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Growth, Survival and Morphometric Measurements of Malabar Grouper (*Epinephelus malabaricus*) Larvae When Co-Fed *Artemia* and an Artificial Diet

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Abstract.- A twelve-day feeding trial was conducted to investigate the effect of four test feeding regimens and one control regimen on the growth, survival and morphometric development of 20 days post-hatch (d.p.h.) malabar grouper (*Epinephelus malabaricus*) larvae while co-fed with *Artemia* nauplii. The five different feeding regimens tested were: *Artemia* nauplii at density of 5 mL^{-1} co-fed with artificial diet (AD) at 5 ppm day^{-1} (control); *Artemia*-3 mL^{-1} / AD-5ppm day^{-1}; *Artemia*-3 mL^{-1} / AD-10 ppm day^{-1}; *Artemia*-3 mL^{-1} / AD-15 ppm day^{-1}; and *Artemia*-3 mL^{-1} / AD-20 ppm day^{-1}. Survival, growth rate and morphometric measurements (*i.e.* total length, standard length, width, 2nd dorsal spine, pelvic spine, eye diameter and upper jaw) of larvae were compared. Larvae were reared in 20L polycarbonate buckets in a static system until 30 d.p.h. Initial stocking density was 5 larvae per litre. High mortalities were observed after transfer of larvae into experimental system however larval mortality plateaued thereafter. Different feeding regimens did not have a marked effect on larval survival ($P > 0.05$), which ranged from 8.0 ± 1.74 % to 16.5 ± 6.21 % at 30 d.p.h. Results showed that a 40% reduction in *Artemia* requirements can be achieved when larvae are co-fed artificial diet at a level of 5 ppm day^{-1} ($P > 0.05$).

Key words: Epinephelus, co-feeding, larval rearing, artificial diets.

INTRODUCTION

The culture of groupers using eggs from either hormone induced or naturally spawning broodstock is in pilot or backyard-hatchery production stage in most of Southeast Asia (Duray *et al.*, 1997; Lim, 1993; Ruangpanit *et al.*, 1993; Sugama *et al.*, 2002b), Japan (Okumura, 1997), the Middle East (James *et al.*, 1998b; Kolkovski, 2000), India (Pillai *et al.*, 2002), the USA (Watanabe *et al.*, 1996) and Australia (Rimmer, 2000). The major constraint regarding further progress of grouper culture is widely reported mortalities in larviculture stages, resulting in a lack of seed supply to grow-out operations (Kohno *et al.*, 1997; Lim, 1993; Rimmer *et al.*, 2002; Toledo, 2002). Larval survival of groupers is generally low (<10% to metamorphosis) and highly variable (Rimmer, 2002).

High mortality of groupers is encountered during the changeover from endogenous to exogenous nutrition (Duray *et al.*, 1997; Hussain and Higuchi, 1980; Lim, 1993; Watanabe *et al.*, 1996). Ordonio-Aguilar *et al.* (1995) found that the groupers *Epinephelus coioides* and *E. fuscoguttatus*, when compared to several other tropical marine fish, were the most disadvantageous to survival during this transition period due to poor endogenous nutrition reserves and low initial feeding on rotifers. Kohno *et al.* (1997) added to this by stating that the difficulties in rearing grouper larvae were also attributable to their small mouth and body size, and the delayed development and small size of the bony elements forming the oral cavity. In light of this, several smaller initial food alternatives have been used in effort to reduce early mortalities of grouper larvae.

A second period of high mortality of grouper larvae is also documented, which occurs between 24 to 35 d.p.h. and may be due to a combination of "shock syndrome", poor nutrition and changeover of food source (Duray *et al.*, 1997; Hussain and Higuchi, 1980; Lim, 1993). During this period of mortality grouper larvae may be deficient in n-3 highly unsaturated fatty acids (n-3 HUFA)

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(Rimmer, 1999), which are essential for the normal growth of marine finfish larvae, in particular docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Watanabe, 1993).

Formulated artificial diets can potentially provide a suitable supplement to conventional live foods, and in some cases co-feeding live and artificial diets can produce growth and survival of larvae superior to that achieved with either live feeds or artificial diets alone (Jones *et al.*, 1993). This can also reduce dependence of live prey production, which is of great technical and economical importance (Person Le Ruyet *et al.*, 1993). For groupers, whose larval period is generally 50-60 days (Liao *et al.*, 2001), live food replacement may be an important cost saving tool.

Several grouper species have already been reared with some success using artificial food as a supplement to live foods. For example, with humpback grouper *Cromileptes altivelis* larvae, administering of artificial food begins 20 d.p.h. (Sugama *et al.*, 2002b), as is with *E. striatus* larvae (Watanabe *et al.*, 1996) while for *E. polyphekadion* larvae a formulated diet is introduced 35 d.p.h. (James *et al.*, 1997). However, in all of these examples artificial food is administered until satiation (a somewhat subjective amount) and may not always be the optimum quantity.

This study aims to determine the effect on the growth, survival and morphometric development of 20 d.p.h. malabar grouper (*E. malabaricus*) larvae when provided with different quantities of artificial food while co-fed with *Artemia* nauplii.

MATERIALS AND METHODS

The experiment was conducted from the 3rd May – 14th May 2002 at the Brackishwater Aquaculture Research Centre (BARC) in Jepara, Indonesia. Approximately 40 locally caught malabar grouper were held outdoors in a 300 m^3 circular concrete tank at a sex ratio of approximately 1:1. Broodstock (approx. 5-10 kg) were fed once daily until satiation on a diet of trash fish. Naturally spawned eggs were obtained the night of the new moon on 13th April 2002. Buoyant eggs were collected the following morning. Eggs were transferred to 7 m^3 rectangular, concrete rearing tanks. Larval rearing prior to the experiment was done in these tanks according to the feeding regimen and water quality management used at BARC at that time. Grouper larvae used were aged 19 d.p.h. on day 1 of the experiment. Larvae of this age were used to allow sufficient time for weaning onto *Artemia* as the major live food source. The experiment was terminated when larvae reached 30 d.p.h. as grouper larvae can become cannibalistic around this time (Lim, 1993; Rimmer, 1999), possibly affecting survival and growth results.

