 0.3	4.7 ± 0.7	7.6 ± 0.2	0.96 ± 0.77
Algae	*R. lens*	33.1 ± 0.7	33.6 ± 0.3	3.4 ± 2.3	7.7 ± 0.4	1.83 ± 2.00*
Species	*Isochrysis sp.*	33.2 ± 0.9	33.6 ± 0.3	4.9 ± 1.0*	7.9 ± 0.3*	0.49 ± 0.73
Algae	9.0×10^4	29.4 ± 2.5	35.1 ± 1.0**	3.8 ± 1.0	7.9 ± 0.1	0.47 ± 0.67
Density	1.35×10^5	29.5 ± 2.4	35.6 ± 1.3	3.3 ± 1.6	7.9 ± 0.1	0.67 ± 0.95
Photoperiod	6L:18D	25.9 ± 0.6**	32.4 ± 0.4	9.5 ± 2.5**	8.3 ± 0.6	0.00 ± 0.00
	12L:12D	26.3 ± 0.8	32.5 ± 0.5	8.7 ± 2.7	8.2 ± 0.4	0.00 ± 0.01
	18L:6D	26.2 ± 0.8	32.4 ± 0.4	5.9 ± 1.8*	7.8 ± 0.3*	0.15 ± 0.23*

*Significantly different when compared to the other treatments of that trial.
**Significantly different compared to the other trials.

5 days (Figure 1). The flasks stocked with 9 mL^{-1} had reached 96 ± 6 mL^{-1} on day 3, which was significantly higher ($p=0.001$) than the other two treatments. After day 3, growth slowed almost completely at the highest stocking density (9 mL^{-1}) treatment. *F. salina* populations stocked at 3 and 6 mL^{-1} continued to increase through 5 days before starting to decline.

The ammonia concentration after 7 days ranged from 1.4 to 2.0 total ammonia nitrogen (TAN) mg L^{-1}, the higher values were from the treatments stocked at higher densities (Figure 2). The growth of *F. salina* slowed or leveled off when ammonia reached 1 TAN mg L^{-1} on Day 4, Day 5 and Day 6 for treatments stocked with 9, 6, and 3 *F. salina* mL^{-1}, respectively (Figure 1). The increase in TAN was positively correlated with the increase in *F. salina* density ($p=0.0001$, $R^2=0.799$). There was a minor decrease in dissolved oxygen correlated ($p=0.0002$, $R^2=0.48$) to an increase in *F. salina* density.

Algae species

In the trial comparing the two algae species, the *F. salina* density increased from 3 mL^{-1} to 216 ± 34 mL^{-1} after 4 days when fed *R. lens* compared to 66 ± 18 mL^{-1} when fed *Isochrysis sp.* (Figure 3). The treatment fed *R. lens* significantly enhanced production compared to the treatment fed *Isochrysis sp.* ($p<0.0001$).

Total ammonia nitrogen was 1.49 mg L^{-1} on day 4 in the *R. lens* treatment and 1.10 mg L^{-1} on day 5 in the *Isochrysis sp.* treatment (Figure 4), which corresponded to the peak day in *F. salina* density (Table 1). Low dissolved oxygen 0.85 mg L^{-1} on both Days 2 and 3 of the trial did not reduce the growth of *F. salina* in the *R. lens* treatment (Figure 5). The *Isochrysis sp.* treatment had significantly higher dissolved oxygen ($p=0.007$) and pH ($p=0.043$) and significantly lower TAN ($p=0.003$) than the *R. lens* treatment

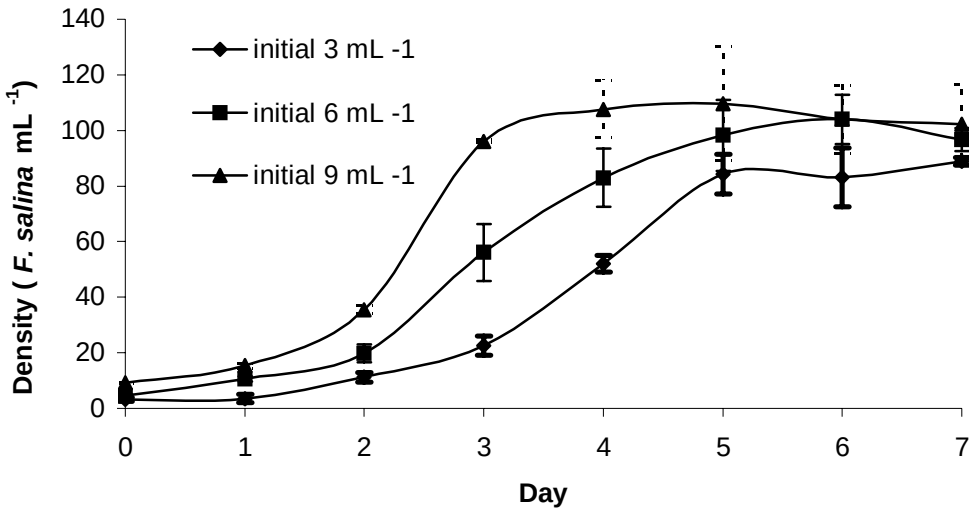

Figure 1. *F. salina* densities ± SE of each treatment in the trial comparing three *F. salina* stocking densities for the 7-day trial period

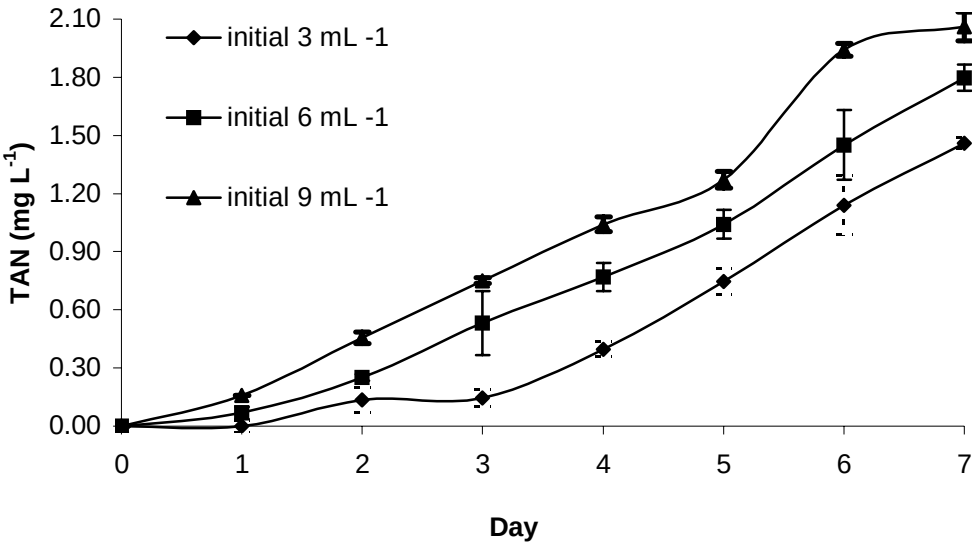

Figure 2. Ammonia concentration ± SE for each treatment in the *F. salina* stocking trial for the 7-day trial period.

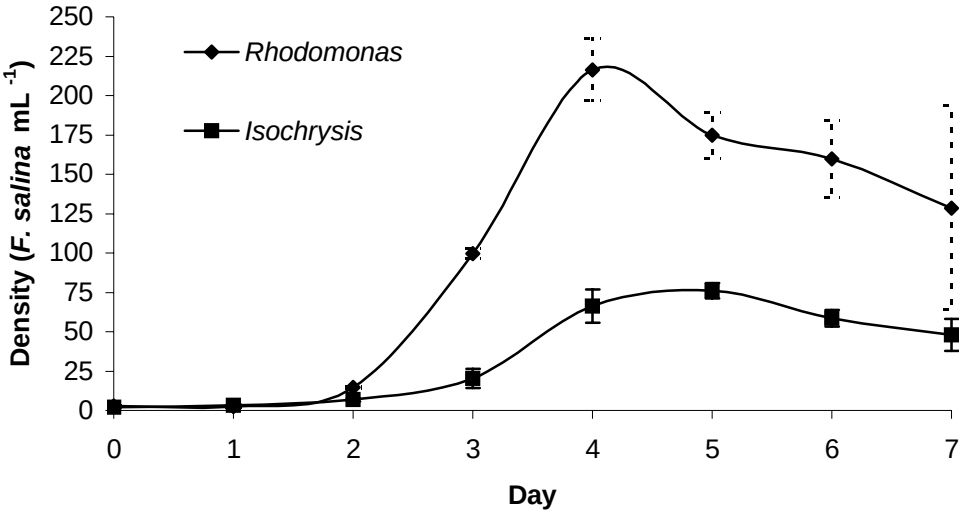

Figure 3. *F. salina* densities ± SE of each treatment in the trial comparing two different algae species as a food source for the 7-day trial period

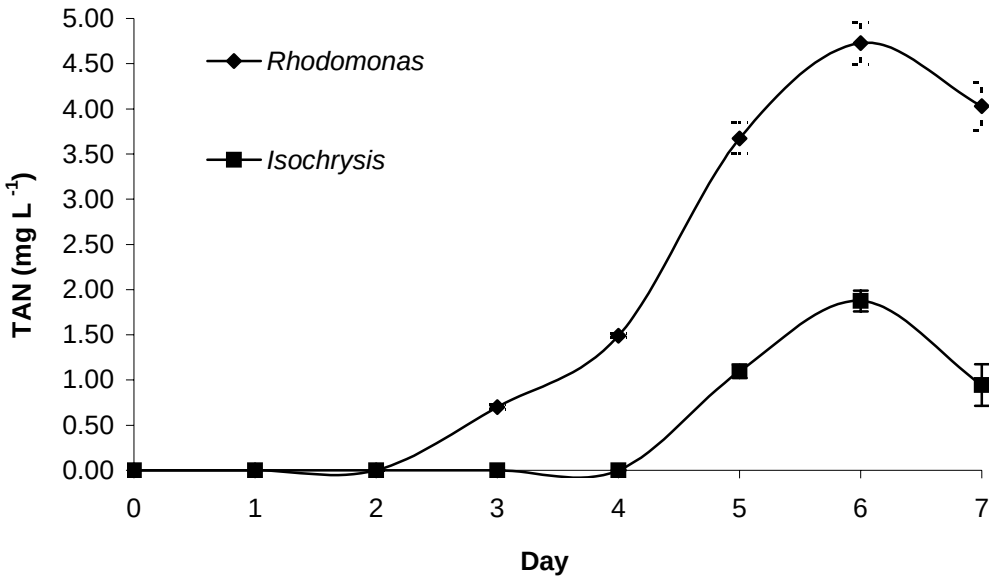

Figure 4. Ammonia concentration ± SE for each treatment in the algae species trial for the 7-day trial period.

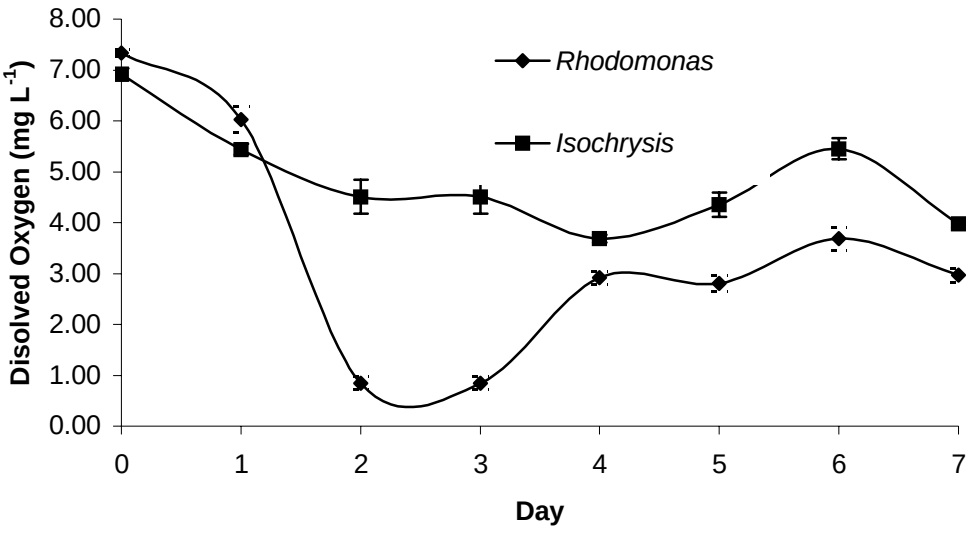

Figure 5. Dissolved oxygen concentration ± SE for each treatment in the algae species trial for the 7-day trial period.

concomittant with less ciliate growth. No correlations were found between TAN or dissolved oxygen and *F. salina* production.

There was no significant difference ($p=0.459$) in *F. salina* maximum production when comparing the daily algae additions of 9.0×10^4 and 1.35×10^5 *R. lens* cells mL^{-1}. The *F. salina* reached densities of 70 ± 11 mL^{-1} with 9.0×10^4 cells mL^{-1} day^{-1} and 51 ± 14 mL^{-1} with 1.35×10^5 cells mL^{-1} day^{-1} (Figure 6). The peak density was not reached during the 7 day trial period, the density of *F. salina* was still on the rise on day 7.

