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Production and use of two marine zooplanktons, *Tigriopus japonicus* and *Diaphanosoma celebensis*, as live food for red sea bream *Pagrus major* larvae

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ABSTRACT**:** We evaluated the effectiveness of two representative marine zooplanktons, harpacticoid copepod *Tigriopus japonicus* and euryhaline cladoceran *Diaphanosoma celebensis* as live food for red sea bream *Pagrus major* larvae. Chicken-dropping extract (CDE) was applied to both zooplankton cultures for improving population growth. Population growth of both animals was significantly enhanced by CDE supplementation (at 1 or 2 ml/l). The highest amount of DHA and higher DHA/EPA ratio was detected in *T. japonicus*, whereas *D. celebensis* showed similar values to that of *Artemia*. Effectiveness of both animals as live food was tested by rearing red sea bream larvae for 28 days and compared with that of *Artemia*. There were no significant differences in total length (8.6±1.1-8.7±0.7 mm) and wet weight (8.2±0.3-9.4±0.1 mg) among fish larvae received three different zooplanktons. Survival rate was significantly higher with *T. japonicus* (39.4±3.1%) than *D. celebensis* (20.8±3.8%) and *Artemia* (16.7±9.8%). Viability was significantly higher in fish fed with *T. japonicus* (60.0±27.8%) and *D. celebensis* (60.0±32.2%) than those with *Artemia* (44.4±12.3%). Fish fed with *T. japonicus* contained higher n-3 highly unsaturated fatty acids than those with *D. celebensis* and *Artemia*. It is concluded that *T. japonicus* and *D. celebensis* have high potential as live food for marine fish larviculture.

KEY WORDS**: copepoda, cladocera, red sea bream, larviculture, growth, survival.**

INTRODUCTION

Food web of hydrosphere utilizes various zooplanktons as energy transporter from photosynthetic sources to series of consumers, e.g. larval animals. Among the zooplanktons in the marine ecosystem, monogonont rotifer *Brachionus plicatilis* sp. complex are widely applied to the commercial hatchery facilities as an initial live food mainly because of their small size which is suitable for the larvae, rapid population growth, and ease to be cultured and nutritionally fortified. Once the larvae are in the advanced stage, brine shrimp (*Artemia* spp.) is also generally supplied to larval animals associated with development [1, 2] because of its convenience in use (i.e., cyst usage to reduce labor-intensive live food availability) and good nutritional value [3]. In spite of the long history of using *Artemia*, many challenges still remain. At present, the most marketed cysts of *Artemi*a are from the Great Salt Lake (GSL), and thus its provision is unpredictable in terms of demand, harvest, cost and nutritional values [4]. Based on these issues, there is a growing interest on the use of other zooplanktons and the need to establish a method of mass culturing them in the hatchery [5].

Copepods are major part of the diet for larval animals in the pelagic food chain and are generally known to match the nutritional requirements of the predators, and have higher nutritional value compared to rotifers (*Brachionus* spp.) and *Artemia* [6-8]. Interest in copepod as a live food for aquaculture has grown since 1980's. Harpacticoid copepod *Tigriopus japonicus* can be cultured at higher density compared to other copepod species and thrive in harsh environmental conditions [9-11]. In addition to these biological characteristics, relatively small size (1 mm of adult body length) zooplanktons attracts attentions for usability as a live food [12, 13], while its epibenthic habitat remained a problem to extend for aquaculture facilities targeted marine fish larvae [14].

Cladocerans comprised the natural diet for many brackish and freshwater larval animals [15] and due to their parthenogenetic reproduction, rapid propagation is possible. The brackishwater cladoceran, *Diaphanosoma celebensis* has the similar size distribution to *Artemia* and strong tolerance to salinity variations [16, 17]. Based on these perspectives, the studies for its application to the larviculture have been tried extensively [18-20].

Seedling production in aquaculture is usually confronted by the cost of producing enough and highly nutritious live foods e.g. zooplankton. To answer this issue, organic fertilizers such as animal manures were suggested as a booster of zooplankton population growth [21, 22] and this method is proven to be useful in many developing countries [23]. Among animal manures, chicken manure is preferred because it is easily soluble and contains high level of nitrogen, phosphorus and potassium [24, 25]. It is indeed known to enhance population growth of zooplankton populations in fishpond setting [26]. In this study, we tested the use of chicken-dropping extracts (CDE) to enhance the population growth of two zooplanktons: *T. japonicus* and *D. celebensis* which have high potential as a live food for intensive larviculture. The mass cultured zooplanktons were fed to red sea bream *Pagrus major* larvae, and growth and survival of the larvae was compared to those fed on *Artemia franciscana* to elucidate the qualification of those zooplanktons as a live food.