Due to such sensitivity, larvae were carefully scooped into the experimental containers directly from mass culture tanks to reduce handling. Larvae were aged 18 d.p.h. on the day of transfer and were given one day to adjust to the experimental system before the experiment commenced. After all larvae had been collected, water levels in each bucket were adjusted so that initial stocking density was 5 larvae L^{-1} as suggested by Duray *et al.*, (1997) for all replicates. Larvae were fed *Artemia* nauplii at a density of 5 individuals mL^{-1} during the adjustment period.

Four test feeding regimens and one control regimen were tested for their efficacy on grouper larvae. The four test regimens differed in the amount of artificial diet to be given, while *Artemia* density remained constant at 3 individuals mL^{-1}. Artificial diet levels of different feeding regimens were set at 5, 10, 15 and 20 ppm day^{-1}. The control feeding regimen was similar to that used by BARC and consisted of *Artemia* at a density of 5 individuals mL^{-1} and artificial diet at a level of 5 ppm day^{-1}. Each treatment was replicated five times.

Grouper larvae were reared in 20L red, polycarbonate buckets. Each tank was filled with 15 L of filtered seawater. The experiment was a static system, with fresh seawater exchanged daily. Seawater was pumped from the Java Sea and passed through a 50 μm sand filter prior to use. Each replicate was slightly aerated by a regulated airstone suspended in the centre of the bucket, approximately 1 inch above the bottom. Placing all of the 20 L buckets into a shallow fibreglass water bath reduced temperature fluctuation and variations among replicates. No heaters were placed into the water bath.

Larvae were fed *Artemia* nauplii (INVE

Artemia GSL 2001 90% Grade AAA) and Lansy Proton 4 (INVE) [T] formulated diet concurrently throughout the experimental period. Lansy Proton 4 is a compound, twin screw extruded diet designed as a partial *Artemia* replacement diet, with a size distribution of 300-500µm. For typical composition analyses for *Artemia* and Lansy Proton 4 used see Table I.

Table I: Typical composition analysis (dry weight) of *Artemia* and formulated diet used in experiment.

Lansy Proton 4	
Protein	58%
Lipid	14%
Ash	12%
N.F.E.	9%
Moisture	7%
Fibre	1%
Phosphorus	1.4%
Antioxidants	Ethoxyquine, BHA, BHT
n3 HUFA	30 mg g^{-1} DW
DHA:EPA	2
Vit. A	30,000 IU kg^{-1}
Vit. D3	2,500 IU kg^{-1}
Vit. E	700 mg kg^{-1}
Vit. C	2,000 mg kg^{-1}
Size	300µm - 500µm
GSL *Artemia* cysts	
Protein	> 45%
Lipid	> 20%
n3 HUFA	< 5 mg g^{-1} DW
Carbohydrates	< 15%
Ash	< 12%
Moisture	< 8%
Size (nauplii)	480µm

Source: INVE product information sheets

Artemia were given to the larvae every morning around 8.00-9.30am. Density levels were checked again in the afternoon and adjusted to orginal densities, if necessary. Artificial diet was given to the larvae 5 times a day at equal intervals between 8.00 am to 4.00 pm. The daily level of artificial diet for required for each feeding regimen was divided equally among the five feedings as per the protocol followed at BARC.

A water quality management protocol consisted of partial water exchanges and siphoning of uneaten food and debris from the bottom of each bucket. As the experiment progressed the volume of water exchanged each day increased as follows: 15% exchanged between 19-21 d.p.h.; 25% between 22-25 d.p.h. and 40% between 26-30 d.p.h. (as per protocol followed at BARC). Bottom siphoning was done every two days. Care was taken not to damage or accidentally suck up larvae while performing such tasks.

Larval samples were measured 19 d.p.h. (initial), 24 d.p.h. and 30 d.p.h. (final). Two larvae from each replicate were randomly sampled 19 and 24 d.p.h. while all larvae remaining in each replicate 30 d.p.h. were taken and used for final measurements. Larvae sampled 19 d.p.h. were pooled together and taken as an initial sample representing all larvae used in the experiment. Larvae were fixed and preserved by placing in a 10% formalin solution for one day and then transferred into 70% ethanol solution.

Survival was recorded every two days, while dry weight and specific growth rate (SGR) of larvae were recorded at 19, 24 and 30 d.p.h.. Seven morphometric features, namely total length (TL), standard length (SL), dorso-ventral width (W), dorsal spine (2^{nd}) length (DS), pelvic spine length (PS), upper jaw (UJ) and eye diameter were measured by a caliper on each larvae sampled. Four ratios of different larval characteristics were used with an intention of making data interpretation clearer and relationships between variables more evident. TL:W and TL:dry weight (DW) ratios were used as an indication of general health and condition of larvae (Suthers, 1991) while TL:DS and SL:DS ratios tell how large the spine is compared to the body length, which is better that analysing spine length alone.

Mixed model analysis was used on morphometric data from larvae 30 d.p.h. (pers. comm. Kevin Murray, Department of Maths and Statistics, University of WA, 2002). Oneway ANOVAs were performed on SGR, dry weight and survival data to see whether any significant differences ($\alpha = 0.05$) occurred among larvae fed different feeding regimens. Oneway ANOVAs were also conducted on variables within treatments to investigate any significant changes ($\alpha = 0.05$) over time. The LSD (least significant difference) post-hoc test was used to identify where significant

differences occurred. Survival data was transformed using the arcsine transformation before any statistical analyses were done. Regression analyses were also used for some variables to investigate possible relationships.

RESULTS

Specific growth rate and dry weight

SGR of larvae from control group was significantly higher in the first half of the experiment ($P < 0.05$) compared to the mean SGR in the second half of the trial and overall SGR (Table II). For all other larvae, mean SGR was greater between 19 and 24 d.p.h. compared to SGR between 24 and 30 d.p.h. and overall SGR. Differences in the mean SGR of larvae in the period from 19 d.p.h. until 24 d.p.h. were not significant ($P > 0.05$). Mean SGR varied among larvae fed different feeding regimens from 6.75 ± 1.72 % mm day^{-1} to 9.82 ± 1.52 % mm day^{-1} for that period. Mean SGR of larvae fed differing feeding regimens varied from 3.89 ± 0.99 to 6.93 ± 0.92 % mm day^{-1} for the period between 24 and 30 d.p.h. but this difference was not significant either ($P > 0.05$).