The *F. salina* densities were increasing even though ammonia was 1.20 and 1.64 TAN mg L^{-1} on Day 6 of the 9.0×10^4 and 1.35×10^5 cells mL^{-1} day^{-1}, respectively (Figure 7). The dissolved oxygen was 2.89 and 2.36 mg L^{-1} on day 4 for the 9.0×10^4 and 1.35×10^5 cells mL^{-1} day^{-1} treatments, respectively (Figure 8). The dissolved oxygen continued to decrease to 2.57 and 0.67 mg L^{-1} on Day 7 (Figure 8). Similar to the *F. salina* stocking density trial, the increase in TAN was positively correlated with the increase in *F. salina* density ($p<0.0001$ $R^2=0.799$). There was a decrease in dissolved oxygen correlated to an increase in *F. salina* density ($p<0.0001$ $R^2=0.584$). As both food addition rates appeared to saturate *F. salina* growth response the daily feeding of algae was selected to be 9.0×10^4 cells mL^{-1} to decrease the amount of algae needed daily.

In the photoperiod trial, the *F. salina* growth and yield in the three different light exposures were not significantly different in any day of the trial ($p=0.3105$). Similar to the algae density trial, the peak density was not reached during the 7-day trial period, the density of *F. salina* was still on the rise on day 7 for all three treatments (Figure 9) ranging from 45 to 84 ciliates mL^{-1}.

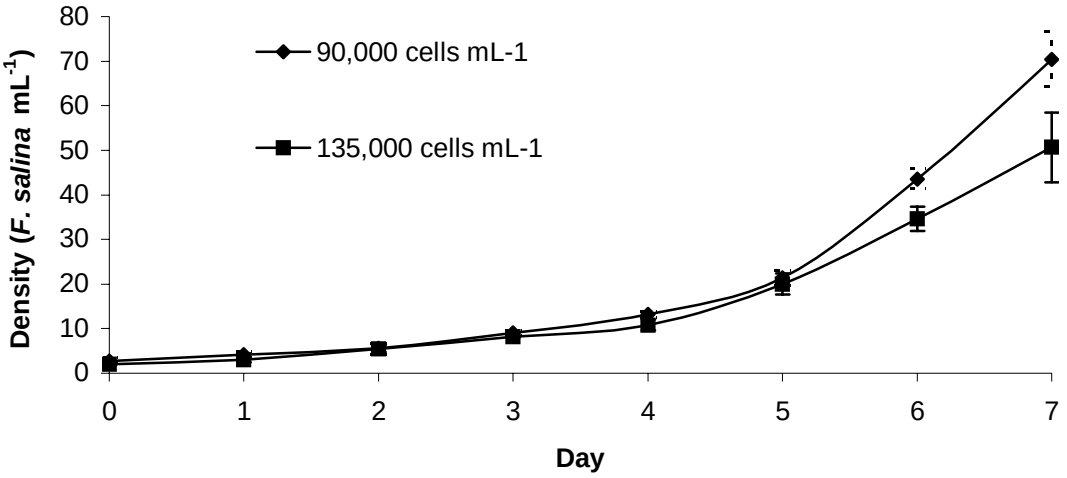

Figure 6. *F. salina* densities ± SE of each treatment in the trial comparing two daily densities of *R. lens* for the 7-day trial period.

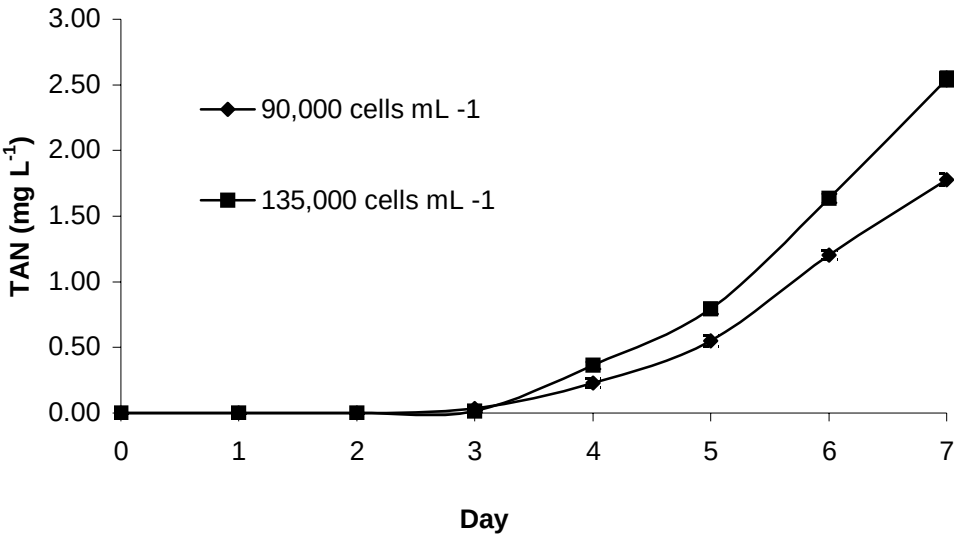

Figure 7. Ammonia concentration ± SE for each treatment in the *R. lens* density trial for the 7-day trial period.

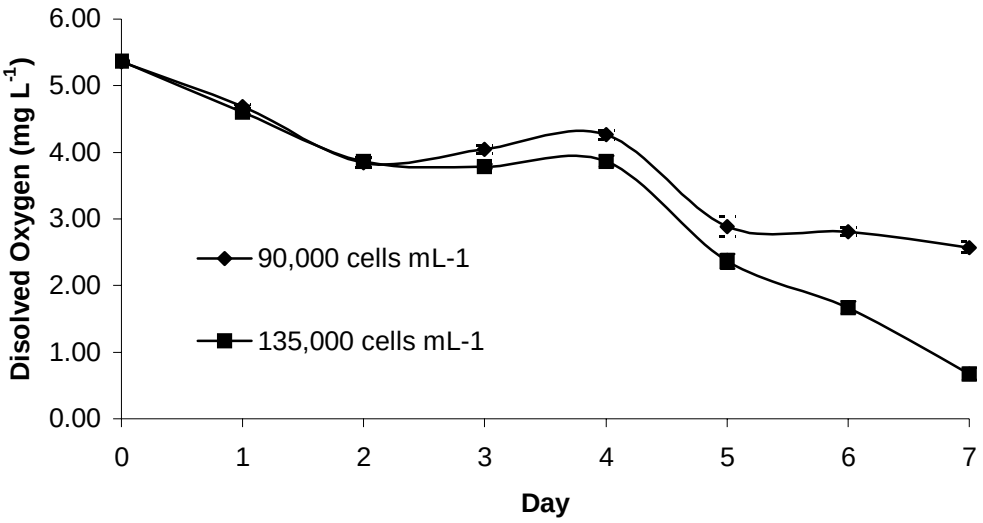

Figure 8. Dissolved oxygen concentration ± SE for each treatment in the *R. lens* density trial for the 7-day trial period.

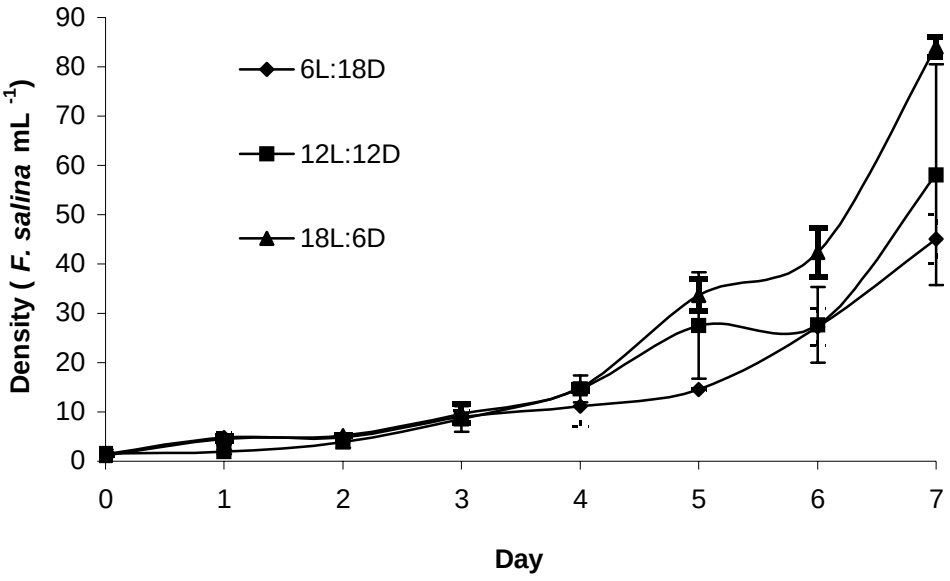

Figure 9. *F. salina* densities ± SE of each treatment in the trial comparing three different photoperiods for the 7-day trial period.

The concentration of TAN was significantly higher ($p=0.0005$) in the cultures provided with 18L:6D when compared to the cultures provided with 12L:12D and 6L:18D (Figure 10). The 18L:6D treatment had significantly lower dissolved oxygen ($p=<0.0001$) and lower pH ($p=0.0003$). The optimum photoperiod was selected as 12L:12D because of the effects on water quality described above and best control of the algae population.

Aside from the dissolved oxygen and ammonia differences between treatments, temperature and salinity were not the same for all trials. The temperature of the photoperiod trial was approximately 26°C, significantly lower ($p=<0.0001$) than the other three trials ranging from approximately 29 to 33°C (Table 2). The salinity in the algae density trial was approximately 35 g L^{-1}, significantly higher ($p=<0.0001$) than the other three trials ranging from approximately 32 to 34 g L^{-1} (Table 2).

Conditions selected for mass culture in 40 L bags or 200 L transparent culture tubes were stocking *F. salina* at 3 mL^{-1}, feeding 9.0 x 10^4 *R. lens* cells mL^{-1} day^{-1} and 12L:12D photoperiod for indoor cultures. *F. salina* was also cultured in 200 L tubes in a greenhouse with natural light conditions (approximately 14L:10D). These conditions resulted in densities of at least 50 ciliates mL^{-1} after 5 days, in all 16 mass culture attempts. There was no significant difference in densities of cultures grown indoors and outdoors ($p=0.237$). The mean intrinsic growth rates of 40 L bag and 200 L culture tube cultures are reported in Table 1.

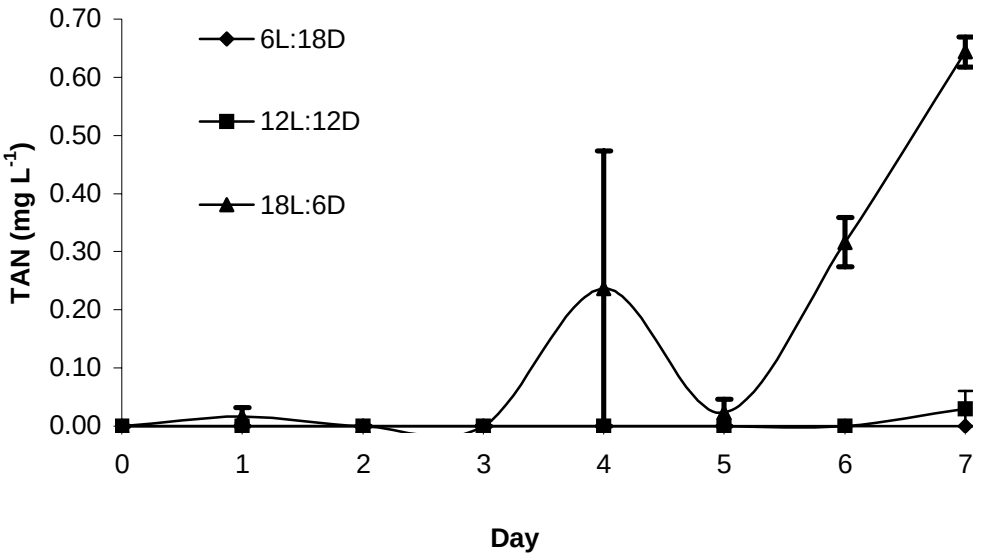

Figure 10. Ammonia concentration ± SE for each treatment in the photoperiod trial for the 7-day trial period.

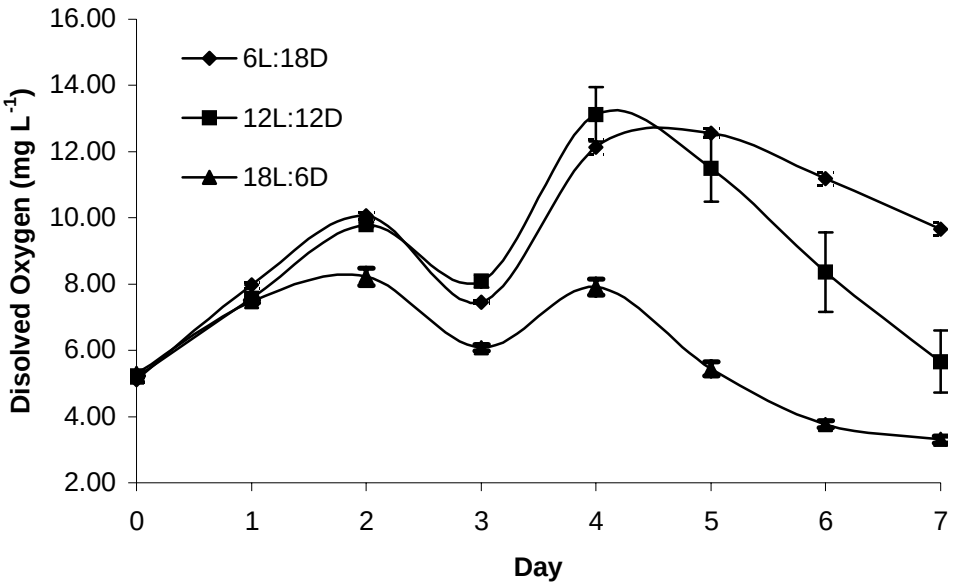

Figure 11. Dissolved oxygen concentration ± SE for each treatment in the photoperiod trial for the 7-day trial period.

