Rearing larvae of dusky grouper, *Epinephelus marginatus* (Lowe, 1834), (Pisces, Serranidae) in a mesocosm of semi-extensive technology

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SUMMARY

One of the major obstacles to propagating dusky grouper, *Epinephelus marginatus*, has been the difficulty of rearing the early larvae. We have successfully raised dusky grouper larvae in mesocosms using a mixed diet of endogenous plankton developed in the rearing tank and an exogenous supply of *Brachionus plicatilis* and *Artemia* sp. Newly hatched larvae at an initial density of 1.3 ind.L^{-1} were stocked in partially shaded 3 m^3 circular outdoor tanks during the summers of 2007 and 2008. Before introducing newly hatched larvae, the water was left for six days to promote plankton growth. Larval growth occurred at two different rates: i.) higher from first feeding to beginning of metamorphosis and ii.) and lower at transformation. Survival at the beginning of metamorphosis was less than 10% (33 DPH) in 2007 and between 25 to 50% (25 DPH) in 2008. High mortalities were observed during larval transformation. Estimated minimum food requirement per grouper

larvae increased more than 300% from the beginning of the notochord flexion to the beginning of metamorphosis. To meet such a high feeding requirement the number of larger prey organisms/copepods in the mesocosm should have an eight fold increase during this time period.

Running title: Rearing larvae of dusky grouper in mesocosm

Keywords: larval rearing, *Epinephelus marginatus*, dusky grouper, feeding requirement, mesocosm

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INTRODUCTION

Dusky grouper, *Epinephelus marginatus* (Lowe, 1834), is a highly prized marine fish important from the perspective of fisheries and game. Globally they are distributed in the Eastern and Southwest Atlantic and Western Indian Ocean where they are associated with rocky bottoms in depths ranging from shallow to 50 m (Heemstra and Randall, 1993). Solitary and territorial, they can be found by spearfishers and scuba divers in rocky caves. They are sequential hermaphrodites, in which the hermaphroditism is protogynous (female–male) and monandric (males are the terminal sex, only produced through sexual transition). In the wild, females are considered to be mature between 2.5 kg (~5 yr) and 11 kg (~7 yr), and only large individuals (more than 9 kg) occur as males. Sexual inversion occurs between 10 and 16 years of age (Pierre *et al.* 2008). With a minimum population doubling time of 4.5 to 14 years this species has low resilience and their vulnerability to exploitation is very high, so they are listed as an endangered species (EN A2d) by the International Union for Conservation of Nature and Natural Resources (IUCN) (IUCN, 2008).

Due to its high market value and the need for seedling production for restocking programs and aquaculture, research on *E. marginatus* started in the mid 1990s (Spedicato *et al.* 1995; Glamuzina *et al.*, 1998; Marino *et al.*, 1998) with studies on breeding, artificial spawning and rearing of early stages. Since then considerable progress has been made on induced reproduction (Marino e*t al.*, 2001; 2003; Cabrita *et al.*, 2008) but larval rearing is still difficult due to poor first feeding with resultant high mortality of larvae (Spedicato *et al.* 1995; Glamuzina *et al.*, 1998).

Obtaining juveniles of dusky grouper produced in aquaculture for stock enhancement is still a major challenge since newly hatched larvae of groupers have mouth gapes too small for traditional live feeds and limited yolk reserves (Kohno *et al.*, 1997; Doi *et al.*, 1997; Glamuzina *et al.*, 1998; Tucker, 1999; Spedicato and Boglione, 2000; Toledo *et al.*, 2002). Consequently successful larval rearing depends not only on the availability of a nutritionally adequate food supply (Watanabe and Kiron, 1994; Rainuzzo *et al*., 1997; Planas and Cunha, 1999) but also on the size of the prey that need to be smaller than rotifers (< 100 μm) (Doi *et al.* 1997; Glamuzina *et al.*, 1998).

In nature a wide range of organisms is available to the fish larvae. This includes not only all stages of copepods, but also much more abundant phytoplankton and protozoan organisms of smaller sizes. The natural bloom method used in mesocosms of semi-extensive technology (van der Meeren and Naas, 1997; Divanach and Kentouri, 2000) is a methodology based on the bloom of a pelagic food chain of wild origin that develops a zooplanktonic segment of highly nutritious individuals (athecate protozoans such as *Strobilidium* sps. and *Synchaeta* sps. as well as copepod nauplii)

(Kentouri and Divanach, 1986). These zooplankters are of appropriate size for dusky grouper larvae that posses 220-250 μm mouth gape (Glamuzina *et al.*, 1998; Russo *et al*., 2009) and a standard length between 2.4 - 2.6 mm at first exogenous feeding (our own data). The duration of the high performing zooplanktonic food chain is long enough for larvae to reach a size compatible with *Brachionus* feeding and later with *Artemia*.

In the 2007 egg production season we tried using the natural food chain method complemented with rotifers (*Brachionus plicatilis*) followed by *Artemia* sp. to raise dusky grouper larvae obtained from artificially fertilised eggs of wild adult groupers kept in captivity. The results were promising and in 2008 we proceeded with a similar technology with improvements based on the previous experience. This work describes the experiments where dusky grouper larvae were raised successfully and presents results on their growth and survival.

MATERIALS AND METHODS

Facilities of the Aquaculture Research Station (EPPO), of the National Institute of Biological Resources (INRB-IPIMAR), based in Olhão, southern Portugal, were used in this study. Fertilized dusky grouper eggs were obtained from a captive broodstock maintained for 5 years in one indoor tank (10.6 m^3) at the EPPO facilities with a mean density of 4.0 Kg m^{-3}. A diet of fresh/frozen squid (*Loligo gahi*) and pilchard (*Sardina pilchardus*) were hand-fed *ad libitum* once a day. Water temperature ranged between 12ºC in winter and 25ºC in summer and during spawning temperature was 22.5ºC. Females were hormonally induced to spawn following the procedure described in Marino *et al.* (2003). Males were obtained from hormonally sex inverted juveniles according to Cabrita *et al.* (2008). Eggs were collected in sterile plastic containers after a gentle pressure on the abdomen in a caudal-cranial direction and fertilization was performed by the wet method (Marino *et al.* 2003).