MATERIALS AND METHODS

CDE effects on the population growth of zooplanktons

The copepod *T. japonicus* was cultured in 800 ml of glass beaker containing 800 ml of natural sea water (34 ppt) with initial density at 0.4 ind./ml (total 320 individuals consisted of 160 nauplii and 160 copepodites). Copepods were fed on vitamin B_{12} enriched *Chlorella vulgaris* (Super fresh chlorella-V12, Chlorella Industry Co. Ltd., Fukuoka, Japan) at 2.5×10^6 cells/ml every 3 days. The culture of cladoceran *D. celebensis* was initiated in 200 ml of glass beaker containing 200 ml of diluted seawater (22 ppt) with 100 individuals (at 0.5 ind./ml), which was randomly selected from a preliminary culture maintained under the same environmental conditions as experimental cultures. Because of the difficulty to conduct small scale batch culture using *Chlorella*, the animals were fed on *Nannochloropsis oculata* at 7×10^6 cells/ml every 2 days to maintain their conditions without external stresses like aeration. The microalgae *N. oculata* was cultured in the modified Erd-Schreiber medium [27] under continuous light with gentle aeration. Prior to feeding, the culture medium of *N. oculata* was centrifuged at 3968×g for 10 min and collected cells were re-suspended in the zooplankton culture medium. Photoperiod and temperature were in the two set up were adjusted at optimal conditions for each species by preliminary tests (personal information): at 18L:6D, 25 °C for *T. japonicus* and under total darkness at 28 °C for *D. celebensis*. Culture media for zooplanktons were prepared by GF/C (CAT No. 1822-047, Whatman) filtration of natural seawater followed by autoclave sterilization at 121 °C for 20 min.

Chicken-dropping extract (CDE) was prepared by the following procedure: 1 kg of fermented chicken droppings (Shitama Inc., Fukuoka, Japan) were mixed with 10 g of fossil coral powder (Coral international Co. Ltd., Okinawa, Japan). The mixture was boiled in 5 l of tap water for 40-50 minutes and then kept overnight at room temperature. The resulting supernatant was filtered in plankton net (150-200 μm of mesh) and mixed with extracted liquid from sludge by the same filtration. The solution (CDE) was preserved at 5 °C until use. To determine the effect of CDE on the population growth of *T. japonicus* and *D. celebensis*, CDE concentration was adjusted in the culture media at 0 (without CDE: control), 1, or 2 ml/l with three replicates and the population density was estimated every 3 days for copepod and every 2 days for cladoceran during culture periods. The culture period lasts for 30 days for copepod, and 18 days for cladoceran.

Potential as live food for marine fish larvae

Different zooplankton species were cultured in order to determine their potential as live food for *Pagrus major* larviculture. L-type rotifer *Brachionus plicatilis* sensu stricto (Makishima strain) was cultured in 50 l of artificial seawater (Marine Art Hi, Tomita Pharmaceutical Co. Ltd., Naruto, Japan) adjusted at 22 ppt and 25 °C under 12L:12D of photoperiod with gentle aeration. The rotifers were daily fed with *C. vulgaris* (Super Fresh Chlorella-V12) at 2.5×10^6 cells/ml twice a day. For *Artemia* feeding, the cyst of *A. franciscana* was incubated in 5 l of 34 ppt artificial sea water at 22 °C of water temperature under 12L:12D of photoperiod with strong aeration. From day 2 after hatching, the nauplii were fed daily with Super Fresh Chlorella-V12 at 2.5×10^6 cells/ml for 24 hours. The copepod *T. japonicus* was semi-continuously cultured in 100 l of 34 ppt artificial sea water at 25 °C with 80 ml/min of aeration under 12L:12D of photoperiod. Food supplement was daily performed at 5.0×10^6 cells of *Chlorella*/ml. The cladoceran *D. celebensis* was semi-continuously cultured in 30 l of 22 ppt artificial seawater at 25 °C with gentle aeration and twice supplementation of *Chlorella* at 2.5×10^6 cells/ml a day. The CDE was supplied to each culture of *T. japonicus* and *D. celebensis* at 1 and 2 ml/l, respectively, due to the limited amount of the prepared CDE.

To compare size distribution of the three zooplanktons tested, 100 individuals of each species were fixed with 5% of neutral formalin for *Artemia* and Lugol solution for copepods and cladocera. The body length of fixed individuals were measured using microscopic measurement system including stereomicroscope (SteREO Discovery V8, ZEISS, Germany) equipped with a digital camera (Axio Cam HSm, ZEISS) and an image-analysis software (Axio Vision Release 4.8.2., ZEISS). The measurement was performed under ×20 of magnification. The fatty acid composition of the cultured zooplanktons was analyzed by the following procedure. Mass cultured zooplanktons were collected by plankton net (45 μm of mesh) and rinsed with distilled water at several days intervals. After removal of remaining water with a paper tissue, the samples were preserved at -40 °C until analysis. Fatty acid analysis was performed at Oita Marine Biological Technology Center, Nippon Suisan Kaisha Ltd., Oita, Japan, and the detailed procedure is same as that used for the fish larvae.

For larviculture trials, fertilized eggs of red sea bream *P. major*were obtained from a local fish farmer hatchery (Ogata Suisan, Kumamoto, Japan). The eggs were incubated in a 100 l of polycarbonate tank containing 34 ppt artificial seawater at 18 °C with 100 ml/min of aeration. Newly hatched larvae (0 days post hatch, dph) were transferred into 9 aquaria each containing 100 l of 34 ppt artificial seawater in a temperature controlled room with 5 l of ceramic sand (grain size: 0.3-0.6 mm, Micros ceramic, NORRA Co. Ltd., Kyoto, Japan) covering the bottom of the tank. Larvae were stocked in each tank following the procedure of Kim et al. [28] in which larval density was adjusted to 10 ind./l. Water temperature in the tank was gradually raised from 18 to 22 °C by daily acclimation of 1°C. Laval rearing was performed at a light-dark cycle of 12L:12D. The microalgae (Super Fresh Chlorella-V12) at 5×10^5

cells/ml was added into the prepared aquaria on 4 dph and this density was maintained until 28 dph [29, 30]. The feeding scheme is shown in Fig. 1. Fish from 4 (mouth opening) to 23dph were fed on the rotifers twice a day and the density was maintained at 10 ind./ml in the larval rearing tanks. On 16 dph, 400 fish were newly transferred into each experimental tank to sort the fish number prior to switch the food items from rotifer to the targeted species. The tanks were assigned to each diet treatment with triplicates. When larvae reached notochord flexion phase (from 20 dph), *Artemia*, copepod, and cladoceran were fed in triplicates at 0.01 ind./ml 3 to 5 times a day according to their growth. To estimate the effectiveness of the zooplanktons as live food for fish larvae, the 6 following parameters were conducted [28, 31].