Discussion

Fabrea salina proved to be a relatively hardy protozoan that could be produced on a large scale (200 L) indoors and outdoors at a mean density of 84 ± 10 mL^{-1}. Maximum densities occurred between day 5 and 8 with variability in peak day presumably due to differences in water quality conditions. Our culture techniques for *F. salina* culture in 40 L bags yielded similar densities to the mass culture attempts of Uhlig (1981). He reported densities of 100 mL^{-1} in 45 L bags after 14 days using the alga, *Dunaliella tertiolecta* as food. Uhlig (1981) calculated a doubling time of approximately 2 days while our bag cultures had a doubling time of 1.73 days.

Repak (1983) found *Rhodomonas lens* to be a better food item nearly doubling the growth rate of *F. salina* when compared to *Isochrysis galbana*. These results were confirmed in this study in which *R. lens* also provided a faster *F. salina* growth rate than *Isochrysis sp.*. These two algae species differ in size (*R. lens* 5 x 12 µm versus 3 x 5 µm for *Isochrysis sp.*) and nutrient profile (Brown, Jeffery, Volkman & Dunstan 1997). Algae are an important source of fatty acids needed for larval fish development. *R. lens* has a higher percentage of polyunsaturated fatty acids (PUFA), 20:4(n-6) and 20:5(n-3) and similar levels of 22:6(n-3) as *Isochrysis sp.* (Brown *et al.* 1997). Ciliates also may provide essential fatty acids, which are passed up the food chain when larval fish consume zooplankton (Watanabe, Tamiya, Oka, Hirata, Kitajima & Fujita 1983).

In addition to algae being an important food for *F. salina* growth and nutrient profile, the fatty acid variation among different species and strains of algae needs to be considered (Lopez-Alonso, Molina-Grima, Sancez Perez, Garcia Sanchez & Garcia Camacho 1992). There is evidence that the culture conditions of the algae such as light

regime (Tzovenis, De Pauw & Sorgeloos 1997) and temperature (Thompson, Guo & Harrison 1992) affects the nutrient profile of algae. The growth phase of the algae, exponential or stationary also changes the proportion of nutrients (Brown *et al.* 1997). Such factors may also impact *F. salina* production when algae is provided as a food.

F. salina can ingest a variety of food sizes from bacteria, algae and other protozoans. Some mass culture attempts have used non-algal diets giving yields similar to algae, fermented rice bran yielded 58 *F. salina* mL^{-1} in 40 L jars after 5 days (Rattan *et al*. 1999) and egg custard yielded 82 *F. salina* mL^{-1} in a 200 L recirculating system after 6 days (Pandey & Yeragi, 2004). Repak (1986) found bacteria to be of minimal value for *F. salina* growth and yeast to have little to no value. An issue with non-algal diets is how *F. salina* can be fed to larval fish without the culture media effecting water quality. Algae are capable of reproducing in the fish culture setting while producing oxygen and consuming ammonia. Non-algal food sources for *F. salina* have a greater potential to negatively effect water quality in the larval rearing system.

It is evident that the food type is important to the growth and nutrient profile of *F. salina* but the optimum density remains unknown. The two densities of algae supplied in this trial 9.0×10^4 and 1.35×10^5 appeared to be above saturating concentrations for growth. Water quality was reduced at 1.35×10^5 cells mL^{-1} resulting in lower dissolved oxygen and higher TAN. Further studies are needed to confirm this reduction water quality of the cultures as much higher densities of algae were used by other investigators. *Dunaliella sp.* densities of $1\text{-}15 \times 10^6$ and $4\text{-}8 \times 10^6$ cells mL^{-1} were offered to *F. salina* by De Winter & Persoone (1975) and Pandey & Yeragi (2004) who found $6\text{-}9 \times 10^6$ cells mL^{-1} (as initial prey densities) provided better growth obtaining densities of 60-120

F. salina mL^{-1}. High densities of prey can inhibit feeding by clogging oral cilia and the infundibulum leading to the cytosome. De Winter & Persoone (1975) found intermittent feeding resulted in better growth of *F. salina*. Vacuole passage time and digestion time are independent of food concentrations ranging from 0 to 1.1 x 10^6 cells mL^{-1} (Capriulo & Degnan 1991) and may be a factor limiting growth. Sherr, Sherr & Rassoulzadegan (1988) also concluded that digestion rates are unresponsive to changes in bacteria concentration. Decreasing the density of algae provided as food may have positive impacts on water quality while providing an adequate abundance when the digestion rate is considered.

The effect of salinity on *F. salina* growth was studied by De Winter & Persoone (1975), Rattan *et al.* (1999), Park & Hur (2001) and Pandey & Yeragi (2004). No wide variations or significant differences were observed at salinity levels ranging from 35 to 90 g L^{-1} in any of the investigators' trials. On the fifth day of the trials, Pandey & Yeragi (2004) reported the highest *F. salina* density in the 65 g L^{-1} treatment. Rattan *et al*. (1999) reported the highest density in the 70 g L^{-1} treatment. In the current study, salinity of the mass cultures ranged from 32 to 35 g L^{-1}, which is likely a physiologically insignificant difference given the salinity tolerance of this ciliate. The doubling time in the present study ranged from 1.19 to 1.47 days (Table 1). De Winter & Persoone (1975) used 70 g L^{-1} salinity for 30 L cultures using *Dunaliella viridis* as food. They obtained a density of 130 *F. salina* mL^{-1} with a generation time of 11.5 h (0.48 days). Being able to culture *F. salina* at salinities similar to those of larval marine fish reduces the need to acclimate *F. salina* before its use as a live feed. It is sensible to use a salinity of the readily available seawater at lower concentration.

De Winter & Persoone (1975) compared continuous light and continuous darkness, for *F. salina* when yeast was given as a food and found no effect of photoperiod. Pandey & Yeragi (2004) obtained densities of 120 mL^{-1} when exposed to light and only 76 mL^{-1} without light when using egg custard as food. No significant difference ($p=0.32$) was found among the three treatments, 6, 12 or 18 hours light in our trial. The photoperiod gives control over the algal growth, dense blooms were evident with the 18L:6D photoperiod. The pH and dissolved oxygen was more stable with less light because algal blooms did not crash during dark periods. Also, having to provide less hours of light is more economical especially using natural lighting outside.

Fabrea salina can be mass cultured and thus has potential as a first-food for fish larvae. Higher densities need to be achieved to facilitate its use. Future studies are needed to improve yields including continuous culture systems to increase production efficiency.

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VI. FORAGING BEHAVIOR OF RED SNAPPER, *LUTJANUS CAMPECHANUS* (POEY, 1860) LARVAE WHEN PRESENTED CILIATED PROTOZOANS AS FIRST FOOD ORGANISMS

Summary

The major challenge of culturing of red snapper, *Lutjanus campechanus* is providing an appropriate prey at the onset of feeding. Many ciliates have no lorica to facilitate their identification in the guts of a larval fish making their significance as a first food poorly understood. It is important to recognize the feeding behavior in the presence of different prey items. Fish larvae may be more successful foragers when given a prey with slower swimming speeds and patterns. Protozoans generally swim slower than traditional live feeds such as rotifers and copepod nauplii. Experiments were conducted in 1 L beakers using the focal animal technique to observe red snapper larvae. Protozoan species of *Fabrea, Strombidium* and *Strombidinopsis* were offered as prey. The occurrence of five modal action patterns including pause, swim, orient, fixate and bite were recorded. Fish were observed in treatments with and without food organisms present. Ciliate densities were enumerated after the experiment in beakers containing fish and control beakers containing ciliates only. The final densities were used to estimate the ingestion. First-feeding larvae are not continuously foraging, very few strikes were observed. The snapper larvae, like many teleost exhibit a saltatory search pattern, pausing before a burst of swimming. There was a significant reduction in *Strombidinopsis* density suggesting it

was the most appropriate food of the three protozoans. Understanding the foraging behavior of fish larvae seems to be an effective way of choosing a prey item.

Introduction

One of the major difficulties in mariculture is selecting a small enough prey for first-feeding larvae. However, food selection is not only constrained by prey size (a larva can only consume those organisms it can mechanically ingest), but also influenced by prey perception, recognition and capture (Govoni et al. 1986). The swimming speed and movement patterns of the prey items are important to stimulating a feed response. Larval fish are primarily sight feeders and suitable prey must attract the attention of the larvae and not swim out of the strike zone before the larvae can react. Behavioral observations have been useful in determining feeding strategies including prey density and type for various marine fish species. Modal action patterns are defined as a spatiotemporal pattern of coordinated movements in which pattern clusters about some mode, making the behavior recognizable (Barlow 1977). Feeding strikes are comprised of the perception and recognition of prey, the flexion of the larva's body axis, and the driving forth of the larva toward the prey (Hunter 1981). The flexion takes place from an S-shaped position from which a lash of the tail forces the larvae towards the prey (Gerking 1994). The first-feeding behavior can be observed to evaluate whether a particular prey will stimulate feeding for a given larval fish. Protozoans are one of the most abundant organisms within the marine microplankton. Densities of greater than 1.0×10^5 ciliates L^{-1} have been reported by sources within Pierce and Turner (1992). The high densities of protozoans make them potentially usable by marine fish larvae as food.

Protozoans not only are the appropriate size range as prey for cultured larval marine fish, they have different swimming patterns and speeds than more commonly used food such as rotifers and copepod nauplii. Protozoans were found to have a mean speed of 0.154 ± 0.06 cm s^{-1} compared to a speed of copepod nauplii 1.65 ± 0.20 cm s^{-1} (Hunt von Herbing and Gallager 2000). Protozoan escape response was found to be slow, 0.27 ± 0.15 cm s^{-1} while nauplii had an escape speed of 2.07 ± 0.5 cm s^{-1} (Hunt von Herbing and Gallager 2000). Copepod nauplii also have a very high rate of change of direction ranging from 589 to 1,931 degrees s^{-1} compared to ciliates with a range of 88 to 502 degrees s^{-1} (Buskey et al. 1993). The slower rate of changing direction may be easier for larvae to capture.

Three protozoans in the class Spirotrichea were choosen as experimental prey organisms. *Fabrea salina* is a heterotrich ciliate in the family Climacostomidae. *F. salina* naturally occurs in extremely high salinity areas. *F. salina* is a relatively large protozoan, ranging from 120-220 μm long by 67-125 μm wide (Kirby 1934). *Strombidium sp.* is an oligotrich ciliate in the family Strombilidiidae. This *Strombidium sp.*was isolated and cultured from the Gulf of Mexico had an average size of 41 μm long by 33 μm wide. *Strombidinopsis sp.* is a choreotrich ciliate in the family Strombidinopsidae. *Strombidinopsis sp.* also isolated and cultured from the Gulf of Mexico measured from 35-82 μm long and 29-49 μm wide. These three aloricate ciliates exhibit different swimming speeds and movement patterns. It was hypothesized that the different sizes and movements would elicit different feeding responses in red snapper larvae. Observation and quantification of feeding response can be used to evaluate the suitablity of a prey item for the production of larval fish. The objective of this study was

to observe the foraging behavior of first-feeding red snapper *Lutjanus campechanus* (Poey, 1860) larvae when given ciliated protozoans as a live food.

Materials and Methods

Red snapper eggs were obtained from natural or induced spawns. Natural spawning occurred from brood stock kept under controlled conditions at Claude Peteet Mariculture Center (CPMC), Gulf Shores, AL. Wild caught brood stock were induced to spawn at Gulf Coast Research Laboratory (GCRL), Ocean Springs, MS, using techniques described by Minton et al. (1983). Larvae that hatched at GCRL were transported to CPMC when they were < 24 hours post hatch (hph). Red snapper larvae, at least 48 hph, with functional mouthparts and fully pigmented eyes were added at 10 fish L^{-1} to1 L beakers filled with 5 μm filtered seawater. Fish were allowed to acclimate in the beakers for at least 1-2 h before observations began.

Each trial had three treatments with four beakers containing fish larvae only, four beakers with fish larvae and ciliates, and four beakers with ciliates only. Ciliates were added to the beakers at 10 mL^{-1}. *F. salina* was originally obtained from the Laboratory of E. Buskey, University of Texas, Marine Science Institute, Port Aransas, TX, USA and *Strombidium sp*. and *Strombidinopsis sp*. were isolated from the Gulf of Mexico and cultured by the Laboratory of R. Snyder, University of West Florida, Department of Biology, Pensacola, FL, USA. Ciliates cultures were then maintained at CPMC. The single cell algae *Isochrysis sp.* (T-ISO strain) was added to all beakers at 9.0 x 10^4 cells mL^{-1} to create a green-water environment. Algae was obtained from CCMP, Bigelow Laboratory for Ocean Sciences, Maine, USA then cultured at CPMC.