Fertilized eggs were transferred to 200 L cylinder-conical fibreglass incubation tanks, with a flow-through of filtered and UV sterilized sea water (T=21ºC, pH=8.0, S=37.5) and gentle aeration (see Table 1 for the detailed experimental settings). Before being freed in the mesocosm tank newly hatched larvae were left to acclimatize to the rearing temperature in 5-L plastics containers floating in the tank.

The mesocosm experiments were performed outdoors in shaded (top covered at 2.5 meters high to protect from direct sun rays) 3 m^3 circular tanks during July and August 2007 and 2008. Only one tank was used each year for the larval rearing experiment. Seawater from the Ria Formosa coastal lagoon (Algarve, Portugal) was filtered throughout 500 µm plankton mesh allowing the seeding of the tanks with natural plankton and eliminating potential predators. After filling, the water was left stagnant for 6 days under natural light and temperature to allow the microzooplankton to develop before seeding the tanks with newly hatched fish larvae. This is a critical step that allows the development of natural plankton blooms that are the main food source for the initial days after the larvae open their mouth. Water circulation, aeration system and water renewal rate for larval rearing in both years is described in Table 1 and Fig. 1. Water renewal was adjusted to guarantee minimum dissolved oxygen levels higher than 5 mg.L^{-1}. A surface skimmer was used between 2 DPH to 15 DPH to prevent formation of oil films on the water surface.

Prey density has a large effect on feeding in marine fish larvae (Hunter, 1981). Since average densities in enclosed coastal areas can exceed 200 L^{-1} (Hunter, 1981) we used this value as a minimum food concentration. Besides the endogenous prey developed in the tanks, food abundance was increased after the opening of the mouth by daily addition of enriched rotifers (*Brachionus plicatilis*) and later on by newly hatched and enriched *Artemia* sp.. The feeding schedule is shown in Fig. 1. In 2007 the minimum food density was checked daily and if the plankton concentration was lower than 200 individuals.L^{-1}, small rotifers were supplied (for the first 3 weeks) followed by *Artemia* sp. nauplii (1.5 weeks) and increasing sizes of metanauplii until the end of the rearing period with live feed, which lasted approximately five weeks. In 2008 the amount of supplied rotifers was 2.5 times higher then in 2007 during an identical period. Co-feeding of rotifers and *Artemia* nauplii was adopted in 2008 with an earlier start and a much shorter period with *Artemia* feeding than in 2007 (2 weeks). Dry feed was also introduced earlier during 2008.

Rotifers were enriched with *Nannochloropsis oculata* for 24h and one day *Artemia* with RICH® (Catvis, Hertogenbosh, Netherlands) following instructions from the manufacturer. Simultaneously with the first addition of rotifers, 10 litres of a mixture of *Nannochloropsis oculata* and *Isochrysis galbana* in equal parts were added to the tanks in a pseudo-green water methodology and whenever the tank bottom was visible more algae were supplied. Addition of algae had three objectives: i) to feed the plankton; ii) to create a more protected environment for early larvae (they avoid direct sunlight) and iii) to reduce cannibalism in older larvae by shading the water. Newly hatched *Artemia* were supplied to the rearing tanks after testing their acceptance by the grouper larvae. In 2007 this happened after 20 days post hatch (DPH) while in 2008 it was after 15 DPH. Five days later there was a shift to one day enriched *Artemia* simultaneously with the delivery of small amounts of commercial formulated feed (Lucky Star®, Hung Kuo Industrial Co. Ltd, Taiwan). Feed rations were manually delivered hourly from 8:00 to 12:00 and before live prey distribution. After larval settlement larger types of natural food, polychaetes *Nereis virens*

(SEABAIT®, Shoreline Polychaetes Farms LLP, Lynemouth, UK) in 2007 and frozen and chopped sardine muscle in 2008 were delivered until complete weaning into artificial diets. The formulated feed in micropellets (≈500 µm) was distributed manually 3 times daily, in early morning, at mid-day and late afternoon.

Local air temperature and solar radiation were determined with a CR 200 Series meteorological station (Quantific®, Coimbra, Portugal). Temperature, salinity, dissolved oxygen, and pH of the water in the tanks was determined daily in the morning and afternoon using a multiparameter water quality portable meter (HI9828 Hanna Instruments Portugal®, Póvoa de Varzim, Portugal). Plankton concentration in the tanks was determined early in the morning prior to larval feeding in one litre water samples from the centre of the tank. The water was filtered through a 55 µm size mesh and the plankton was preserved in 4% formaldehyde and analysed *in toto* under a stereomicroscope.

We needed to have an indication of whether the grouper larvae were ingesting the food particles present in the tanks. Since we did not want to sacrifice nor handle the larvae we used the attrition of food particles as an indication of feeding. The daily attrition of food particles in the tanks was the result of several undetermined factors such as zooplankton natural mortality, ingestion by the larvae and washing out due to water exchange. Zooplankton intrinsic production in the tanks counteracts the decrease of food particles but this is also an unknown factor.

Daily Attrition of Food Particles during day n ($AtFP_n$) was calculated as the number of Total Available Food Particles during day n ($TAFP_n$) minus the number of food particles present in the water before feeding (8:30) the next day (Counted Food Particles - CFP_{n+1}). The formula was:

$$AtFP_n = TAFP_n - CFP_{n+1} \quad (1)$$

with $TAFP_n = (CFP_n + DDFP_n)$, the sum of Counted Food Particles in the water at 8:30 during day n (CFP_n) and the Daily Delivered Food Particles during the same day n ($DDFP_n$). This last parameter is equal to the Delivered Food Particles at 9:00 (DFP_{n9}) plus the Delivered Food Particles at 16:00 (DFP_{n16}) during day n.

Growth in length was determined from the exponential regression of standard length on days after hatching:

$$L_t = L_0 e^{Gt} \quad (2)$$

where L_t is the length at time t days, G is the instantaneous daily growth (in length) coefficient and L_0 is standard length at hatching. Specific growth rate, in length, (percent per day) was determined as $100(e^G - 1)$.

Larval measurements were done under a binocular microscope using an incorporated micrometer with an accuracy of 0.1 mm.

