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Ciliated protozoans as food for first-feeding larval grouper, *Epinephelus septemfasciatus*: Laboratory experiment

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Abstract: The survival from hatching to day 8 of larval grouper, *Epinephelus septemfasciatus*, was examined in two rearing experiments. In Experiment 1, the larvae were fed with aloricate ciliates, *Euplotes* sp. 1 or *Euplotes* sp. 2, at different concentrations. Significantly higher survival of the larvae was observed in the treatment with the highest density of *Euplotes* sp. 1 (1.4×10^4 cells l^{-1}) on days 5 and 6 after hatching. Experiment 2 consisted of different prey concentrations of a loricate ciliate, *Favella taraikaensis*. Significantly higher larval survival was observed in the treatment having the highest density of *F. taraikaensis* (4.8×10^3 cells l^{-1}) on days 4–6 after hatching. Larval gut content analyses failed to show any ciliates in Experiment 1, whereas hard body parts of *F. taraikaensis* were detected in Experiment 2. However, feeding on *Euplotes* by grouper larvae was confirmed by labelling the former with fluorescent microspheres. The lack of ciliate remains in Experiment 1 might be due to complete digestion by the larvae. The present experiments demonstrated that the higher ciliate densities enhanced larval survival until 4–6 days after hatching, suggesting the importance of ciliates as initial food for first-feeding *E. septemfasciatus*. It is likely that ciliates in the marine ecosystem may bridge the gap until grouper larvae can encounter foods such as copepod nauplii.

Key words: ciliate, first-feeding, larval grouper, rearing, survival

Introduction

Although most first-feeding marine fish larvae feed chiefly upon copepod nauplii (Arthur 1976; Last 1978), the densities of copepod nauplii are usually too low to totally satisfy larval growth requirements (Lasker et al. 1970; Arthur 1977; Hunter 1981). Moreover, fish larvae characterized by a small mouth (such as grouper and snapper larvae) may suffer restrictions in available food size compared with fish larvae with a large mouth (Hussain & Higuchi 1980; Doi et al. 1997). As an alternative food source to copepod nauplii, ciliates 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; Uye et al. 1996, 1998), and (2) most of the ciliates in the plankton are of a similar or smaller size than copepod nauplii (Taniguchi 1977).

Tintinnid ciliates have often been found during gut content analyses of field-collected marine fish larvae (Jenkins 1987; Watson & Davis 1989; Govoni & Chester 1990). Pierce & Turner (1992) suggested that the main importance of tintinnids as food for fish larvae might be at the larval first-feeding stage. Aloricate (naked) ciliates also may be important prey of fish larvae because they occur in large numbers, generally several times more abundant than tintin-

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nid ciliates (Pierce & Turner 1992), although there is no evidence that fish larvae feed upon naked ciliates in the field.

There have been only a few laboratory experimental studies which have determined the effect of dietary ciliates on larval survival. Korniyenko (1971) found that consumption of ciliates by first-feeding larvae of freshwater fishes enhanced their survival. Larval sea bream, *Lithognathus mormyrus* (Linnaeus), grew faster with lower mortality when microzooplankton, including ciliates, were available as prey (Kentouri & Divanach 1983). However, there have been no reports yet on larval rearing using only ciliates to evaluate their potential as a food. In this study, survival of first-feeding larval grouper (Serranidae), *Epinephelus septemfasciatus* (Thunberg), reared with different densities of three kinds of ciliates (*Euplotes* sp. 1, *Euplotes* sp. 2 and *Favella taraikaensis*) were examined.

Materials and Methods

Experiment 1

Experiment 1 was conducted using two unidentified aloricate ciliates, *Euplotes* sp. 1 and *Euplotes* sp. 2. Both ciliates were isolated from Miyazaki Harbor, Miyazaki, Japan, and cultured in autoclaved filtered (Whatman GF/F) seawater. Cultures were enriched with 0.1% peptone (Difco) to stimulate growth of the mixed natural bacterial assemblage. Mean dimensions of *Euplotes* sp. 1 and *Euplotes* sp. 2 were $63\times38\,\mu m$ ($n=30$) and $34\times22\,\mu m$ ($n=30$), respectively. Eggs of artificially fertilized grouper, *Epinephelus septemfasciatus*, were provided by the Miyazaki Prefectural Fisheries Experimental Station on 14 July 1997. Approximately 70 fertilized eggs were introduced to each 1-liter glass beaker filled with filtered (Whatman GF/F) seawater. The rearing water was aerated for a day before the experiment so as to be saturated with dissolved oxygen. The beakers were then placed in a heater-regulated water bath, and were protected from rain and strong sunshine by a translucent roof and dark mesh. Two days after hatching (2 DAH) (i.e. one day before mouth opening), ciliates were fed to the larvae. The number of larvae in each beaker at 2 DAH varied between 48 and 52. Immediately before feeding, ciliate cultures were concentrated on a 5-μm-mesh Nitex screen and rinsed with GF/F-filtered seawater to remove any dissolved peptone.