The largest difference in mean dry weight of larvae 30 d.p.h. was between those fed the 10 ppm regimen (4.97 ± 0.959 mg) and larvae fed the control regimen (3.29 ± 0.387 mg) (Fig. 1). However, this difference was not significant ($P > 0.05$).

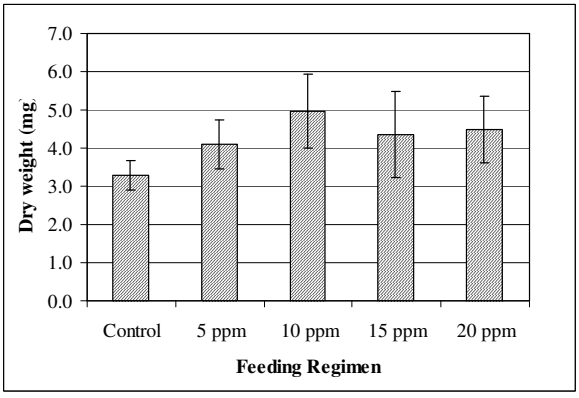

Fig. 1. Mean dry weight (mg) of malabar grouper larvae, 30 d.p.h.

Survival

The range in survival of 30 d.p.h. larvae was greater for larvae fed the 5 ppm and 20 ppm feeding regimens (5.3-33.3% and 4.0-37.3% respectively (n=5)) compared to those fed the 10 ppm, 15 ppm and control regimens (6.7-16.0%, 4.0-13.3% and 4.0-16.0% respectively (n=5)).

Larval survival greatly decreased between 19 and 21 d.p.h. and then slowly decreased until 30 d.p.h. for all treatments (Fig. 2). On any given day when survival was recorded there were no significant differences in mean survival rates of grouper larvae, regardless of feeding regimen ($P > 0.05$). For all feeding regimens larval survival significantly decreased ($P < 0.05$) as time progressed however, the pattern/rate in which mortality occurred differed among larvae fed different regimens.

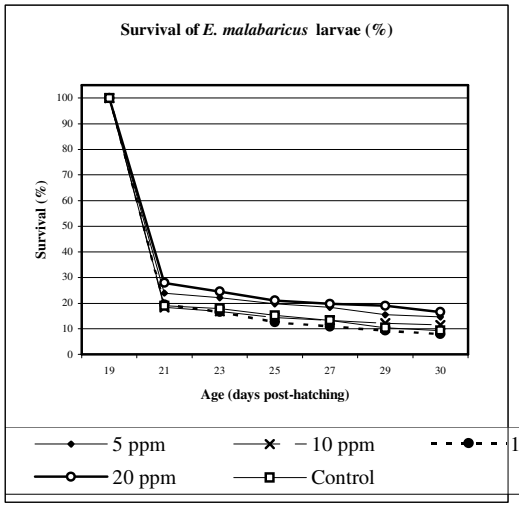

Fig. 2. Comparison of survival of malabar grouper larvae over different feeding regimens.

Morphometric variables

Larvae 24 d.p.h. and 30 d.p.h. showed no significant differences ($P > 0.05$) in TL or SL throughout the five feeding regimens (Table III). The largest difference in mean TL was between larvae fed the control regimen (mean TL ± S.E. = 11.03 ± 0.60 mm) and those fed the 10 ppm regimen (12.66 ± 0.79 mm) (Fig. 3). The largest difference in mean SL also occurred between larvae fed these two regimens (SL = 9.14 ± 0.47 mm and 10.42 ± 0.64 mm for control and 10 ppm regimens respectively).

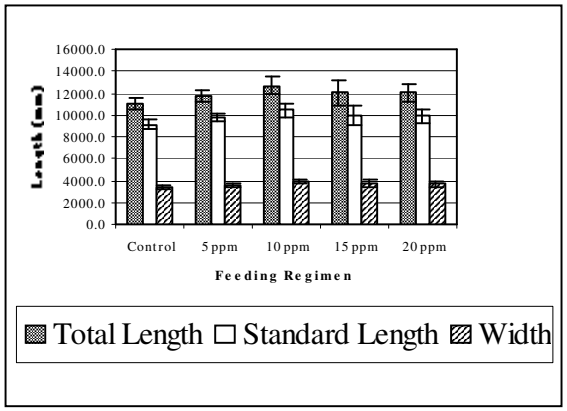

Fig. 3. Mean, total length, standard length and width of malabar grouper larvae (30 d.p.h.) after five different feeding regimens (Error bars indicate standard error of mean)

Mean TL and SL of larvae throughout all feeding regimens were significantly larger ($P < 0.05$) for larvae 30 d.p.h. than those 24 d.p.h., which was also the case between larvae 24 d.p.h. and 19 d.p.h. Similarly, 24 d.p.h. and 30 d.p.h. larvae showed no significant differences ($P > 0.05$) in any morphometric variable (*i.e.* W, DS, PS, UJ and eye) when fed any feeding regimen. Larvae from each feeding regimen showed significant increases ($P < 0.05$) in mean W, UJ and eye measurements during the period between 19 to 24 d.p.h. and also from 24 to 30 d.p.h. (Table III).

In general, larvae showed a significant increase ($P < 0.05$) in mean DS and PS lengths during the period between 19 to 24 d.p.h. and did not increase significantly ($P > 0.05$) during the period between 24 to 30 d.p.h. However, this was not the case for larvae fed the 10 ppm regimen and the 20 ppm regimen, where mean DS lengths (10 and 20 ppm regimens) and PS lengths (20 ppm regimen only) showed significant increases in the period from 19 to 24 d.p.h. and from 24 to 30 d.p.h. (Table III).

Biological ratios

Twenty four and 30 d.p.h. larvae showed no significant differences ($P > 0.05$) in mean TL:DS, SL:DS, TL:W and TL:Dry Weight (DW) values regardless of feeding regimen (Table IV). There were no significant changes ($P > 0.05$) in mean TL:W values among larvae 19, 24 and 30 d.p.h. fed any feeding regimen. Mean TL:DS and SL:DS values of larvae, irrespective of feeding regimen, decreased significantly ($P < 0.05$) in the period between 19 and 24 d.p.h. Mean SL:DS values of all larvae except those fed the control regimen further decreased significantly ($P < 0.05$) during the period of 24 d.p.h. to 30 d.p.h., while a significant decrease in mean TL:DS values during the same period was only found in larvae fed the 5 ppm and 10 ppm feeding regimens (Table IV).