Hatching and Survival activity index

The fertilized fish eggs (30 eggs) were incubated in a 500 ml of glass beaker containing 500 ml of artificial seawater at 18°C under total darkness to calculate hatching rate and survival activity index (SAI) with triplicate observations. The hatching rate was determined by the number of hatchlings after 24 h. The SAI was estimated by the following equation [31]:

$$\mathrm{SAI} = \frac{1}{N}\sum_{i=1}^{K}(N - hi) \times i$$

where N is the total number of examined larvae, hi is the cumulated mortality by i-th day, K is the number of days elapsed until all fish larvae died due to starvation.

Survival

The survival rate was calculated from the mean number of surviving fish larvae in three aquaria for each zooplankton species on the last day of larviculture (28 dph). The adjusted density at 400 individuals on 16 dph was applied as an initial number of fish larvae.

Viability

On the last day of larviculture, air exposure test was conducted to compare the viability of the larvae fed on each zooplankton. We caught fish larvae on a net (130×345 mm, Super net M, SANY co., Ltd., Kanagawa, Japan) from each tank (n=3) and exposed them to air for 1 minute. After this, the fish were immediately returned to seawater and their survival was observed every 2 hours for 24 hours.

Growth

Larval growth was determined by measuring the total length and wet weight. On 20, 23, 26, and 28 dph, 20 larvae were randomly collected from each aquarium, and anaesthetized with MS222 followed by 5% neutral formalin fixation. The total length was measured with all the fixed larvae under digital microscope (VH-6300, Keyence, Japan). Wet weight of fish on 28 dph was measured using an analytical balance (AB204-S, Mettler-Toledo International Inc., United Kingdom). Using these data, the total biomass of fish larvae (i.e., production) was calculated with the number of surviving larvae on the last day of the experiment. To estimate optimal prey size, upper jaw length (JL) was measured using the larvae of 20 dph, and the mouth diameter was determined by the following equation: $\sqrt{2}(JL)$, where the assumption is that the mouth opens to an angle of 90° during prey capture [32].

Fatty acid analysis

For the fatty acid composition of fish larvae fed on three different diets, the reared larvae were sampled at the end of the experiment and preserved at -40 °C until analysis. The analysis was performed at Central Research Laboratory of Nippon Suisan Kaisha, Ltd. with the following detailed method. Pooled cultured zooplankton or fish larvae (on 20 and 28 dph) homogenates were precisely weighted in glass tubes. An internal standard consisting of 20 μg tricosanoic-acid (C23:0) and 50 μg butylated hydroxytoluene dissolved in 2 ml of methanol-hexane 4:1 (v/v) was added to biological samples and methylated in the presence of 200 μl acetyl chloride at 80 °C for 1 h, based on the method of Lepage and Roy [33]. After cooling on ice, 5 ml of 6% (w/v) aqueous potassium carbonate was added to each tube to stop the methylation reactions, and centrifuged at 2000 rpm for 5 minutes. The upper organic phase containing the fatty acid methyl ester was collected, and analyzed on a DB-Wax column (30 m length, 0.32 mm id, 0.25 μm film) (Agilent Technologies) coupled to a GC System 6850N (Agilent Technologies). The gas chromatography oven temperature was 180 °C and increased at a rate of 3 °C/min to a final temperature of 230 °C.

Statistical analysis

The CDE effects on the population growth of the both zooplanktons related to its concentrations and culture day were analyzed by two-way repeated-measures ANOVA using Statview version 5.0 (SAS Institute Inc., USA). When significant differences were detected ($P<0.05$), Tukey's HSD test was performed by R version 3. 1. 2 [34]. For the fish larviculture, the mean body length of the three zooplankton species, and survival, wet weight and biomass of fish larvae on 28 dph associated with the targeted live food species were compared with one-way ANOVA followed by Tukey-Kramer *post hoc* test as the first test showed significant differences ($P<0.05$). To compare the viability of fish larvae, Log-rank test were performed. The variation of larval total length was analyzed by two-way repeated-measures ANOVA followed by Tukey-Kramer *post hoc* test associated with the food types and culture days (20, 23, 26, and 28 dph). These analyses for fish larviculture were performed by Statview (SAS institute).

RESULTS

Effects of CDE on zooplankton populations

The population growth of copepods was observed with developmental stages: nauplius, copepodite, and nauplius+copepodite (Fig. 2). The population growth of each treatment increased with the culture days ($P<0.0001$) but the pattern was different among CDE concentrations ($P<0.0001$). At 2 ml/l of CDE, active population growth was obtained regardless of developmental stages ($P<0.0001$) and population growth decreased at lower CDE concentration. On the last day of culture, three developmental groups showed the highest count at 2 ml/l ($P<0.0001$): 4408.9±321.1 ind. of nauplii, 7768.9±635.5 ind. of copepodites, and 12177.8±694.6 ind. of total population.