Experiment 1 was performed with 7 treatments each having 3 replicates, as outlined in Table 1. Aeration was not provided in order to avoid physical shock to the larvae during the experiment. Larval survival by percentage was estimated from 3 DAH (when the larvae are expected to feed on food organisms) by counting the number of surviving larvae in each beaker each morning from 0900 to 1100 h. Mean survival from the three replicate beakers in each treatment was used for comparisons of the treatments, differences in those survivals being tested by analysis of variance (Okuno 1986). Dead and malformed larvae were removed from each beaker by gentle pipetting during the experiment. Because grouper larvae stop feeding at night (Yamamoto 1996), concentrations of ciliates for each treatment were monitored every night and maintained at the initial food concentrations. The experiment was continued until all of the larvae died.

Table 1. Experimental designs for Experiment 1; 3 replicates for each of 7 treatments were prepared.

Treatment No.	Initial prey densities (cells l^{-1})	
	Euplotes sp. 1	*Euplotes* sp. 2
1	1.2×10^4	0
2	1.0×10^3	0
3	1.3×10^2	0
4	0	1.4×10^4
5	0	1.2×10^3
6	0	1.3×10^2
7 (Control)	0	0

Simultaneously, gut content analyses of fish larvae were conducted on another 3 replicates of 6 treatments so as to determine whether or not grouper larvae could ingest ciliates, the experimental conditions being the same as the above rearing experiment. At least 5 to 10 larvae were collected from each treatment on each day (following sunset) and preserved with 1% phosphate-buffered glutaraldehyde solution. Guts of grouper were opened using fine insect pins under a dissecting microscope at 6.6–10×magnification. The gut contents were carefully observed under a light microscope at 100× or 200× magnification after the diagnostic morphological characteristics of each ciliate species had been established.

Additional feeding experiments were also conducted on 15 June 1999. Fertilized grouper eggs were provided by the Ehime Prefectural Fisheries Experimental Station. Feeding on *Euplotes* by grouper larvae was detected using a fluorescent microsphere technique, which had been previously described for the detection of the ingestion of ciliates by other predacious ciliates (Dolan & Coats 1991). Fluorescent microspheres (1.0 μm in diameter) were used (Yellow Green Fluoresbrite Microspheres, Polysciences Inc., Warrington, PA, USA) to label *Euplotes*. Microspheres were added to cultures of *Euplotes* sp. 1 and sp. 2 at a final concentration of 1×10^7 microspheres ml^{-1} and the culture incubated for 30 min. Microspheres which were not ingested were removed by rinsing the culture with three washes of GF/F-filtered seawater over a Nuclepore filter (5-μm pore size). The ciliates were then resuspended with GF/F-filtered seawater, those labelled with microspheres being visible under an epifluorescence microscope. *Euplotes* sp. 1 and *Euplotes* sp. 2 were labelled with microspheres at levels of 92 and 94%, respectively. The labelled ciliates were fed to the larvae at 4 DAH, the larvae having been pre-acclimated to the food by adding unlabelled prey organisms prior to the feed-

Table 2. Experimental designs for Experiment 2; 3 replicates for each of 5 treatments were prepared.

Treatment No.	Initial prey densities (cells l^{-1})	
	Favella taraikaensis	*Prorocentrum minimum*
1	4.8×10^3	3.2×10^6
2	1.0×10^3	3.2×10^6
3	5.0×10^2	3.2×10^6
4 (Control)	0	0
5 (Control)	0	3.5×10^6

ing experiment. Three replicates of 6 treatments (Treatments A–F) were prepared, with respective ciliate concentrations being the same as in Treatments 1–6. After 2 h, 10 larvae were removed from each beaker, fixed with 1% phosphate-buffered glutaraldehyde solution and their gut contents examined under an epifluorescence microscope.

