Ciliated Protozoans as Alternative Live Food for First Feeding Red Snapper, *Lutjanus campechanus*, Larvae

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ABSTRACT

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. One of the major challenges of culturing of red snapper, *Lutjanus campechanus* is providing an appropriate food source at onset of feeding. Possible food organisms include species of *Fabrea, Strombidium* and *Strombilidium* with average sizes of 40 x 100, 33 x 41, and 35 x 80 µm, respectively. Optimum growth conditions evaluated were photoperiod, stocking densities of ciliates and algae, and algal food type. Techniques were developed for the consistent mass culture of *Fabrea salina* using 200 L solar tubes, resulting in 75 ciliates/ml in seven days.

Growth and survival of red snapper larvae was evaluated in a production setting using 1 m^3 tanks. Larvae were stocked at 10/L, 36 hours post hatch, and prior to functioning mouthparts. Three treatments were fed:

i) Copepod nauplii, 20-75µm only from days 1 to 10;
ii) Copepod nauplii from days 1 to 10 plus *Fabrea* from days 1 to 5; and
iii) *Fabrea* 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. Two larvae per tank were removed daily from day 1 to 5 and once per week thereafter and photographed for morphometrics. Survival after 28 days was 0.28% and 2.39% in treatment 1 and 2, respectively. Treatment 3 did not have any survival after six days post-hatch. Larvae were more active feeders in the tanks given *Fabrea* and copepods as first foods with 34.6 ± 58.1% average daily reduction in copepod nauplii compared to 15.8 ± 107% reduction when only nauplii were provided. Survival was positively related to the presence of *Fabrea* and nauplii at first feeding.

KEY WORDS: Ciliate, first feeding, larviculture

Los Protozoos Ciliados como Alternative Viven Alimento para Alimentar Primero Larvas Pargo Rojo *Lutjanus campechanus*

Los ciliados son abundantes en aguas marinas pero su importancia como primer alimento para larvas de peces se desconoce escasamente, especialmente debido a que los ciliados no tienen un exoesqueleto que facilite su identifica-

ción en el intestino de una larva depez. Uno de los principales desafíos se encuentra en el levante de larvas de pargo rojo *Lutjanus campechanus*, en cuanto a que proporcione un alimento adecuado en su primera comida. Los organismos que pueden servir como posible alimento inicial incluyen especies tales como *Fabrea, Strombidium, y Strombilidium*, con tamaños promedio de 40x100, 33x41, y 35x80 µm respectivamente. Los condiciones ambientales que se evalvaron durante los experimento de crecimento fueron: temperatura, salinidad, intensidad de luz y fotoperiodo, aireación, densidad de cultivo y tipo de alga utilizada como alimento. Para los experimentos se usaron tubos solares de 200L de capacidad, con una producción promedio aproximada de 50 ciliados/ml en 7 días.

El crecimiento y la soberevivencia de las larvas de pargo rojo fueron evaluados en tanques de fibra de vidrio de $1m^3$. Las larvas fueron sembradas en proporcion de 10/L, 36 horas post-eclosión, antes de la formación de las partes bucales. Se hicieron tres tratamientos alimenticios:

i) Nauplios de copepodo de 20-75 µm del dia 1 hasta el dia 10,
ii) Nauplios de copepodo del día 1 hasta el día 10 más *Fabrea* durante los días 1 hasta el día 5, y
iii) *Fabrea* solamente del días1 al 3 más nauplios de copepodo desde el día 4 al 10.

Los nauplios de copepodo se adicionaron en proporcion de 5/ml. Se extrajeron dos larvas por tanque por dia durante los dias 1 al 5 para ser fotografiados con fines morfometricos. La sobrevivencia despues de 28 días fue del 0.28% y del 2.39% en los tratamientos 1 y 2, respectivamente. En el tratamientos 3 no se observó ninguna supervivencia después del día 4 de haberse formado las partes bucales. Los larvas mostraron mayor actividad alimenticia en los tanques alimentados con *Fabrea* y copepodos como primer alimento con un promedio de reducción de 45.4 ± 18.7% en nauplios de copepodo comparado con el 15.7 ± 9.1% de reducción registrado en el tratamiento en el que se aplicaron solamente nauplios de copepodo. La sobrevivencia estuvo positivamente relacionada con el porcentaje diario de reducción en abundancia de nauplios ($R^2 = 0.81$; $p = 0.002$).

