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Oil extraction from microalgae for live prey enrichment and larviculture of clownfish *Amphiprion percula*

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The present study investigates the potential of algal oil (extracted from Nannochloropsis salina*), cod liver oil, olive oil and yeast for live prey enrichments in* A. percula *larviculture. After hatching, larvae were divided into six experimental groups as follows. Larvae fed on non-enriched (control), cod liver oil enriched, olive oil enriched, algal oil enriched, yeast enriched live prey and wild collected mixed plankton. Growth (total length, standard length, body depth, head depth and weight), survival, carotenoid and PUFAs content were observed at higher levels in juveniles fed on wild plankton and algal oil enriched diets. Thyroid hormones (T_3, T_4 and TSH) levels were also higher in the juveniles fed on wild plankton followed by algal oil enriched diet. Based on the present study, it can be concluded that mixed zooplankton and algal oil enriched rotifers* Brachionus plicatilis *and* Artemia *nauplii may be considered suitable live prey for clownfish larviculture.*

Keywords: algal oil extraction, carotenoid, fatty acid, thyroid hormones, *A. percula*

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INTRODUCTION

Larval rearing of marine ornamental fishes is relatively complicated due to their small size and initial feeding problems. Most of the fish larvae show deformed growth and inability to swim and consume the feed if they do not initiate nutritious first feeding, soon after the mouth opening (Dou *et al*., 2002). One of the main reasons for mortality at this stage is considered to be the lack of several essential nutrients in feed, such as fatty acids, vitamins and minerals (Kamler, 1992). Several practical methods for enrichment of live feed with essential fatty acids and vitamins have been developed to enhance survival during this stage. The majority of ornamental fishes are reared using rotifers *Brachionus plicatilis* and *Artemia* nauplii, but they are not the best live prey for marine fish larvae, as they are not their natural food.

Marine larvae in general require polyunsaturated fatty acids such as eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3) for their normal development and survival. However, EPA is present in low amounts in *Artemia* nauplii and DHA is practically absent. For this reason, the nauplii need to be enriched before they can be used for feeding marine larvae. The enrichment is commonly achieved by placing the nauplii in a medium, generally an emulsion, containing EPA and DHA. The nauplii are passive filter feeders and thus incorporate the emulsion in their digestive tract, acting as live vehicles. This enrichment process has also been termed bioencapsulation and is successful enough to allow the use of *Artemia* nauplii as larval feed for marine organisms, at least during certain phases of their rearing. But enriched *Artemia* nauplii are still far from being an optimal diet for marine fish larvae as compared with marine plankton.

Despite its widespread use, the enrichment process is not well understood in terms of the mechanisms of lipid assimilation and efficiency in long chain PUFA enrichment (Olivotto *et al*., 2011). Usually, high survival and growth rates are characteristic of marine fish larvae reared on natural assemblages of marine zooplankton or copepods (Olivotto *et al*., 2008). In fish larvae, fatty acids provided through live prey are essential to satisfy the high energy demand and are required to promote their growth. Fish oils are more complex than vegetable oils, having a complex nature of saturated, unsaturated and polyunsaturated fatty acids (Immanuel *et al*., 2007). In addition to these, fish oil contains free fatty acids, carotenoids, cholesterol and lipid-soluble vitamins A and D (Aidos *et al*., 2002).

Micro-algae are the fastest growing photosynthesizing organisms and they can complete an entire growing cycle in a few days. Among all the numerous classes of natural colours, carotenoids are the most widespread and structurally diverse pigmenting agents (Britton *et al*., 1981). The carotenoid pigmentation pattern of the fish is the result of the pigments present in the diet that the fish metabolizes. Minerals are inorganic elements necessary in the diet for normal body functions. They can be divided into two groups (macro-minerals and micro-minerals) based on the quantity required in the diet and the amount present in fish. Common macro-minerals are sodium, calcium, potassium and phosphorus and these minerals regulate osmotic balance and aid in bone formation and integrity. Micro-minerals (trace minerals) are required in small amounts as components in enzyme and

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hormone systems. Common trace minerals are copper, magnesium, manganese, iodine, zinc, iron and selenium. Fish can absorb many minerals directly from the water through their gills and skin, allowing them to compensate to some extent for mineral deficiencies in their diet.

Hormones play an important role in regulating larval developmental processes. The importance of the thyroid hormones (THs), thyroxine (T_4) and triiodothyronine (T_3), in vertebrate development is well established (Power *et al.*, 2001). In fish, prior to the maturation of the larval thyroid gland, fish eggs and, subsequently, the larval yolk sac contain significant amounts of THs of maternal origin (Tagawa *et al.*, 1990). This source of THs is likely to be of importance for the physiological regulation of growth, development and osmoregulation in larvae prior to the development of functional endogenous thyroid follicles. In fish, THs are also involved in the transition from the larval to the juvenile stage.

Currently, larval rearing of marine fish has been facing various bottlenecks such as low survival and growth due to less nutritious food. Percula clownfish *Amphiprion percula* is a typical marine ornamental fish with a short larval phase, hardy in captivity and with specific nutritional requirements. In view of the importance of these factors, this study throws light on how the selected enrichment media enrich the live prey and ensure higher survival and faster growth of *A. percula* larvae and juveniles.

MATERIALS AND METHODS

Micro-algal culture, harvesting and oil extraction

The unicellular marine micro-alga *Nannochloropsis salina* was cultured as stock using Walne's medium. Temperature, salinity, light intensity and photoperiod were maintained for the culture at 25–26°C, 23–28 gL^{-1}, 4000 lx, 12 h light and 12 h dark respectively. The mass culture was maintained by using commercial fertilizers (ammonium sulphate, urea and super phosphate). For settling the micro-algal biomass, sodium hydroxide (NaOH) was added to algae up to pH 9. After 2 days, the settled algal mass was collected and centrifuged at 3000 rpm for 30 min. The algal residue was then collected in glass beakers and dried in a hot air oven at 50°C for 48 h. The oil was extracted following the method of Folch *et al.* (1957). The dried biomass was obtained after drying and ground using a mortar and pestle, a solution of chloroform: methanol (2:1 ratio) was then added along with distilled water and homogenized. It was kept on ice for 10 min, and then chloroform: methanol solution (2:1 ratio) and water was added again. After centrifugation (2000 rpm for 10 min), the supernatant layer was collected and passed through anhydrous sodium sulphate (sodium sulphate was taken in a glass funnel with No. 1 Whatman filter paper). The rotator evaporator was used to get the oil for the further studies.

$$\text{Per cent oil in the micro-alga (\%)} = \frac{\text{Weight of oil (g)} \times 100}{\text{Weight of sample (g)}}$$

Physical and chemical properties of algal, cod liver and olive oils

SPECIFIC GRAVITY

$$\text{Specific gravity oil} = \frac{W_3 - W_1}{W_2 - W_1}$$

where, W_1 = Weight of the bottle, W_2 = Weight of the bottle with water, W_3 = Weight of the bottle with oil

SAPONIFICATION VALUE

Saponification value is defined as the amount of potassium hydroxide (KOH) in milligrams required to saponify one gram of fat or oil under alkaline conditions (AOAC, 1995).

$$\text{Saponification value} = \frac{56.1\ (B - S) \times N}{W}$$

where, B = Volume in mL of standard HCl required for blank, S = Volume in mL of standard HCl required for sample, N = Normality of standard HCl, W = Weight of oil in g taken for the test.

ACID VALUE

The acid value is the mass of potassium hydroxide (KOH) in milligrams that is required to neutralize one gram of oil. It is determined according to the method of AOAC (1995).

$$\text{Acid value} = \frac{56.1\ VN}{W}$$

where, V = Volume in mL of standard KOH used, N = Normality of KOH, W = Weight of sample in g.

IODINE VALUE

The iodine value is the amount of iodine, measured in grams, absorbed by 100 mL of the given oil (AOAC, 1999). A blank was also determined in the same manner as a test sample.

$$\text{Iodine value} = \frac{12.69\ (B - S)N}{W}$$

where, B = Volume in mL of standard sodium thiosulphate solution required for the blank, S = Volume in mL of standard sodium thiosulphate solution required for the sample, N = Normality of standard sodium thiosulphate solution, W = Weight in gram of the sample.

TOTAL CAROTENOID VALUE

Oil samples were dissolved separately in a known volume of hexane. These samples were scanned under the visible range of a UV spectrophotometer.

$$\text{Carotenoid yield } (\mu g\, g^{-1} \text{ sample}) = \frac{\text{Absorption at maximum wavelength (450 nm)} \times \text{dilution factor}}{\text{Extinction coefficient} \times \text{sample weight}}$$

where, Dilution factor = 10, Extinction coefficient = 0.25.

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Larval rearing of *A. percula*

After hatching, the larvae (TL, 3.4 ± 0.2 mm; mouth gap, 0.35 ± 0.5 mm) were kept for 2 days within the brooder tank. Larvae from the same brooder fish were used for the entire experiment. They were initially fed with non-enriched rotifer *Brachionus plicatilis* along with wild collected mixed zooplankton up to 2 days after hatching (DAH) at a density of 5–10 individuals mL^{-1}. On the third day, the larvae were transferred to rectangular fibreglass tanks (capacity, 50 L), having 30 L of filtered (through sand and UV filtration) brackish water, with a salinity of 24 ± 1 gL^{-1} and 7.8–8.3 pH (larvae density, 2 individuals L^{-1}). Light intensity was ~600–1000 lx in each tank with 12 h light and 12 h dark photoperiod. Dissolved oxygen was varied from 4.11 to 6.94 mg L^{-1}. Survival (%) rates of the larvae in each experimental tank were monitored daily over an experimental period of 30 days. The tanks were provided with mild aeration and 60–70% water exchange was done daily along with bottom cleaning.

Experimental setup

Larvae were divided into six groups (each of 60) fed on rotifers *B. plicatilis* cultured by yeast (from 1 to 10 DAH), *Artemia* nauplii (from 11 to 30 DAH) and wild zooplankton. Four groups were used for the enrichment study and of the remaining two groups, one was fed on wild zooplankton and the other considered as control (fed on 11–12 h starved rotifer). The experiment was done in triplicate.