Logarithmic regression analysis of TL and TL:DS of larvae 30 d.p.h. found a strong relationship among larvae from the 10 ppm group, while only a moderate relationship was found in larvae from 5 ppm group (Table V).

DISCUSSION

Although 30 d.p.h. grouper larvae fed different feeding regimens showed no significant differences ($P > 0.05$) in SGR, dry weight, survival and several morphometric variables, a definite trend was apparent. Generally, larvae fed the 10, 15 or 20 ppm feeding regimens were faster growing (therefore larger) than those larvae fed on the 5 ppm or control feeding regimens.

For grouper species, whose larval period is relatively long, it is of great commercial advantage to have a short hatchery period as it can save considerable hatchery space, time and manpower (James *et al.*, 1998a). Mean SGR (between 19 and 30 d.p.h.) of larvae in the present study regardless of feeding regimen, is greater than that of malabar grouper larvae in previous publications (Ruangpanit, 1993). It is one of the faster growth rates compared with several grouper species over a similar period (Table VI).

Mean dry weight and TL:DW of 30 d.p.h. larvae was greatest in larvae fed the 10 ppm feeding regimen and lowest for larvae fed the control regimen. This shows that not only were larvae fed the 10 ppm regimen heavier than larvae fed any other regimen but were also heavier per unit of length (*i.e.* fatter). Again the differences were not significant ($P > 0.05$) and the similar trend seen here could just be due to the relationship of dry weight as

Table II: Specific growth rate (SGR) of malabar grouper larvae at different intervals of the experiment. Values are expressed as mean ± SE (% mm day^{-1}, n = 5).

Treatment	SGR 19-24 d.p.h.	SGR 24-30 d.p.h.	SGR 19-30 d.p.h.
Control	^{a} 9.82 ± 1.52 $_{1}$	^{b} 3.89 ± 0.99 $_{1}$	^{b} 6.09 ± 0.53 $_{1}$
5 ppm	^{a} 6.75 ± 1.72 $_{1}$	^{a} 6.64 ± 0.72 $_{1}$	^{a} 6.69 ± 0.41 $_{1}$
10 ppm	^{a} 7.79 ± 1.28 $_{1}$	^{a} 6.93 ± 0.92 $_{1}$	^{a} 7.32 ± 0.63 $_{1}$
15 ppm	^{a} 8.72 ± 1.33 $_{1}$	^{a} 5.18 ± 1.35 $_{1}$	^{a} 6.78 ± 0.92 $_{1}$
20 ppm	^{a} 7.98 ± 1.00 $_{1}$	^{a} 5.91 ± 0.77 $_{1}$	^{a} 6.85 ± 0.66 $_{1}$

Means within a row (a,b) having different superscript letters are significantly different ($P < 0.05$). Means within a column (1) followed by same subscript numbers are not significantly different ($P > 0.05$).

a function of the overall size of the larvae which, as mentioned previously, may only be the result of chance and natural variation rather than the effect of different feeding regimens.

Dry weight of 35 d.p.h. *E. coioides* larvae was 4.9 ± 0.96 mg (TL = 11.96 ± 0.34) when fed *Artemia* at 3 individuals mL^{-1} three times a day (Duray *et al.*, 1997). This is comparable to the mean dry weight of malabar grouper larvae 30 d.p.h. from the present study.

It is widely reported that survival of grouper larvae is highly variable and generally very low. From hatching to metamorphosis, survival of groupers is generally <10% (Liao *et al.*, 2001; Rimmer, 2002) though a survival of 53.9% has been achieved in *C. altivelis* larvae (Sugama *et al.*, 2002b). Survival of *E. tauvina* larvae between 13 and 24 d.p.h. may range anywhere from 0% to as high as 90.7% (Lim, 1993).

Survival did not seem to be influenced by feeding regimen. In the present study, high mortalities were observed in larvae between 19 and 21 d.p.h. however it is thought that this was largely caused by stress due to handling and transfer into the experimental system, even though great care was taken. Grouper larvae are very sensitive to physical handling at the time they were transferred into the experimental system, and can easily die from 'shock syndrome' when handled (Duray *et al.*, 1997; Lim, 1993). The problem of high larval mortalities immediately after transfer might be avoided if larvae are transferred at a younger age, before the dorsal and pelvic spines become too long. At this point larvae may not yet be too sensitive for handling (Ruangpanit, 1993). After 21 d.p.h., larvae fed any of the different feeding regimens showed no other substantial peaks in mortality, contrary to the fore mentioned peaks around 24 d.p.h. previously reported in other grouper species. A mortality pattern very similar to the present study was observed in *E. coioides* larvae after transferring to different tanks aged 20 d.p.h. (Duray *et al.*, 1997). By 30 d.p.h. larval survival was around 20%. In the same study, larvae transferred into new tanks 14 d.p.h. did not exhibit a high mortality after transfer however did show increased mortality 27 to 34 d.p.h., consistent with the mortality peaks mentioned previously.

Survival rate is not the only factor subjected to a high level of variation in grouper larviculture. Post-metamorphic *E. striatus* juveniles reared in a pilot-scale trial were characterized by considerable size variations, the result of which was a high incidence of cannibalism (Watanabe *et al.*, 1996). High size variations also caused serious cannibalism in *E. tauvina* and *E. polyphekadion* larvae after 35 d.p.h. (James *et al.*, 1997; Lim, 1993). Cannibalism in grouper larviculture is a major cause of larval mortalities from 30 to 35 d.p.h. onwards (Rimmer, 1999), which is why the current study was terminated at 30 d.p.h. If the experiment was allowed to continue to a point were cannibalism did occur, results might have been compromised, especially in a feeding trial with small larval population such as this. A large size variation also occurred among 30 d.p.h. larvae in the present study (Fig. 4) however no incidence of cannibalism was observed.

Table III: Morphometric variables of malabar grouper larvae aged 19, 24 and 30 d.p.h. Values are expressed as mean ± SE (n=5).