PALABRAS CLAVES: Ciliados, alimento para larvas de peces, pargo rojo, *Lutjanus campechanus*

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. The problem lies with the relatively small mouth size, limited yolk reserve, and late development of functional mouthparts. Typically, rotifers or copepod nauplii are offered as first foods. The importance of other microplankton including dinoflagellates and ciliates as available prey is poorly understood. Ciliated protozoans may be

important for first-feeding fish larvae because: 1) ciliates often dominate such communities and are more abundant than copepod nauplii in coastal waters (Kamiyama 1994), and 2) most of the ciliates in the plankton are of a similar or smaller size than copepod nauplii (Taniguchi 1978). 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). 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 the predation of protozoans by fish larvae, except for the loricate tintinnids, which have indigestible hard parts that can be identified in the guts (review by Pierce and Stoecker 1992). There is less information about the predation of naked ciliates by fish larvae. Korniyenko (1971) described a relationship between protozoans and carp larvae; Howell (1972) reared lemon sole with protozoans; Nagano et al., (2000 a,b) detected feeding on *Euplotes sp.* by larval grouper and surgeonfish, respectively; and Ohman, et al. (1991) observed larval anchovy feeding on *Strombidium sp.* when presented as a first food. Fukami et al. (1999), investigated predation on naked protozoa by 52 different taxonomical groups of fish larvae collected in the field. They found fish in the group Acanthopterygii consumed the greatest quantity of protozoans (> 30 protists/individual). Red snapper have a small mouth gape at first feeding similar to the fish mentioned above. A protozoan may be of a more appropriate size than other marine organisms as a first food.

Fabrea salina is a heterotrich ciliate in the family Climacostomidae. It naturally occurs in estuarine environments and high saline areas. *Fabrea* is a relatively large protozoan, its size ranging from 120 - 220µm by 67 - 125µm. A few attempts have been made to culture it using single cell algae, bacteria or yeast (De Winter et al. 1975, 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, 1975 as follows:

i) It is one of the few truly “pelagic” ciliates,
ii) It has the appropriate dimensions as a live food: depending on culturing conditions its size can vary from 50 - 500 µm,
iii) The smooth cell wall and the absence of appendages facilitate its uptake by the predators,
iv) The generation time is very short,
v) As a particle feeder it can be cultured on live algae as well as inert foods,
vi) According to the literature data its nutritional value for fish larvae seems to be excellent, and
vii) As many other protozoans it forms a tough cyst membrane when submitted to unfavorable environmental conditions. The cysts can be kept viable for a certain period of time without losing their hatchability.

The following work consists of two parts, 1) to develop culture techniques for ciliated protozoans and 2) evaluate the use of the ciliate *Fabrea salina* as a first food for larval red snapper.

MATERIALS AND METHODS

Ciliate Culture

Initially, several ciliates were considered for culture, and *Fabrea salina* showed to be more reliable in culture than *Strombilidium sp.* and *Strombidium sp* and is, therefore, the focus of this paper. The *Fabrea salina* culture was obtained from the University of Texas, Marine Science Institute, Port Aransas, TX. Stock cultures were maintained at Claude Peteet Mariculture Center, Gulf Shores, AL. Algae provided as food was cultured with Gulliard's F/2 in the laboratory. All of the experiments were carried out for seven days in 2 L Erlenmeyer flask initially containing 1 L of chlorinated then dechlorinated, 1 µm filtered seawater (32 - 34 ‰). Each treatment contained three replicates. No aeration was supplied, flasks were swirled before sampling to evenly distribute the ciliates and resuspend algal cells. The temperature was not controlled for the experiments; the normal range was 25 - 30°C. The trials were conducted under continuous light ranging from 1,240 to 1,680 lux. Daily counts for organisms were performed in triplicate using Sedgwick-Rafter slides on a compound microscope; samples preserved and stained using Lugol's iodine solution. Samples for water quality measurements including ammonia, temperature, salinity, dissolved oxygen, and pH were taken daily from each replicate.