The beakers were randomly placed on a 30 cm black countertop with 30 cm white coutertop behind the beakers with a black wall to improve visibility for observations. The environmental conditions in the hatchery for all trials ranged from 26.5-27 °C temperature, 31-33 ppt salinity, 5.6-6.7 mg L^{-1} dissolved oxygen, and 7.6-8.0 pH measured with a YSI 556 MPS (YSI Incorporated, Yellow Springs, CO, USA). The hatchery had continuous lighting by a combination of 60 W (F96T12CW WM and F96T12DX) and 34 W F40 CW/SS fluorescent bulbs with an intensity range of 520 to 840 lux measured with a light meter (Fisherbrand Traceable®, Fisher Scientific, Pittsburgh PA USA).

All containers were monitored to determine if larvae were seen striking at objects in the water column. Behavior observations were made using the focal animal technique (Altmann 1974), where a larva was arbitrarily selected and monitored for 1 min. The occurrence of five modal action patterns (MAPs) similar to observations of redfish larvae by Laurel et al. (2001) were recorded (Table 1). The MAPs were recorded using a five-button laboratory counter with one minute timed by a stopwatch. The bite was recorded on all attacks (both successful and unsuccessful captures).

After the 1-min observation the fish larva was removed from the beaker. If a fish left the field of view before the end of the 1-min period the data was not used in the analysis and a different fish larva in the same beaker was then focused upon and observed. One fish was observed from each replicate then the fish was removed. After one fish from each of eight beakers was observed, another fish from each beaker was observed until five fish from each beaker had been observed for 1 min each.

Table 1. Definition of the Modal Action Patterns (MAPs) observed for red snapper larvae.

MAP	Definition
Pause	No observable movement of the larva
Swim	A quick undulation in the caudal region of the larva resulting in forward movement
Orient	A head or eye movement towards a prey item
Fixate	The larva is stationary and bends its caudal region into a 'S' shape position; typically follows orient
Bite	The larva lunges forward from the fixate position in an attempt to ingest of prey item

In addition to MAPs, the location in water column, vertical distance traveled and the orientation of the fish were noted in three of the four trials. The position of the fish observed was noted at the beginning and end of the observation period. The position in the beaker was divided into three levels: surface (top 4 cm), middle and bottom. The distance traveled was based on the approximate starting and ending position, according to the beaker's volume markings. The distance between volume markings was then measured to calculate the vertical movement of the fish. The vertical distance traveled occurred by fish swimming or by sinking or rising. It was noted when no vertical movement was achieved. The horizontal movement and fish swimming speed were not measured.

Water samples were taken with a glass pipette at study termination to determine ciliate densities. Samples were preserved and stained using Lugol's iodine solution for triplicate counts using Sedgwick-Rafter slides on a compound microscope with 40x magnification. The final ciliate densities were used to estimate ingestion.

Observations were conducted with first feeding fish larvae that were, 54.7 ± 3.1 hph during the study period with *Fabrea salina*, *Strombidium sp.* and *Strombidinopsis sp*. as prey. A second trial was conducted with *Strombidinopsis sp*. using larvae 78.1 ± 1.2 hph, approximately 24 hours past first feeding trials. These larvae were not previously exposed to any food. The trial using *Strombidium sp.* was the only trial that used larvae that were transported from an induced spawn at Gulf Coast Research Laboratory, the other three trials were stocked with larvae from natural spawns at CPMC.

All statistical analysis were preformed using SAS version 8.02 (SAS Institute, Cary, North Caroloina, USA). The data was compared with a two sample t-test. The

equality of variances was tested using the Folded F method. If the variances were found to be unequal than the Satterthwaite's approximation was used to compare the two samples. The total number of actions per minute for each of the two treatments containing fish were compared for each trial. The percent change in ciliate density in beakers containing fish were compared to those without fish. The vertical distance traveled for each group of fish was also compared.

Results

Each of the five MAPs and the total number of actions per minute are listed for each treatment of all trials in Table 2. The total MAPs percentage of searching events (pause, swim and orient) was higher than striking events (fixate and bite) in all four of the trials (Table 3). The *F. salina* trial resulted in the lowest percentage of searching events and the highest percentage of striking events (Figure 1a and 1b).

The percent completion from one MAP to the next was calculated for pause to bite, orient to fixate and fixate to bite. The percent of pauses that resulted in a bite was 13.9 ± 4.2% in *F. salina* trial, 0.5 ± 0.5% in first *Strombidinopsis sp*. trial and 1.9 ± 0.8% in the second *Strombidinopsis* trial. The percent of orientation followed by fixation was 16.55 ± 5.42% for *F. salina* compared to 1.28 ± 0.89% for the *Strombidinopsis sp*. trial with the same age fish and 2.08 ± 0.96% for the *Strombidinopsis sp*. trial with 24 h older fish. The fish offered *F. salina* had a higher percent fixation to orientation than the two *Strombidinopsis sp*. trials (p=0.002). The percent of fixation that lead to a bite in the *F. salina* trial was 80.1 ± 10.3%, first *Strombidinopsis sp*. trial was 50 ± 50% and second *Strombidinopsis sp*. trial was 85.7 ± 14.3%. There were no fixate or bite patterns observed in the *Strombidium sp*. trial.

Table 2. Mean number of actions per minute ± SE for each trial, Treatment 1, fish larvae only (unfed) and Treatment 2, fish larvae provided with ciliates.

MAP	*F. salina* Trial 1		*Strombidium* Trial 1		*Strombidinopsis* Trial 1		*Strombidinopsis* Trial 2	
Treatment	1	2	1	2	1	2	1	2
Pause	6.5 ± 1.0	4.6 ± 0.4	3.4 ± 0.6	3.2 ± 0.2	8.8 ± 1.2	6.4 ± 1.0	10.1 ± 0.9	12.2 ± 1.0
Swim	10.5 ± 2.2	5.8 ± 1.4	3.5 ± 1.2	2.6 ± 0.4	9.3 ± 1.7	7.6 ± 1.5	15.5 ± 2.1	20.0 ± 2.1
Orient	8.7 ± 1.9	7.1 ± 1.7	1.0 ± 0.4	1.1 ± 0.2	2.6 ± 0.8	3.3 ± 1.1	9.3 ± 1.3	11.5 ± 1.3
Fixate	0.5 ± 0.2	0.9 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.4 ± 0.2
Bite	0.4 ± 0.2	0.7 ± 0.3	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.4 ± 0.2
Fish observed	17	18	20	20	16	16	19	19
Total	26.6 ± 4.6	19.0 ± 3.7	7.8 ± 2.1	6.9 ± 0.8	20.7 ± 3.4	17.4 ± 2.8	35.1 ± 4.0	44.5 ± 4.1

Table 3. Mean percent frequency of each action ± SE for each trial, Treatment 1, fish larvae only (unfed) and Treatment 2, fish larvae provided with ciliates.

MAP	*F. salina* Trial 1		*Strombidium* Trial 1		*Strombidinopsis* Trial 1		*Strombidinopsis* Trial 2	
Treatment	1	2	1	2	1	2	1	2
Pause	31.2 ± 3.6	36.6 ± 5.0	62.2 ± 5.1	50.7 ± 3.1	46.9 ± 2.6	44.0 ± 3.9	31.9 ± 2.0	29.1 ± 2.2
Swim	35.2 ± 3.0	27.3 ± 2.4	30.6 ± 3.9	34.1 ± 2.5	43.3 ± 1.2	39.5 ± 2.2	41.5 ± 1.9	43.6 ± 1.3
Orient	29.9 ± 3.7	28.7 ± 3.5	7.3 ± 2.1	14.9 ± 2.4	9.9 ± 2.2	15.8 ± 2.7	26.3 ± 1.8	25.8 ± 1.6
Fixate	1.9 ± 0.8	4.4 ± 1.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.3	0.2 ± 0.1	0.8 ± 0.4
Bite	1.8 ± 0.8	3.0 ± 1.2	0.0 ± 0.0	0.3 ± 0.3	0.0 ± 0.0	0.2 ± 0.2	0.1 ± 0.1	0.8 ± 0.4

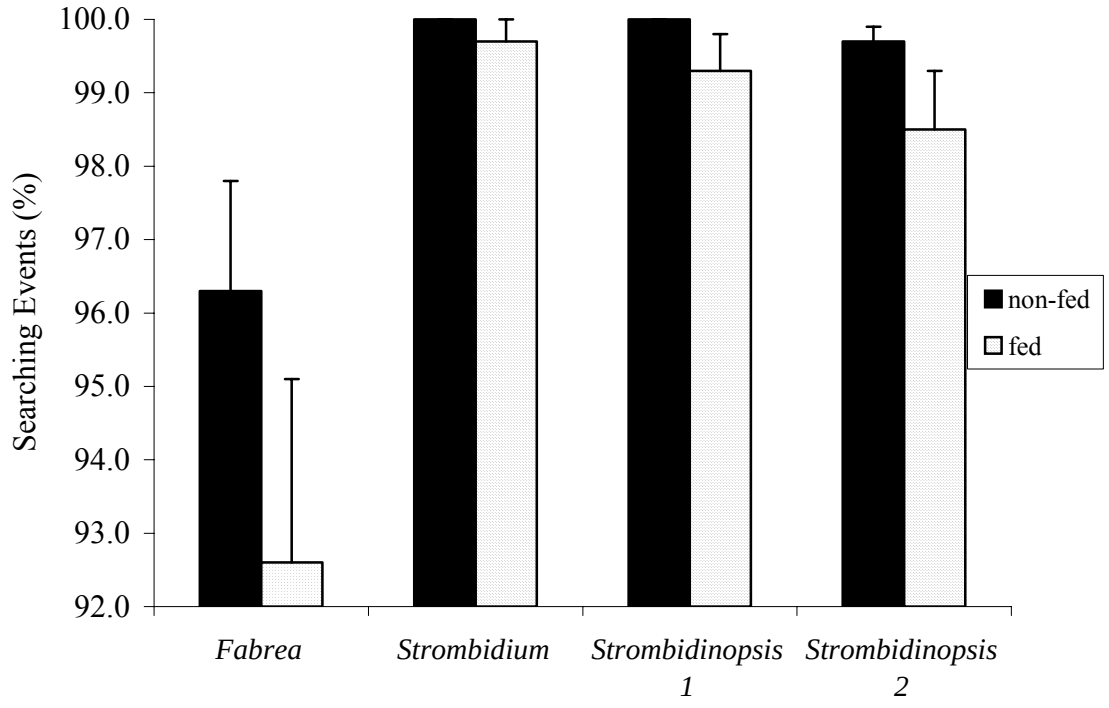

Figure 1a. The percentage of searching events (sum of pause, swim and orient) of each trial.

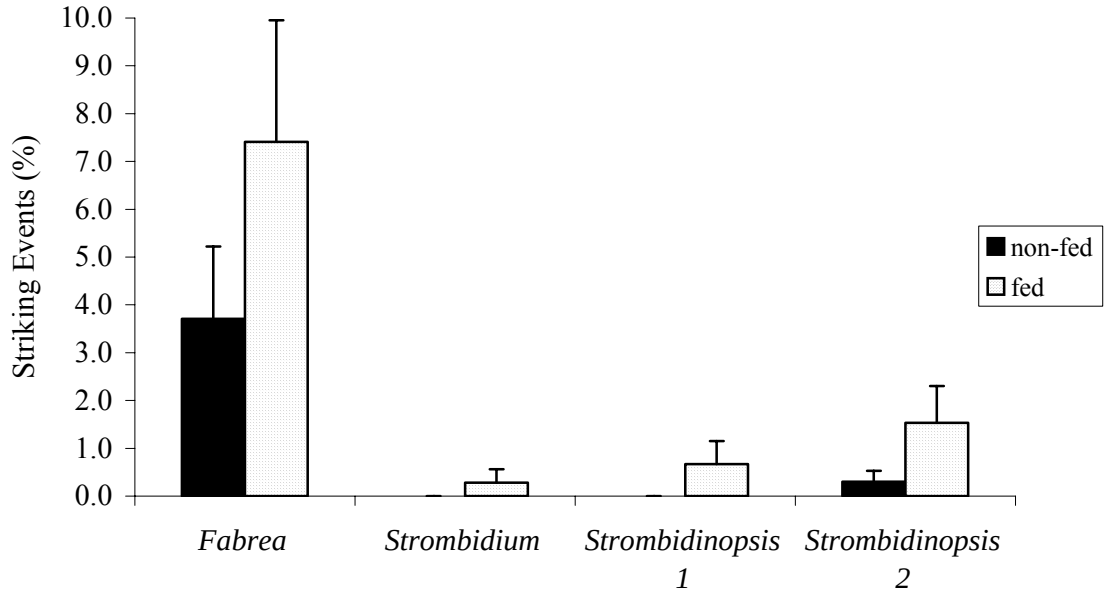

Figure 1b. The percentage of striking events (sum of fixate and bite) for each trial of the fed and unfed treatments.

In addition to the number of bites observed, the change in ciliate abundance (Table 4) was used to infer feeding response. The percent change was not found to be significantly different (p=0.552) between treatments of the *F. salina* trial. The *F. salina* density increased in both the fed and unfed treatments. There was also no significant difference (p=0.931) in the density of *Strombidium sp.* The density of *Strombidinopsis sp*. in the first trial was significantly less (p=<0.001) in the treatment with fish. The mean reduction of *Strombidinopsis sp*. in the second trial was also significantly greater (p=0.014) in the treatment with fish.