Experimental groups:

Group A: Non-enriched live prey (control).
Group B: Live prey enriched with cod liver oil (Sea cod).
Group C: Live prey enriched with olive oil.
Group D: Live prey enriched with algal oil.
Group E: Live prey enriched with yeast.
Group F: Wild collected mixed zooplankton (*Cyclops, Daphnia, Sagitta, Mysis, Cladocerans, Ostracod, Zoea* etc.) (size range: <300 μm).

One mL of each type of oil (cod liver, olive and algal oils) was taken in a Petri dish and emulsified with 1 mL of egg yolk by thorough homogenization for 2–3 min. It was then added to a 1 L bottle containing rotifer/*Artemia* [~20,000–25,000 rotifers (20–25 individuals mL^{-1}) or 15,000–20,000 *Artemia* nauplii (15–20 individuals mL^{-1})] with a moderate aeration. For Group E, approximately 100 ± 5 mg of Baker's yeast (*Saccharomyces cerevisiae*) was used as enrichment medium without emulsification. 2 DAH *Artemia* nauplii were used for the enrichment since newly hatched nauplii have closed mouths. After 11–12 h of enrichment process, the live prey were filtered (using a 30 μm net), washed 2–3 times with brackish water and given to larvae/juveniles (2 times day^{-1} at 0700 and 1900 h). The larvae and juveniles of Group F were fed on mixed zooplankton (15,000–20,000 individuals) collected from the Vellar estuary (11°29′N 79°46′E) using a 100–150 μm net. Excess live feeds in the treatment tank were removed with water changes. Live feeds were enriched prior to each feeding.

Morphometric analysis

Larval sampling was done (5 individuals) at 10 days after hatch (DAH) (rotifer phase) and 30 DAH (*Artemia* phase) before feeding the larvae. Total length (TL), head depth (HD), body depth (BD), standard length (SL), eye diameter (ED) and mouth gape were measured using an ocular micrometer (Erma, Tokyo) in a light microscope at 10× magnification (Novex, the Netherlands). Wet weight (W) was recorded, using an electronic weighing balance (Shimadzu, Japan). Attaining adult colouration by the larva was considered to be metamorphosis.

Survival rate and specific growth rate (SGR)

At the end of 30 days of the experiment, the growth parameters were estimated by measuring the length and weight of the larvae and juveniles from each group. The weight gain was calculated by deducting the initial weight from final weight.

$$SGR = \frac{(\text{In} W_2 - \text{In} W_1)}{(t_2 - t_1)} \times 100$$

where: In = natural log, W_1 = Initial weight at time t_1 and W_2 = Final weight at time t_2.

$$\text{Survival rate } (\%) = \frac{N_2}{N_1} \times 100$$

where, N_1 and N_2 are the initial and final number of larvae/juveniles.

Cumulative Mortality Index (CMI)

Cumulative percentage of mortality was calculated by summing the number of dead larvae/juveniles at each day interval over the experimental period. The cumulative mortality index was calculated by multiplying the cumulative mortality with the respective day.

$$CMI = Dx_1 + Dx_2 + Dx_3 + \cdots + Dx_n(\text{final day})$$

where, D is the number of dead individuals at day x_1, x_2, $x_3 \ldots x_n$. Using the CMI value, the reduction of mortality percentage was calculated (Immanuel *et al*., 2007).

$$\text{Reduction of mortality percentage} = \frac{\text{CMI of sample}}{\text{CMI of control}} \times 100 - 100$$

Carotenoid analysis

Carotenoid content of the fish was determined, following the method of Olson (1979). About 1 ± 0.01 g of the body tissue of the fish was put in a 10 mL screw cap test tube and 2.5 g of anhydrous sodium sulphate was added. The sample was gently crushed with a glass rod and 5 mL of chloroform was added and incubated overnight at 0–4°C. Two layers were visible and 0.3 mL aliquots of the upper layer were taken and diluted to 3 mL with absolute ethanol. The optical density

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was read at 380, 450, 475 and 500 nm in a UV spectrophotometer (UV-1800-Shimadzu, Japan). A blank was also prepared in a similar manner for comparison. Wavelength at the maximum absorption was used for the calculation.

$$\text{Carotenoid yield } (\mu g\, g^{-1} \text{ sample}) = \frac{\text{Absorption at maximum wavelength} \times \text{dilution factor}}{\text{Extinction coefficient} \times \text{sample weight}}$$

where, Dilution factor = 10, Extinction coefficient = 0.25.

Fatty acid analysis

Total lipids of wild zooplankton, enriched and non-enriched rotifers, *Artemia* nauplii and larval fish samples (Group A, B, C, D, E and F) were extracted by adopting the method of Folch *et al.* (1957). Samples were rinsed with tap water to remove the residual emulsion. For fatty acid analysis, each sample was oven dried below 67°C for 24 h and ground finely with a mortar and pestle. Preparation and analysis of fatty acid methyl esters (FAMEs) from the fish tissues were performed. About 50 ± 0.1 mg of tissue samples were added to 1 mL of 1.2 M NaOH in 50% aqueous methanol in a screw-cap tube and then incubated at 100°C for 30 min in a water bath. Saponified samples were cooled at room temperature for 25 min and were acidified and methylated by adding 2 mL 54% 6 N HCl in 46% aqueous methanol and incubated at 80°C for 10 min in water bath. After rapid cooling, methylated FAs (fatty acids) were extracted with 1.25 mL 50% diethyl ether in hexane. Each sample was mixed for 10 min and the bottom phase was removed with a glass syringe. Top phase was washed with 3 mL 0.3 M NaOH. After mixing for 5 min, the top phase was removed for analysis. Following the base wash step, the FAMEs were cleaned in anhydrous sodium sulphate and then transferred to GC sample vial for analysis.

Algal oil, cod liver oil and olive oil were esterified, following the method of Metcalfe *et al.* (1966). One mL of the oil was mixed with 5 mL of BF_3 methanol and refluxed in a water bath (60°C) for 5 min. It was cooled and 6 mL of saturated NaCl was added and then transferred to a separating funnel. It was then extracted three times with petroleum ether and finally the extracts were combined. Subsequently, it was washed three times with distilled water and filtered through anhydrous Na_2SO_4 and made up to 1 mL with petroleum ether and transferred to a GC sample vial for analysis. FAMEs were separated by gas phase chromatography (Network gas chromatograph – 6890 N, Agilent Technologies, USA) and were identified by comparing the commercial Eucary database with the MIS Software package (MIS Ver. No. 3.8, Microbial ID. Inc., Newark, DE). Samples were injected by Split injector (split ratio 100:1), and Ultra-2 capillary column and Flame Ionization Detector (FID) were used as column and detector respectively.

Mineral analysis

Whole bodies of juveniles (N = 15) of each experimental group were cut into small pieces and dried in a hot air oven at 60°C for 24 h. After complete drying, samples were finely powdered using a pestle and mortar and weighed to 500 mg using an electronic weighing balance (Denver, USA). The weighed samples were digested in 100 mL glass beakers with concentrated nitric acid (10 mL) overnight. They were then heated on a hotplate at 100°C up to complete dryness. The residue was then dissolved and diluted with 10 mL of a solution of deionized water and concentrated nitric acid (4:1) (v:v) and this solution filtered through Whatman filter paper (11 μm). Mineral concentrations were determined by using an Inductively Coupled Plasma Optical Emission Spectrometer (Software – WinLab 32) (Perkin Elmer, Optima 2100DV). The precision of the analytical procedure was checked by analysing standard reference materials of commercially available standards (Merck KGCA, 64271 Darmstadt, Germany, ICP-Multielement standard solution IV, 23 elements in nitric acid). Deionized water was obtained using a Millipore water system. All acids and chemicals were of analytical reagent grade. Metal concentrations were calculated in micrograms per gram dry weight (μg metal g^{-1} d.w.). All the glassware was kept overnight in 10% nitric acid solution and rinsed with deionized water and air dried before use.

Hormone level analysis

Thyroid hormones were extracted from the whole bodies of clownfish larvae (up to ~50 mg), following the method of Olivotto *et al.* (2011). Samples were placed in Teflon tubes on ice. Larvae were extracted in 1 mL of 95% ethanol with homogenization and vortexing. All samples were centrifuged (10 min, 9000 rpm, 4°C) and supernatants were decanted into clean Teflon tubes. Further, 1 mL of 95% ethanol was added to larval sample pellets. Tubes were vortexed and re-centrifuged (10 min, 9000 rpm, 4°C). The supernatants were collected and kept at −20°C to analyse the thyroid hormones.

The quantitative determinations of T_3, T_4 and TSH were performed using Microplate Enzyme Immunoassay Kit (ACCU-BIND ELISA MICROWELLS, Monobind Inc., USA). 50 μL of the supernatant of each sample was added into the assigned microplate well; 100 μL of the respective enzyme reagent solutions (fT_3, fT_4 and TSH) were added to each well and swirled on a microplate gently for 20–30 s to mix. They were then incubated for 60 min at room temperature. The contents of the microplates were discarded by decantation and 350 μL of wash buffer decanted. This was repeated two more times for a total of three washes. 100 μL of working substrate solution was added to all the wells without shaking the plates. These were then incubated at room temperature for 15 min; 50 μL of stop solution was added to each well and gently mixed for 15–20 s. Absorbance in each well was read at 450 nm in a microplate reader (Automated ELISA strip reader, Span Autoreader 3011, USA). The absorbance value for each sample was plotted *vs* the concentration of hormones (fT_3, fT_4 and TSH) on linear graph paper. The intersecting point on the curve was considered as the concentration.

Statistical analysis

Pearson correlation coefficient was used for establishing the relationship among the percentages of growth rate of larvae, juveniles and fatty acid profiles of juveniles using the statistical package SPSS 16.0. One-way ANOVA was also employed to understand the variations in the above-mentioned parameters.

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RESULTS

Oil extraction from algae

About 1700 g dried biomass of the micro-alga *N. salina* was obtained, which gave about 332 mL oil. The oil was collected in a bottle and stored in a refrigerator for further use. The total yield of oil obtained from the micro-algae was 19.52%. Physical and chemical properties of the algal, cod liver and olive oils are given in Table 1. Algal oil had comparatively higher specific gravity (0.95 kg m^{-3}) than the cod liver (0.93 kg m^{-3}) and olive oils (0.9 kg m^{-3}). Saponification value was high in cod liver oil as compared with the other oils. The level of carotenoid was assessed to determine the quality of the oils. The cod liver oil exhibited thehighest total carotenoid content (2.64%) while olive oil showed the lowest content at 0.6%. The properties of the algal, cod liver and olive oils were not significantly different with each other ($F_{(2,12)} = 0.988$).