Before and during notochord flexion, standard length (SL) was measured from the tip of the upper jaw to the end of the notochord. After notochord flexion, SL was measured from the tip of the upper to the posterior margin of the hypurals. All the individuals were photographed. In 2007 only a small numbers of individuals (13 in total) were measured from days 10 throughout 35 post hatch. In 2008, 140 larvae were measured from days 1 until 35 post hatch with each daily mean being determined for 10 individuals. During this year sampling was performed daily during the first 4 DPH, every other day until 16 DPH, and every 5 days until 35 DPH. All specimens were collected near the surface. Terminology used to refer to dusky groper early life history stages generally follows Kendal *et al.* (1984) and Colin *et al.* (1996).

The minimum energy requirements of larvae for growth was estimated by adapting the methodology followed by Yoshinaga *et al.* (1994) where they assess metabolism and ingestion through respiration and dry weight. To calculate grouper larvae dry weight (DW) we first converted the larval length into ungutted wet weight using a power regression obtained with older larvae and juveniles (33 mm to 123 mm) and assumed the DW to be equal to 20% the WW. Respiration was estimated based on the following regression equation between respiration rate, R (in µl O_2 ind. h^{-1}), and body dry weight, DW (in mg.ind^{-1}) for red sea bream (*Pagrus major*) post larvae and juveniles at 20^{o}C (Yoshinaga *et al.*, 1994):

$$R=3.75DW^{0.92} \quad (3)$$

These respiration rates can be considered as routine metabolism (R_{rout}) since they did not include either active swimming or feeding energy loss as described by Yoshinaga *et al.* (1994). To account for daily energy losses of larvae R_{rout} was multiplied by 2.0 to get the active feeding metabolic rate and this conversion factor was applied only to daytime (14.5 light hours during the rearing period). The resulting equation to calculate food ingestion was:

$$F= 2.0 \bullet 1.6\ R_{rout} = 3.2\ R_{rout} \quad (4)$$

Grouper is a carnivorous fish and therefore the respiration quotient should be close to 0.8 [Morioka, (1985) as given by Yoshinaga *et al.*, (1994)]. When the respiration quotient is 0.8, 1 µl of oxygen combusts 0.43 µg of organic carbon. Similarly for *Pagrus major* (Yoshinaga *et al.*, 1994) we assumed a carbon weight:dry weight conversion factor of 0.4.

Based on the estimated respiration rates and body weights and on the assumptions described above, minimum daily food requirements (in terms of carbon) were calculated for grouper larvae at the time of: mouth opening, oil globule exhaustion, beginning of notochord flexion and beginning of metamorphosis.

Individual dry weight of the main zooplankters (groups or species) present in the mesocosms was estimated according to the weight/length relationships in van der Meeren (1991) and in Uye

(1982) and the mean length of the pertinent taxa was taken from the literature. Dry weight was converted into carbon content assuming a conversion of 43 % and 53% in the case of copepods and bivalvia and polychaeta, respectively (Uye, 1982). Carbon content for rotifers and *Artemia* nauplii was based in Lubzens and Zmora (2003) and in Dhont and Van Stappen (2003) respectively.

All biotic and abiotic data were analysed for normality and transformed when necessary and tests of significance for means were carried out after checking for equality of variances and the rejection level for the null hypothesis was $p < 0.05$. With the exception of rotifers and Artemia, that were square root transformed, all the biotic variables were Naperian log transformed.

RESULTS

The rearing of dusky grouper larvae in outdoor mesocosms took place in southern Portugal during summer with mean values of solar radiation and air temperature during the rearing periods of 2007 and 2008 of 538±36.9(SD) Wm^{-2} and 513±40.2 Wm^{-2} and 24.1±2.06ºC and 24.2±1.17ºC respectively. Since during rearing the variation in temperature between day and night was relatively small (3.3±1.72ºC in 2007 and 3.2±1.44ºC in 2008), water temperatures in the tanks were only slightly lower than the air temperatures with values of 23.6±1.07ºC and 23.5±0.86ºC in 2007 and 2008 respectively and were not significantly different (t = -0.446, df = 59, p = 0.657). Salinity of the water was 36.7±0.11 in 2007 and 37.1±0.30 in 2008, slightly but significantly different (t = 6.237, df = 32, p < 0.001). Mean dissolved oxygen decreased with rearing time and was significantly lower (t = 7.473, df = 55, p < 0.001) when the larvae were fed with *Artemia* (5.50±0.88 $mg.L^{-1}$) than when they were fed with rotifers (6.99±0.69 $mg.L^{-1}$).

Abundance of live feed available to the larvae during the two rearing experiments (Table 2) show that mean daily total prey in 2007 was half that in 2008. Feeding particles were composed mainly by a mix of endogenous plankton and rotifers (the rotifer period) for slightly more than half of the rearing period and by endogenous prey and *Artemia* (the *Artemia* period) in the second half (Fig. 1 and Table 2). In 2008 the amount of rotifers delivered to the larvae was much higher than in 2007 while *Artemia* was lower. Endogenous prey was not significantly different (t = -0.615, df = 56, p = 0.541) between the two years when the whole period is considered, but in 2008 it was significantly higher (t = 4.704, df = 27, P < 0.001) during the first half of rearing (the rotifer period) in comparison to the second half (the *Artemia* period).

Natural zooplankton grown in the tanks made up part of the live food available for dusky grouper larvae during rearing. These endogenous preys were mainly composed of different developmental stages of copepods belonging to several species of

Acartia (*A. clausi*, *A. grani*, and *A. margalefi*) and Harpacticoids (*Tisbe tenera*, *T. furcata* and Euterpina *acutifrons*) and by other groups including bivalve trocophors, nectoquets of polychaetes, tintinnids and natural rotifers. The succession of zooplankton populations during the two rearing periods is shown in Fig. 2. The first peak of zooplankton abundance in 2007 took place two weeks after the beginning of rearing while in 2008 it occurred one week after. In 2007 (Fig. 2 upper graph) the initial zooplankton population was mainly composed of copepod nauplii that developed into copepodites and adult copepods. These laid eggs that produced a large number of nauplii that, in addition to bivalve trocophors and nectoquets of polychaetes, constituted the second peak occurring 1 month after the incubation of the dusky grouper larvae. At the beginning of larval rearing in 2008 (Fig. 2 lower graph) blooms of *Strobilidium* sps. and *Synchaeta* sps. occurred followed by the development of a large number of copepod nauplii. The bloom of copepod nauplii occurred after the first week of rearing and lasted for almost one week. Table 3 summarizes the abundance of the different copepod stages and other groups available to the larvae during the rearing periods of 2007 and 2008 and give information on their size range.