The population growth of cladocera varied with the two parameters: culture days and CDE concentrations (Fig. 3). The cladocera population maintained steady growth until day 12 but sharply decreased thereafter. The highest density (14.0±2.6 ind./ml)on day 12 was observed with 2 ml/l of CDE supplementation and it was decreased with the reduction of CDE concentration: 10.3±1.5 ind./ml at 1 ml/l and 8.7±3.2 ind./ml in the control group ($P<0.05$).

Total fatty acid level of each animal was described as follows: 0.76% of *Artemia* wet weight, 1.16% of copepod, and 1.04% of cladocera. The highest proportion of n-3 HUFA and DHA (C22:6n-3) / EPA(C20:5n-3) ratio were in copepods cultured with CDE (Table 2).

Fish larviculture

The employed fish eggs showed 94.4±5.1% of hatching rate and 13.6±6.6 of SAI, respectively. Fish larvae from these eggs showed significantly higher survival rate with the copepods compared to those reared with cladocerans or *Artemia* (Table 1)($P<0.05$). The larval diets of copepod and cladoceran induced higher viability compared to *Artemia* (Table 1) ($P<0.05$).

Total length of fish larvae on 28 dph was shown as 8.7±0.7 mm with *Artemia*, 8.5±1.1 mm with copepod, and 8.8±0.7 mm with cladoceran, respectively without significant differences among diet treatments. The calculated mouth diameter of 20 dph fish larvae was 0.97±0.08 mm, therefore the size of optimal prey was ranged from 0.5 to 0.7

mm which is similar to the mean size of each zooplankton species: 0.8± 0.1 mm for *Artemia*, 0.9± 0.1 mm for copepods, and 0.7± 0.0 mm for cladoceran (Fig. 5). There were no significant differences in wet weight of fish (Table 1) and the total biomass (i.e., production of 28 dph, Fig. 6) of fish larvae among those with three larval diets. The larval production on the last day of larviculture was shown as follows: 552.0±325.4 mg with *Artemia* feeding, 1395.6±564.2 mg with copepod, and 780.3±134.4 mg with cladoceran.

The total fatty acids were estimated to compose 1.3% of larval wet weight with *Artemia* and copepod diet, and 1.5% with cladoceran which value was slightly higher than initial rotifer-fed larvae (1.4%) on the last day of larviculture. Among these fish larvae (on 28 dph), only the copepod-fed one showed higher proportion of total n-3 HUFA and DHA/EPA ratio compared to the initial larvae on 20 dph (Table 3).

DISCUSSION

Intensive larviculture system of red sea bream has been stably established with rotifers and *Artemia* as live foods related to the developmental stages [35, 36]. Previous studies were conducted in small scale larviculture and obtained high survival, viability and growth with copepod diet, but it has not been applied to intensive culture in larger scale. Feasibility of the tested zooplanktons: *T. japonicus* and *D. celebensis* depends on the competitive cost to culture them at higher densities for the intensive larviculture system. This study made an attempt to mass culture of these zooplanktons with the addition of CDE, because we further aimed to promote cost-effective method of mass production of these zooplanktons to fish culturists. Our results clearly showed the efficiency of CDE to enhance production achieve high density culture of the employed species with proper feeding dosages (Fig. 2 and 3). Many studies on the use of animal manure showed that indeed, CDE could enhance zooplankton population growth [37-39]. The chicken manure is known to contain water-soluble natural 17β-estradiol (E2) [40, 41]. In addition, supplementation of synthetic E2 (10-1000μg/l) increased reproduction of *D. celebensis* [42], but not that of *T. japonicus* [43]. The increase in the population growth in *D. celebensis* with the addition CDE should be viewed as direct effect, but other mechanisms may be involved in *T. japonicus*. *T. japonicus* is known as omnivorous and shift feeding resources to detritus when living particles become limited [44]. Detritus contains bacteria which are important decomposer of organic matters, and its population increased with chicken manure dosage [45]. The accelerated bacterial growth with the CDE is expected to induce the better growth of copepod population in this study. Not only to the copepods, several studies also reported that bacteria contribute to the diet of cladoceran [46, 47]. Therefore, the high population growth of cladoceran is probably due to the build-up of bacterial populations by CDE.

Hatching rate of fertilized eggs and SAI are used to estimate initial larval quality [48]. These values in this study are comparable to those reported by Kim et al. [28] and are higher than those of other fishes [49]. SAI of fish larvae is influenced by ambient environmental conditions and *Epinephelus akaara* larvae exhibited about 12 of SAI under optimal conditions for stable growth and development [50]. It should indicate that the set-up conditions for the present larval rearing are suitable for the targeted larval fish. Under this condition, the effective larval rearing of red sea bream was constructed with copepod and cladoceran cultured with CDE, and these fish larvae showed higher survival and viability (Table 1) compared to those with *Artemia*in the present study.