Four trials were conducted to examine *Fabrea* stocking density, algal species *Isochrysis* and *Rhodomonas*, *Rhodomonas* algal density, and photoperiod. To determine stocking density of *Fabrea*, three treatments were used 3, 6 and 9/ml, being fed 120,000 *Isochrysis* cell/ml daily. Based on the results, the remaining experiments were initially stocked at 3 ciliates/ml. Determining the optimum food type and density was another series of studies. One trial compared growth when fed 90,000 *Rhodomonas* or *Isochrysis* cells daily. With *Rhodomonas* providing a better growth rate, the next trial compared the algal density of 90,000 to 135,000 cells/ml. Photoperiod was another variable, three treatments included 6 hours light and 18 hours dark (6L:18D), 12L:12D and 18L:6D. Continuous light was not compared with these three because the other trials were conducted at 24L:0D. Sampling was conducted as described earlier. Conditions selected for mass culture in 40 L bags or 200 L solar tubes included, stocking ciliates at 3/ml, 12 to 14 hours light/day, and feeding 90,000 *Rhodomonas* cells/ml/day.

Snapper Rearing

Wild caught brood stock were induced to spawn using techniques described by Minton et al. (1983). Fertilized eggs were incubated in the hatchery. Larvae were stocked 12 - 24 hours post-hatch, prior to functioning mouthparts into 1 m^3 tanks at 10 larvae/L to evaluate the value of *Fabrea* as a larval food. Fish were fed according to one of the following protocols using

four replicates/protocol. The feeding protocols were:

i) Copepod nauplii only, 20 - 75µm from days 1 to 10;
ii) Copepod nauplii from days 1 to 10 plus *Fabrea* from days 1 to 5; and
iii) *Fabrea* from days 1 to 5 plus copepod nauplii from days 4 to 10.

From days 7 to 21, all of the treatments were fed copepod nauplii ranging from 40 - 100µm. On day 12, when all treatments were being fed the same copepod diet and the ciliates were no longer present, the recirculation of water began. A commercial feed by INVE, Proton-1 was given starting on day 16. Adult copepods were added to all treatments from days 18 to 28.

Daily additions of *Isochrysis galbana* were made to maintain 90,000 cells/ml, which created a greenwater environment. Daily additions of zooplankton were made to maintain copepod nauplii at 2/ml and ciliates at 5/ml. *Fabrea* was produced in 40 L bags in the laboratory or 200 L solar tubes in the laboratory or greenhouse. Mixed zooplankton was collected from 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 samples were randomly collected using a Stempel pipette and pooled per tank. Counts of algae were performed using a hemacytometer and zooplankton counts including protozoans, rotifers, and copepod nauplii were conducted in triplicate using Sedgwick-Rafter counting slides. Water quality measurements including temperature, salinity, dissolved oxygen and pH were taken twice daily from each replicate using a YSI 556 MPS. 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 weekly thereafter on day 12 and day 18. 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 stage micrometer. At harvest, total length was measured (± 1 mm) using a metric ruler. All statistical analyses were performed using SAS Version 8.2 software and/or StatView 5.0.

RESULTS

Ciliate Culture

Based on several *F. salina* culturing trials that altered stocking density, algal species and density, and photoperiod, the following results were found. After seven days of the stocking density trial, there were no significant differences ($p = 0.001$) in the growth when stocked at 3, 6 or 9 *Fabrea*/ml. The mean maximum density reached 97 ± 9/ml after five days. The ammonia production after seven days ranged from 1.4 to 2.0 ppm, the higher values were from the treatments stocked at higher densities. The flasks stocked at 9/ml had reached 96 ± 6/ml on day, 3 then growth slowed almost completely. There was

a drop in dissolved oxygen corresponding to the spikes in ammonia. The flasks stocked at 3 and 6/ml grew more consistently through five days before starting to decline.

The trial comparing *Isochrysis* and *Rhodomonas* resulted in *Rhodomonas* providing higher growth. The density increased from 3/ml to 216 ± 34/ml for *Rhodomonas* compared to 66 ± 18/ml when being fed *Isochrysis* (Figure 1). When comparing 90,000 and 135,000 *Rhodomonas* cells/ml, the best density of algae to feed daily was found to be 90,000 *Rhodomonas* cells/ml. The treatment fed higher densities of algae had lower dissolved oxygen and higher ammonia and decreased growth. In the photoperiod trial, the three exposures to light had similar results. In all treatments, the density approximately doubled daily and peaked after seven days. The light controlled the algae from blooming, which had an effect on the overall water quality.