Daily attrition of food particles during the two rearing periods were highly correlated with food availability in the respective tanks ($R^2 = 0.823$; $df = 37$; $p < 0.001$ in 2007 and $R^2 = 0.937$; $df = 28$; $p <$

0.001 in 2008) with a mean value for prey attrition in 2007 lower but not significantly different (t = -1.455, df = 52, p = 0.076) from 2008 (Table 2). The amount of attrition during the rotifer period was 2.6 times lower in 2007 than in 2008. When the attrition of food particles per day was normalised to the amount of total available food (the attrition rate in Fig. 3), it is evident in Fig. 3 (lower graph) that attrition is low during the first days of rearing and progressively increases until day 10 post hatch. However after this initial stage and during a subsequent period of seven days most of the food (90 to 95%) in the tank was disappearing. This period of high attrition corresponded to rotifer feeding (R in Fig. 3) after which there was a sharp decrease that seemed to coincide with the change of food from rotifers to *Artemia* which also involved higher water renewal. A few days later the attrition rate increased but never to levels seen during the rotifer period.

The growth in length of the individuals during the two rearing periods is shown in Fig. 4. During 2008 there were two different trend lines in the growth curve identified in the figure as a) and b). a) corresponds to the growth of larval stages from first feeding to beginning of metamorphosis and b) to growth of larval stages during transformation. The number of samples collected in 2007 was not enough to construct a similar curve because we did not sample enough individuals during transformation. Growth from first feeding to beginning of metamorphosis was significantly higher in 2008 (ANCOVA, df = 113, $p < 0.001$).

Rearing characteristics of dusky grouper larvae during the two years (Table 4) were very different although the initial densities of newly hatched larvae were similar (1.3 L^{-1} in 2007 and 1.5 L^{-1} in 2008). During 2008 the larvae took less time to start metamorphosis which occurred when larvae were 25 days old and 20±1.2 mm (SD) in total length than in 2007 when the larvae took 33 days to reach a similar length (Fig. 4). Settlement, that started to occur at 22±1.3 mm, was also earlier in 2008 (30 DPH) than in 2007 (37 DPH). This is reflected in the percentage of daily growth that in 2007 was 7% during the first larval stages (before metamorphosis) and in 2008 was 9% (Table 5). During transformation the growth rate declined significantly (Fig. 4), and was 3% per day in 2008 (Table 5). Survival at the beginning of transformation was estimated to be less then 10 percent in 2007 and 25-50% in 2008 (Table 4). Sixty days post hatch, when all benthic juveniles were already well weaned onto dry food, survival was 1% in 2007 and 6% in 2008. High mortality during transformation was observed during both years.

The relationship between juvenile grouper body wet weight and standard length (SL) was highly significantly and the power coefficient for SL was 3.156±0.020 SE ($t = 154.648$, $df = 276$, $p < 0.001$) and the intercept (log a) was -2.114±0.039 SE ($t = -54.675$, $df = 276$, $p < 0.001$). This relation was used to determine individual dry weight of the smaller larvae that was then converted into carbon weight assuming a conversion factor of 40%. Individual dry weight was also used to calculate the respiration rate as described in the Methods section using equation (3).

Information on mean larval length, body weight, metabolism and minimum daily food requirements (in terms of carbon) for larvae at the time of mouth opening (2 DPH), oil globule exhaustion (5 DPH), beginning of notochord flexion (15 DPH) and beginning of metamorphosis (25 DPH) is shown in Table 6. Body carbon weight at each larval stage was, respectively, 15, 32, 412 and 5,311 μgC larvae^{-1}. With increasing body size both metabolism and ingestion per unit body weight decline from 12.6 to 7.9 and from 40.3 to 25.2 % respectively, although the minimum daily carbon requirement also increases with age. Assuming that at the opening of the mouth, i.e., at day 2 post hatch, grouper larvae eat only individuals between 100 and 160 μm, each larva will need to eat 96 individuals within this size range to fulfil its minimum daily carbon requirement (Table 7). As the larvae growth they eat larger prey and the minimum number of prey required per day decreases. However at the beginning of metamorphosis (≈25 DPH) the daily food requirement is high and, even considering only the larger zooplankters in the mesocosms and *Artemia*, the minimum number of prey per larvae is very high at 513 individuals.

DISCUSSION

Dusky grouper larvae are characterized by small mouth gape and body size and poor reserves of endogenous nutrition (Glamuzina *et al.* 1998; Spedicato and Boglione, 2000; Gracia López and Castelló-Orvay, 2003). The results of this study show that larvae of dusky grouper can be successfully reared in mesocosms, using the natural bloom method with addition of rotifers and *Artemia* in later developing stages. In the mesocosm, grouper larvae had available a large variety of prey of different sizes from phytoplankton to different larval stages of copepods to meet their basic nutritional needs. The daily attrition of food particles in the tanks might be related to zooplankton natural mortality, washing out due to water exchange and ingestion by the larvae. However at the onset of rearing, a time when production of endogenous prey in the mesocosm is elevated and the water exchange was very low, attrition rates of 40 to 60 % (Fig. 3) suggest that ingestion might be the determining factor. If so, dusky grouper might capture prey after the second day post hatch (DPH). Day 2 to 5 post hatch corresponded to the period of mixed feeding, when the larvae had not yet exhausted their own energetic reserves but already started feeding on exogenous prey. This was the phase of the beginning of the zooplankton bloom. In 2008, after the initial period with high numbers of athecate protozoans (Tintinnids, *Strobilidium* sps. and *Synchaeta* sps.) (Fig 2 (lower graph)), copepod eggs, especially from the genus *Acartia*, started to hatch producing a large number of nauplii. Daily sampling coverage during the rearing period of 2008 revealed that the attrition rate increased progressively after 5 DPH and at 10 DPH reached a maximum of 95% and remained high during the time of rotifer feeding (Fig. 3 – lower graph). Specific growth rates in length of dusky grouper larvae (Table 5) were high (9% day^{-1}), and at 10 DPH the larvae attained an average length of 5.2 mm, a size at which *Brachionus* spp. could easily be ingested (we observed that 15 DPH larvae were already able to eat instar I *Artemia* nauplii).