	Feeding Regimen	Age 19 d.p.h.†	Age 24 d.p.h.	Age 30 d.p.h.
Total Length (mm)	Control	$^{a}5.61 \pm 0.16$	$^{b}9.24 \pm 0.68^{\ddagger}{}_{1}$	$^{c}11.03 \pm 0.60{}_{1}$
	5 ppm	$^{a}5.61 \pm 0.16$	$^{b}7.98 \pm 0.68{}_{1}$	$^{c}11.76 \pm 0.52{}_{1}$
	10 ppm	$^{a}5.61 \pm 0.16$	$^{b}8.35 \pm 0.54{}_{1}$	$^{c}12.66 \pm 0.79{}_{1}$
	15 ppm	$^{a}5.61 \pm 0.16$	$^{b}8.74 \pm 0.54{}_{1}$	$^{c}12.06 \pm 1.15{}_{1}$
	20 ppm	$^{a}5.61 \pm 0.16$	$^{b}8.40 \pm 0.42{}_{1}$	$^{c}12.04 \pm 0.83{}_{1}$
Std Length (mm)	Control	$^{a}5.02 \pm 0.08$	$^{b}7.68 \pm 0.53^{\ddagger}{}_{1}$	$^{c}9.14 \pm 0.47{}_{1}$
	5 ppm	$^{a}5.02 \pm 0.08$	$^{b}6.68 \pm 0.53{}_{1}$	$^{c}9.77 \pm 0.41{}_{1}$
	10 ppm	$^{a}5.02 \pm 0.08$	$^{b}6.96 \pm 0.42{}_{1}$	$^{c}10.42 \pm 0.64{}_{1}$
	15 ppm	$^{a}5.02 \pm 0.08$	$^{b}7.25 \pm 0.43{}_{1}$	$^{c}9.97 \pm 0.91{}_{1}$
	20 ppm	$^{a}5.02 \pm 0.08$	$^{b}7.01 \pm 0.33{}_{1}$	$^{c}9.89 \pm 0.68{}_{1}$
Width (mm)	Control	$^{a}1.79 \pm 0.03$	$^{b}3.02 \pm 0.18^{\ddagger}{}_{1}$	$^{c}3.44 \pm 0.15{}_{1}$
	5 ppm	$^{a}1.79 \pm 0.03$	$^{b}2.52 \pm 0.21{}_{1}$	$^{c}3.57 \pm 0.16{}_{1}$
	10 ppm	$^{a}1.79 \pm 0.03$	$^{b}2.66 \pm 0.16{}_{1}$	$^{c}3.91 \pm 0.18{}_{1}$
	15 ppm	$^{a}1.79 \pm 0.03$	$^{b}2.81 \pm 0.17{}_{1}$	$^{c}3.73 \pm 0.35{}_{1}$
	20 ppm	$^{a}1.79 \pm 0.03$	$^{b}2.53 \pm 0.15{}_{1}$	$^{c}3.71 \pm 0.25{}_{1}$
Dorsal Spine (mm)	Control	$^{a}3.23 \pm 0.07$	$^{b}4.29 \pm 0.12^{\ddagger}{}_{1}$	$^{b}4.52 \pm 0.09{}_{1}$
	5 ppm	$^{a}3.23 \pm 0.07$	$^{b}3.95 \pm 0.25{}_{1}$	$^{b}4.33 \pm 0.10{}_{1}$
	10 ppm	$^{a}3.23 \pm 0.07$	$^{b}4.05 \pm 0.13{}_{1}$	$^{c}4.54 \pm 0.04{}_{1}$
	15 ppm	$^{a}3.23 \pm 0.07$	$^{b}4.13 \pm 0.16{}_{1}$	$^{b}4.39 \pm 0.05{}_{1}$
	20 ppm	$^{a}3.23 \pm 0.07$	$^{b}3.91 \pm 0.13{}_{1}$	$^{c}4.48 \pm 0.11{}_{1}$
Pelvic Spine (mm)	Control	$^{a}2.99 \pm 0.06$	$^{b}3.53 \pm 0.24^{\ddagger}{}_{1}$	$^{b}3.79 \pm 0.09{}_{1}$
	5 ppm	$^{a}2.99 \pm 0.06$	$^{b}3.39 \pm 0.21{}_{1}$	$^{b}3.57 \pm 0.08{}_{1}$
	10 ppm	$^{a}2.99 \pm 0.06$	$^{b}3.48 \pm 0.15{}_{1}$	$^{b}3.81 \pm 0.06{}_{1}$
	15 ppm	$^{a}2.99 \pm 0.06$	$^{b}3.45 \pm 0.18{}_{1}$	$^{b}3.73 \pm 0.10{}_{1}$
	20 ppm	$^{a}2.99 \pm 0.06$	$^{b}3.38 \pm 0.13{}_{1}$	$^{c}3.76 \pm 0.11{}_{1}$
Upper Jaw (µm)	Control	$^{a}796 \pm 21$	$^{b}1203 \pm 86^{\ddagger}{}_{1}$	$^{c}1410 \pm 98{}_{1}$
	5 ppm	$^{a}796 \pm 21$	$^{b}1085 \pm 101{}_{1}$	$^{c}1489 \pm 52{}_{1}$
	10 ppm	$^{a}796 \pm 21$	$^{b}1142 \pm 79{}_{1}$	$^{c}1665 \pm 83{}_{1}$
	15 ppm	$^{a}796 \pm 21$	$^{b}1219 \pm 115{}_{1}$	$^{c}1587 \pm 117{}_{1}$
	20 ppm	$^{a}796 \pm 21$	$^{b}1119 \pm 36{}_{1}$	$^{c}1609 \pm 132{}_{1}$
Eye (µm)	Control	$^{a}556 \pm 9$	$^{b}807 \pm 28^{\ddagger}{}_{1}$	$^{c}949 \pm 49{}_{1}$
	5 ppm	$^{a}556 \pm 9$	$^{b}747 \pm 66{}_{1}$	$^{c}1031 \pm 30{}_{1}$
	10 ppm	$^{a}556 \pm 9$	$^{b}744 \pm 40{}_{1}$	$^{c}1087 \pm 65{}_{1}$
	15 ppm	$^{a}556 \pm 9$	$^{b}781 \pm 65{}_{1}$	$^{c}1034 \pm 65{}_{1}$
	20 ppm	$^{a}556 \pm 9$	$^{b}764 \pm 49{}_{1}$	$^{c}1039 \pm 45{}_{1}$

Means within a row having different superscript letters (a, b, c) are significantly different ($P < 0.05$). Means within a column (of a particular variable) followed by the same subscript numbers (1) are not significantly different ($P > 0.05$).