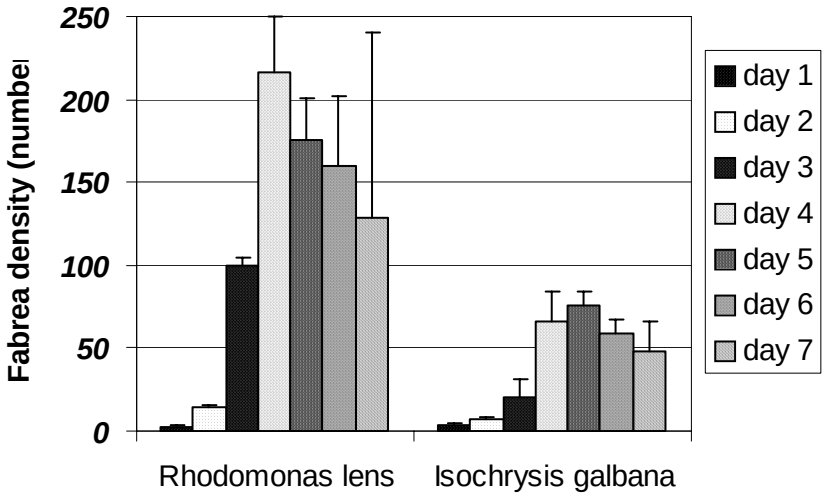

Figure 1. Results of *F. salina* growth trial when either *Rhodomonas* or *Isochrysis* was stocked at 90,000 cells/ml and Fabrea at 3/ml.

Optimum culturing conditions, stocking at 3/ml, 12 to 14 hours light/day, and feeding 90,000 *Rhodomonas* cells/ml were used to mass culture *Fabrea* in 40 L bags or 200 L solar tubes. These conditions lead to growth of at least 50 ciliates/ml in no more than seven days, in 100% of culture attempts. In 200 L tubes the overall mean density was 75 ± 31/ml with some variability in day of peak growth (Table 1).

Table 1. Average densities of mass cultures from past and present studies and the densities from culture trials (parameter with highest density cited).

	Food type	No Days Cultured	Avg. density (#/ml)	Culture Volume (L)	Source
Past					
	Rice bran	5	59	40	Rattan, 1999
	Egg custard	5	60	50	Pandey and Yeragi, 2004
	Egg custard	5	82	200	Pandey and Yeragi, 2004
	Duniellia sp.	14	100	45	Uhlig,1981
	Duniellia sp.	4	130	30	De Winter and Persone, 1975
Present					
	Rhodomonas	7	70 ± 11	1	Algal density trial-90,000 cells/ml
	Rhodomonas	7	84 ± 9	1	Lighting trial-18 hours light
	Isochrysis	7	110 ± 12	1	Stocking density trial-9/ml
	Rhodomonas	7	216 ± 34	1	Algal comparison trial
	Rhodomonas	7	100 ± 36	40	Bag cultures
	Rhodomonas	7	75 ± 31	200	Solar tube cultures

Snapper Rearing

Survival of red snapper after 28 days was 0.28 ± 0.15% in treatment 1, fed copepod nauplii only; 2.39 ± 2.75% in treatment 2, fed copepods nauplii and ciliates; no survival in treatment 3, fed *Fabrea* as the only food source for the first three days of feeding. Survival was highly variable within treatments, but the addition of *Fabrea* had a positive impact on survival p = 0.12. Standard lengths, and body depths length are reported for each treatment (Table 2). The final standard lengths were 11.45 ± 1.1mm and 13.45 ± 5.6mm for treatments 1 and 2, respectively (p = 0.020).

Larvae were more active feeders in the tanks given *Fabrea* and nauplii as first foods, with an average daily reduction in copepod nauplii of 34.6 ± 58.1%, compared to 15.8 ± 107.0% reduction when only nauplii were provided. Survival was positively related to the percent daily reduction in nauplii abundance (p = 0.19). The total number of nauplii available for days 1-7 was 2.49 ± 1.02/ml in treatment 2, nauplii and *Fabrea* and was significantly higher (p = 0.05) than treatment 1, containing nauplii only at 1.92 ± 1.03/ml.

The average daily reduction in *Fabrea* was 37.01 ± 50.05% in treatment 2 and 17.7 ± 49.36% in treatment 3 (p = 0.18). The average density was 3.12 ± 2.4/ml in treatment 2 and 6.51 ± 4.35/ml in treatment 3 (p = 0.002). There was a difference in the average density and a trend towards a difference in the daily reduction.

There was no difference in the average daily reduction of rotifers or total average density. The average daily reduction in rotifers was 14.4 ± 49.2% in

treatment 1 and 9.78 ± 77.29% in treatment 2 (p = 0.80). The average density was 14.03 ± 4.99/ml in treatment 1 and 13.86 ± 5.14/ml in treatment 2 (p = 0.91).