Experiment 2

A culture of the tintinnid ciliate, *Favella taraikaensis* Hada, isolated from the Inland Sea of Japan, was used as the initial food for larvae in Experiment 2. Mean dimensions of the lorica were $183\times76\,\mu m$ ($n=25$). *Favella taraikaensis* was cultured in a seawater medium with 3×10^3 cells ml^{-1} of a dinoflagellate, *Prorocentrum minimum* (Pavillard) Schiller, as food. In Experiment 2, fertilized grouper eggs which were provided by the Ehime Prefectural Fisheries Experimental Station were dispersed into the 5 rearing treatments as outlined in Table 2. Three replicates were made for each treatment. Larval rearing was performed in the same manner as in Experiment 1. Larval gut content analyses were also conducted in the same manner as in Experiment 1 until termination at 6 DAH due to an abrupt decrease in the number of surviving larvae.

Results

Experiment 1

Water temperature varied from 21.0 to 22.8°C throughout Experiment 1. Larvae opened their mouths at 3 DAH and entirely consumed their yolk by 4 DAH. The range of survival at 4, 5, 6 and 7 DAHs varied at 74–100, 34–88, 6–46 and 0–21%, respectively. Figure 1 shows the mean survival rate of larvae for the 7 treatments. At 4 DAH, the highest mean survival rate (92%) was recorded for Treatment 1 with the highest concentration of *Euplotes* sp. 1. However, survival at 4 DAH was not significantly different between the 7 treatments. A sudden decrease in survival was observed in Treatments 2–7 at 5 DAH (Fig. 1). Significant differences in survival between Treatments 1–2 ($p<0.05$), 1–3 ($p<0.01$), 1–5 ($p<0.01$), 1–6 ($p<0.01$) and 1–7 ($p<0.01$) were recorded (Table 3). Mean survival at 6 DAH was significantly higher ($p<0.05$) for Treatments 1 and 4 (38% and 33%, respectively), compared with Treatments 2, 3, 5, 6 and 7 (Table 3). At 7 DAH, there was no significant difference between treatments, and all remaining larvae died the following day. Neither *Euplotes* sp. 1 nor

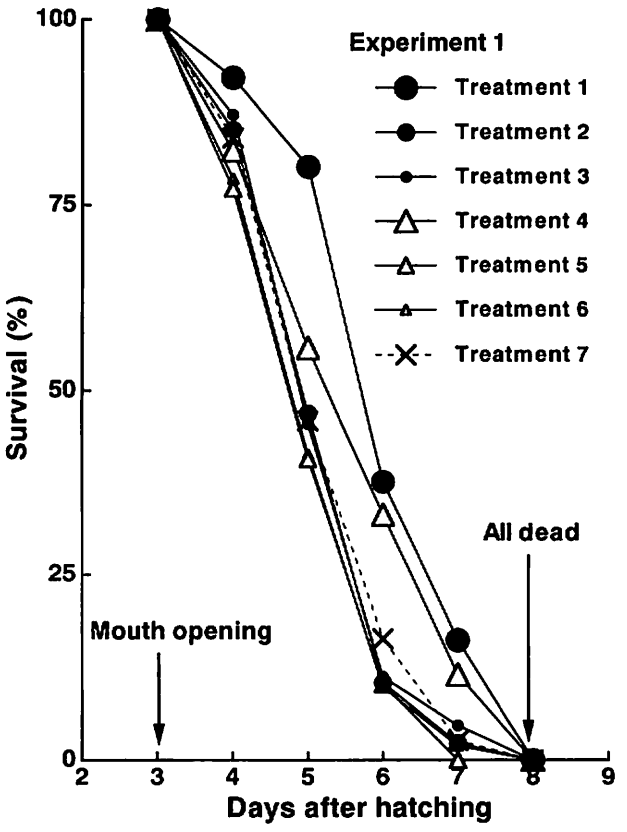

Fig. 1. Survival of larval grouper, *Epinephelus septemfasciatus* (Experiment 1). Treatments are defined in Table 1.

Table 3. Tests of differences in survival among 7 treatments at 5 and 6 d after hatching (DAH); results from Experiment 1 (++, significant at the level of $p<0.01$; +, significant at the level of $p<0.05$; −, not significant).