We observed that dusky grouper late larvae are visual feeders and that when near the food particle the feeding mode is "sucking/grasping". The same feeding mode is reported by Kohno *et al*. (1997) for *E. coioides* and Yoseda *et al.* (2008) suggests that leopard coral groupers (*Plectropomus leopardus*) are also visual feeders with food intake increasing with light intensity. Although visual feeders, food preferences of dusky grouper larvae appear to be determined mostly by the encounter rate. The higher the abundance of endogenous prey and rotifers the higher the attrition of prey (Table 2). During the *Artemia* feeding period, when lower numbers of food organism were delivered to the larvae, the attrition rate decreased drastically (Fig. 3) even considering higher prey losses due to higher water exchange. This prey reduction may be the cause of the high larval mortality that we perceived starting at metamorphosis and during transformation. We observed at this time that a large number of the larvae became lethargic and there was cannibalism. Pierre *et al.* (2008) in their review about grouper aquaculture reported that cannibalism during the stage of metamorphosis is one of problems affecting commercial larval rearing in Taiwan where they feed with high protein pellets as prevention. In *Gadus morhua* large mortalities also occur around and after metamorphosis (Øiestad, 1985). As an explanation for this Folkvord (1991) suggested that cannibalism in combination with prey depletion during the period after metamorphosis could explain most of the mortalities.

Growth rates of *E. marginatus* to metamorphosis in our experiments were much higher then those observed for other species like *E. coioides* (Toledo *et al.* 1999), *E. bruneus* (Sawada *et al*., 1999) and *E. suilus* (Duray *et al.*, 1997) among others. However within the same species there is variability as indicated by the larval growth and survival of *E. marginatus* during our 2007 and 2008 rearing periods. Although environmental conditions and initial densities of newly hatched larvae in the tanks were similar (Table 4), survival and growth (Table 5) were very different. This seemed to be related to the much higher amount of food available to the larvae during 2008 in comparison to 2007 (Table 2) since prey density has been reported to have a large effect on feeding in marine fish larvae (Hunter, 1981). In 2008 not only was the bloom of endogenous plankton earlier then in 2007 (Fig. 2) but also numbers of available nauplii were higher (Table 3). As reported by van der Meeren and Mæss (1993), fish larvae need copepod nauplii to start growing and they actively select these nauplii for their energetic needs. Copepods have high nutritional value, particularly n-3 highly unsaturated fatty acids (n-3 HUFA), indispensable amino acids and vitamins that are essential for growth and survival of marine fish larvae (Sargent and Falk-Peterson, 1988; McEvoy *et al.* 1998; Støttrup, 2003; van der Meeren *et al*. 2008). Late (in the rearing period) production of nauplii in 2007 (Fig. 2) and in lower abundances (Table 3) may have retarded larval growth and possibly affected larval viability. To add to this lower nauplii production in 2007, the amount of rotifers delivered to the larvae during the rotifer period (Table 2) was also lower in comparison to the amount delivered in 2008. Consequently survival was higher at the onset of metamorphosis during 2008. However, during transformation mortality became high (Table 4), probably due to the reduced amount of available food during the *Artemia* period in 2008. Not only was the number of endogenous prey low but also the quantity of *Artemia* delivered was lower than in 2007.

Our results demonstrate that early larvae of dusky grouper can be successfully reared at temperatures close to 23 ^{o}C in semi-intensive systems (van der Meeren and Naas, 1997; Divanach and Kentouri, 2000). The tanks for rearing the larvae should be prepared in advance and filled with coarsely filtered (500 µm) sea water 6 days before seeding with newly hatched larvae. This permits the development of microzooplankton whose maximum abundance coincides with the initial days after the opening of the mouth of the larvae. In parallel with larval seeding exogenous phytoplankton should be added to the tanks at a density of 3 mL.L^{-1} of water to create shading and food should be supplemented with increasing amount of rotifers up to a density of 3,000 rotifers.L^{-1} at 5 DPH during the first 2 weeks. Although first feeding larvae may not feed directly on rotifers they have small rotifer eggs available. In Fig. 5 we suggest a feeding plan based on our experimental results. Considering a density of 1 larva per litre the density of endogenous prey at seeding should be at least 100 ind.L^{-1} (Table 7) of which 25% are small ciliates and 60% of small copepod nauplii. These numbers appear to be appropriate to propagate nauplii for first feeding larvae up to a size compatible with rotifer feeding at the exhaustion of the oil globule. If the concentration of endogenous zooplankton does not reach the above densities additional filtered zooplankton (55 µm) may be added to fulfil this requirement. At an age of 10 DPH small amounts of recently hatched *Artemia* (5 ind. L^{-1}) should start to be delivered to the tanks in order to check their acceptability by the larvae. If *Artemia* are well accepted the density should be increased steadily so that the total density of prey larger than 275 µm should be at least 70 ind. L^{-1} per larvae at 15 DPH.

The minimum food requirement from this time until the beginning of metamorphosis (≈ 25 DPH) increases exponentially and the number of larger prey organisms in the mesocosm at this age should be adjusted daily to attained a value higher than 520 per larvae at 25 DPH. Rotifer feeding should not be discontinued until 20 DPH in order to keep the level of encounter rates high. Specific growth rates of dusky grouper are high and at this age they are well into the post-flexion stage and have a mean total length of 11.8 mm, a size compatible with larger prey like 24h enriched *Artemia*. Simultaneously shredded polychaetes and/or fish muscle pellets and high protein formulated feed in micropellets should be distributed to the larvae in controlled quantities in order to meet the high energy requirement of larvae at these ages and to decrease mortality and cannibalism during larval metamorphosis.

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Legend for TABLES

Table 1. – Experimental settings for dusky grouper egg incubation and larval rearing during 2007 and 2008.

Table 2. – Larval live feeding abundance during 2007 and 2008: time duration of each period (days), mean daily total prey number per litre, mean daily cleared prey and gross food composition (percentage) during the entire rearing (Whole period) period and during mix feeding periods with rotifers (Rotifer period) and *Artemia* (*Artemia* period).