MORPHOMETRIC ANALYSIS

An enriched diet was able to accelerate the development of *A. percula* larvae, since in groups B, C, D and E, the larvae started metamorphosis at 9 DAH whereas the larvae from the control group started at 12 DAH. Metamorphosis in larvae of Group F, fed on wild collected live mixed zooplankton, started at 7 DAH. Morphometric analysis was carried out at 10 and 30 DAH. Morphometric characters (mean ± SD) of 10 DAH larvae fed on enriched and non-enriched diets are plotted in Figure 1. Total length (TL) of the 10 DAH larvae was maximum in the larvae fed on wild plankton (8.15 ± 0.07 mm) and least in the control group, which was without enrichment (7.15 ± 0.07 mm). The total length of larvae fed on enriched and without enriched diet was not significantly different from each other ($F_{(5,6)} = 1.27$). According to the Pearson correlation coefficient, significant positive correlation was observed between the olive oil and cod liver oil fed larvae ($r = 0.999$, $P < 0.05$) but negative correlation was noticed between yeast enriched counterparts and controls ($r = -0.999$, $P < 0.05$).

In the case of standard length (SL), the maximum increment was observed in the larvae fed on wild plankton (6.21 ± 0.13 mm) and least in the control (5.3 ± 0.14 mm). Standard lengths of larvae fed on enriched and non-enriched diet were not significantly different with each other ($F_{(5,6)} = 0.791$). Standard length of the wild plankton fed juveniles was negatively correlated with those fed on cod liver ($r = -0.862$), olive ($r = -0.778$) and algal oils ($r = -0.583$) enriched diet and control group ($r = -0.693$), but positively correlated with yeast enriched diet ($r = 0.970$).

Body depth (BD) of the larvae was maximum in those fed on wild plankton (2.76 ± 0.06 mm) and was lowest in controls (1.85 ± 0.07 mm). Body depth of the larvae was not significantly different among the various enrichments ($F_{(5,6)} = 0.689$). Body depth of juveniles fed on olive oil enriched diet was positively correlated with those fed on cod liver oil fed diet ($r = 0.998$, $P < 0.05$). Eye diameter (ED) was maximum in the larvae fed on olive oil enriched diet (0.6 ± 0 mm) and least development in eye diameter was observed in the larvae fed with yeast enriched diet and control (0.5 ± 0 mm). Eye diameter was not significantly different among the larvae fed on enriched and non-enriched diets ($F_{(5,6)} = 1.13$). Eye diameter of the larvae fed on wild plankton was negatively correlated with those fed with algal oil enriched diet ($r = -0.866$).

Head depth (HD) was maximum in larvae fed on wild plankton (1.7 ± 0.14 mm) and larvae fed on non-enriched diet showed least increment (1.1 ± 0.14 mm). Head depth was not significantly different among larvae fed on enriched and non-enriched diets ($F_{(5,6)} = 0.359$). Head depth of larvae fed on yeast enriched rotifer was positively correlated with those fed on algal oil enriched rotifers ($r = 0.756$). Mouth gap of all larvae was similar (0.35 ± 0.07 mm) except in the control group (0.3 ± 0 mm). It was not significantly different among the larvae fed on enriched and non-enriched diet ($F_{(5,6)} = 0.2$). Mouth gap of larvae fed on algal oil enriched rotifer was positively correlated with those fed on cod liver oil enriched rotifers ($r = 0.866$) but negatively correlated with larvae fed on olive oil enriched rotifer ($r = -0.866$). Weight of the larvae was maximum when they were fed on wild plankton (20.5 ± 0.71 mg) and among all the experimental groups, the control group showed the least weight gain (10.5 ± 0.71 mg). Weight of larvae was not significantly different with the different enrichments and the not enriched diet ($F_{(5, 6)} = 1.49$). Weight of the larvae fed on algal oil enriched rotifers was positively correlated with those fed on olive oil enriched rotifers ($r = 0.277$).

Juveniles of 30 DAH (N = 3) were randomly collected from each experimental tank and measured their morphometric characters (Figure 2). Juveniles fed on wild plankton showed better growth rate in terms of TL (14.5 ± 0.71 mm), SL (11.9 ± 0.14 mm), BD (5.3 ± 0.28 mm), ED (1.4 ± 0.42 mm), HD (4.2 ± 0.28 mm), mouth gap (1.15 ± 0.07 mm) and weight (47.5 ± 7.79 mg), than those fed on algal oil, yeast, cod liver and olive oil enriched diets. Maximum values of total length (TL), standard length (SL), body depth (BD), head depth (HD) and mouth gap were observed in the juveniles fed on plankton followed by algal oil (12.35 ± 0.78 mm, 9.4 ± 0.71 mm, 4.1 ± 0.14 mm, 2.9 ± 0.14 mm, respectively) and yeast (11.8 ± 1.67 mm, 9.23 ± 1.58 mm, 3.95 ± 0.45 mm, 2.8 ± 0.28 mm, respectively) enriched diets. In the case of eye diameter (ED), the

Table 1. Properties of the algal, cod liver and olive oils were not significantly different from each other.

	Physical property	Chemical properties			
Type of oil	Specific gravity (kg m^{-3})	Saponification value (mg KOH g oil^{-1})	Acid value (mg KOH g oil^{-1})	Iodine value (g I_2 100 g^{-1})	Total carotenoid value (%)
Algal oil	0.95	173.5	2.54	52	1.8
Cod liver oil	0.93	187.4	2.1	64.93	2.64
Olive oil	0.9	185	6.6	76	0.6

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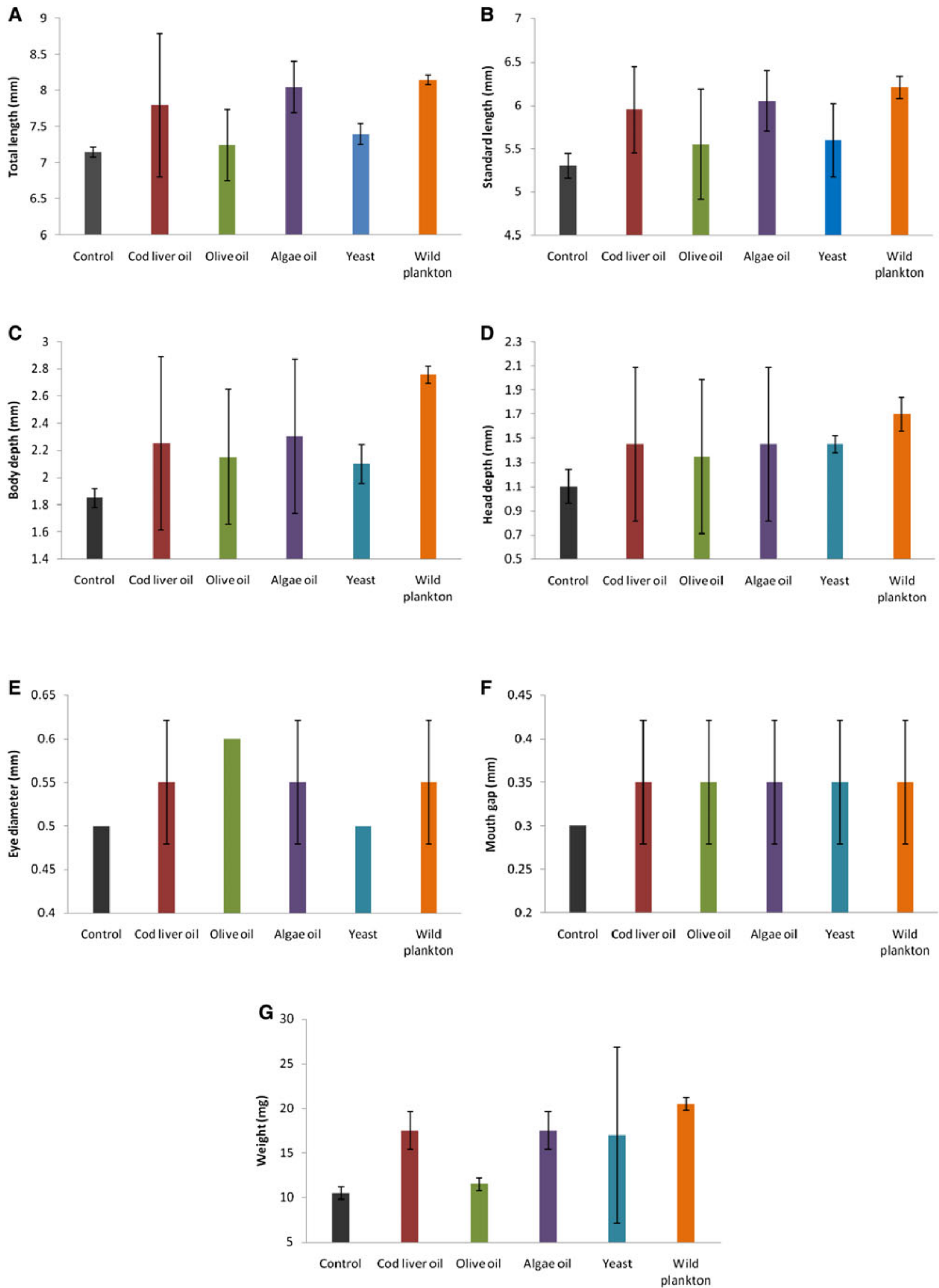

Fig. 1. (A–G) Morphometric characters (mean ± SD) of 10 DAH larvae fed on enriched and non-enriched diets. Morphometric characters were not significantly different among the larvae fed on enriched and non-enriched diets.