The fish's position in the water column was noted in three of the four trials. The beaker was divided into three levels: surface (top 4 cm), middle and bottom. The position of the fish observed in the fed and unfed treatments were similar. The percent of fish observed searching in the three levels of the beaker only varied slightly between trials (Figure 2a.). The fish that were observed striking were seen throughout the beaker (Figure 2b.). The rate of displacement was similar for both fed and unfed treatments. The mean ± SE rate of searching fish was 1.41 ± 0.27 cm min^{-1} in the *F. salina* trial and 1.38 ± 0.26 cm min^{-1} *Strombidinopsis sp*. trial using older fish. The mean ± SE rate of striking fish was 0.53 ± 0.22 cm min^{-1} in the *F. salina* trial and 1.79 ± 0.86 cm min^{-1} *Strombidinopsis sp.* Trial using older fish.

Discussion

The foraging behavior of red snapper larvae can be defined as a saltatory search pattern similar to that described by O'Brien et al. (1989). Saltatory searchers have long pauses punctuated by burst of swimming. This type of foraging is a mid-point between a cruise predator and a sit and wait predator. The red snapper larvae often sank between

Table 4. Percent change in ciliate abundance of each trial for the treatment containing fish and the ciliate only control.

Trial	Treatment	
	fish and ciliates	ciliates only
F. salina	151.7 ± 205.2	83.3 ± 70.1
Strombidium sp.	-19.2 ± 30.5	-1.7 ± 28.9
Strombidinopsis 1	-81.7 ± 8.8	-15.8 ± 17.7
Strombidinopsis 2	-69.2 ± 8.8	-35.8 ± 17.3

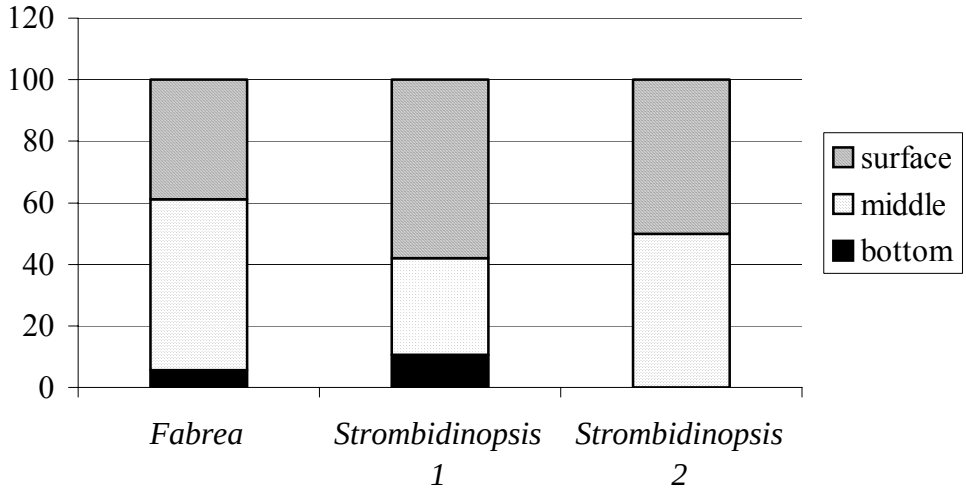

Figure 2a. The percent of fed fish observed searching in the surface, middle and bottom of the beaker for three different trials

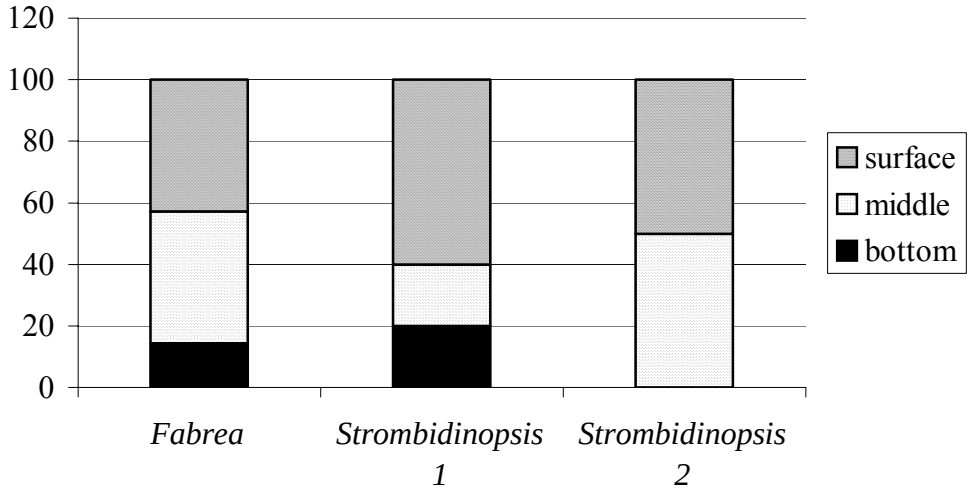

Figure 2b. The percent of fed fish observed striking in the surface, middle and bottom of the beaker for three different trials.

burst of swimming as Coughlin et al. (1992) noticed with clownfish larvae. An active pattern was needed for larvae to remain in the water column. The sinking rate of inactive herring larvae was about 0.3 cm s^{-1} (Blaxter and Ehrlich 1974). The sinking could be due to the fact that, the red snapper gas bladder is not inflated until 4 days after hatch (DAH) (Drass et al. 2000).

The first step in the visual selection process is encountering prey. Saltatory predators scan for prey only during the frequent stationary pauses between repositioning movements (O'Brien et al. 1989). The percent completion from pause to bite confirms that larvae are searching for prey while motionless (Table 3). When no prey are recognized the larvae must swim to an unsearched area or change orientation to increase the total area searched. For white crappie and golden shiner larvae only 1-10% of pauses were followed by attacks (Browman and O'Brien 1992 a, b).

The encounter rate is not only affected by prey density but also the prey's swimming speed and pattern. Based on the percent of striking events, *F. salina* elicited the most feed responses. *F. salina* was the largest of the three ciliates used in the behavior observation trials. The larger size may have increased the visual encounter rate. The slow swimming pattern may also make *F. salina* an easier target to strike.

In all trials the percent of striking events is much less than the percent of searching events. The number of swim actions min^{-1} was higher in the treatment without prey. This pattern of swimming rates was also seen with redfish larvae (Laurel et al. 2001). This suggests that larvae continue to swim to new locations to search for prey. Larval fat snook mean swimming duration was found to be 6.41 s min^{-1} (Temple et al. 2004). Capture frequencies of larval fat snook was low at all prey densities (5-30 prey L^{-}

^{1}) with a mean of 0.0113 captures min^{-1} (Temple et al. 2004). This is less than 1% of time in orient, lunge and capture which is similar to the results of this study for red snapper. The highest percent of striking events (fixate and bite) was 7.4% in the treatment fed *F. salina*, the other trials had much lower percentage (Table 3).

Capture success is a critical factor determining whether first-feeding larvae succeed in consuming enough prey to survive. Snapper larvae capture success was not measured, the bite was recorded for both successful and unsuccessful captures. Capture success is low initially and increases with age (Drost 1987). First-feeding bay anchovy and sea bream attacks were only successful 50% of the time (Houde and Schekter 1980). Hunter (1972) reported capture efficiency as low as 10% for anchovy larvae. Bay anchovy search time decreased from 0.4 h at 100 prey L^{-1} to only 0.04 h at 1,000 prey L^{-1} with the difference in consumption being 1.2 prey h^{-1} compared to 12.5 prey h^{-1} (Houde and Schekter 1980). The snapper attempted bites ranged from 6 to 42 bites h^{-1}, with the higher occurance corresponding to higher ciliate density.

Final ciliate density is the only proof of ingestion for this study. The density of *F. salina* was much higher than the other two ciliates by the end of the trial. The *F. salina* doubling rate was found to be less than 1 day in the previous chapter, which could account for the increase in *F. salina* density. The percent of reduction of *Strombidinopsis* density was significant in the both trials. Suggesting ingestion occurred at a higher rate than the other two ciliates. Perhaps the larvae preferred *Strombidinopsis sp.* to *F. salina* even though more strikes were recored in the *F. salina* trial. The high *F. salina* density could have increased the feed response but it also suggest the bites observed were unsuccessful attacks as there was no notable reduction in *F. salina* density. The unfed

control group of the *F. salina* trial was more active than the other trials. The differences between control groups of different trials could be due to the variable quality of the larvae, which potentially influences foraging behavior.

Although feeding ability and maneuverability quickly increase with age, there were no observed differences in the trial containing fish that were 24 h older than the first-feeding fish. The frequencies of bites in the fed groups of the *Strombidinopsis sp.* trials with 54 hph and 78 hph fish were 0.2 ± 0.2% and 0.8 ± 0.4%, respectively. The older fish were not previously exposed to food, they didn't have any practice catching prey. This suggests that although the modal action patterns are automatic, foraging behavior is also learned, getting better with age given practice.

In all trials, the treatment containing prey had a higher percent of striking events than the treatment with fish only. Over the course of each trial (4 to 6 h), there was no significant difference in foraging events. This study was helpful in understanding the foraging behavior of larval red snapper. More trials with other food sources and several stocking densities may be useful in selecting a feeding protocol for fish larvae. The observation of feeding response alone may not be a useful tool to evaluate potential food sources. If the observation time period was increased, the limited feeding activity of first-feeding fish larvae could be seen more completely. The reduction in ciliate abundance together with behavior observations seemed to give a better picture of the value of the food source. There was a reduction in *Strombidinopsis sp.* but also a low occurrence of prey striking events. Since the fish were only observed for 1 min (a total of 5 min per beaker), it is possible that the striking events occurred during the non-observed period of the trial.

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V. EVALUATION OF THE CILIATED PROTOZOA, *FABREA SALINA* AS A FIRST FOOD FOR LARVAL RED SNAPPER, *LUTJANUS CAMPECHANUS* IN A PRODUCTION SETTING

Abstract

One of the major challenges of culturing of red snapper, *Lutjanus campechanus* is providing an appropriate food source at onset of feeding. Ciliates are abundant in marine waters but their significance as a first food for fish larvae is poorly understood, as many have no lorica to facilitate their identification in the gut of a larval fish. Mass cultures of *Fabrea salina* in 200 L solar tubes resulting in 84 ± 10 ciliates/mL in 7 days. Growth and survival of red snapper larvae was evaluated in a production setting using $1m^3$ tanks. Larvae were stocked at 10/L, 36 hrs post hatch, before first-feeding commences. Three treatments were fed with: 1) copepod nauplii, 20-75μm only from days 1 to 10; 2) copepod nauplii from days 1 to 10 plus *F. salina* from days 1 to 5; and 3) *F. salina* only from days 1 to 3 plus copepod nauplii from days 4 to 10. Copepod nauplii were added at 2/mL and ciliates were added at 5/mL. Survival after 28 days was 0.28 ± 0.15% and 2.39 ± 2.75% in treatment 1 and 2, respectively. Treatment 3 did not have any survival after 4 days after the trial began. Larvae were more active feeders in the tanks given *F. salina* and copepods as first foods with 34.6 ± 8.5% average daily reduction in copepod nauplii compared to 15.8 ± 16.2% reduction when only nauplii were provided.

Introduction

Red snapper, *Lutjanus campechanus* is the target of commercial and recreational fishing in the Gulf of Mexico. Culture and stock enhancement efforts are constrained due to low larval survival. Development of suitable feeding regimens for mass rearing of larval fish is one of the major barriers to successful propagation of this and other marine species. Several factors include small mouth size and limited yolk reserve at the time of functional mouthparts and digestive tract. At the onset of feeding the digestive tract only has a few functional enzyme systems, so that a proper food source should: be at least partially and easily digestible, contain enzyme systems which allow autolysis, and supply the essential nutrients required by the larval fish (Lavens and Sorgeloos 1996). Ciliated protozoans fit the requirements for a first-food source.

Typically rotifers or copepod nauplii are offered as first foods for marine fish larvae. The importance of other microplankton including dinoflagellates and ciliates as available prey is poorly understood although some evidence suggests they are eaten by larval fish. Planktonic marine ciliates are conventionally divided into loricate (tintinnid) and aloricate (naked) forms. Naked ciliates may be more important food for fish larvae than tintinnids, because naked ciliates occur in considerably larger numbers than tintinnids (Pierce and Turner 1992). There is little information on fish larvae predation on protozoans, except for the loricate tintinnids, which have indigestible hard parts that can be identified in the gut (review by Pierce and Turner 1992). There is less information about predation on aloricate ciliates due to the lack of identifiable gut contents by fish larvae. *Fabrea salina* is a naked heterotrich ciliate in the family Climacostomidae (Repak 1972). *F. salina* naturally occurs in estuarine environments and high saline areas.

F. salina is a relatively large protozoan, its size ranging from 120-220 µm long by 67-125 µm wide (Kirby 1934). It has been cultured in the laboratory using single celled algae, bacteria or yeast (De Winter et al. 1975, 1976; Uhlig 1981; Rattan et al. 1999; Park and Hur 2001; Pandey and Yeragi 2004). *F. salina* can be mass produced in 200 L transparent tubes as described in the previous chapter at densities of 84 ± 10/mL in 5-8 d facilitating its use as a larval food. The goal of this study was to evaluate the importance of *F. salina* as a first food organism for red snapper larvae at a production scale.

Materials and Methods

Brood stock were wild caught with hook and line and transported to the hatchery at Claude Peteet Mariculture Center, Gulf Shores, AL, USA, where they were induced to spawn using techniques described by Minton et al. (1983). Fertilized eggs were incubated in 100 L tanks with an aeration ring around the 200 µm screened standpipe. A temperature of 27 C and salinity of 33 ppt were maintained in the incubators. The hatchery had continuous lighting by 60 W F96T12 CW WM fluorescent bulbs (General Electric, Fairfield, CT USA) with an intensity range of 370 to 520 lx measured with a light meter (Fisherbrand Traceable®, Fisher Scientific, Pittsburgh, PA USA) at the water's surface.