Table 2. Growth of red snapper larvae reported in standard length and body depth in the three treatments fed 1=nauplii only, 2=nauplii and *Fabrea*, 3=*Fabrea* only.

	Treatment 1		Treatment 2		Treatment 3	
Day	**Length (mm)**	**Depth (mm)**	**Length (mm)**	**Depth (mm)**	**Length (mm)**	**Depth (mm)**
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
2	2.72±1.08	0.18±0.11	2.68±0.25	0.19±0.02	1.90±0.15	0.19±0.17
3	2.66±0.09	0.20±0.02	2.57±0.29	0.18±0.01	2.34±0.21	0.16±0.05
4	2.47±0.80	0.20±0.01	2.57±0.54	0.19±0.03	2.48±0.59	0.19±0.02
5	2.78±0.17	0.20±0.04	2.55±0.27	0.18±0.02	--[2]	--[2]
6	2.16±0.39	0.17±0.02	2.33±0.25	0.17±0.02	--[2]	--[2]
12	3.47±0.36	0.39±0.09	3.63±0.26	0.44±0.08	--[2]	--[2]
20	8.34±1.10	2.37±0.43	7.17±0.41	1.91±0.22	--[2]	--[2]
28	11.45±1.1	--[1]	13.45±5.6	--[1]	--[2]	--[2]

[1]-Body depth was not measured on the final day.
[2]-Treatment 3 did not have any survival after day 4.

DISCUSSION

Fabrea proved to be a relatively hardy protozoan that could be produced in a large volume (200 L) at densities of 75 ± 31/ml. The culture techniques determined for *Fabrea salina* yielded similar densities to other mass culture attempts ranging from 59 to 130/ml (Table 1). *Fabrea* multiplies relatively fast. Therefore, differences in initial stocking rates 3 to 9/ml had little impact on the final densities obtained. Mass cultures were initially stocked with 3/ml to decrease the initial volume needed to inoculate the culture. *Rhodomonas* supported better *Fabrea* growth than *Isochrysis.* These two algae species differ in size, *Rhodomonas* 9 - 12 µm versus 5 - 6 µm for *Isochrysis* and nutrient content (Repak 1983). Repak (1983) evaluated 45 species of algae as food for *Fabrea* and found *Rhodomonas lens* to provide the best growth rate; seventeen other species of algae also proved to be nutritious, including *Isochrysis*. Other authors have used non-algal diets such as egg custard, fermented rice bran, and wheat bran that provide bacteria and yeast (Table 1). Protozoans can grow often just as well on inert foods, which is easier for aquaculture purposes. However, Repak (1986) found bacteria to be of minimal value for *Fabrea* growth and yeast to have little to no value. Major fatty acids required for larval fish are present in high numbers in *Fabrea*, the nutritional value was found to vary with diet (Harvey et al. 1997, Pandey et al. 2004).

Lighting trials conducted by DeWinter and Persone (1975) using continuous light and continuous darkness, showed no difference. Pandey and Yeragi (2004) obtained densities of 120/ml when exposed to light and only 76/ml without light. No significant difference (p = 0.32) was found amongst the three treatments, 6, 12, or 18 hours light. This study did not contain a 24 or 0

hour light treatment. The photoperiod gives control over the algal growth, which affects the water quality.

The presence of *Fabrea* along with copepod nauplii gave an approximate 100% improvement in survival than when only copepod nauplii were added. However, *Fabrea* alone is not adequate to support snapper growth and development. Similar results were observed with the dinoflagellate *Gymnodinium splendens* as first food for the red spotted grouper and the Japanese stripe knife jaw (Rodriguez and Kirayama 1997). The survival was higher when fed both *G. splendens* and rotifers than when fed exclusively *G. splendens* or rotifers. The diversity of food organisms leads to better survival. 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 *Fabrea*. There were no other reports mentioning the use of *Fabrea* 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. F. salina* may be better suited for larval fish requiring a smaller first feed or the transition is so fast that larger food may be needed sooner.

The treatment containing both nauplii and *Fabrea* were more active feeders based on the reduction in copepod nauplii when compared to the treatment containing only nauplii and more active feeders on *Fabrea* when compared to the treatment being fed *Fabrea* only for the first three days. There was no difference in the average number of rotifers available to each treatment or the average daily reduction of rotifers. The fish lengths began to decrease in the treatment being fed *Fabrea* 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 six days 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 feed 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 it being more commonly used.

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