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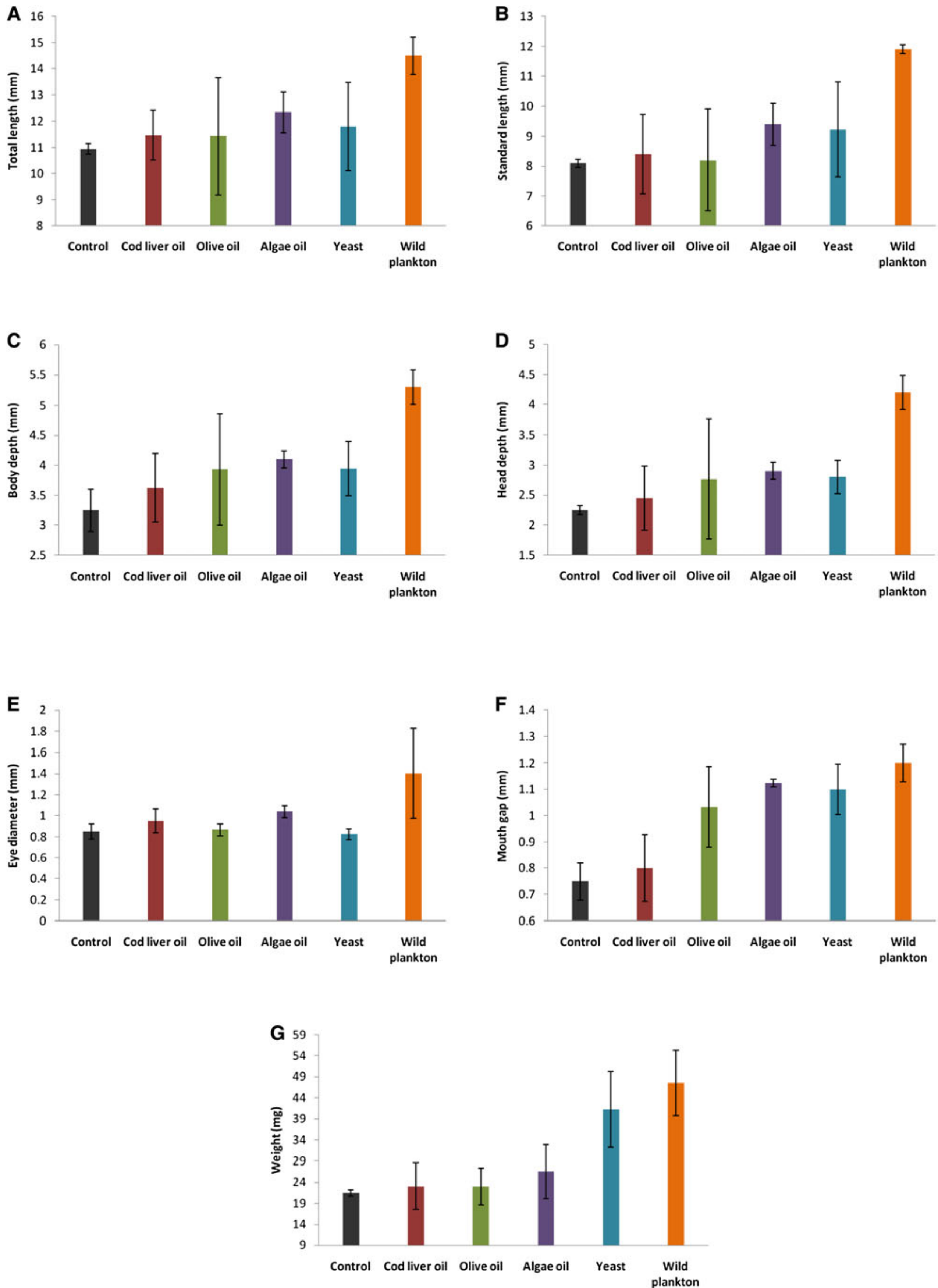

Fig. 2. (A–G) Morphometric characters (mean ± SD) of 30 DAH juveniles fed on enriched and non-enriched diets. Morphometric characters of the juveniles were significantly varied with respect to different groups.

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maximum values were observed in juveniles fed on wild plankton followed by those fed with algal oil (1.04 ± 0.06 mm), cod liver oil (0.95 ± 0.11 mm) and olive oil (0.87 ± 0.06 mm) enriched diets. Maximum values of weight of juveniles were observed in those fed on wild plankton followed by yeast (41.25 ± 8.99 mg), algal oil (26.5 ± 6.36 mg) and cod liver oil (23 ± 5.48 mg) enriched diets.

The morphometric characters of the juveniles varied significantly with respect to different groups. Total length (TL) of juveniles was significantly varied with various experimental groups ($F_{(5,12)} = 3.448$, $P < 0.05$). According to the Pearson correlation coefficient, juveniles fed on wild plankton were positively correlated to those fed on algal ($r = 0.997$) and olive oils enriched *Artemia* ($r = 0.994$), but negatively correlated with the yeast enriched *Artemia* fed juveniles ($r = -0.603$). Similarly, other morphometric characters in terms of standard length ($F_{(5,12)} = 6.74$, $P < 0.05$), body depth ($F_{(5,12)} = 6.5$, $P < 0.05$), eye diameter ($F_{(5,12)} = 18.8$, $P < 0.05$), head depth ($F_{(5,12)} = 6.137$, $P < 0.05$), mouth gap ($F_{(5,12)} = 9.55$, $P < 0.05$) and weight ($F_{(5,12)} = 16.12$, $P < 0.05$) were also significantly different with respect to different diets. While considering the standard length of juveniles fed on wild plankton, it was positively correlated to olive ($r = 0.728$) and algal oils ($r = 0.994$) fed juveniles. Body depth of yeast fed juveniles was positively correlated to cod liver oil fed juveniles ($r = 0.854$) and control group ($r = 0.564$). In the case of eye diameter of juveniles fed on wild plankton, it was positively correlated to those fed on cod liver oil enriched *Artemia* ($r = 0.998$) at 0.05 level.

Survival rate and specific growth rate (SGR)

Higher survival was observed in the juveniles fed on wild plankton (94%) followed by those fed on yeast (92%) and cod liver oil (90%). The lowest survival was observed in the control group (80%). The percentage of survival was significantly different with different enrichments ($F_{(5,12)} = 40.8$, $P < 0.05$). Specific growth rate of the wild plankton fed juveniles was higher among the experimental groups (7.56%), whereas the SGR was decreased to 6.53, 5.5, 5.06, 5.06 and 4.83% in yeast, algal, cod liver and olive oils fed juveniles and control respectively.

CUMULATIVE MORTALITY INDEX (CMI) AND REDUCTION OF MORTALITY

Cumulative mortality index for the control group of *A. percula* larvae/juveniles was 4583 while those fed with wild plankton, algal, cod liver, olive oils and yeast enriched diet was 1394, 2733, 2269, 3663 and 1859 respectively. The reduction of mortality was highest (69.58%) in wild plankton fed larvae/juveniles.

CAROTENOID ANALYSIS OF JUVENILES

Highest carotenoid content, 9.75 μg g^{-1} was recorded in the juveniles fed on wild plankton, followed by cod liver oil fed juveniles (6.93 μg g^{-1}), yeast fed juveniles (6.34 μg g^{-1}), algal oil fed juveniles (6.3 μg g^{-1}) and olive oil fed juveniles (5.85 μg g^{-1}); and the least, in the juveniles fed with non-enriched diet (3.62 μg g^{-1}).

Fatty acid analysis

Tables 2–4 show the fatty acid values of different diets. Among the four enrichment media, better fatty acid profiles were obtained in the algal and cod liver oils. Considering the essential fatty acids, linoleic acid (LA, C.18:2ω−6), alpha-linolenic acid (ALA, C.18:3ω−3), eicosapentaenoic acid (EPA, C.20:5ω−3), docosahexaenoic acid (DHA, C.22:6ω−3), arachidonic acid (AA, C.20:4ω−6) and decosapentaenoic acid (DPA, C.22:5ω−3); algal oil showed higher ALA (8.12%), EPA (10.11%) and AA (3.62%) content, while the cod liver oil showed higher DHA (8.12%) and DPA (1.2%) content. LA was highest in olive oil (13%) as compared with other enrichments.

In addition, during the rotifer enrichment, algal oil showed higher LA, EPA and AA contents (5.51, 8.12 and 5.12% respectively) while higher ALA (12.11%), DHA (8.42%) and DPA (1.35%) were observed in cod liver and olive oils enriched rotifer and control groups respectively. Besides, EPA, DHA and DPA were higher in wild plankton (16.19, 29.58 and 1.78% respectively). During *Artemia* enrichment, ALA, EPA and AA contents (8.11, 11.16 and 4.54% respectively) were higher in algal oil enriched *Artemia*, whereas cod liver oil fed *Artemia* showed higher LA (7.11%), DHA (3.84%) and DPA (1.41%) levels. Juveniles fed on wild plankton showed higher EPA and DHA contents (9.49 and 21.45% respectively) but those fed on the algal oil enriched diet provided higher ALA and AA (8.14 and 3.51% respectively), as compared with the juveniles fed on other enriched diets.

Saturated fatty acid (Σ SFA) content was higher in algal oil (40.07%), followed by cod liver (30.53%) and olive oils (20.71%). Yeast showed higher percentage of monounsaturated fatty acid (Σ MUFA) (67.28%), and olive (53.35%), cod liver (35.12%) and algal oils (24.99%) showed lower content of Σ MUFA. Higher percentage of polyunsaturated fatty acids (Σ PUFA) was found in cod liver (32.31%), algal (32.16%) and olive oils (21.51%). Fatty acids such as SFA, MUFA and PUFA present in all media, enriched with live preys and juveniles were not significantly different from each other. According to the Pearson correlation coefficient, the SFA contents in enrichment medium were positively correlated with each other. The SFA present in yeast was significantly correlated with those present in algal ($r = 0.936$), cod liver ($r = 0.946$) and olive oils ($r = 0.936$) at 0.01 level. The MUFAs of cod liver oil were significantly correlated with algal oil ($r = 0.466$) at 0.05 level, while those present in olive oil were significantly correlated with algal ($r = 0.539$) and cod liver oils ($r = 0.807$) at the 0.01 level. In terms of PUFA, the algal oil was significantly correlated to cod liver oil ($r = 0.565$) at the 0.05 level.

In the enriched and non-enriched live prey, algal oil fed rotifers showed higher saturated fatty acids (38.10%) followed by cod liver oil fed rotifer (35.19%) and *Artemia* (34.12%). The monounsaturated fatty acids (Σ MUFA) were higher in the yeast fed *Artemia* (58.13%) and lowest in wild plankton (13.02%). The wild plankton showed highest polyunsaturated fatty acid content (Σ PUFA) (58.83%) followed by algal oil fed *Artemia* (41.01%) and olive oil fed rotifer (32.12%). The SFAs present in rotifers and wild plankton were significantly correlated to each other at the 0.01 level. In the case of MUFAs, the

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Table 2. Fatty acid profiles of enrichment media, enriched and non-enriched live preys.