† data obtained from one sample (n=39), assumed to be representative of malabar grouper larvae 19 d.p.h.

‡ n = 4

Table IV: Ratios of some morphometric variables of malabar grouper larvae aged 19, 24 and 30 d.p.h. Values are expressed as mean ± SE (n=5).

	Feeding Regimen	Age 19 d.p.h†	Age 24 d.p.h	Age 30 d.p.h
TL : DS (mm:mm)	Control	$^{a}0.584 \pm 2.01\text{x}10^{-2}$	$^{b}0.478 \pm 3.78\text{x}10^{-2}$ ‡ $_{1}$	$^{b}0.425 \pm 1.90\text{x}10^{-2}{}_{1}$
	5 ppm	$^{a}0.584 \pm 2.01\text{x}10^{-2}$	$^{b}0.499 \pm 1.09\text{x}10^{-2}{}_{1}$	$^{c}0.380 \pm 1.04\text{x}10^{-2}{}_{1}$
	10 ppm	$^{a}0.584 \pm 2.01\text{x}10^{-2}$	$^{b}0.491 \pm 1.86\text{x}10^{-2}{}_{1}$	$^{c}0.376 \pm 2.32\text{x}10^{-2}{}_{1}$
	15 ppm	$^{a}0.584 \pm 2.01\text{x}10^{-2}$	$^{b}0.481 \pm 1.36\text{x}10^{-2}{}_{1}$	$^{b}0.384 \pm 3.48\text{x}10^{-2}{}_{1}$
	20 ppm	$^{a}0.584 \pm 2.01\text{x}10^{-2}$	$^{b}0.470 \pm 1.17\text{x}10^{-2}{}_{1}$	$^{b}0.389 \pm 2.35\text{x}10^{-2}{}_{1}$
SL : DS (mm:mm)	Control	$^{a}0.643 \pm 1.08\text{x}10^{-2}$	$^{b}0.571 \pm 4.04\text{x}10^{-2}$ ‡ $_{1}$	$^{b}0.512 \pm 2.10\text{x}10^{-2}{}_{1}$
	5 ppm	$^{a}0.643 \pm 1.08\text{x}10^{-2}$	$^{b}0.594 \pm 1.04\text{x}10^{-2}{}_{1}$	$^{c}0.456 \pm 1.22\text{x}10^{-2}{}_{1}$
	10 ppm	$^{a}0.643 \pm 1.08\text{x}10^{-2}$	$^{b}0.588 \pm 1.93\text{x}10^{-2}{}_{1}$	$^{c}0.456 \pm 2.74\text{x}10^{-2}{}_{1}$
	15 ppm	$^{a}0.643 \pm 1.08\text{x}10^{-2}$	$^{b}0.579 \pm 1.53\text{x}10^{-2}{}_{1}$	$^{c}0.464 \pm 4.12\text{x}10^{-2}{}_{1}$
	20 ppm	$^{a}0.643 \pm 1.08\text{x}10^{-2}$	$^{b}0.562 \pm 1.22\text{x}10^{-2}{}_{1}$	$^{c}0.473 \pm 2.82\text{x}10^{-2}{}_{1}$
TL : W (mm:mm)	Control	$^{a}0.323 \pm 9.94\ \text{x}10^{-3}$	$^{a}0.331 \pm 1.73\text{x}10^{-2}$ ‡ $_{1}$	$^{a}0.313 \pm 3.44\text{x}10^{-3}{}_{1}$
	5 ppm	$^{a}0.323 \pm 9.94\ \text{x}10^{-3}$	$^{a}0.315 \pm 3.33\text{x}10^{-3}{}_{1,2}$	$^{a}0.305 \pm 6.17\text{x}10^{-3}{}_{1}$
	10 ppm	$^{a}0.323 \pm 9.94\ \text{x}10^{-3}$	$^{a}0.319 \pm 1.96\text{x}10^{-3}{}_{1,2}$	$^{a}0.312 \pm 6.71\text{x}10^{-3}{}_{1}$
	15 ppm	$^{a}0.323 \pm 9.94\ \text{x}10^{-3}$	$^{a}0.321 \pm 3.35\text{x}10^{-3}{}_{1,2}$	$^{b}0.309 \pm 1.14\text{x}10^{-3}{}_{1}$
	20 ppm	$^{a}0.323 \pm 9.94\ \text{x}10^{-3}$	$^{a}0.299 \pm 1.26\text{x}10^{-2}{}_{2}$	$^{a}0.310 \pm 1.55\text{x}10^{-3}{}_{1}$
TL : DW (mm:mg)	Control	Not recorded	Not recorded	$0.294 \pm 2.45\ \text{x}10^{-2}{}_{1}$
	5 ppm			$0.342 \pm 4.16\ \text{x}10^{-2}{}_{1}$
	10 ppm			$0.379 \pm 5.83\ \text{x}10^{-2}{}_{1}$
	15 ppm			$0.338 \pm 6.70\ \text{x}10^{-2}{}_{1}$
	20 ppm			$0.358 \pm 5.00\ \text{x}10^{-2}{}_{1}$

Means within a row having different superscript letters (a, b, c) are significantly different ($P < 0.05$). Means within a column (of a particular variable) followed by different subscript numbers (1,2) are significantly different ($P < 0.05$).

† data obtained from one sample (n=39), assumed to be representative of malabar grouper larvae 19 d.p.h.