Table 3. – Characteristhics of endogenous live feed produced in the tanks during 2007 and 2008: mean abundance (number.L^{-1}) of prey items during the entire rearing (Whole period) period and during the mix feeding periods with rotifers (Rotifer period) and *Artemia* (*Artemia* period). The size range of each group (adult copepods and copepodites – cephalothorax length; nauplii – trunk length; other groups – total length) is given in brackets.

Table 4. – Characteristics of larval rearing during 2007 and 2008.

Table 5. - Regression coefficients describing growth in length of dusky grouper larvae before metamorphosis in 2007 and 2008 and during transformation in 2008. Percent gain - specific growth rate.

Table 6. – Carbon budget for metabolism and ingestion of dusky grouper larvae based on estimated respiration rate at 20°C.

Table 7. – Characteristics of prey size and estimated minimum number of daily prey requirement for different stages of dusky grouper larvae until the beginning of metamorphosis.

Legend for FIGURES

Figure 1. - Water exchange and feeding schedule.

Figure 2. – Taxa and abundance succession of endogenous prey during the rearing period of 2007 (upper graph) and 2008 (lower graph).

Figure 3. - Attrition rates during 2007 (a) and 2008 (b). Numbers above each graph correspond to rates of water exchange: 1 – 5 to 10 %; 2 – 25 to 50 %; 3 – 70 to 100 %; and letters to feeding scheduled: R – rotifer; A – *Artemia*.

Figure 4. – Growth in length of dusky grouper larvae during 2007 (black squares) and 2008 (white circles). Exponential lines: a) – before metamorphosis; b) – during transformation.

Figure 5. – Idealized feeding scheme for rearing dusky grouper larvae in mesocosm of semi-extensive technology. (Exogenous phytoplankton and formulated microdiets are not to scale)

Table 1.

	Egg incubation	Larval rearing
Rearing units	Indoor; 200 L cylinder-conical fibreglass	Outdoor; 3,000 L circular (Ø 2.10 m) fibreglass
Water inlet	Bottom, near the tip of the conical edge	Bottom, near the wall
Water outlet	Top, outlet protected with 500 μm nylon mesh banjo type screen	Top, outlet diametrically opposed to inlet and protected with removable screen of ≈ 500 cm2 open area made of: 1. 80 μm nylon mesh, before Artemia co-feeding; 2. 150 μm nylon mesh during Artemia co-feeding and wet feed 3. 500 μm and 1000 μm nylon mesh during dry feed regime
Aeration system	Bottom and around outlet filter to prevent clogging.	Air bubbling with no diffusers: one at centre, 2 laterals diametrically opposed and one below outlet filter. Wooden diffusers used during dry feed regime
Water renewal	One total renewal per hour before hatching Two total renewals per hour after hatching	See Figure 1
Photoperiod	16 hours light, 8 hours dark	Natural, 14 hour light, 10 hours dark

Table 2.

Dates:	07 Jul - 14 Ag 2007	02 – 31 Jul 2008
Whole period	**38**	**32**
Total prey	890±422	1,850±1,628
Endogenous prey	240 (18%)	242 (12%)
Rotifers	782 (59%)	1,591 (81%)
Artemia	294 (22%)	138 (7%)
Prey attrition	556±385	1,359±1,274
Rotifer period	**20**	**17**
Total prey	1,238±400	2,928±1,287
Endogenous prey	251 (20%)	308 (11%)
Rotifers	963 (80%)	2,614 (89%)
Artemia	0 (0%)	6 (0%)
Prey attrition	835±385	2,185±1,012
***Artemia* period**	**18**	**15**
Total prey	558±183	326±81
Endogenous prey	226 (39%)	149 (46%)
Rotifers	61 (11%)	10 (3%)
Artemia	281 (50%)	166 (51%)
Prey attrition	242±162	158±89

Table 3.

Dates:	07 Jul - 14 Ag 2007	02 – 31 Jul 2008
Whole period		
Adult copepods (500>870 μm)	54±70.7	22±20.6
Copepodites (390>830 μm)	78±98.4	51±41.8
Nauplii (110>320 μm)	76±80.2	171±87.9
Other groups (20>400 μm)	38±47.6	11±22.4
Rotifer period		
Adult copepods (500>870 μm)	73±91.6	28±22.8
Copepodites (390>830 μm)	109±127.5	62±37.8
Nauplii (110>320 μm)	62±59.5	200±98.3
Other groups (20>400 μm)	10±9.8	12±29.1
***Artemia* period**		
Adult copepods (500>870 μm)	38±44.4	13±12.8
Copepodites (390>830 μm)	42±14.4	2±1.3
Nauplii (110>320 μm)	88±94.2	128±46.6
Other groups (20>400 μm)	60±54.2	6±4.8

Table 4.

Dates:	09 Jul - 14 Ag 2007	02 – 31 Jul 2008
Initial larval density (number L^{-1})	1.3	1.5
Larval period (days until metamorphosis)	33	25
Time to settlement (days)	37	29
Survival at beginning of metamorphosis (%)	<10	25-50
Survival at 60 DPH (%)	1	6

Table 5.

Year:	2007	2008	2008
Ontogenic stage:	Larva	Larva	Transformation
Stage duration (days):	33	25	10
Percent gain (% d^{-1}):	7.5	9.2	2.9
n	12	114	31
Intersection [ln (L_0)]	0.612 **	0.795 ***	2.2636 ***
Slope (G)	0.0719 ***	0.0880 ***	0.0286 ***
$SE_{[\ln(Lo)]}$	0.1516	0.0129	0.0919
SE_G	0.0055	0.0011	0.0031
R^2	0.945	0.985	0.752

Signif. codes: $p<0.001$ ***; $p<0.01$ **; $p<0.05$ *

Table 6.

Age (DPH)	Standard length (mm)	Body weight (μg C ind.$^{-1}$)	Routine metabolism		Ingestion	
			(μg C ind. day^{-1})	(% body C day^{-1})	(μg C ind. day^{-1})	(% body C day^{-1})
2	2.7	14.8	1.9	12.6	6.0	40.3
5	3.5	31.9	3.8	11.8	12.1	37.9
15	7.9	411.9	39.8	9.7	127.2	30.9
25	17.7	5,311.7	417.9	7.9	1,337.2	25.2

Table 7.