Carbon chain	Fatty acid	Algal oil	Cod liver oil	Olive oil	Yeast	Rotifer enriched with algal oil	Rotifer enriched with cod liver oil	Rotifer enriched with olive oil	Rotifer enriched with yeast	Wild plankton	Rotifer without enrichment
C10:0	Capric acid	1.11	0.12	–	–	0.87	–	0.17	–	–	–
C11:0	Undecylic acid	1.32	0.31	–	0.15	0.11	0.13	–	0.14	0.17	–
C12:0	Lauric acid	1.2	0.93	0.13	0.14	0.53	0.11	0.18	0.13	0.13	0.17
C13:0	Tridecylic acid	1.32	0.81	0.11	0.61	1.11	0.17	0.13	0.51	0.11	0.11
C14:0	Myristic acid	3.5	4.1	0.51	1.32	0.59	2.71	1.82	1.21	3.11	2.13
C15:0	Pentadecyclic acid	0.7	1.11	–	0.14	2.81	0.11	0.13	0.17	1.81	1.11
C16:0	Palmitic acid	20.12	12	13	9.9	21.01	21.13	18.11	17.23	12.87	12.21
C17:0	Margaric acid	2.11	1.11	2.21	0.52	1.19	0.41	0.13	0.51	0.54	0.55
C18:0	Stearic acid	1.91	2.8	3.1	4.1	1.1	6.17	5.11	5.53	3.14	3.61
C19:0	Nonadecyclic acid	0.5	1.92	–	–	4.21	0.11	1.81	0.17	1.01	0.18
C20:0	Arachidic acid	1.12	0.93	0.62	–	0.81	0.43	0.71	1.00	2.11	0.41
C21:0	Heneicosanoic acid	0.62	0.52	0.51	0.17	0.71	0.35	0.31	0.63	0.18	0.53
C22:0	Behenic acid	0.82	0.89	0.31	–	0.63	0.73	0.42	0.12	0.25	–
C23:0	Tricosanoic acid	1.81	1.62	–	0.93	1.69	0.81	0.91	1.21	0.11	–
C24:0	Lignoceric acid	1.91	1.36	0.21	1.1	0.73	1.82	1.11	0.55	0.44	1.17
Σ of SFA		**40.07**	**30.53**	**20.71**	**19.08**	**38.1**	**35.19**	**31.05**	**29.11**	**25.98**	**22.18**
C14:1ω−3	Cis-3 Myristoleic acid	0.11	0.13	–	–	0.10	–	0.13	–	0.77	–
C14:1ω−5	Trans-5 Myristoleic acid	0.12	–	–	0.17	–	0.13	–	0.13	–	0.16
C14:1ω−7	Cis-7 Myristoleic acid	0.81	0.51	0.14	–	0.80	0.71	0.61	0.41	0.11	0.51
C15:1ω−6	Cis-6-Pentadecenoic	5.1	2.11	0.81	0.67	3.1	2.11	1.17	0.83	0.13	0.17
C16:1ω−5	Cis-5-Palmitoleic acid	0.32	0.31	0.31	0.16	0.11	0.17	0.61	0.71	3.13	0.8
C16:1ω−6	Cis-6-Palmitoleic acid	0.91	0.42	–	–	0.17	0.13	0.17	0.11	–	–
C16:1ω−7	Trans-7-Palmitoleic acid	0.82	4.11	0.91	10.13	3.33	6.91	8.12	7.12	–	6.51
C16:1ω−9	Trans-9-Palmitoleic acid	0.83	0.19	0.13	21.1	–	0.11	–	4.21	–	0.13
C17:1ω−7	Cis-7- Heptadecenoic acid	1.01	–	–	–	0.89	–	0.84	0.19	1.11	1.11
C17:1ω−8	Trans-8-Heptadecenoic acid	2.56	0.18	0.17	0.51	0.51	0.17	0.51	0.71	0.11	–
C18:1ω−5	Cis-5-Octadecenoic acid	1.11	0.4	0.31	0.16	0.51	0.15	0.64	1.21	0.18	0.11
C18:1ω−7	Cis-7-Octadecenoic acid	1.32	2.2	–	–	1.13	–	0.17	1.00	1.85	2.51
C18:1ω−9	Oleic acid	4.11	10.12	45.1	32.11	5.11	11.21	13.26	31.84	2.13	38.11
C19:1ω−8	Nonadecenoic acid	0.82	–	1	0.51	–	0.55	–	–	–	0.13
C20:1ω−7	Cis-7-Eicosenoic acid	0.11	0.52	–	–	0.78	0.13	0.19	–	–	0.18
C20:1ω−9	Cis-9-Eicosenoic acid	0.53	4.01	0.93	0.16	1.81	3.84	2.84	0.56	0.58	2.9
C20:1ω−11	Trans-7-Eicosenoic acid	0.90	1.35	0.53	0.51	–	0.84	0.16	–	0.43	0.11
C22:1ω−7	Trans-7-Docosenoic acid	0.52	5.4	1.21	–	–	3.31	1.81	0.14	–	–
C22:1ω−9	Cis-9-Docosenoic acid	0.31	0.32	0.17	0.16	1.11	0.71	0.61	–	0.19	1.11
C24:1ω−3	Cis-3-Tetracosenoic acid	0.72	0.94	–	–	–	0.14	0.13	–	0.17	0.18
C24:1ω−6	Cis-6-Tetracosenoic acid	0.81	0.81	0.51	0.61	0.49	–	1.01	0.18	–	0.53
C24:1ω−9	Trans-9-Tetracosenoic acid	1	0.4	0.31	0.21	0.85	1.01	0.29	0.62	0.33	0.14
Σ of MUFAs		**24.99**	**35.12**	**53.35**	**67.28**	**20.80**	**32.33**	**34.11**	**50.11**	**13.02**	**55.4**
C16:2ω−6	Hexa decenoic	0.11	0.16	0.61	1.12	1.11	1.10	–	1.10	–	–
C18:2ω−3	Trans-3-linoleic	0.13	0.51	0.17	3.01	–	0.17	0.81	1.12	0.13	1.13
C18:2ω−6	Linoleic	3.3	5	13	4	5.51	0.53	0.83	0.81	1.05	10.25
C18:3ω−3	Alfa-linolenic	8.12	1	1	1.1	4.13	12.11	11.11	2.00	2.57	1.16
C18:3ω−6	Gammalinoleic	0.52	0.42	0.52	–	0.13	0.57	0.41	1.00	0.17	–
C18:4ω−3	Stearidonic	0.43	2.41	0.41	0.71	0.17	0.13	0.43	0.84	1.11	–
C19:2ω−6	Octadecenoic	0.41	–	0.91	0.11	0.40	0.11	–	1.1	0.13	0.11
C20:2ω−6	Eicosadienoic	1	0.52	0.13	0.13	1.31	–	0.81	0.91	1.85	1.11
C20:3ω−6	Dihomogammalinoleic	0.81	0.81	0.19	–	1.10	0.53	0.63	0.64	1.98	1.32
C20:4ω−6	Arachidonic acid	3.62	0.7	0.51	0.17	5.12	0.49	0.19	0.17	0.75	0.11

Continued

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Table 2. Continued

Carbon chain	Fatty acid	Algal oil	Cod liver oil	Olive oil	Yeast	Rotifer enriched with algal oil	Rotifer enriched with cod liver oil	Rotifer enriched with olive oil	Rotifer enriched with yeast	Wild plankton	Rotifer without enrichment
C20:5ω−3	Eicosapentaenoic	10.11	8.41	0.11	0.10	8.12	3.32	4.21	4.10	16.19	2.21
C20:5ω−6	Cis-6 Eicosapentaenoic	0.13	0.91	1.11	0.12	–	2.81	3.13	0.16	–	–
C22:3ω−3	Docosatrienoic	1.11	1.51	1.51	0.11	0.17	0.54	0.16	0.69	1.01	0.11
C22:4ω−6	Docosatetraenoic	0.12	0.63	0.41	0.12	0.33	0.13	0.17	0.21	0.53	0.18
C22:5ω−3	Decosapentaenoic	0.11	1.2	0.82	1.1	1.00	0.1	0.81	1.16	1.78	1.35
C22:6ω−3	Docosahexaenoic	2.13	8.12	0.1	0.1	8.22	7.64	8.42	2.12	29.58	1.31
Σ of PUFAs		**32.16**	**32.31**	**21.51**	**12**	**36.82**	**30.28**	**32.12**	**18.13**	**58.83**	**20.35**
Σ PUFA ω−3		22.14	23.16	4.12	6.23	21.81	24.01	25.95	12.03	52.37	7.27
Σ PUFA ω−6		10.02	9.15	17.39	5.77	15.01	6.27	6.17	6.1	6.46	13.08
Σ HUFA ω−3		12.24	16.53	0.21	0.2	16.34	10.96	12.63	6.22	45.77	3.52
Σ ω−3/ω−6		2.21	2.53	0.24	1.08	1.45	3.83	4.21	1.97	8.11	0.56
Σ ω−6/ω−3		0.45	0.4	4.22	0.93	0.69	0.26	0.24	0.51	0.12	1.8
C14:0Iso		0.13	0.11	–	–	–	–	0.10	–	–	–
C15:0Iso		0.11	–	0.52	–	0.78	–	–	0.17	–	0.17
C15:0Anteiso		0.31	0.19	0.41	–	0.71	0.17	0.18	–	0.17	–
C16:0Iso		0.32	–	0.51	0.17	–	–	0.14	0.18	–	0.11
C17:0Iso		0.21	0.43	0.42	0.61	0.63	0.61	0.51	–	0.13	–
C17:0Anteiso		0.13	0.11	–	–	0.41	–	0.61	0.91	0.11	–
C19:0Iso		0.37	–	0.63	0.13	0.58	0.54	–	0.13	0.51	0.43
C20:0Iso		0.51	0.52	0.81	0.11	0.13	0.11	0.56	–	0.41	–
C20:0Anteiso		0.17	0.28	0.54	0.12	0.65	0.57	–	0.61	–	0.24
Σ of Branched		2.26	1.64	3.84	1.14	3.89	2.00	2.10	2.00	1.33	0.95
Unknown & Others		0.52	0.40	0.59	0.5	0.38	0.20	0.62	0.65	0.84	0.5

wild plankton was not significantly correlated with other enriched rotifers at the 0.01 level. The PUFA level in wild plankton was significantly correlated with cod liver oil ($r = 507$) and olive oil fed rotifers ($r = 601$) at the 0.05 level. In enriched *Artemia*, SFAs in all the enrichments were significantly correlated with each other at the 0.01 level and MUFA content in the olive oil fed *Artemia* was significantly correlated with *Artemia* enriched with algal oil and cod liver oil at the 0.01 level. PUFA level in the yeast fed *Artemia* was significantly correlated with *Artemia* enriched by cod liver and olive oil at the 0.01 level.