The study was conducted in 1 m^3 black fiberglass tanks in a greenhouse with shadecloth above for a maximum light intensity of 11,200 lx at the water's surface with natural photoperiod (14L:10D). Six tanks were filled with full strength seawater filtered to 5µm 5 d before the trial began. Each tank was chlorinated using 7.4 g dry chlorine granules (70% Calcium Hypochlorite) aerated for 1 h then dechlorinated with 7.7 g sodium thiosulfate. Four of the tanks were then inoculated with 2 *F. salina*/mL and

5.0 x 10^4 *Isochrysis sp*. cells/mL (as a starter culture). Two days before the trial initiation the remaining 6 tanks were filled and sterilized as described for the other tanks. *F. salina* was then added to four of the newly filled tanks at 2/mL. *Isochrysis sp*. was added to all of the tanks at 9.0 x 10^4 cells/mL for green-water conditions.

Larvae were stocked 32-36 h post-hatch, prior to functioning mouthparts, at 10 larvae/L. Stocking of fish larvae was named Day 1 of the trial. Fish were fed according to one of following protocols using four replicates/protocol (Figure 1), each protocol differing in the first ten days. The protocols were: 1) copepod nauplii only, (20-75 μm) from days 1 to 10; 2) copepod nauplii from days 1 to 10 plus *F. salina* from days 1 to 5; and 3) *F. salina* from days 1 to 5 plus copepod nauplii from days 4 to 10. After 7 days until day 21, all of the treatments were fed copepod nauplii ranging from 40-100 μm. A commercial feed, Proton-1 (INVE, Aquaculture nv, Dendermonde, Belgium) was given starting on Day 16. Adult copepods were added to all treatments from days 18 to 28. The trial lasted 28 d (30 DAH). The tanks were static (no exchange of water) until Day 12, when all treatments were being fed the same copepod diet and the ciliates were no longer present. At this time recirculation of water had a flow rate of 500 mL/min (1 turnover/day).

Daily additions of *Isochrysis sp*. (T-ISO strain) were made to maintain 9.0 x 10^4 cells/mL. Algae was obtained from CCMP, Bigelow Laboratory for Ocean Sciences, Maine, USA and cultured in the laboratory with Gulliard's F/2 media. Gentle aeration was initially provided at a rate of 0.1 L/min then increased to 0.5 L/min after 12 d. Daily additions of zooplankton were made to maintain copepod nauplii at 2/mL and ciliates at 5/mL.

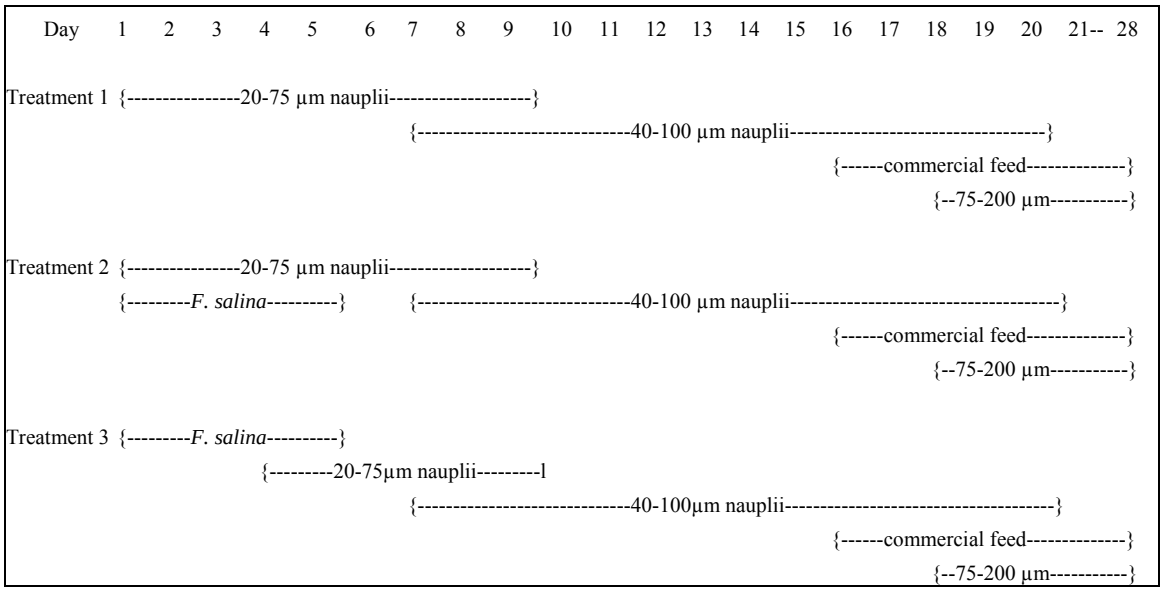

Figure 1. Feeding protocols for each of the three treatments for the entire study period.

F. salina was produced in 40 L bags in the laboratory or 200 L transparent tubes in the laboratory or greenhouse as described in the previous chapter. Mixed zooplankton was collected from full strength saltwater ponds following the procedures of Lam et al. (2000) for supplying copepod nauplii. Rotifers were collected along with the nauplii and were incidentally added to the tanks when nauplii were given. No attempt was made to maintain a specific rotifer density in the larval rearing tanks.

Five 10 mL aliquots were randomly collected using a Stempel pipette and pooled per tank. Daily counts of algae (Days 1 to 6) were performed using a hemacytometer with a compound microscope using 100x magnification. Zooplankton were preserved and stained using Lugol's iodine solution, counts of protozoans (Days 1 to 9), copepod nauplii and rotifers (Days 1 to 20) were conducted in triplicate using Sedgwick-Rafter counting slides with a compound microscope using 40x magnification. This allowed for the calculation of daily reduction of algae and zooplankton. Water quality measurements including temperature, salinity, dissolved oxygen and pH were taken twice daily from each replicate using a YSI 556 MPS (YSI Incorporated, Yellow Springs, CO, USA). Samples for ammonia were also collected twice a day and analyzed using Nesslerization method, read using a spectrophotometer.

Two fish were randomly selected from each replicate daily from Days 2 to 6 and on Day 12 and Day 18. Each larva was photographed live to circumvent fixative induced shrinkage using a digital Olympus® Camedia 3040 zoom camera outfitted to an Olympus® stereomicroscope (Olympus America Inc., Melville, NY, USA). Morphometric measurements of standard length and body depth at the anus were made using Image-Pro® Version 4.0.1 (Media Cyberetics Inc. Silver Spring, MD, USA) image

analysis software calibrated with a stage micrometer. The larvae were then fixed in 5 % formalin to later examine gut contents for presence or absence of food organisms. At harvest, Day 28, the total length was measured (± 1 mm) using a metric ruler.

All statistical analyses were performed using SAS Version 8.2 software (SAS Institute, Cary, North Carolina, USA). Levene's test for homogeneity of variances was used to check the assumption that variances were equal. Differences in mean zooplankton reductions, water quality and morphometrics were analyzed by one-way ANOVA repeated measures since the data sets were normally distributed. If Levene's test failed, Welch's ANOVA was used. The student's t-test was used to determine the significant differences ($p \leq 0.05$) in survival between the two treatments. Correlations between water quality parameters and survival as well as mean zooplankton reductions and survival were determined using linear regression analysis.

Results

Snapper survival and growth

Survival of red snapper after 28 d was 0.28 ± 0.15% in treatment 1, fed copepod nauplii only, 2.39 ± 2.75% in treatment 2, fed copepods nauplii and ciliates, and no survival in treatment 3, fed *F. salina* as the only food source for the first 3 d of feeding (Figure 2). Survival was highly variable within treatments but the addition of *F. salina* with copepod nauplii had a positive impact on survival ($p=0.12$).

Standard lengths and body depths are reported for each treatment in Table 1. The slower growth of larvae in treatment fed *F. salina* only was evident soon after the time of first-feeding (Days 2 and 3). There were no fish observed in treatment 3 after Day 4 of the trial (6 DAH). The final standard lengths on Day 28 were 11.45 ± 1.1 mm

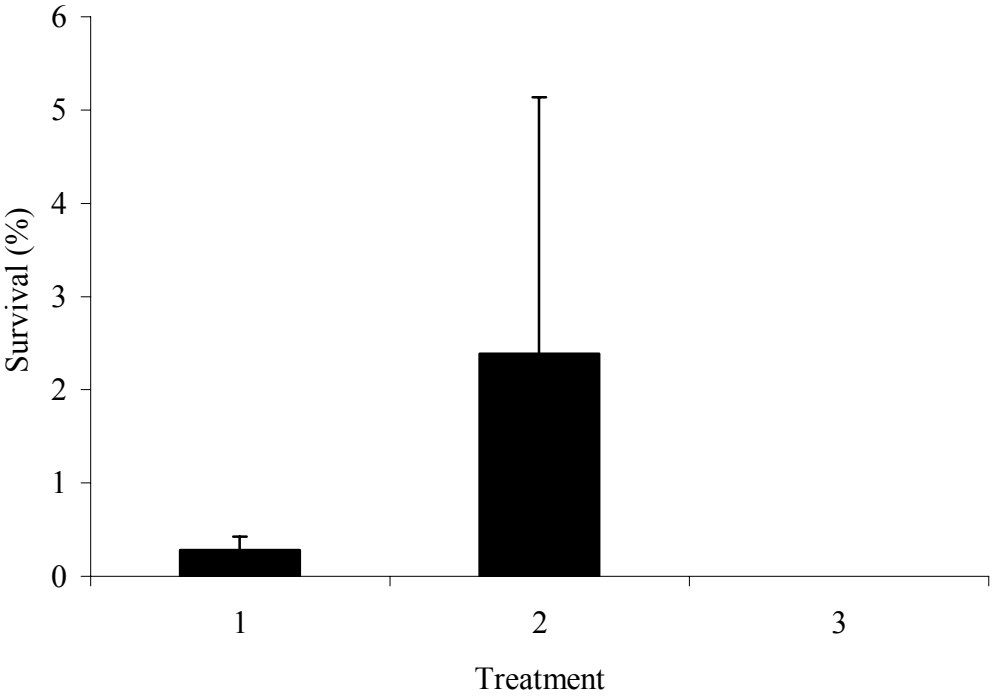

Figure 2. Final survival of the three treatments fed 1 (nauplii only), 2 (nauplii and *F. salina*), 3 (*F. salina* only)after 28 days.

and 13.45 ± 5.6 mm for treatments 1 and 2, respectively (p=0.020), opposite of the results on Day 20 where treatment 1 was significantly larger than treatment 2 (Table 1).

The gut contents from the first 6 d of the trial were examined for the presence or absence of food. The number of fish guts with food present was low in both treatments with survival, 15.2% (n=33) in treatment 1, fed copepod nauplii only and 5.9% (n=34) in treatment 2, fed *F. salina* and copepod nauplii. All of the fish guts (n=17) from the treatment fed *F. salina* only were absent of food.

Plankton Counts

Based on daily counts, the mean daily reduction (MDR) of *Isochrysis sp*. from days 1 to 6 of the study was significantly higher ($p<0.0001$) in treatments containing pond collected zooplankton, 80.6 ± 4.0% and 83.2 ± 4.2% in treatments 1 and 2, respectively than treatment 3, containing *F. salina* only having a 39.7 ± 7.6% MDR. Final fish survival in the eight tanks of treatments 1 and 2 was negatively correlated with algae reduction ($R^2=0.58$, $p=0.03$).

Larvae were more active feeders in the tanks given *F. salina* and nauplii as first foods with MDR of copepod nauplii from days 1-20, 34.6 ± 8.5%, compared to 15.8 ± 16.2% reduction when only nauplii were provided (p=0.085). Survival was positively correlated to the MDR in nauplii abundance ($R^2=0.83$, $p= 0.02$). There was no difference in the mean number of nauplii present from days 1-20 in the two treatments. The mean number of nauplii present for days 1-7 was 2.49 ± 1.02/mL in treatment 2 (nauplii and *F. salina*) and was significantly higher (p=0.05) than treatment 1, containing nauplii only at 1.92 ±1.03/mL. The mean number of nauplii present in each treatment is further broken down into first-feeding from days 1-3 (Figure 3) and days 4-7 (Figure 4).

Table 1. Growth of red snapper larvae reported in standard length and body depth in the three treatments fed 1 (nauplii only), 2 (nauplii and F. salina), 3 (F. salina only).