Age (DPH)	Standard length (mm)	Minimum food required (μg C ind. day^{-1})	Size of prey		Minimum prey required (ind. day^{-1})
			Length range (μm)	Mean weight (μg C ind.$^{-1}$)	
2	2.7	6.0	100-160	0.06	94
5	3.5	12.1	100-275	0.24	51
15	7.9	127.2	275-860	1.9	67
25	17.7	1,337.2	610-860	2.6	513

Figure 1.

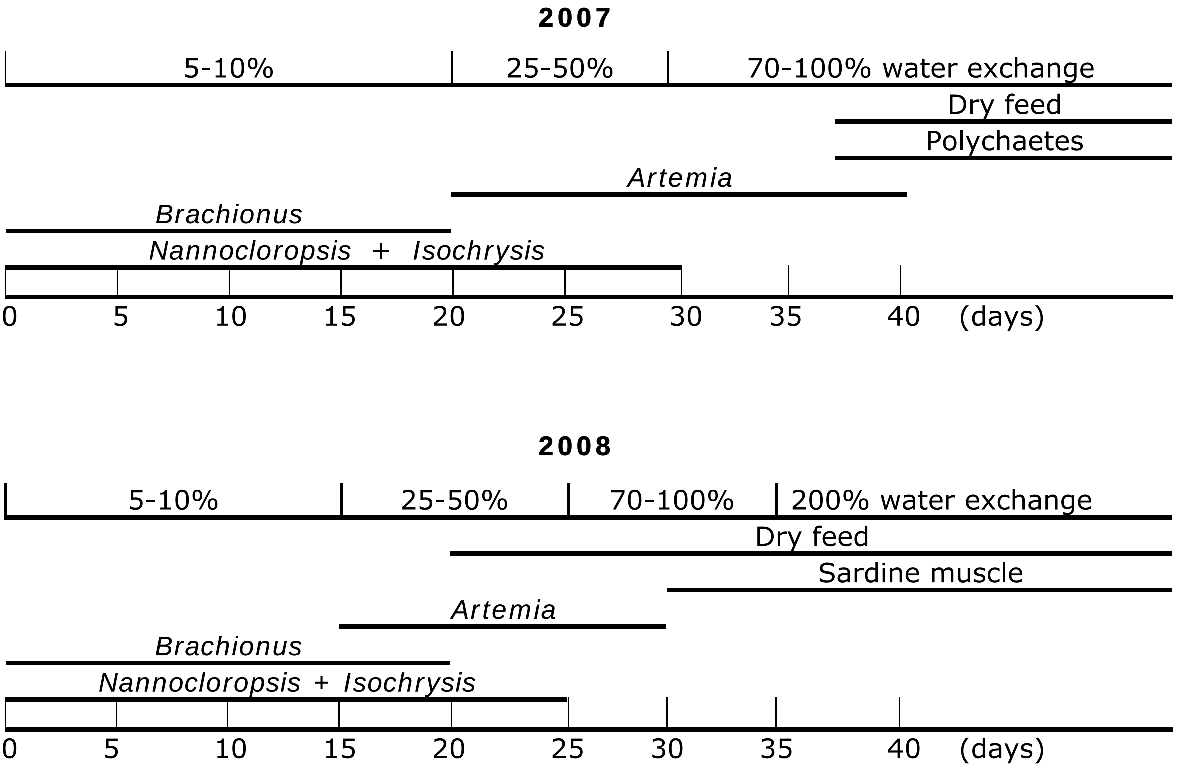
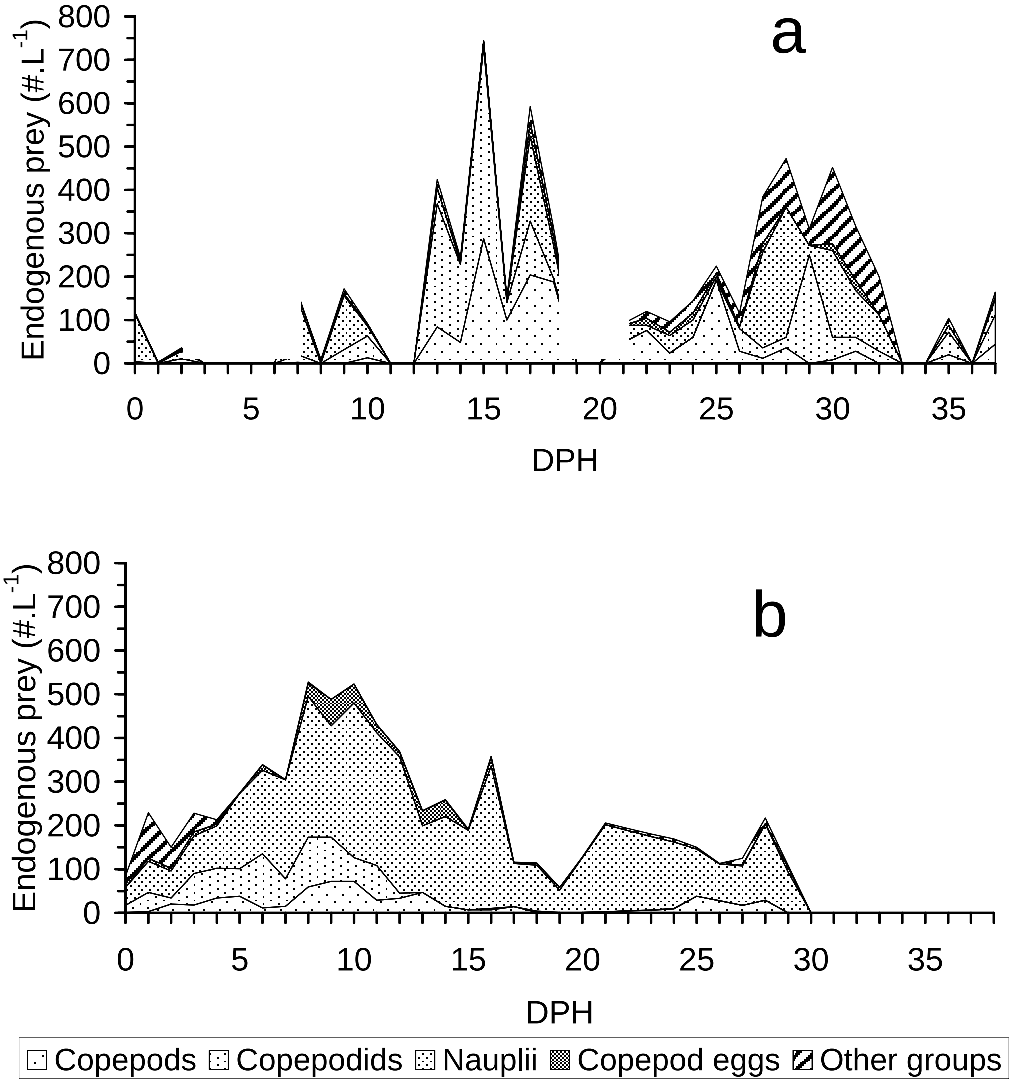
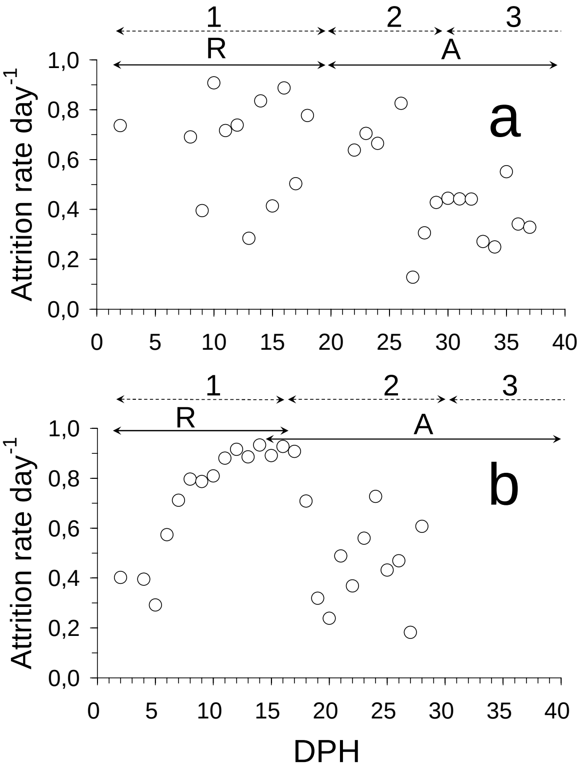