In the juveniles, saturated fatty acids were higher in those fed on algal oil enriched diet (31.13%) followed by wild plankton (29.05%). The lowest SFA percentage was observed in the control group (27.16%). MUFA was higher in the juveniles fed on non-enriched diet (43.5%) followed by those fed on olive oil enriched diet (34.53%) and the lowest levels were observed in the wild plankton fed juveniles (23.43%). Polyunsaturated fatty acids were observed at the highest level in the wild plankton fed juveniles (45.4%), followed by the algal enriched diet fed juveniles (39.91%) and were lowest in the control group (28.2%). The SFAs, MUFAs and PUFAs of the juveniles which were fed with all enriched diets and wild plankton showed significant correlation with one another at the 0.01 level.

Among the four enrichment media, highest percentage of omega-3 ($\sum \omega-3$) was recorded in cod liver oil (23.16%) while highest omega-6 ($\sum \omega-6$) was in olive oil (17.39%). In the case of enriched and non-enriched live preys, $\sum \omega-3$ was higher in wild plankton (52.37%) and higher percentage of $\sum \omega-6$ was in *Artemia* enriched with cod liver oil (16.51%). After 30 days of enrichment, higher percentage of $\sum \omega-3$ and $\sum \omega-6$ was found in the juveniles fed on wild plankton (40.77%) and yeast (15.73%), respectively. In the case of enrichment media, cod liver oil has highest $\sum$ HUFA-3 content (16.53%) than others. While considering HUFA-3 in live preys, highest content was in wild plankton (45.77%). After completing the enrichment experiments, the wild plankton fed juveniles showed maximum $\sum$ HUFA-3 content (30.94%) when compared with others.

MINERAL ANALYSIS

Levels of trace minerals in juveniles fed on enriched and non-enriched diets are shown in Table 5. Levels of Cu (92 $\mu g\ g^{-1}$ d.w.), Mg (11,440 $\mu g\ g^{-1}$ d.w.), Mn (105 $\mu g\ g^{-1}$ d.w.) and Zn (1371 $\mu g\ g^{-1}$ d.w.) were higher in juveniles fed on cod liver oil enriched diet compared with those fed on other diets, while Fe level (2294.6 $\mu g\ g^{-1}$ d.w.) was higher in juveniles fed on wild plankton. Each type of mineral present in the body of the fish at various enrichment groups was significantly different from other ($F_{(4,\ 25)} = 4.939$, $P < 0.05$). According to the Pearson correlation coefficient, Mg present in the juveniles was significantly correlated with Cu and Zn at the 0.05 level while Zn was significantly correlated to Cu at the 0.01 level.

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Table 3. Fatty acid profiles of enriched and non-enriched live prey.

Carbon chain	Fatty acid	*Artemia* enriched with algal oil	*Artemia* enriched with cod liver oil	*Artemia* enriched with olive oil	*Artemia* enriched with yeast	*Artemia* without enrichment
C10:0	Capric acid	0.11	–	–	–	–
C11:0	Undecyclic acid	0.76	0.17	0.16	0.11	–
C12:0	Lauric acid	0.89	2.18	0.11	0.13	0.13
C13:0	Tridecyclic acid	0.87	1.12	0.13	0.11	0.12
C14:0	Myristic acid	2.15	2.11	0.19	1.53	1.32
C15:0	Pentadecyclic acid	0.19	0.79	1.13	1.21	1.11
C16:0	Palmitic acid	16.16	13.91	11.13	7.11	9.16
C17:0	Margaric acid	1.11	1.91	1.93	1.81	0.18
C18:0	Stearic acid	0.91	3.78	5.11	3.22	5.13
C19:0	Nonadecyclic acid	0.40	2.54	1.12	1.31	–
C20:0	Arachidic acid	0.79	1.12	0.97	0.84	1.21
C21:0	Heneicosanoic acid	0.97	1.34	0.81	0.52	0.93
C22:0	Behenic acid	1.13	1.21	0.13	0.48	0.81
C23:0	Tricosanoic acid	0.10	1.34	0.87	0.61	1.11
C24:0	Lignoceric acid	0.99	0.60	0.36	0.11	1.92
Σ of SFA		**27.53**	**34.12**	**24.15**	**19.10**	**23.13**
C14:1ω−3	Cis-3 Myristoleic acid	–	–	–	–	–
C14:1ω−5	Trans-5 Myristoleic acid	0.17	0.15	0.14	0.19	–
C14:1ω−7	Cis-7 Myristoleic acid	0.18	0.41	0.11	0.13	0.19
C15:1ω−6	Cis-6-Pentadecenoic	0.18	2.10	0.43	0.31	0.17
C16:1ω−5	Cis-5-Palmitoleic acid	0.81	0.17	0.51	0.84	0.13
C16:1ω−6	Cis-6-Palmitoleic acid	–	0.21	0.49	0.91	0.54
C16:1ω−7	Trans-7-Palmitoleic acid	9.53	6.00	4.12	4.81	8.62
C16:1ω−9	Trans-9-Palmitoleic acid	5.61	5.21	5.63	20.31	10.11
C17:1ω−7	Cis-7- Heptadecenoic acid	0.19	–	0.17	0.16	0.17
C17:1ω−8	Trans-8- Heptadecenoic acid	0.18	0.19	0.81	0.15	–
C18:1ω−5	Cis-5-Octadecenoic acid	–	0.17	0.13	0.84	0.41
C18:1ω−7	Cis-7-Octadecenoic acid	1.31	1.11	1.21	3.33	2.21
C18:1ω−9	Oleic acid	10.11	18.13	28.11	23.11	22.13
C19:1ω−8	Nonadecenoic acid	0.11	0.13	0.12	0.13	–
C20:1ω−5	Cis-5-Eicosenoic acid	0.53	–	0.17	0.16	0.51
C20:1ω−7	Cis-7-Eicosenoic acid	0.19	0.49	0.41	0.43	1.13
C20:1ω−9	Cis-9-Eicosenoic acid	–	0.17	0.52	0.51	–
C20:1ω−11	Trans-7-Eicosenoic acid	0.13	0.16	0.86	0.43	0.18
C22:1ω−7	Trans-7-Docosenoic acid	–	0.19	1.33	0.19	0.14
C22:1ω−9	Cis-9-Docosenoic acid	0.11	–	0.81	0.17	1.21
C24:1ω−3	Cis-3-Tetracosenoic acid	–	0.56	0.71	0.21	0.13
C24:1ω−6	Cis-6-Tetracosenoic acid	0.54	0.43	0.13	0.13	–
C24:1ω−9	Trans-9-Tetracosenoic acid	0.25	0.16	0.26	0.68	0.13
Σ of MUFA s		**30.13**	**36.14**	**47.18**	**58.13**	**48.11**
C16:2ω−6	Hexadecenoic	0.13	0.17	0.13	0.37	0.41
C18:2ω−3	Trans-3-linoleic	0.17	–	2.11	1.11	–
C18:2ω−6	Linoleic	1.34	7.11	6.12	3.16	6.16
C18:3ω−3	Alpha-linolenic	8.11	2.92	3.00	1.92	3.12
C18:3ω−6	Gammalinoleic	1.92	5.11	4.12	2.93	4.32
C18:4ω−3	Stearidonic	4.31	0.78	0.84	0.91	1.68
C19:2ω−6	Octadecenoic	0.82	0.11	0.19	0.74	–
C20:2ω−6	Eicosadienoic	0.54	0.17	0.56	0.36	0.18
C20:3ω−6	Dihomogammalinoleic	0.83	0.54	0.44	0.84	1.11
C20:4ω−6	Arachidonic acid	4.54	2.56	0.31	1.99	1.36
C20:5ω−3	Eicosapentaenoic	11.16	2.11	3.11	0.83	0.18
C20:5ω−6	Cis-6 Eicosapentaenoic	0.17	0.74	0.91	0.96	0.81
C22:3ω−3	Docosatrienoic	0.63	0.18	1.10	1.34	0.14
C22:4ω−6	Docosatetraenoic	1.84	–	0.12	–	1.96
C22:5ω−3	Decosapentaenoic	1.07	1.41	0.93	0.6	0.11
C22:6ω−3	Docosahexaenoic	3.43	3.84	2.13	0.71	0.32
Σ of PUFAs		**41.01**	**27.75**	**26.12**	**18.77**	**21.86**
Σ PUFA ω−3		**28.88**	**11.24**	**13.22**	**7.42**	**5.55**
Σ PUFA ω−6		12.13	16.51	12.9	11.35	16.31

Continued

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Table 3. Continued

Carbon chain	Fatty acid	*Artemia* enriched with algal oil	*Artemia* enriched with cod liver oil	*Artemia* enriched with olive oil	*Artemia* enriched with yeast	*Artemia* without enrichment
Σ HUFA ω−3		14.59	5.95	5.24	1.54	0.5
Σ ω−3/ω−6		2.38	0.68	1.02	0.65	0.34
Σ ω−6/ω−3		0.42	1.47	0.98	1.53	2.94
C14:0Iso		–	–	0.17	–	–
C15:0Iso		–	0.16	–	0.17	–
C15:0Anteiso		0.17	–	0.64	0.13	0.81
C16:0Iso		–	0.55	–	0.18	0.17
C17:0Iso		0.16	–	0.53	–	0.13
C17:0Anteiso		–	0.19	–	0.17	0.14
C19:0Iso		0.19	0.44	–	0.19	0.5
C20:0Iso		0.33	0.33	0.11	–	0.18
C20:0Anteiso		0.25	0.33	0.37	0.30	0.38
Σ of Branched		1.10	2.00	1.82	1.14	2.31
Unknown & Others		0.23	–	0.73	2.86	4.59

Hormone levels

Levels of T_3, T_4 and TSH were determined in 30 DAH juveniles and the results are shown in Table 6. T_3, T_4 and TSH levels were higher in the juveniles fed on wild plankton (23.6 pg mL^{-1}, 22.8 ng dL^{-1} and 45.2 μlU mL^{-1} respectively). In enrichments, T_3 level was higher in the juveniles fed on algal oil enriched diet (9.4 pg mL^{-1}) and it was comparatively lower in the juveniles fed on cod liver (9 pg mL^{-1}) and olive oil enriched diets (7.8 pg mL^{-1}). Higher T_4 level was recorded in the juveniles fed on algal oil (7.6 ng dL^{-1}) and it was subsequently lowered in the juveniles fed on cod liver (6.8 ng dL^{-1}) and olive oils (5.8 ng dL^{-1}). Compared with T_3 and T_4 levels, TSH content in the juveniles was far higher in all the treatments. The TSH content was highest in the juveniles fed with yeast (29 μlU ml^{-1}), followed by algal (21 μlU ml^{-1}) and olive oils (19.6 μlU ml^{-1}). Levels of T_3, T_4 and TSH in *A. percula* juveniles were significantly different from each other ($F_{(2,15)} = 5.639$, $P < 0.05$). According to the Pearson correlation coefficient, T_3 level is significantly correlated with T_4 at the 0.01 level and in TSH it was significantly correlated with T_3 and T_4 at the 0.05 level.