	Treatment 1		Treatment 2		Treatment 3		p-value	
Day	Length (mm)	Depth (mm)	Length (mm)	Depth (mm)	Length (mm)	Depth (mm)	Length	Depth
1	2.81 ± 0.09	0.20 ± 0.02	2.81 ± 0.09	0.20 ± 0.02	2.81 ± 0.09	0.20 ± 0.02	--[c]	--[c]
2	2.59 ± 0.10	0.28 ± 0.02	2.55 ± 0.25	0.27 ± 0.03	1.85 ± 0.15	0.22 ± 0.02	<0.0001	0.0003
3	2.63 ± 0.10	0.27 ± 0.02	2.57 ± 0.19	0.26 ± 0.02	2.27 ± 0.20	0.27 ± 0.03	0.0012	0.6512
4	2.43 ± 0.35	0.28 ± 0.07	2.5 1± 0.51	0.25 ± 0.07	2.47 ± 0.57	0.27 ± 0.08	0.9394	0.8451
5	2.78 ± 0.17	0.26 ± 0.05	2.55 ± 0.27	0.24 ± 0.03	--[b]	--[b]	0.2349	0.2327
6	2.16 ± 0.39	0.17 ± 0.02	2.33 ± 0.25	0.17 ± 0.02	--[b]	--[b]	0.3437	0.8968
12	3.55 ± 0.36	0.41 ± 0.09	3.63 ± 0.26	0.44 ± 0.08	--[b]	--[b]	0.3364	0.3024
20	8.34 ± 1.10	2.37 ± 0.43	7.17 ± 0.41	1.91 ± 0.22	--[b]	--[b]	0.0189	0.0214
28	11.45 ± 1.10	--[a]	13.45 ± 5.60	--[a]	--[b]	--[b]	0.0200	--[a]

[a]-Body depth was not measured on the final day.
[b]-Treatment 3 did not have any survival after day 4.
[c]-Fish larvae for measurements were taken from incubator at time of stocking.

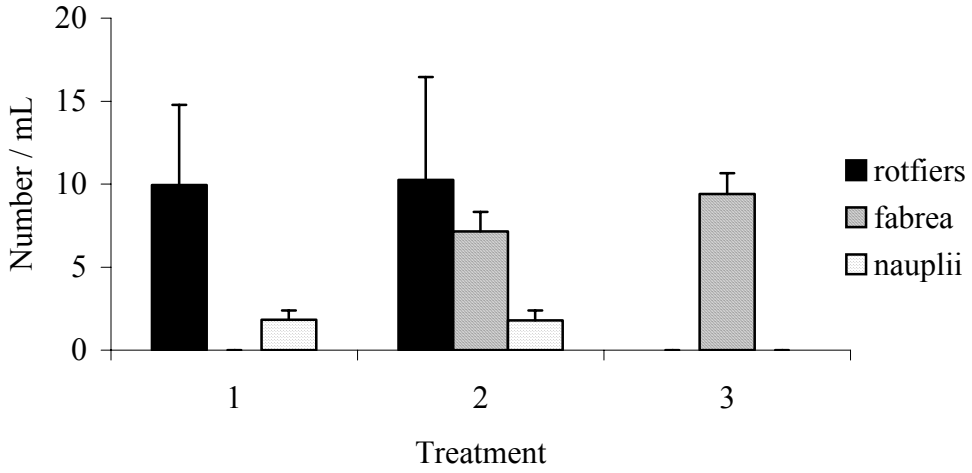

Figure 3. The mean number/mL of each zooplankton present from Days 1 to 3 for each of the three treatments fed 1 (nauplii only), 2 (nauplii and *F. salina*), 3 (*F. salina* only).

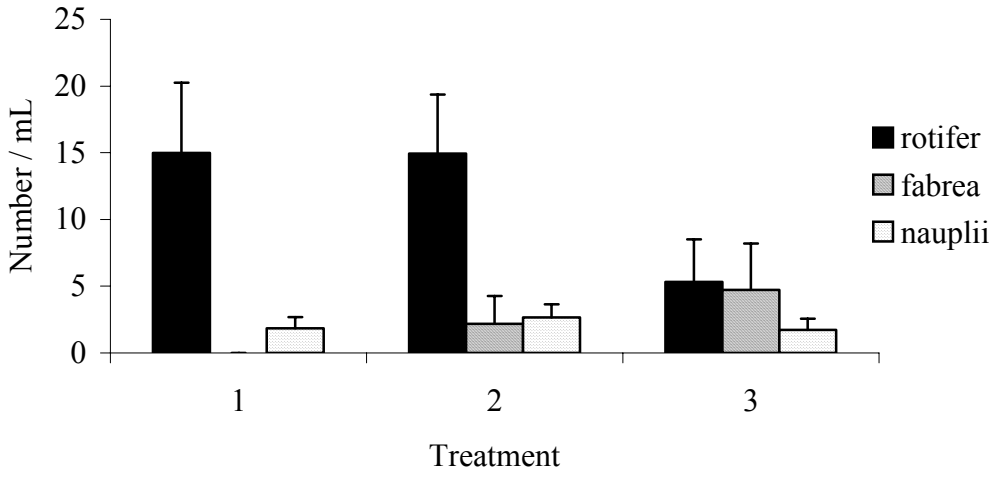

Figure 4. The mean number/mL of each zooplankton present from Days 4 to 7 for each of the three treatments fed 1 (nauplii only), 2 (nauplii and *F. salina*), 3 (*F. salina* only).

Fish larvae were only present through day 4 of the trial in treatment 3. The MDR of *F. salina* days 1-4 was 13.6 ± 8.0% in treatment 2 and –4.8 ± 11.4% (*F. salina* density increasing) in treatment 3 which was significantly less reduction (p=0.038). The mean number of *F. salina* present in treatment 3 was higher than that in treatment 2 in both days 1-3 (Figure 3) and days 4-7 (Figure 4). With no evidence of reduction in treatment 3, the mean *F. salina* density from days 1-9 was significantly higher 6.51 ± 4.35/mL than treatment 2 with only 3.12 ± 2.4/mL (p=0.002). There was no correlation between MDR of *F. salina* and fish survival.

There was no difference in the mean number of rotifers/mL present from days 1-20 in the two treatments with fish survival. There was also no significant difference in the MDR in rotifers from days 1-20, 20.0 ± 11.3% in treatment 1 and 12.0 ± 17.3% in treatment 2 (p=0.47). The mean number of rotifers present was similar in both treatments with survival (treatments 1 and 2) from days 1-3 (Figure 3) and days 4-7 (Figure 4). Treatment 3 did not have any rotifers from days 1-3 (Figure 3) and less rotifers were available on days 4-7 (Figure 4) but there were no fish remaining in this treatment. There was no correlation with MDR of rotifers and fish survival.

Water quality

The tanks were static the first 11 days of the study (Table 2). The mean temperature for all 12 tanks was 26.87 C with a range of 25.13 to 29.32 C with no significant differences (p=0.999) between tanks. The mean dissolved oxygen was 5.78 mg/L ranging from 4.20 to 7.51 with no significant differences (p=0.213) between tanks. Dissolved oxygen was also positively correlated with fish survival (R^2=0.75, p=0.01) ranging from 5.72 to 6.09 mg/L.

Table 2. Mean water quality ± SD values for each parameter of the three treatment groups from Days 1-11 the water was static and the two treatment groups remaining from Days 12-28, water was recirculating as one system.

Days	Treatment	Temperature (C)	DO (mg/L)	Salinity (ppt)	pH	TAN (ppm)
1-11	1	26.9 ± 1.1	5.82 ± 0.40	34.8 ± 0.6[a]	7.86 ± 0.20	0.27 ± 0.31
	2	27.0 ± 1.1	5.88 ± 0.60	33.9 ± 0.4	7.88 ± 0.19	0.29 ± 0.30
	3	26.7 ± 1.2	6.05 ± 0.45[a]	33.8 ± 0.4	8.01 ± 0.14[a]	0.05 ± 0.12[a]
12-28	1	27.8 ± 0.9	5.69 ± 0.36	34.2 ± 0.7	7.86 ± 0.20	0.15 ± 0.18
	2	27.7 ± 0.9	5.88 ± 0.33	34.2 ± 0.7	7.86 ± 0.19	0.14 ± 0.17

[a]-Significantly different than the other two treatments

There were differences in salinity, pH and ammonia among the three treatments. The pH was significantly higher ($p<0.0001$) in treatment 3 than treatments 1 and 2 (Table 2). pH was positively correlated with fish survival, showing higher survival at a higher pH ($R^2=0.63$, $p=0.02$). The range of pH was only from 7.84 to 7.9. The mean salinity in treatment 1 was significantly higher ($p<0.0001$) than treatments 2 and 3 (Table 2). The mean TAN concentration of treatment 3 was significantly less ($p=0.003$) than treatments 1 and 2 (Table 2). Salinity, ammonia and temperature did not show any correlations with fish survival.

The tanks in the system began recirculating as one system on Day 12 of the study. Only the eight tanks of treatments 1 and 2, remained at this point. None of the parameters (temperature, DO, pH, salinity and ammonia) were significantly different ($p>0.05$) between the two treatments (Table 2).

Discussion

The presence of *F. salina* along with copepod nauplii gave an approximate 100% improvement in survival than when only copepod nauplii were added (Figure 2). However, *F. salina* alone is not adequate to support snapper growth and development. Similar results were seen with the dinoflagellate *Gymnodinium splendens* as first food for the red spotted grouper, *Epinephelus akaara*, the Japanese stripe knife jaw, *Oplegnathus fasciatus* and the red sea bream, *Pagrus major* (Rodriguez and Kirayama 1997). The survival of all three species was higher when fed both *G. splendens* and rotifers than when fed exclusively *G. splendens* or rotifers. The diversity of food organisms including dinoflagellates lead to better survival of first-feeding Japanese anchovy, *Engraulis japonica* larvae (Yamashita et al. 1989). They found better larval survival when fed two

naked dinoflagellates and rotifers than rotifers alone. Larvae fed with only dinoflagellates did not grow and survive (Yamahita et al. 1989).

Early reports by Renè (1974) and Barnabè (1974) concluded *F. salina* could be used instead of rotifers as a food for marine fish larvae. Renè (1974) used *F. salina* as a successful substitute to young *B. plicatilis* to feed larval gilthead, *Sparus aurata*, 3-7 days after hatching. Barnabè (1974) reported no difference in survival when feeding larval sea bass, *Dicentrachus labrax*, rotifers or *F. salina*. However, Park and Hur (2001) found higher mortality of larval ayu, *Plecoglossus altivelis* when fed *F. salina* compared *Brachionus plicatilis*. Park and Hur (2001) fed *Brachionus* with a size range of 220-260 μm and *F. salina* ranging from 80-130 μm. This suggests that larval ayu require a food larger than *F. salina* for survival. Larvae of gilthead and sea bass have larger egg diameters and length at hatch (Jones and Houde, 1981) than red snapper (Papanikos et al. 2003). This study demonstrated that *F. salina* could be fed to smaller marine fish larvae. The transition to a larger food may be needed sooner than was provided to the treatment fed *F. salina* only.

The treatment containing both nauplii and *F. salina* were more active feeders than when presented either prey item alone. The reduction of copepod nauplii was greater compared to the treatment containing only nauplii. Reduction of *F. salina* was also greater compared to the treatment being fed *F. salina* only for the first 3 days. It is not uncommon that mixed diets improve growth and survival. Payne et al. (2001) reported greater growth and survival of West Australian dhufish, *Glaucosoma herbaicum* and pink snapper, *Pagrus auratus* when fed both copepod nauplii and rotifers. They observed a preferential selection of copepod nauplii over rotifers.

The percent of protein and major fatty acids required for larval fish are present in high numbers in *F. salina* (Harvey et al. 1997; Pandey et al. 2004). Amino acids and vitamins are also important components of larval fish diets (Fyhn 1989; Merchie et al. 1997) however, these analyses are not currently known of *F. salina*. The high nutrient contents and soft exoskeleton of *F. salina* may provide a highly dependable source of food needed during the first few days of feeding by a larval fish.

There was no difference in the average number of rotifers available to each treatment or the average daily reduction of rotifers. This is not to say that rotifers were not consumed by the snapper larvae. Based on the MDR the reduction ratio of copepods were 34.6 ± 8.5 % and 15.8 ± 16.2 % for treatments 1 and 2, while the rotifers were 20.0 ± 11.0 % and 12.0 ± 17.3 %, respectively. The copepod nauplii and *F. salina* may have been preferred over the rotifers.

The treatment provided with the combined diet of copepod nauplii and *F. salina* had an overall higher density of prey available for the first 4 days of the trial than the other two treatments containing *F. salina* or copepods only. The higher density and range of prey sizes may have contributed to better growth. The fish fed *F. salina* only may have needed to be supplemented with other zooplankton earlier than 4 d after first-feeding. The larvae at this age (6 days after hatch) reached the point of no return (Blaxter and Hempel 1963) also called the age of irreversible starvation (May 1974). Typically mortality occurs within 5-10 days after hatch due to starvation. The fish lengths began to decrease in the treatment being fed *F. salina* only as early as Day 2 (Table 2), this is due to the fish using energy without acquiring enough nutrients from the food provided which results in resorption of tissues. The decrease in standard length during the first 6 d seen

in Table 2 was described by Williams et al. (2004), as growth is channeled into further development of the jaw and increased body depth.

Fabrea salina can be important prey item for marine fish larvae particularly those with small mouth gapes when other foods are available to support additional growth. *F. salina* can be mass cultured but higher densities must be achieved to facilitate its use.