Age	Treatment No.	1	2	3	4	5	6	7
5 DAH	1		+	++	−	++	++	++
	2			−	−	−	−	−
	3				−	−	−	−
	4					−	−	−
	5						−	−
	6							−
	7							
6 DAH	1		+	+	−	+	+	+
	2			−	+	−	−	−
	3				+	−	−	−
	4					+	+	+
	5						−	−
	6							−
	7							

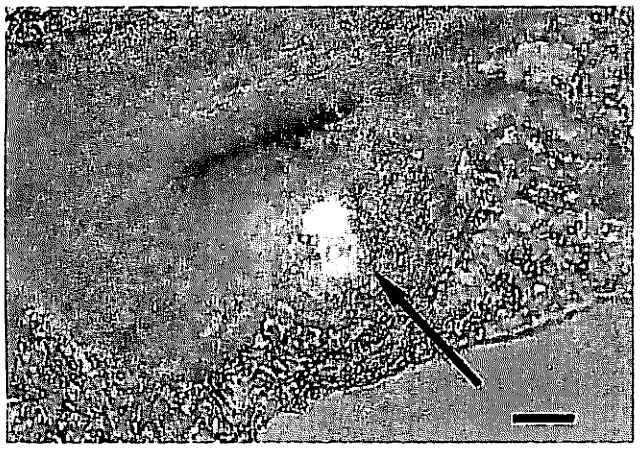

Fig. 2. Photomicrographs of larval *Epinephelus septemfasciatus* with ingested fluorescent microspheres. Arrow indicates microspheres. Scale bar=50 μm.

Euplotes sp. 2 were found in the gut contents of preserved larval specimens.

On the other hand, an additional feeding experiment on 15 June 1999 showed that the ingested fluorescent microsphere-labelled *Euplotes* sp. 1 and sp. 2 cells were easily visualized inside the guts of larvae under an epifluorescence microscope (Fig. 2). The percentage of larvae which contained fluorescent microspheres for Treatments A–F were 73, 57, 40, 77, 57 and 43%, respectively.

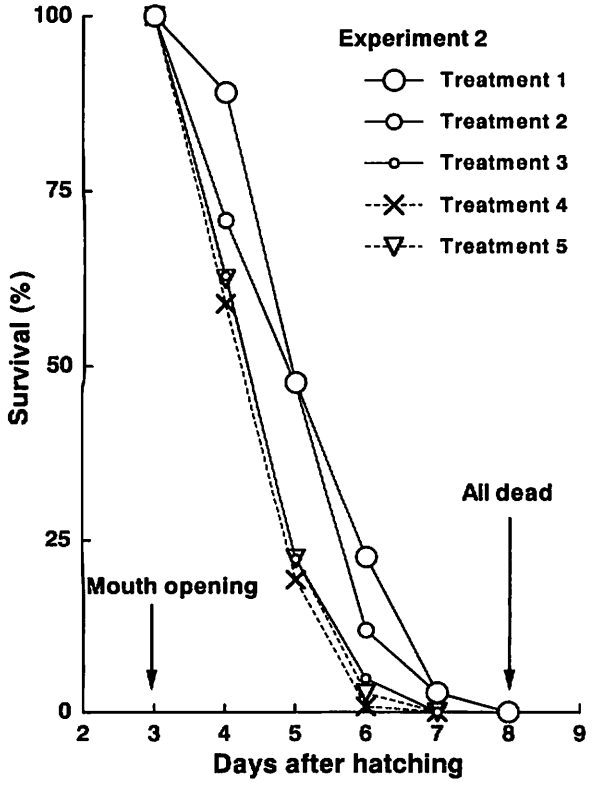

Fig. 3. Survival of larval grouper, *Epinephelus septemfasciatus* (Experiment 2). Treatments are defined in Table 2.

Table 4. Tests of differences in survival among 7 treatments at 4, 5 and 6 d after hatching (DAH); results from Experiment 2 (symbols as in Table 3).

Age	Treatment No.	1	2	3	4	5
4 DAH	1		−	++	++	++
	2			−	−	−
	3				−	−
	4					−
	5					
5 DAH	1		−	+	+	+
	2			++	+	+
	3				−	−
	4					−
	5					
6 DAH	1		−	+	++	+
	2			−	−	−
	3				−	−
	4					−
	5					

Experiment 2

Water temperature ranged from 22.5 to 23.6°C throughout Experiment 2. Larvae opened their mouths at 3 DAH and had entirely consumed their yolk by 4 DAH. The survival at 4, 5, 6 and 7 DAH fluctuated between 56–91, 16–50, 0–25 and 0–3%, respectively. Figure 3 shows the daily changes in mean survival of larvae for the 5 treatments. Changes in mean survival showed a similar pattern to those in Experiment 1, the survival being highest in the treatment with the highest food concentration, but nevertheless decreasing abruptly at 5 DAH. The survival at 4 DAH was significantly ($p<0.01$) different between Treatment 1 and Treatments 3–5 (Table 4). Significantly higher ($p<0.05$) survival was also found at 5 DAH for Treatments 1 and 2, compared with Treatments 3–5 (Table 4), but significant ($p<0.05$) differences were observed only between Treatments 1 and Treatments 3–5 at 6 DAH (Table 4). By 7 DAH, the survival in each treatment ranged from 0 to 3%, and the remaining larvae died the following day.