‡ n = 4

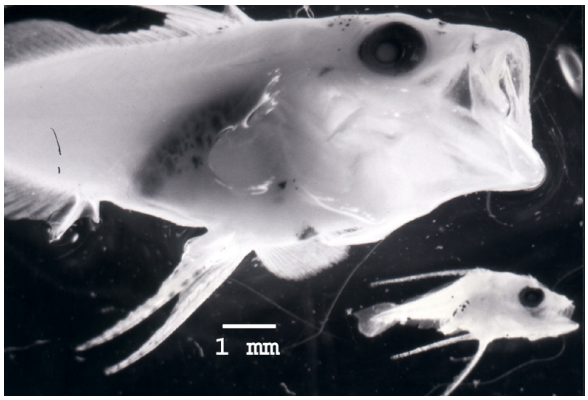

Fig. 4. Malabar grouper larvae (30 d.p.h.) from the present study illustrating the extent of size variation that may be encountered in grouper larviculture. These two larvae were taken from the same tank, and fed the same feeding regimen.

Table V: Regression analysis of the variables TL and TL:DS of malabar grouper larvae, 30 d.p.h. Where y = TL ; x = TL:DS and ln signifies natural logarithm.

Feeding Regimen	Regression Equation	R^2 value
Control	y = -0.281 ln(x) + 3.03	0.7909
5 ppm	y = -0.2057 ln(x) + 2.3038	0.6804
10 ppm	y = -0.3001 ln(x) + 3.2014	0.9226
15 ppm	y = -0.2854 ln(x) + 3.0514	0.8349
20 ppm	y = -0.3024 ln(x) + 3.2171	0.8859

The mean TL recorded for 19 d.p.h. larvae (5.61 ± 1.58 mm) is not unreasonable when compared to a previously quoted range of 5-8 mm (TL) for malabar grouper larvae 18 d.p.h. (Ruangpanit, 1993). Interestingly, Ruangpanit (1993) showed that TL of 32 d.p.h. malabar grouper larvae is 8-10 mm. This is considerably

Table VI: Reported total lengths and SGR of various grouper species at similar age to malabar grouper larvae in present study.

Species	Age (d.p.h.)	Total Length (mm)	SGR	Diet between 20 and 30 d.p.h.	Reference/s
C. altivelis	21 30	~ 7.0 12.0-13.0	~5.99-6.88 (21-30 dph)	*Artemia* nauplii, artificial food	(Sugama *et al.*, 2002a; Sugama *et al.*, 2002b)
E. bruneus	20 30	7.0† 12.8†	6.08 (20-30 dph)	n/a	(Sawada *et al.*, 1999)
E. coioides	21 36	9.3 17.6	4.26 (21-36 dph)	Copepods, enriched *Artemia*	(Toledo *et al.*, 1999)
E. coioides (as *E. suillus)*	21 28	9.1 11.1	2.84 (21-28 dph)	*Artemia* nauplii	(Duray, 1994)
E. fuscoguttatus	20 25 30	7.2 9.2 13.1	5.99 (20-30 dph)	n/a	(James *et al.*, 1998a)
E. fuscoguttatus x E. polyphekadion	20 25 30	6.1 11.9 18.1	10.88 (20-30 dph)	n/a	(James *et al.*, 1998a)
E. malabaricus	18 32 39	5.0-8.0 8.0-10.0 10.0-12.0	1.06-4.17 (18-39 dph)	Rotifers, *Artemia* nauplii	(Ruangpanit, 1993)
E. polyphekadion	20 25 30	6.7 10.5 11.9	5.74 (20-30 dph)	n/a	(James *et al.*, 1998a)
E. striatus	20 35	6.4-7.6‡ 10.4-10.9‡	2.10-3.54 (20-35 dph)	Enriched *Artemia*, artificial food	(Watanabe *et al.*, 1996)
E. tauvina	25 31	9.6 17.8	10.29 (25-31 dph)	Copepods, *Artemia* nauplii	(Hussain and Higuchi, 1980)

† standard length
‡ notochord length
n/a – not available

Table VII: Physical variables of malabar grouper larvae 30 d.p.h. cultured using a mass culture technique. These larvae were from the same spawning batch as larvae used in the present study.

Parameter	Mean ± SE	95 % Confidence Interval	
		Lower Limit	Upper Limit
Total Length (mm)	11.02 ± 2.92	10.43	11.61
Standard Length (mm)	9.19 ± 2.36	8.71	9.67
Width (mm)	3.39 ± 0.86	3.21	3.56
Dorsal Spine (mm)	4.13 ± 0.52	4.03	4.24
Pelvic Spine (mm)	3.56 + 0.50	3.46	3.66
Upper Jaw (mm)	1.41 ± 0.36	1.34	1.48
Eye (mm)	1.03 ± 0.24	0.98	1.08
TL:DS (mm:mm)	$0.381 \pm 7.28 \times 10^{-3}$	0.366	0.396
SL:DS (mm:mm)	$0.456 \pm 8.57 \times 10^{-3}$	0.439	0.473
Dry Weight (mg)	2.33 ± 0.28	1.13	3.53

ower than mean TL of 30 d.p.h. larvae observed in the present study, regardless of feeding regimen. 30 d.p.h. larvae that attained the largest mean TL (12.66 ± 1.1 mm from larvae fed 10 ppm regimen) is larger than *E. striatus* larvae of similar age, smaller than *E. tauvina* larvae and comparable to *C. altivelis, E. bruneus* and *E. polyphekadion* larvae (Table VI).

Width, eye diameter and upper jaw length of 30 d.p.h. larvae all varied with the same trend as TL and SL, indicating that value of these variables differ as a function of the overall size of the larvae and are not individually effected by simple factor such as feeding regimen.

Nineteen d.p.h. larvae exhibited a larger SL:DS ratio ($0.643 \pm 1.08 \times 10^{-2}$) compared to a previously quoted maximal value of 0.60 for malabar grouper larvae (Sugama and Ikenoue, 1999). Furthermore, the maximum TL:DS value for *E. akaara* larvae occurred when TL was 3.3mm (Kusaka *et al.*, 2001), which indicates that the maximum SL:DS and TL:DS values of the larvae used in the present study may well have occurred prior to 19 d.p.h.

A striking feature of grouper larvae is the presence of large spinelets protruding from the dorsal and pelvic fins during certain stages of larval development. Larvae at this stage can be easily shocked, causing mortalities (Ruangpanit, 1993). These elongated spines may function as antipredatory devices (Kusaka *et al.*, 2001) however grouper species that possess relatively long spines, such as *C. altivelis*, may encounter a higher incidence of mortality caused by entanglement with filamentous algae and other larvae (Sugama and Ikenoue, 1999). This supports the research by Ruangpanit *et al.* (1993) who claim that the spines of grouper larvae may trap particles suspended in the water causing larvae to stick together and die.