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VI. CONCLUSION

The value of ciliates as alternative live foods for first-feeding red snapper *Lutjanus campechanus*, larvae was evaluated. The foraging behavior of first-feeding larvae was observed when presented with three aloricate ciliates, *Fabrea salina, Strombidinopsis sp*. and *Strombidium sp*. Larval red snapper exhibited a saltatory search pattern similar to most teleost larvae pausing to search before a burst of swimming. Optimum culture conditions for *F. salina* were investigated. Conditions of 12 hours light/day, an initial *F. salina* stocking rate of 3/mL and daily addition of algae at 9.0×10^4 *Rhodomonas lens* cells/mL were applied to mass cultures of 40 and 200 L. The 200 L *F. salina* cultures provided 84 ± 10 ciliates/mL after 7 days. Survival and growth of the larval fish was significantly improved when ciliates were fed in combination with pond grown copepod nauplii compared to pond grown copepod nauplii alone. The larval fish fed only *F. salina* did not survive beyond 6 days after hatching. This study provided evidence that suggest better growth and survival can be achieved when first-feeding larvae are supplemented with ciliates but not with ciliates alone. The mass culture techniques for *Fabrea salina* need to be further improved before the application to production of fish is more practical. Observations of foraging behavior may be helpful in choosing a diet before attempting to rear larval marine fish on a large-scale. Behavior observations used in combination with traditional measures of prey consumption and feeding response may provide better understanding of the nutritional requirements of first-feeding fish larvae

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VIII. APPENDIX. ADDITIONAL LARVAL REARING TRIALS ATTEMPTED

Small-Scale Rearing Trials

The goal of the aquaria trials was to evaluate the effectiveness of using protozoans as a first food organism for red snapper larvae in small-scale experiments.

Materials and Methods

Wild caught brood stock were induced to spawn using techniques described by Minton et al. (1983). Two trials used fish larvae from an induced spawn at Claude Peteet Mariculture Center (CPMC), Gulf Shores, AL. Fertilized eggs were incubated in 100 L tanks with an aeration ring around the 200 µm screened standpipe. The temperature of 27 C and salinity of 33 ppt were maintained in the incubators. The hatchery had continuous lighting by a combination of 60 W F96T12CW WM and F96T12DX fluorescent bulbs (General Electric Fairfield CT USA) with an intensity range of 370 to 520 lx measured with a light meter (Fisherbrand Traceable®, Fisher Scientific, Pittsburgh PA USA) at the water's surface. One trial was conducted with larvae that were transported from Gulf Coast Research Laboratory (GCRL), Ocean Springs, MS to CPMC.

Larvae were stocked 36-48 hours post-hatch (hph), prior to functioning mouthparts into 70 L aquaria at 10 larvae/L. The aquaria flow rates were set for 80 mL/min (~2 turnovers/day). There was a 40 µm screened standpipe to prevent the loss of zooplankton. Daily additions of *Isochrysis sp*. (T-ISO) were made to maintain

9.0 x 10^4 cells/mL, which created a green-water environment. Two different experimental designs (different batches of fish) were used with two different ciliates, *Fabrea salina* and *Strombidium sp.*

Two separate trials used *Fabrea salina* as a potential food organism. *F. salina* was originially obtained from the University of Texas, Marine Science Institute, Port Aransas, TX. Stock cultures of *F. salina* were maintained at CPMC as mentioned in the previous chapters. The experimental design included, four aquaria contained fish that were presented with 10 ciliates/mL, four aquaria contained fish without ciliates and four with ciliates only. The treatments were randomly assigned to aquaria in hatchery. The treatment without fish was to monitor the change in abundance of ciliates from the flow through system not predation. These fish larvae were from the induced spawn at CPMC.

One trial was conducted using *Strombidium sp*., isolated from the Gulf of Mexico by University of West Florida, Pensacola, FL. For the experiment, four aquaria contained fish that were presented with 10 ciliates/mL, four aquaria contained fish with mixed zooplankton and four with ciliates and mixed zooplankton. The treatments were randomly assigned to aquaria in hatchery. Mixed zooplankton was collected from full strength saltwater ponds following the procedures of Lam et al. (2000) for supplying copepod nauplii. Fish larvae were stocked at 8/L for this trial not 10/L as in the previous trials. These fish larave were transported from the induced spawn at GCRL.

Five 10 mL samples were randomly collected using a Stempel pipette and pooled per tank. Counts of algae were performed using a hemacytometer and protozoans were counted in triplicate using Sedgwick-Rafter counting slides. Zooplankton densities were maintained at 10 ciliates/mL and 2 copepod nauplii/mL with daily additions. Water

quality parameters including temperature, salinity, dissolved oxygen and pH were measured daily from each replicate using an YSI 556 MPS (YSI Incorporated, Yellow Springs, CO, USA). Samples for ammonia were also collected and analyzed using Nesslerization method, read using a spectrophotometer.

Five fish were randomly selected from each replicate daily with a pipette; each live larva was photographed with an Olympus digital camera outfitted to an Olympus stereomicroscope. Morphometric measurements of standard length and body depth at the anus were made using Image-Pro® Version 4.0.1, image analysis software calibrated with a photograph of a stage micrometer.

All statistical analyses were performed using SAS Version 8.2 software. Two sample t-tests were used to compare the two similar treatments of each *F. salina* trial. One way ANOVA was used to compare the three treatments of the *Strombidium* trial.

Results

In the first trial using *F. salina* as a food source, there was no fish survival after Day 3 (~96 hph). On the final day fish were observed, there was no significant difference ($p=0.966$) in the fish standard lengths, 2.37 ± 0.14 mm between the fed and unfed treatments however the body depth was significantly less ($p=0.028$) in the treatment fed ciliates, 0.24 ± 0.02 mm than the unfed treatment, 0.22 ± 0.01 mm. This could be due to small sample size; there were only 5 fish from the fed treatment and 13 fish from the unfed treatment. There were no differences in the first two days fish samples. There was no significant difference ($p=0.295$) between the mean daily reduction (MDR) of *F. salina* in the treatment containing fish, 36.3% and the treatment containing *F. salina* only, 28.8%. There were no differences in any water quality parameter between any of the

treatments. The overall mean temperature was 26.98 C with a range of 26.35 to 27.37 C. The mean dissolved oxygen was 6.28 mg/L with a range of 5.70 to 8.32 mg/L. The mean pH was 8.02, ranging from 7.95 to 8.15. The mean ammonia was 0.01 ppm with a range of 0.00 to 0.10 ppm. Salinity was not measured for the first *F. salina* trial.

In the second trial with *F. salina*, there was no survival after Day 3. There was no significant difference in the length, 2.22 ± 0.11 mm or depth, 0.22 ± 0.02 mm, of the fish from the fed and unfed treatments (p=0.761 and p=0.224, respectively). The MDR of *F. salina* was slightly higher (p=0.065) in the treatment with fish, 42.6% compared to 20.7% reduction in the treatment with ciliates only. There werer no differences in any water quality parameter between any of the treatments. The overall mean temperature was 26.99 C, with a range from 26.7 to 27.37 C. The mean dissolved oxygen was 6.25 mg/L with a range of 6.01 to 6.49 mg/L. The mean pH was 7.88 with a range of 7.50 to 8.17. The mean salinity was 32.34 ppt with a range of 31.57 to 33.57 ppt. The mean TAN was 0.04 ppm, with a range of 0 to 0.17 ppm.

The *Strombidium* trial also only had fish present until Day 3 in all treatments. There were no significant differences in mean length, 2.30 ± 0.19 mm or body depth, 0.23 ± 0.02 mm in the three treatments (p=0.148 and 0.212, respectively). The MDR in *Strombidium* was 92.9% in the ciliate and nauplii treatment and 95.8% in the ciliate only treatment (p=0.558). The MDR of copepod nauplii was 0.90% in the nauplii only treatment and 1.85% in the nauplii and ciliate treatment (p=0.972). There werer no differences in any water quality parameter between any of the treatments. The overall mean temperature was 26.77 C with a range of 25.09 to 27.89 C. The mean dissolved oxygen was 5.84 mg/L, with a range of 4.95 to 6.50 mg/L. The mean pH was 8.09 with a

range of 7.97 to 8.23. The mean salinity was 31.02 ppt with a range of 30.7 to 31.35 ppt. The mean ammonia was 0.07 ppm with a range of 0 to 1.705.

No survival was achieved past 96 hph in any of the three small-scale rearing trials. Chigbu et al. (2000) reported the only successful rearing of red snapper has been in containers ≥ 200 L.

Large-Scale Rearing Trial

The goal of the study was to compare the effectiveness of protozoans to mixed zooplankton (copepod nauplii and rotifers) as first-food organisms for red snapper larvae in a large-scale setting.

Materials and Methods

Brood stock were induced to spawn using techniques described by Minton et al. (1983). Fertilized eggs were incubated in 100 L tanks with an aeration ring around the 200 µm screened standpipe. The temperature of 27 C and salinity of 33 ppt were maintained in the incubators. The hatchery had continous lighting by 60 W F96T12 CW WM fluorescent bulbs (General Electric, Fairfield, CT USA) with an intensity range of 370 to 520 lx measured with a light meter (Fisherbrand Traceable®, Fisher Scientific, Pittsburgh, PA USA) at the water's surface.

The study was conducted in 1.5 m diameter tanks in a greenhouse exposed to natural photoperiod (14L:10D). Tanks were filled with 1 µm filtered Gulf seawater, 2 days before stocking of algae, ciliates and fish. The initial water volume was 160 L based on available protozoan cultures and to allow for additions of algae and protozoans.

Larvae were stocked 39 h post-hatch, prior to functioning mouthparts at 10 larvae/L. Stocking of fish larvae was named Day 1 of the trial. Fish were fed according

to one of following protocols using three replicates/protocol: 1) copepod nauplii only, 20-75 µm from days 1 to 7; 2) copepod nauplii from days 1 to 7 plus *Strombidinopsis* from days 1 to 5; and 3) *Strombidinopsis* from days 1 to 5 plus copepod nauplii from days 4 to 7.

Daily additions of *Isochrysis sp*. (T-ISO strain) were made to maintain 9.0 x 10^4 cells/mL to create a green-water environment. Algae was obtained from CCMP, Bigelow Laboratory for Ocean Sciences, Maine, USA and cultured in the laboratory with Gulliard's F/2 media. Daily additions of zooplankton were made to maintain copepod nauplii at 2/mL and ciliates at 10/mL. *Strombidinopsis* stock culture was isolated from the Gulf of Mexico by University of West Florida, Pensacola, FL. *Strombidinopsis* was then produced in 40 L bags in the laboratory or 200 L transparent tubes in the laboratory or greenhouse at Claude Peteet Mariculture Center, Gulf Shores, AL. Mixed zooplankton was collected from full strength saltwater ponds following the procedures of Lam et al. (2000) for supplying copepod nauplii. Rotifers were collected along with the nauplii and were incidentally added to the tanks when nauplii were given. No attempt was made to maintain a specific rotifer density in the larval rearing tanks. After Day 2, the water level in the 9 tanks balanced out (with the sump) and additions to each tank, for a mean volume of 280 L. The tank volumes ranged from 180 to 565 L on Day 3.

Five 10 mL aliquots were randomly collected using a Stempel pipette and pooled per tank. Daily counts of algae (Days 1 to 8) were performed using a hemacytometer with a compound microscope using 100x magnification. Zooplankton were preserved and stained using Lugol's iodine solution, counts of protozoans (Days 1 to 6), copepod nauplii and rotifers (Days 1 to 6) were conducted in triplicate using Sedgwick-Rafter

counting slides with a compound microscope using 40x magnification. This allowed for the calulation of daily reduction of algae and zooplankton. Water quality measurements including temperature, salinity, dissolved oxygen and pH were taken twice daily from each replicate using a YSI 556 MPS (YSI Incorporated, Yellow Springs, CO, USA). Samples for ammonia were also collected twice a day and analyzed using Nesslerization method, read using a spectrophotometer.

Two fish were randomly selected from each replicate on Days 2 and 3. Each larva was photographed live to prevent shrinkage using a digital Olympus® Camedia 3040 zoom camera outfitted to an Olympus® stereomicroscope (Olympus America Inc., Melville, NY, USA). Morphometric measurements of standard length and body depth at the anus were made using Image-Pro® Version 4.0.1 (Media Cyberetics Inc. Silver Spring, MD, USA) image analysis software calibrated with a stage micrometer.

All statistical analyses were performed using SAS Version 8.2 software (SAS Institute, Cary, North Carolina, USA). Differences in mean zooplankton reductions, water quality and morphometrics were analyzed by one-way ANOVA.

Results

There were no fish observed after Day 3 (<96 hph) of the trial. On Day 3 there were no significant differences between the overall mean length, 2.56 ± 0.08 mm or body depth, 0.27 ± 0.02 mm ($p=0.434$ and $p=0.508$, respectively) of the three treatments.

There were no differences ($p=>0.05$) between treatments for any water quality parameter. The mean temperature was 26.8 C with a range of 24.6 to 30.4 C. The mean dissolved oxygen was 6.07 mg/L with a range of 5.00 to 8.85 mg/L. The mean pH was 8.2 with a range of 7.65 to 8.9. The mean salinity was 30.0 ppt with a range of 28.6 to

32.4 ppt. The mean total ammonia nitrogen concentration was 0.10 ppm with a range of 0 to 0.748 ppm.

The mean daily reduction (MDR) ± SE of algae was 3.9 ±17.0% in treatment 1 containing copepod nauplii only, 17.0 ± 13.3% in treatment 2 containing copepod nauplii and ciliates and 50.0 ± 8.3% in treatment 3 containing ciliates only. The MDR of *Strombidinopsis* was 94.8 ± 1.8% in treatment 2 containing ciliates and nauplii and the *Strombidinopsis* increased in treatment 3 containing ciliates only by 257.1 ± 152.0%. The MDR of copepod nauplii was 54.5 ± 10.3% in treatment 1 containing nauplii only, 54.2 ± 16.4% in treatment 2 containing copepod nauplii and cilates and 39.0 ± 9.8% in treatment 3 which had copepods added on day 4 (there were no fish remaining at this point).