In the gut content analyses, the mean number of food organisms per larva in Treatment 1 ranged from 2.5 to 3.2 cells (Fig. 4A). The frequency of larvae containing *F. taraikaensis* in the gut varied from 74 to 85% (Fig. 4B). In Treatment 2, the mean number of food organisms per larva ranged from 1.5 to 2.5 cells (Fig. 4A). The feeding incidence of larvae ranged from 55 to 60% (Fig. 4B). No *F. taraikaensis* were found in the guts in Treatments 3–5 when larvae were dissected.

Discussion

Higher survival of larval grouper was recorded from treatments with higher food concentrations (>10^4 ciliates

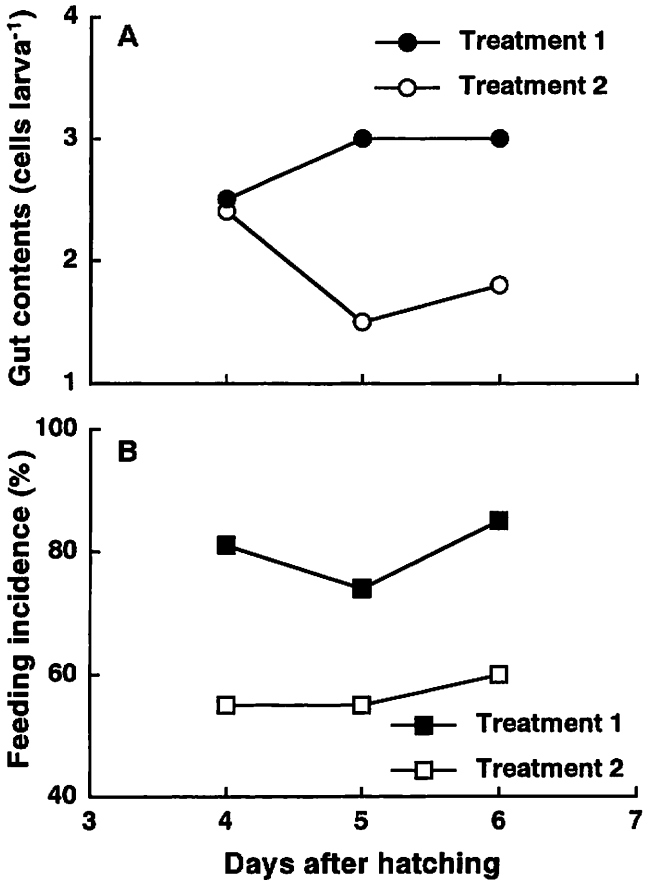

Fig. 4. Daily changes in (**A**) gut contents (cells larva^{-1}) and (**B**) feeding incidence (%) of larval grouper, *Epinephelus septemfasciatus* (Experiment 2). Treatments are defined in Table 2.

l^{-1} for Experiment 1 and $>10^3$ ciliates l^{-1} for Experiment 2), especially between 4–6 DAH (Figs 1, 3, Tables 3, 4). Many studies have shown that larval survival is directly related to food concentration (e.g. Houde 1975, 1977; Bailey & Stehr 1986). The large numbers of ciliates, therefore, must have enhanced larval survival over the period preceding 5 to 6 DAH (Figs 1, 3, Tables 3, 4). In Experiment 2 (Treatments 1 and 2) where higher survival was attained (Fig. 4), an average of 1.5 to 3.2 cells of *F. taraikaensis* were found in the guts of the larvae. Whereas aloricate *Euplotes* were not observed in larval gut contents in Experiment 1, it is still reasonable to consider that larval grouper may have fed upon *Euplotes* spp., as described below. In Experiment 2, only the loricae of *F. taraikaensis* remained in the guts of larvae but other components inside the loricae were not observed, indicating that ciliates are readily digested and assimilated by larval fishes (Stoecker & Govoni 1984). In fact, additional feeding experiments showed *Euplotes* spp. was detectable in the guts of grouper larvae by using ciliates labelled with fluorescent microspheres (Fig. 2). Therefore, the reason that the grouper larvae contained no recognizable ciliates in the gut might be due to immediate digestion by the larvae.