Although the differences are not significant ($P > 0.05$), results indicate that 30 d.p.h. larvae fed the control regimen possess a larger dorsal spine, as a percentage of SL or TL, compared to larvae fed other regimens. Also, larvae fed the control regimen were the only fish group not to show a significant decrease ($P < 0.05$) in SL:DS ratio between 24 d.p.h. and 30 d.p.h. which leads to the suggestion that these larvae may not develop at the same rate as larvae fed the other feeding regimens.

When the logarithmic relationship between TL and TL:DS was investigated in 30 d.p.h. larvae, it was found that the strength of this relationship varied according to what feeding regimen the larvae were fed. R^2 values (*i.e.* the fraction of the variation in TL:DS that is explained by the logarithmic regression of TL:DS on TL) showed a stronger relationship between these two variables in larvae fed the 10, 15 or 20 ppm feeding regimens compared to larvae fed either the control or 5 ppm feeding regimens (Table V). Note that the correlation of these two variables does not necessarily imply causation, however, the association between TL and TL:DS could possibly be explained in terms of a third, unmeasured variable. In this situation it is plausible to suggest that both TL and TL:DS are related (perhaps causally) to the nutritional status of the larvae. In which case, if the nutritional status of the larvae (which could be a direct result of the feeding regimen) was low, it is reasonable that the response of both TL and TL:DS may vary more. A similar trial over a longer time period may accentuate these differences further.

Grouper larvae are usually reared in 3 to 30 m^3 rectangular concrete tanks (James *et al.*, 1998b; Lim, 1993; Okumura, 1997; Rimmer, 1999; Ruangpanit, 1993; Sugama *et al.*, 2002b; Watanabe *et al.*, 1996). Survival is generally better in larger tanks, which could due to lower chance of larvae encountering the tank walls (Duray *et al.*, 1997). Malabar grouper larvae from the same spawning batch as those in the present study were reared in a 7 m^3 concrete tank since hatching. Thirty d.p.h. larvae from the 7 m^3 tank were generally smaller (Table VII) when compared to larvae of the same age from the present study. Larvae from the 7 m^3 tank were fed on rotifers (5-10 individuals mL^{-1}) and *Artemia* nauplii (4-5 individuals mL^{-1}). This raises the point of whether size differences observed between these two groups of 30-d.p.h larvae were due to tank size, water quality, diet or other unknown variables. A trial similar to the present using a larger tank size could be helpful in answering these questions and would also be more applicable to commercial practices.

Feeding levels of artificial diets vary among previous research attempts from 1 ppm to 33 ppm per day (Chu and Ozkizilcik, 1999; Khemis *et al.*, 2000; Kolkovski *et al.*, 1997; Koven *et al.*, 2001; Person Le Ruyet *et al.*, 1993; Walford *et al.*, 1991; Yufera *et al.*, 1995; Yufera *et al.*, 1999), with some researchers choosing to give artificial food to larvae until satiation and some are given at set levels. This makes setting feeding levels in an experiment such as this difficult, therefore artificial diet feeding levels used in this experiment were set on an incremental basis only. Thus, the optimal level of artificial diet required in grouper larviculture may indeed be outside the levels used in the present study.

n-3 HUFA levels of the artificial diet (30 mg g^{-1} DW) were far greater when compared to the *Artemia* nauplii (<5 mg g^{-1} DW) used in the present study (Table I). However, one of the major problems in the success of artificial foods in larval culture is its acceptability by the larvae (Jones *et al.*, 1993). If an artificial diet is not well accepted, any nutritional superiority is effectively negated. Kolkovski *et al.* (1997) found that the presence of *Artemia* will considerably increase ingestion rates of formulated diets. The use of artificial diets in the culture of *C. altivelis* larvae has shown to be more effective if administered prior to the introduction of *Artemia* (Kawahara *et al.*, 2000). Once grouper larvae are in the selective feeding stage (*i.e.* when larvae can swim freely to search for food, usually after the unpaired fins differentiate from the fin fold) the feeding regimen should change to frequency oriented rather than the previous density oriented (Sugama and Ikenoue, 1999). The artificial diet feeding frequency in the present study of five times per day may not be optimal. The desired feeding frequency of artificial diets should be investigated further.

When co-fed with an artificial diet, the *Artemia* requirement of malabar grouper larvae (3 mL^{-1}) is considerably lower than reported by Ruangpanit (1993), who used n-3 HUFA enriched *Artemia* at concentrations of 7-10 ml^{-1} (from 25 d.p.h. onwards). *C. altivelis* larvae have been successfully cultured with *Artemia* concentrations as low as 0.2-0.5 mL^{-1} if co-fed with artificial diet between 20 and 45 d.p.h. (Sugama *et al.* 2002b). This indicates that the *Artemia* requirement in grouper larviculture can possibly be reduced substantially when co-fed artificial diets. The subsequent reduced dependence on live prey production, is of great technical and economical importance (Person Le Ruyet *et al.*, 1993).

E. coioides larvae showed an immediate preference for *Artemia* nauplii over rotifers when introduced 21 d.p.h. (Duray *et al.*, 1997). In light of this, rotifers were deemed not essential in the present study, which would then help reduce unnecessary variables and possible sources of experimental error.

Water quality management implemented in this study was assumed to be sufficient enough to avoid any water quality problems. In larger scale grouper culture water exchange and bottom siphoning are delayed for as long as the water quality is tolerable due to the increased chance of larval mortality from stress (Sugama and Ikenoue, 1999). In this situation, higher levels of artificial diets such as those used in some feeding regimens in the present study may not be beneficial considering the possible associated water quality problems.

CONCLUSION

Growth, survival and morphometric measurements of malabar grouper larvae were not affected by a 40% reduction in *Artemia* concentration when supplemented with an artificial diet between 20 d.p.h. and 30 d.p.h. Additionally, there is evidence to suggest that increasing the amount of artificial diet may further enhance larval growth, although extra research is needed to validate this. The need for research into new artificial diets for use in the early larval stages of groupers is of high priority (Rimmer, 2002).

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