The survival rate associated with the dietary sources was the highest with the copepod *T. japonicus* (Table 1) even though it has epibenthic features. Influences of turbulence by aeration and predator presence may also change swimming behavior of copepods (e.g., frequency and speed) [14, 51] and should be examined. The size distribution of the employed cladoceran was estimated most favorable for the targeted fish larvae (Fig. 5) to induce the high capture efficiency. The larval capture efficiency with copepod is lower than with cladocerans, although, the earlier stage larvae prefer the copepod adults and nauplii because copepods yield substantial energy to larval fish because of the minimal handling time [52]. This phenomenon is expected to induce the highest survival rate with copepod diet (Table 1). All the mass cultures of zooplanktons were maintained with *C. vulgaris* which has similar cell component to *N. oculata* [53]. The cultivated copepods with CDE contained the highest amount of fatty acids compared to the other zooplanktons (Table 2), although, the level was comparatively lower than the natural one [54]. However, this amount is enough to maintain the stable growth and development of red sea bream larvae [55], especially n-3 HUFAs was highly contained in copepods. It was expected that the copepod feeding induced stable survival of the targeted fish larvae [56]. In addition, the parameter of DHA/EPA ratio is regarded as an effective factor to determine food and larva quality. The optimal ratio of marine fish larval food is estimated more than 1 by comparing with natural diet [57] and the ratio of reared fish larvae is more than 5 which was determined by grunt *Plectorhynchus cinctus* [58]. The effects of DHA/EPA ratio on the larval survival was clearly observed in this study and the copepod-fed larvae showed the highest survival rate (Table 1) associated with the higher ratio of it (5.6 in Table 3).

P. major larvae fed with *T. japonicus* and *D. celebensis* showed the better resistance than those fed with *Artemia* (Table 1). Successful larval rearing generally depends on first feeding regimes with live food species and its nutritional quality. The dietary lipids especially the essential fatty acid (EFA) is recognized as one of the most important nutritional factor that influence larval growth and survival [59, 60] and its deficiency will result to various symptoms including decrease of larval health, poor growth, low feed efficiency, anaemia and high mortality [61-63]. It was also reported that red sea bream during larval development especially utilized the neutral lipids 16:0, 18:1(n-9), and 22:6(n-3) to maintain their growth and survival [64]. While DHA has an important role in stress resistance of mahimahi

Coryphaena hippurus [65], DHA content with larval stress resistance is not demonstrated in the present study (Table 1). Free amino acids (FAA) is mainly used as metabolic fuel and for body protein synthesis, thus it is regarded as important nutritional components influencing the viability of early stage marine fish [66]. In the wild, various copepods contain more than twice of FAA per gram of wet mass than *Artemia* [6, 67, 68]. Thus, it is expected that FAA composition of the cultured copepods and cladocerans affects the higher viability of fish larvae.

The growth of the *P. major* was not significantly influenced by the zooplanktons tested (Fig. 4). To date, studies have shown that larvae fed with copepods or cladocerans achieved better growth than those fed with *Artemia.* This is in the cases of sea bass *Lates calcarifer* [69], yellowtail clownfish *Amphiprion clarkia* [70], barber goby *Elacatinus Figaro* [71], mangrove killifish *Kryptolebias marmoratus* [72] and kuruma prawn *Marsupenaeus japonicus* [18]. Pandey et al. [13] and Grageda et al. [72] detected that the larval growth was significantly related to feeding behavior of the mangrove killifish larvae in terms of food size preference. Until 26 dph, copepod-fed *P. major* larvae showed low morphometric growth compared to cladoceran and *Artemia*-fed, but on 28 dph the larvae grew comparatively fast caused by the expected reason; the shifting food size preference to more than 0.8 mm (Fig. 5). Similar trends were found with former studies which determined food size selectivity by larval gut analyses [73, 74]. The supply of fatty acids is expected the other reason why the reared fish larvae showed no differences in larval wet weight (individual) and growth (Table 1; Fig. 4, 6). The supplied n-3 HUFA which is consisted of EPA (20:5n-3), DHA (22:6n-3), has activities in the fatty acid metabolism [75]. The requirement of these fatty acids was estimated as 0.5% of diet dry weight for juvenile [76] and 0.4% of diet wet weight for larvae of red sea bream [77]. The total n-3 HUFA contained in *Artemia*, *T. japoicus*, *D. celebensis* were calculated to be 0.03, 0.46, and 0.05% of total fatty acids and thus, the level of *T. japonicas* only satisfies the minimum requirement of the targeted fish larvae (Table 2).

The present study showed the enhanced survival and viability of red sea bream *P. major* larvae fed on copepod *T. japonicus* and cladoceran *D. celebensis* which were mass-cultured with CDE. We opined that these were due to the optimum nutritional contents of these live foods and their appropriate size that stimulate appetite of the larvae. Thus, we recommend the use of *T. japonicus* and *D. celebensis* as substitute of *Artemia* for intensive marine larviculture.

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Table 1 Larval characteristics of red sea bream *Pagrus major* (28 dph) fed with three different diets

Diet	Survival (%)	Viability (%)	Wet weight (mg/ind.)
A. franciscana	16.7±9.8^{b}	44.4±12.3^{B}	8.2±0.3
T. japonicus	39.4±3.1^{a}	60.0±27.8^{A}	8.8±3.4
D. celebensis	20.8±3.8^{b}	60.0±32.2^{A}	9.4±0.1

Values are mean±SD of triplicate observations (n=3). Different alphabetical letters in a same column represent significant differences among three different diets (a>b, Tukey-Kramer *post hoc* test, P<0.05, n=3; A>B, Long-rank test, P<0.05, n=3).