All of the larvae had died by 8 DAH in both Experiments (Figs 1, 3). One possible explanation for this is related to food quality, which is considered to be one of the most important parameters in larval rearing. Fyhn (1989) suggested that free amino acids might be an important energy source for development of eggs and first-feeding larvae. Marine ciliates, as well as other marine invertebrates (Yancey et al. 1982), contain high intracellular concentrations of free amino acids (Kaneshiro et al. 1969). Stoecker & Govoni (1984) determined the C : N ratio of an herbivorous tintinnid, *Favella* sp., as 4.0, being similar to that of nauplii of the copepod *Acartia tonsa*, which has been regarded as a primary food for fish larvae. A lower C : N ratio may indicate a particularly rich source of nitrogen compounds, such as amino acids and proteins. Accordingly, the quality of ciliates, or at least that of *F. taraikaensis* used in Experiment 2, may have been sufficient for larval survival. Bacterivorous naked ciliates, *Euplotes* spp., as used in Experiment 1, are possibly a poor source of nitrogen compounds compared with *F. taraikaensis*, because the C : N ratio of the bacterivorous ciliate, *Uronema*, has been determined as 6.2 (Le Gall et al. 1997), higher than in algivorous ciliates. However, simple generalizations about food quality, based on free amino acids or C : N ratios, are unlikely to be sufficient for determining the overall food quality of ciliates. Data are needed to determine other biochemical constituents, such as fatty acids and sterols.

A second possible explanation reflects the problems of larval grouper rearing due to their own biological characteristics. Although many attempts have been made to rear larval grouper, they usually result in large mortality rates (Hussain & Higuchi 1980; Tseng & Chan 1985; Kohno et al. 1990), including *Epinephelus septemfasciatus* (Kitajima et al. 1991). Kohno et al. (1994) indicated that difficulties in larval grouper rearing resulted from disadvantageous characteristics of the larvae, including a smaller mouth and body, and fewer reserves of endogenous nutrition. A recent study, which revealed that administration of thyroid hormones to larval grouper lessened larval mortality, suggested that the lower potential survivorship of grouper larvae may be due to an insufficient nutritional condition in artificially reared brood stock (Yamanoi, pers. comm.). In the present study, massive mortality of the larvae occurred at 7 or 8 DAH, in spite of various food conditions. Hence, these potential biological problems might be responsible for larval mortality.

Grouper larvae have poor feeding and swimming abilities due to the slow development of functional bony elements (Kohno et al. 1997; Narisawa et al. 1997). No functional feeding or swimming abilities were observed until 9 and 13 DAH, respectively (Narisawa et al. 1997). On the basis of the preceding statement, it might be difficult for the larvae to capture copepod nauplii. Ciliates should be more easily captured by fish larvae than copepod nauplii because of their relatively slower movement rates (Stoecker & Govoni 1984). Ciliates could be manageable prey, in terms of their mobility, until swimming- and feeding-related attributes are

developed.

It has been established that insufficient food at first-feeding leads to irreversible starvation, resulting in death, of the fish larvae (Lasker et al. 1970; Bailey & Stehr 1986). Larvae of northern anchovy had a 100% mortality rate due to their inability to feed exogenously within 1.5 d of yolk absorption (Lasker et al. 1970). According to van der Meeren and Nass (1993), mesocosm-reared first-feeding larval cod maintained normal growth and survival by feeding mostly on ciliates, when ciliates were abundant (<7130 indiv. l^{-1}) and copepod nauplii were scarce (<5 indiv. l^{-1}). They also inferred that feeding on ciliates under such conditions might enable the larvae to avoid death due to immediate starvation. In this study, grouper larvae exposed to a large density of ciliates (>10^4 ciliates l^{-1} for Experiment 1 and >10^3 ciliates l^{-1} for Experiment 2) fed on ciliates immediately after yolk absorption (3 DAH) and survived better than the starved larvae in the control between 4 and 6 DAH (Figs 1, 3). It is likely that ciliates in the marine ecosystem play an important role for first-feeding fish larvae, being an alternative food source to copepod nauplii, thereby enhancing the survival of the larvae. Therefore, if fish larvae can feed on ciliates at first-feeding, ciliates may bridge the gap until larvae encounter copepod nauplii.

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