DISCUSSION

The rate of biomass gain and duration of larval period of a marine fish can be significantly affected by diet. Fish have shown specific events during the larval phase and these can have a profound impact on the number of successful individuals reaching the juvenile stage (Avella *et al.*, 2010). Individuals can either allocate the energy towards growth or allocate it in important biological processes such as metamorphosis (Metcalfe & Monaghan, 2001). In marine fish larvae, a proper diet is essential to satisfy high energy demand (Tocher *et al.*, 2003), promote growth and metamorphosis. The majority of this energy derives from fatty acid oxidation and thus they are particularly sensitive to lipid deficiency (Izquierdo *et al.*, 2008). Several studies have reported that lipid enriched live prey may improve the survival and growth and reduce time to reach metamorphosis of fish larvae (Olivotto *et al.*, 2008, 2011).

In the current study, the larvae fed on enriched live prey metamorphosed at 9 DAH which was earlier than the control group (12 DAH in control), where they fed on a non-enriched diet. But larvae fed on wild plankton metamorphosed at 7 DAH, thus it is clear that HUFAs were able to improve growth performance and shorten the larval phase in percula clownfish larvae fed on enriched live prey. Juveniles which fed on wild plankton showed maximum $\sum$ HUFA-3 content (30.94%) compared with others.

The present study shows that live wild plankton (majority of copepods) is an essential live feed for clownfish larvae and juveniles. This study has comprehensively proved the superiority of copepods over rotifer and *Artemia*. Recent studies have suggested that carotenoids including β-carotene, astaxanthin and canthaxanthin are potent antioxidants for *in-vitro* membrane models and they work synergistically with vitamin E (Bell *et al.*, 2000). In the present study, higher carotenoid content in clownfish juveniles (9.75 μg g^{-1}) which fed on mixed live wild zooplankton may be attributed to a high amount of astaxanthin. The experiment clearly indicated that the astaxanthin present in marine copepods not only enhances the pigmentation but its growth as well. However, the larvae fed on *Artemia* nauplii have not shown much growth and it might be due to the lack of astaxanthin and other fatty acids. It is known that the nutritional strength that includes pigments (astaxanthin) besides essential fatty acids of copepods may be an explanation for higher growth (Rajkumar & Vasagam, 2006). In the present study, considering HUFA-3 in live prey, highest content was observed in wild plankton (45.77%). The nutritional quality of *Artemia* nauplii is often unpredictable, thus making a quality strain is costly. Hence, several enrichment diets such as micro-algae, highly unsaturated fatty acid (HUFA)-modified yeast, compound diets with coated micro-particles, oil-based emulsions and micro-encapsulated feeds have been successfully used. The supplement of HUFAs positively influenced the growth and metabolism, inducing faster development, a shorter larval phase and better survival.

A number of beneficial effects have been linked to copepod nutrient composition in relation to early larval nutrition. In particular, emphasis has been put on lipid composition,

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Table 4. Fatty acid profiles of fish fed with enriched and non-enriched live prey.

Carbon chain	Fatty acid	Juveniles fed on algal oil enriched rotifer and *Artemia*	Juveniles fed on cod liver oil enriched rotifer and *Artemia*	Juveniles fed on olive oil enriched rotifer and *Artemia*	Juveniles fed on yeast enriched rotifer and *Artemia*	Juveniles fed on wild plankton	Juveniles fed on non-enriched diet
C10:0	Capric acid	0.13	–	–	–	–	–
C11:0	Undecyclic acid	0.14	0.17	–	0.11	–	0.17
C12:0	Lauric acid	0.54	0.18	0.13	1.78	1.13	–
C13:0	Tridecyclic acid	0.81	1.11	0.81	0.54	1.01	1.81
C14:0	Myristic acid	2.19	2.13	0.62	2.23	6.17	3.11
C15:0	Pentadecyclic acid	0.17	0.17	1.31	0.19	1.61	1.13
C16:0	Palmitic acid	20.16	14.13	14.62	15.64	11.13	12.12
C17:0	Margaric acid	1.13	1.17	1.61	0.61	1.11	1.11
C18:0	Stearic acid	0.97	4.21	2.71	4.32	4.51	1.31
C19:0	Nonadecyclic acid	1.45	1.19	1.91	0.19	1.17	1.98
C20:0	Arachidic acid	0.84	0.28	0.84	–	0.59	1.11
C21:0	Heneicosanoic acid	0.64	0.68	0.51	0.18	0.17	0.19
C22:0	Behenic acid	0.72	0.71	0.11	–	0.28	1
C23:0	Tricosanoic acid	0.61	1.27	1.00	0.84	0.17	0.81
C24:0	Lignoceric acid	0.63	1.31	1.59	0.54	–	1.31
Σ of SFA		**31.13**	**28.71**	**27.77**	**27.17**	**29.05**	**27.16**
C14:1ω−3	Cis-3 Myristoleic acid	0.11	–	–	–	0.13	0.16
C14:1ω−5	Trans-5 Myristoleic acid	0.17	0.61	0.13	0.19	0.14	–
C14:1ω−7	Cis-7 Myristoleic acid	0.16	0.18	0.14	0.18	–	0.11
C15:1ω−6	Cis-6-Pentadecenoic	2.16	1.71	0.84	0.34	–	–
C16:1ω−5	Cis-5-Palmitoleic acid	0.15	0.55	1.21	1.00	5.11	–
C16:1ω−6	Cis-6-Palmitoleic acid	1.17	0.64	1.31	1.21	0.17	1.19
C16:1ω−7	Trans-7-Palmitoleic acid	2.13	4.33	5.12	6.12	–	1.17
C16:1ω−9	Trans-9-Palmitoleic acid	3.12	0.71	1.84	4.13	0.19	1.33
C17:1ω−7	Cis-7- Heptadecenoic acid	0.16	–	1.21	0.84	4.56	0.81
C17:1ω−8	Trans-8- Heptadecenoic acid	–	0.68	0.97	0.73	0.13	0.59
C18:1ω−5	Cis-5-Octadecenoic acid	0.19	1.77	1.61	0.19	–	0.51
C18:1ω−7	Cis-7-Octadecenoic acid	0.64	1.81	0.84	0.81	1.11	1.11
C18:1ω−9	Oleic acid	9.12	15.44	13.61	14.61	9.17	25.11
C19:1ω−8	Nonadecenoic acid	0.51	0.91	0.84	0.11	0.17	–
C20:1ω−5	Cis-5-Eicosenoic acid	0.62		1.71	0.84	–	2.81
C20:1ω−7	Cis-7-Eicosenoic acid	0.17		0.91	0.71	0.89	0.93
C20:1ω−9	Cis-9-Eicosenoic acid	–		0.81	0.33	0.63	1.16
C20:1ω−11	Trans-7-Eicosenoic acid	–	0	0.21	0.21	–	–
C22:1ω−7	Trans-7-Docosenoic acid	0.64	0.13	0.14	0.17	0.79	1.13
C22:1ω−9	Cis-9-Docosenoic acid	0.51	0.41	0.11	–	–	1.3
C24:1ω−3	Cis-3-Tetracosenoic acid	0.43	0.11	0.13	0.84	0.13	0.51
C24:1ω−6	Cis-6-Tetracosenoic acid	1.24	0.12	–	0.19	0.11	0.83
C24:1ω−9	Trans-9-Tetracosenoic acid	0.51	0.17	0.84	0.61	–	2.74
Σ of MUFA s		**23.91**	**30.28**	**34.53**	**34.36**	**23.43**	**43.5**
C16:2ω−6	Hexadecenoic	–	0.11	0.16	0.13	0.17	0.18
C18:2ω−3	Trans-3-linoleic	–	–	0.14	–	0.14	1.11
C18:2ω−6	Linoleic	3.31	4.12	6.11	5.89	0.53	5.19
C18:3ω−3	Alpha-linolenic	8.14	4.12	3.12	2.77	5.43	0.74
C18:3ω−6	Gammalinoleic	0.84	1.31	1.81	0.81	0.11	–
C18:4ω−3	Stearidonic	1.12	2.23	1.71	1.33	0.17	0.63
C19:2ω−6	Octadecenoic	0.94	–	–	0.84	0.13	–
C20:2ω−6	Eicosadienoic	–	1.31	0.81	1.21	1.11	0.91
C20:3ω−6	Dihomogammalinoleic	0.53	1.62	0.68	0.91	1.03	2.84
C20:4ω−6	Arachidonic acid	3.51	2.71	1.81	2.24	0.19	2.13
C20:5ω−3	Eicosapentaenoic	4.12	3.16	2.47	2.31	9.49	2.12
C20:5ω−6	Cis-6 Eicosapentaenoic	0.19	2.17	0.64	1.92	1.03	1.17
C22:3ω−3	Docosatrienoic	0.36	1.81	1.11	0.96	2.06	2.1
C22:4ω−6	Docosatetraenoic	1.00	1.36	0.91	1.78	0.33	1.79
C22:5ω−3	Decosapentaenoic	1.72	0.84	1.97	1.98	2.03	2.18
C22:6ω−3	Docosahexaenoic	14.13	10.85	12.24	12.11	21.45	5.11
Σ of PUFAs		**39.91**	**37.72**	**35.69**	**37.19**	**45.4**	**28.2**