Table 2 Total fatty acids (mg/g WW) and fatty acid composition (%) of the three employed zooplanktons: *Artemia franciscana*, *Tigriopus japonicus*, and *Diaphanosoma celebensis* under the experimental conditions

	A. franciscana	*T. japonicus*	*D. celebensis*
Total	7.6	16.1	10.4
C14:0	0.6	0.6	2.0
C16:0	10.4	12.1	13.9
C16:1n-7	3.4	2.5	7.6
C18:0	5.6	3.3	4.4
C18:1n-9	17.6	8.3	5.7
C18:1n-7	8.6	1.8	3.5
C18:2n-6	5.5	16.2	22.6
C18:2n-4	0.0	0.1	0.1
C18:3n-6	0.4	0.2	0.4
C18:3n-4	0.1	0.1	0.1
C18:3n-3	25.0	3.9	6.6
C18:4n-3	3.3	0.2	0.4
C20:0	0.1	0.1	0.1
C20:1n-9	0.5	0.3	0.1
C20:2n-6	0.2	0.1	0.0
C20:3n-6	0.2	1.0	0.4
C20:4n-6	1.9	0.9	1.4
C20:3n-3	0.7	1.2	0.2
C20:4n-3	0.5	0.4	0.0
C20:5n-3 (EPA)	3.6	4.9	4.8
C22:1n-9	0.2	0.3	0.1
C21:5n-3	0.0	0.9	0.1
C22:5n-3	0.0	1.5	0.1
C22:6n-3 (DHA)	0.3	22.3	0.3
unknown	11.3	17.0	25.2
DHA/EPA	0.1	4.6	0.1
Σn-3HUFA	3.9	28.7	5.2

Table 3 Total fatty acids (mg/g WW) and fatty acid composition (%) of the fish larvae on 20 (initial) and 28 days post hatch (dph) with three different diets: *Artemia franciscana*, *Tigriopus japonicus*, and *Diaphanosoma celebensis*

	20 dph (Initial)	28 dph		
		A. franciscana	*T. japonicus*	*D. celebensis*
Total	13.8	13.3	13.0	14.7
C14:0	0.6	0.5	0.4	0.8
C16:0	14.5	15.2	15.1	15.4
C16:1n-7	1.4	1.7	1.6	2.6
C18:0	8.9	10.3	8.9	9.3
C18:1n-9	3.9	7.8	6.0	4.8
C18:1n-7	1.2	2.7	1.6	2.9
C18:2n-6	13.7	8.7	11.3	13.9
C18:2n-4	0.0	0.0	0.2	0.1
C18:3n-6	0.1	0.2	0.1	0.1
C18:3n-4	0.1	0.1	0.0	0.0
C18:3n-3	2.4	4.5	1.5	2.2
C18:4n-3	0.0	0.4	0.1	0.4
C20:0	0.2	0.2	0.2	0.2
C20:1n-9	1.5	0.8	0.5	0.5
C20:2n-6	0.2	0.2	0.2	0.2
C20:3n-6	2.4	1.3	1.3	1.2
C20:4n-6	0.9	1.8	1.2	1.0
C20:3n-3	0.9	0.6	0.7	0.4
C20:4n-3	0.5	0.4	0.2	0.2
C20:5n-3 (EPA)	5.4	4.5	3.9	7.4
C22:1n-9	0.2	0.2	0.2	0.2
C21:5n-3	0.5	0.2	0.4	0.2
C22:5n-3	6.5	5.4	3.4	3.9
C22:6n-3 (DHA)	13.0	13.2	21.8	10.8
unknown	20.9	19.0	19.25	21.5
DHA/EPA	2.4	2.9	5.6	1.5
Σn-3HUFA	24.9	23.2	29.1	22.1

Figures

Fig. 1 Experimental scheme for the larviculture of red sea bream *Pagrus major* with the three targeted zooplanktons: *Artemia franciscana*(A), *Tigriopus japonicus*(T), *Diaphanosoma celebensis*(D) associated with the developmental stage of fish larvae and rearing days (dph, days post hatch).

Fig. 2 Population growths of three different developmental groups (a) nauplius,(b) copepodite,and (c) nauplius+copepodite at different concentrations of chicken droppings extract (0, 1, and 2 ml/l) in *Tigriopus japonicus*. Each plot and error bar indicates the mean and standard deviation of triplicate, respectively. Different alphabetical letters represent significant differences (a>b>c>d>e>f>g>h>i>j>k>l, Tukey's HSD test, $P<0.05$, $n=3$).

Fig. 3 Population growths of *Diaphanosoma celebensis*at different concentrations of chicken droppings extract (0, 1, and 2 ml/l). Each plot and error bar indicates the mean and standard deviation of triplicate, respectively. Different alphabetical letters represent significant differences (a>b>c>d>e>f, Tukey's HSD test, $P<0.05$, $n=3$).

Fig. 4 Larval growth of red sea bream *Pagrus major* with the three live zooplanktonic diets: *Artemia franciscana*, *Tigriopus japonicus*, and *Diaphanosoma celebensis* associated with rearing days. Each plot and error bar indicates the mean and standard deviation of triplicates, respectively.

Fig. 5 Size distribution of the three employed zooplanktons: *Artemia franciscana* (a), *Tigriopus japonicus* (b), *Diaphanosoma celebensis* (c). The arrow indicates the estimated range of optimal food size [32]which can be eaten by the targeted fish larvae of red sea bream *Pagrus major* on 20 days post hatch. A superscript letter on the mean size of each zooplankton indicates significant difference (a>b>c, Tukey-Kramer *post-hoc* test, $P<0.0001$, $n=100$).