2007
5-10%
25-50%
70-100% water exchange
Dry feed
Polychaetes
Artemia
Brachionus
Nannocloropsis + *Isochrysis*
0
5
10
15
20
25
30
35
40
(days)
2008
5-10%
25-50%
70-100%
200% water exchange
Dry feed
Sardine muscle
Artemia
Brachionus
Nannocloropsis + *Isochrysis*
0
5
10
15
20
25
30
35
40
(days)

Figure 2.


a
Endogenous prey (#.L^{-1})
0
100
200
300
400
500
600
700
800
0
5
10
15
20
25
30
35
DPH
b
Endogenous prey (#.L^{-1})
0
100
200
300
400
500
600
700
800
0
5
10
15
20
25
30
35
DPH
Copepods
Copepodids
Nauplii
Copepod eggs
Other groups

774 Figure 3.

775



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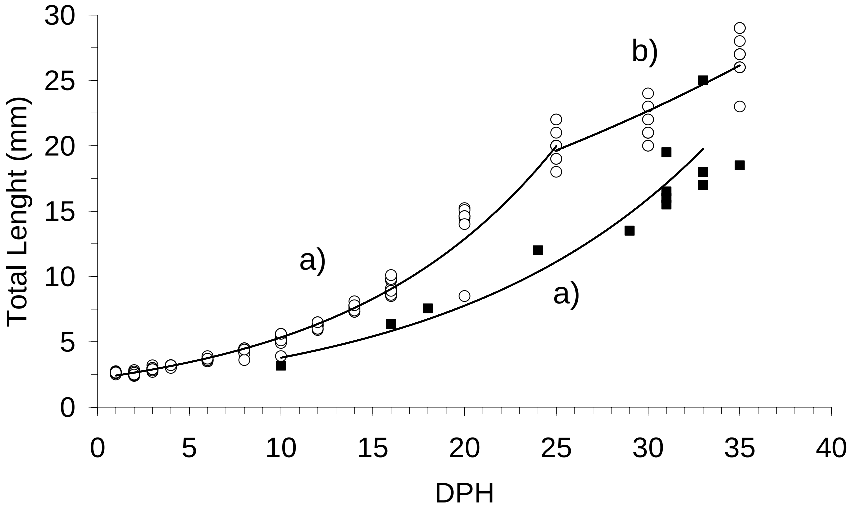

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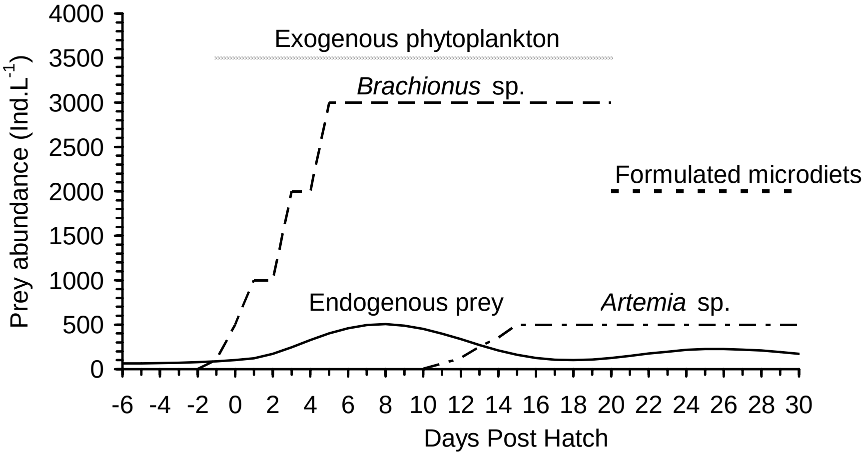
779 Figure 5.



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