Continued

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Table 4. Continued

Carbon chain	Fatty acid	Juveniles fed on algal oil enriched rotifer and *Artemia*	Juveniles fed on cod liver oil enriched rotifer and *Artemia*	Juveniles fed on olive oil enriched rotifer and *Artemia*	Juveniles fed on yeast enriched rotifer and *Artemia*	Juveniles fed on wild plankton	Juveniles fed on non-enriched diet
Σ PUFA ω−3		29.59	23.01	22.76	21.46	40.77	13.99
Σ PUFA ω−6		10.32	14.71	12.93	15.73	4.63	14.21
Σ HUFA ω−3		18.25	14.01	14.71	14.42	30.94	7.23
Σ ω−3/ω−6		2.87	1.56	1.76	1.36	8.81	0.99
Σ ω−6/ω−3		0.35	0.64	0.57	0.73	0.11	1.02
C14:0Iso		–	–	0.11	–	0.17	0.18
C15:0Iso		–	–	–		–	0.19
C15:0Anteiso		–	–	0.14	0.13	0.54	0.17
C16:0Iso		–	–	–		–	1.11
C17:0Iso		0.15	0.81	–	–	0.43	0.13
C17:0Anteiso		0.19	–	0.21		–	–
C19:0Iso		0.56	0.43	–	0.11	–	0.59
C20:0Iso		0.19	–	–		–	0.42
C20:0Anteiso		0.15	0.20	–	0.17	0.55	–
Σ of Branched		1.24	1.44	0.46	0.41	1.69	2.79
Unknown & Others		3.81	1.85	1.55	0.71	0.43	1.35

content and ratio of the polyunsaturated fatty acids (PUFA) such as docosahexaenoic (DHA), eicosapentaenoic (EPA) and arachidonic (ARA) acids. Using copepods as feed compared with intensive rearing of cod larvae with rotifers has indicated a significant nutritional influence on juvenile quality and growth (Imsland *et al*., 2006). Recent advances in knowledge about lipid and fatty acid requirements of marine fish larvae have pointed out the importance of phospholipids, DHA, EPA, ARA and the ratios of PUFA for optimal lipid digestion, normal larval development, survival and growth, and stress tolerance (Stottrup, 2003). Considering the fraction of phospholipids relative to total lipid, copepods are rich in phospholipids (57–63%) compared with rotifers (40%) and particularly compared with *Artemia* (15–20%).

Depending on species, microalgae can produce different lipids, hydrocarbons and other complex oils (Banerjee *et al*., 2002). The microalgae can be a suitable alternative because algae are the most efficient biological producers of oil and a versatile biomass source and may soon be one of the most important renewable fuel crops (Campbell, 1997). Average oil yield from microalgae can be 10–20 times higher than the yield obtained from seeds and vegetable oils (Chisti, 2007). Pankaj Kumar *et al*. (2011) extracted 10–15% of oil (total yield) from six species of algae and they reported that all the micro-algal lipids were mainly composed of 50–60% unsaturated fatty acids. These findings match the results of the present study, in which the maximum oil extracted from *Nannochloropsis salina* was 19.52% and also contributed with 55% unsaturated fatty acids.

Agh & Sorgeloos (2005) reported that the algae was used for boosting the fatty acids like eicosapentaenoic (EPA 20:5*n*-3) and docosahexaenoic acids (DHA 22:6*n*-3) in rotifers, *B. plicatilis*. A common practice is to enrich the rotifers overnight and feed the fish larvae the following morning. The implications of these results are concerned with general health and well-being of the fish larvae and have been significantly improved by the feeding with algae enriched rotifers.

Table 5. Minerals (macro and micro) in juveniles fed on enriched and non-enriched diets.

Samples	Metals ($\mu g\ g^{-1}$ d.w.)				
	Cu	Fe	Mg	Mn	Zn
Juveniles fed with non-enriched diet	39.69	1669.07	1573.2	37.01	146.19
Juveniles fed with algal oil enriched diet	49	1187	1424	82	157
Juveniles fed with cod liver oil enriched diet	92	1811	11,440	105	1371
Juveniles fed with olive oil enriched diet	77	1457	4892	67.5	1122.5
Juveniles fed with yeast enriched diet	45	1734	1625	57	162
Juveniles fed with wild plankton	34.05	2294.6	2313.51	56.62	178.65

Table 6. Free Triiodothyronine (T3), Thyroxine (T4) and Thyroid Stimulating Hormones (TSH) content in *A. percula* juveniles (each weighed approximately 15–50 mg) fed on enriched and non-enriched diets.

	T_3 (pg ml^{-1})	T_4 (ng dl^{-1})	TSH (μIU ml^{-1})
Algal oil fed juveniles	9.4	7.6	21.0
Cod liver oil fed juveniles	9.0	6.8	17.4
Olive oil fed juveniles	7.8	5.8	19.6
Yeast fed juveniles	7.2	5.4	29.0
Wild plankton fed juveniles	23.6	22.8	45.2
Control juvenile	6.6	5.1	12.8

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According to previous reports, algae enriched rotifers can enhance the development of larval growth compared with rotifers enriched by PUFA (Immanuel *et al.*, 2007).

Fish oil is used for fish feed mainly because it offers a wide range of fatty acid classes, including long chain $\omega-3$ PUFA, that contribute energy, growth and reproductive demands of the fish. Among the four enrichment media used in the present study, highest percentage of $\sum \omega-3$ was recorded in cod liver oil (23.16%) when compared with algal, olive oils and yeast. However, the level of $\omega-3$ PUFA in fish oil varies depending on species, extraction procedure and storage conditions. Consequently, the marine oils where the DHA levels are particularly high due to its origin in specific fish tissues (cod liver and tuna orbital oil), or through special extraction procedures (silage, cold acetone) have been recommended in broodstock diets and larval rearing enrichment preparations (Sargent *et al.*, 1999).

Trace minerals are essential chemical elements involved in the normal metabolism of fish. The information currently available is very patchy so a detailed mineral budget is yet to be worked out and more research has to be done on the uptake, function and biological availability of many minerals. In the present study, levels of Cu (92 $\mu g\ g^{-1}$ d.w.), Mg (11,440 $\mu g\ g^{-1}$ d.w.), Mn (105 $\mu g\ g^{-1}$ d.w.) and Zn (1371 $\mu g\ g^{-1}$ d.w.) were higher in juveniles fed on cod liver oil enriched diet, while Fe level (2294.6 $\mu g\ g^{-1}$ d.w.) was higher in juveniles fed on wild plankton. In soft tissues, macro-minerals such as Mg occur both intra- and extracellularly and are essential for maintenance of intra- and extracellular homeostasis in fish (Moyle & Cech, 1988). Seawater typically contains high levels of magnesium (1350 $mg\ L^{-1}$) and it is excreted by marine fish, resulting in blood levels lower than that of the external medium (Davis, 1996).

Copper is important for animals as it is involved in the activity of enzymes such as cytochrome oxidase, superoxide dismutase, lysyl oxidase, dopamine hydroxylase and tyrosinase. In addition, copper-proteins and chelates also have metabolic roles. In the present study, Cu content in the fish in all the experimental groups ranged between 34.05 and 92 $\mu g\ g^{-1}$. Levels of manganese varied from 37.01 to 105 $\mu g\ g^{-1}$. Manganese is important for fish and is widely distributed in fish and animal tissues. Mitochondria have a greater concentration of manganese than cytoplasm or other cell organelles. Manganese is necessary for the normal functioning of the brain and for proper lipid and carbohydrate metabolism. An inadequate supply of manganese usually results in retardation of growth.

Zinc is an important trace element in fish nutrition as it is involved in various metabolic pathways. It exists in marine and fresh waters and can be utilized by all living aquatic organisms. Growth of rotifers under artificial conditions such as those found in the sites of marine finfish hatcheries may lead to inadequate concentration of zinc and other trace elements because the variety of foods available under these circumstances is limited. In the present study, higher content of Zn (1371 $\mu g\ g^{-1}$) was found in juveniles fed on the cod liver oil enriched diet.

Iron has an active part in oxidation/reduction reactions and electron transport, associated with cellular respiration. It is found bound to proteins such as haem, in enzymes such as microsomal cytochromes, catalase, etc., and in non-haem compounds such as transferrin, ferritin and flavin iron enzymes. Haemoglobin occurs in erythrocytes while transferrin is found in plasma; the latter is the principal carrier of iron in blood. The iron content of fish is very low compared with that of mammals (Van Dijk *et al.*, 1975). Information on absorption and metabolism of iron in fish is very limited, but the process is generally the same as in other vertebrates. In the present study, Fe content (2294.6 $\mu g\ g^{-1}$ d.w.) was higher in the juveniles fed on wild plankton and among all experimental groups it ranged between 2294.6 and 1187 $\mu g\ g^{-1}$ d.w.

It is well established that hormonal secretion and action of the thyroid hormones and those of the GH/IGF axis are interdependent (Akin *et al.*, 2009). Thyroid hormone influences growth in part by increasing the GH/IGF secretion that, in turn, affects growth and function of the thyroid as well as its metabolism (Akin *et al.*, 2009). Several studies have been conducted on alterations of the GH/IGF axis in animals showing hypothyroidism, with the main alterations reported in hypothyroidism being related to low IGF levels and slower growth (Akin *et al.*, 2009). Similar results were also obtained in this study; which reveals highest levels of IGFs were always associated with highest free T3, T4 and TSH contents. Solbakken *et al.* (1999) reported that thyroxine is a mediator of metamorphosis of the Atlantic halibut, affecting growth, skeletal formation, pigmentation and number and size of thyroid follicles.

CONCLUSION

In the present study, it was found that HUFA was able to improve the growth performance and shorten the larval phase in *A. percula* larvae fed with enriched live prey. The study also revealed the potential of wild zooplankton and algal oil enriched rotifers and *Artemia*, as an essential live feed for clownfish larvae and juveniles. It can be stated that during the larval phase, survival may be strongly influenced by the energy status of the larvae. Thus the fatty acids provided through live prey are essential to satisfy high energy demand and are required to promote growth.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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