Fig.6 Total biomass (i.e., production) of red sea bream *Pagrus major* larvae with the three live zooplanktonic diets: *Artemia franciscana*, *Tigriopus japonicus*, and *Diaphanosoma celebensis*. Each column and error bar indicates the mean and standard deviation of triplicates, respectively.

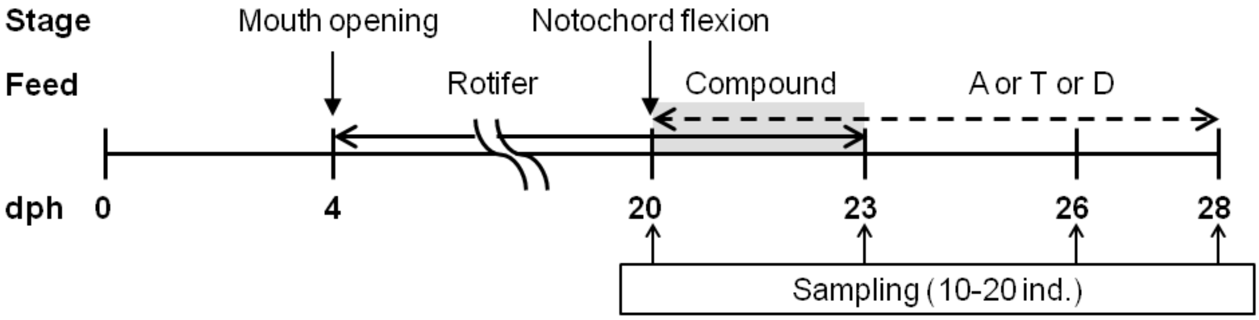
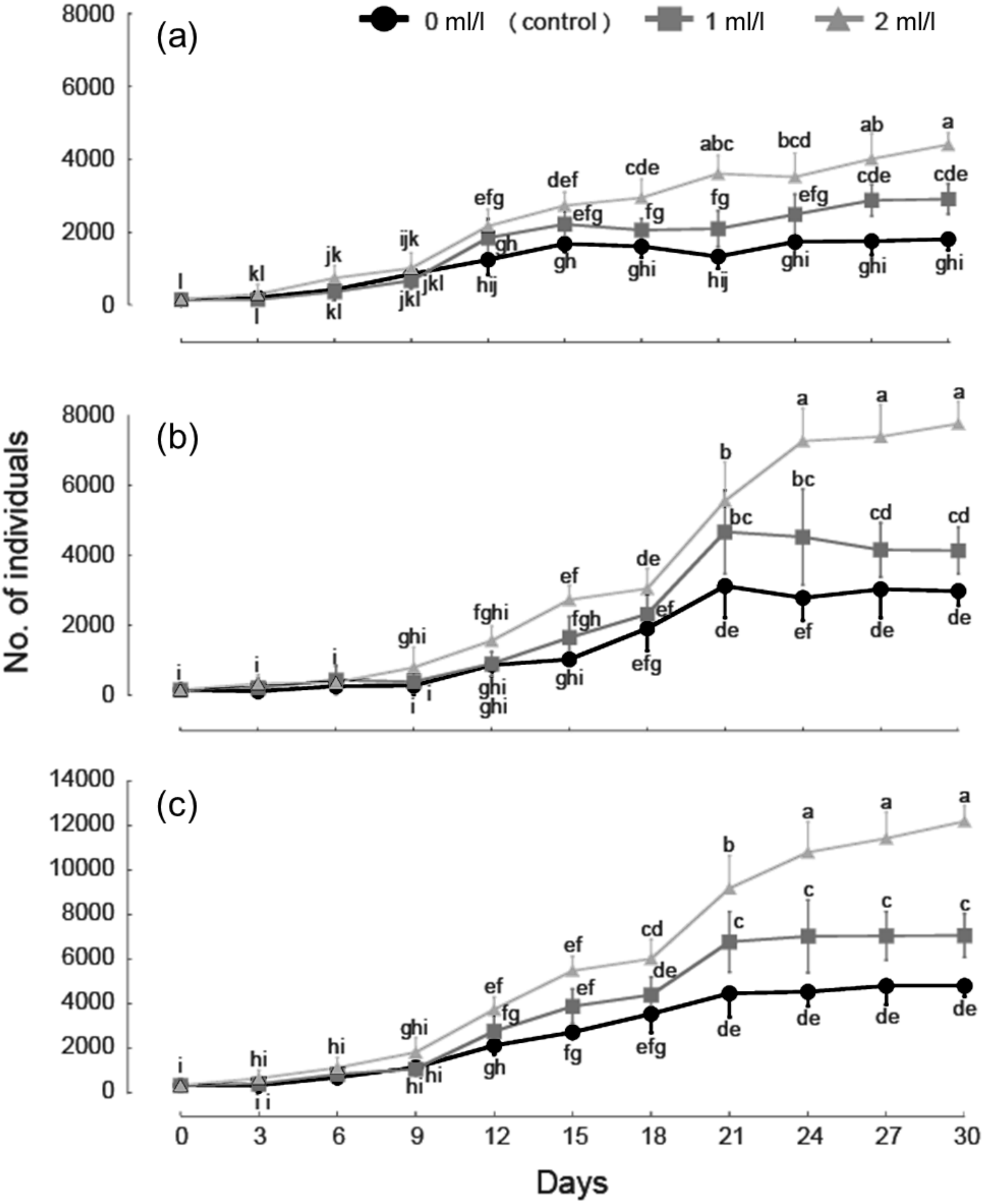

Stage
Mouth opening
Notochord flexion
Feed
Rotifer
Compound
A or T or D
dph
0
4
20
23
26
28
Sampling (10-20 ind.)

Fig. 1.


0 ml/l （control）
1 ml/l
2 ml/l
(a)
8000
6000
4000
2000
0
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jk
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jkl
jkl
efg
gh
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def
efg
gh
cde
fg
ghi
abc
fg
hij
bcd
efg
ghi
ab
cde
ghi
a
cde
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(b)
8000
6000
4000
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No. of individuals
i
i
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ghi
i
i
fghi
ghi
ghi
ef
fgh
ghi
de
ef
efg
b
bc
de
a
bc
ef
a
cd
de
a
cd
de
(c)
14000
12000
10000
8000
6000
4000
2000
0
i
hi
i i
hi
ghi
hi
hi
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fg
gh
ef
ef
fg
cd
de
efg
b
c
de
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c
de
a
c
de
a
c
de
0
3
6
9
12
15
18
21
24
27
30
Days

Fig. 2.

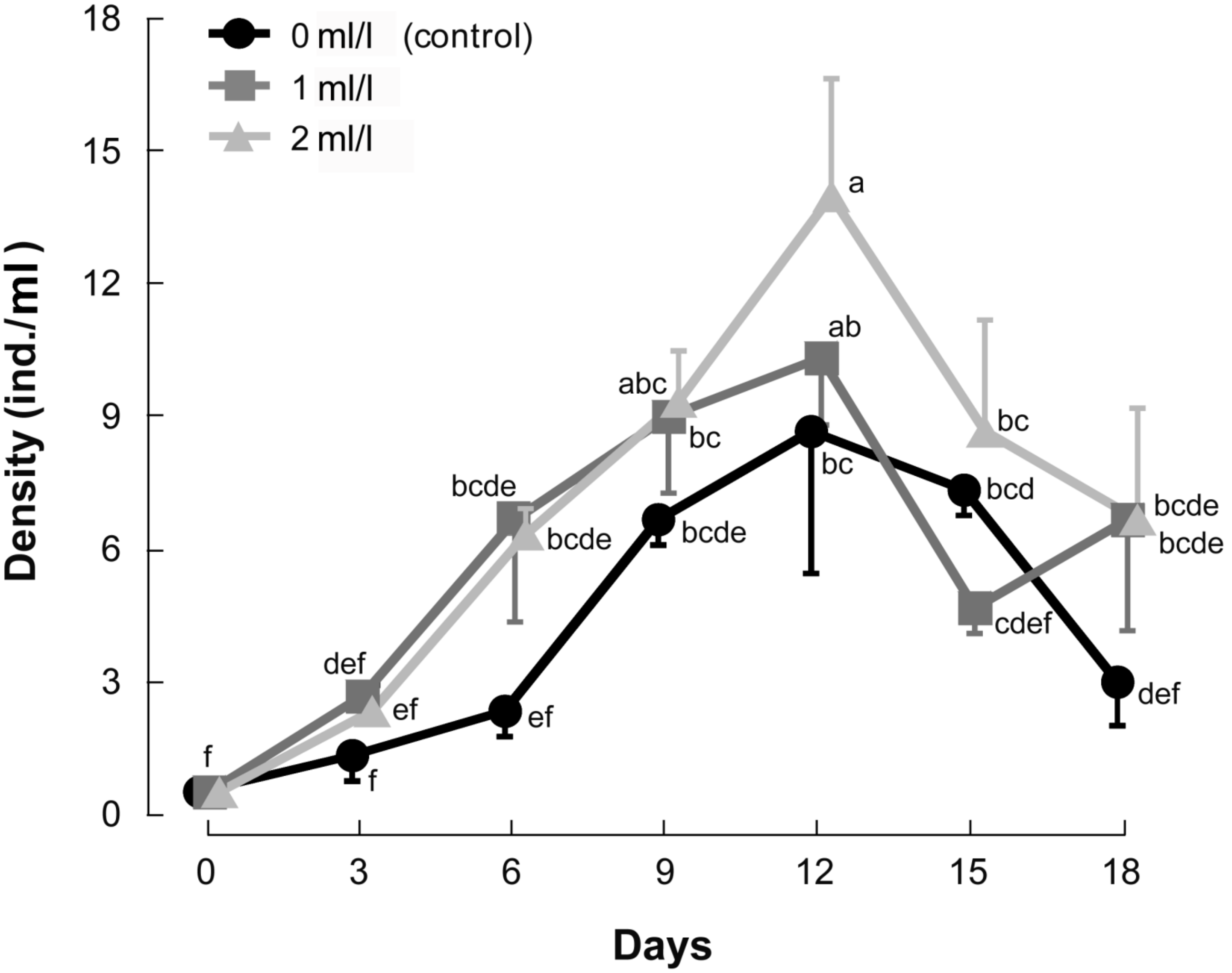
0 ml/l (control)
1 ml/l
2 ml/l
Density (ind./ml)
Days
0
3
6
9
12
15
18
0
3
6
9
12
15
18
a
ab
abc
bc
bc
bc
bc
bcde
bcde
bcde
bcd
bcde
bcde
def
ef
ef
f
f
cdef
def

Fig. 3.

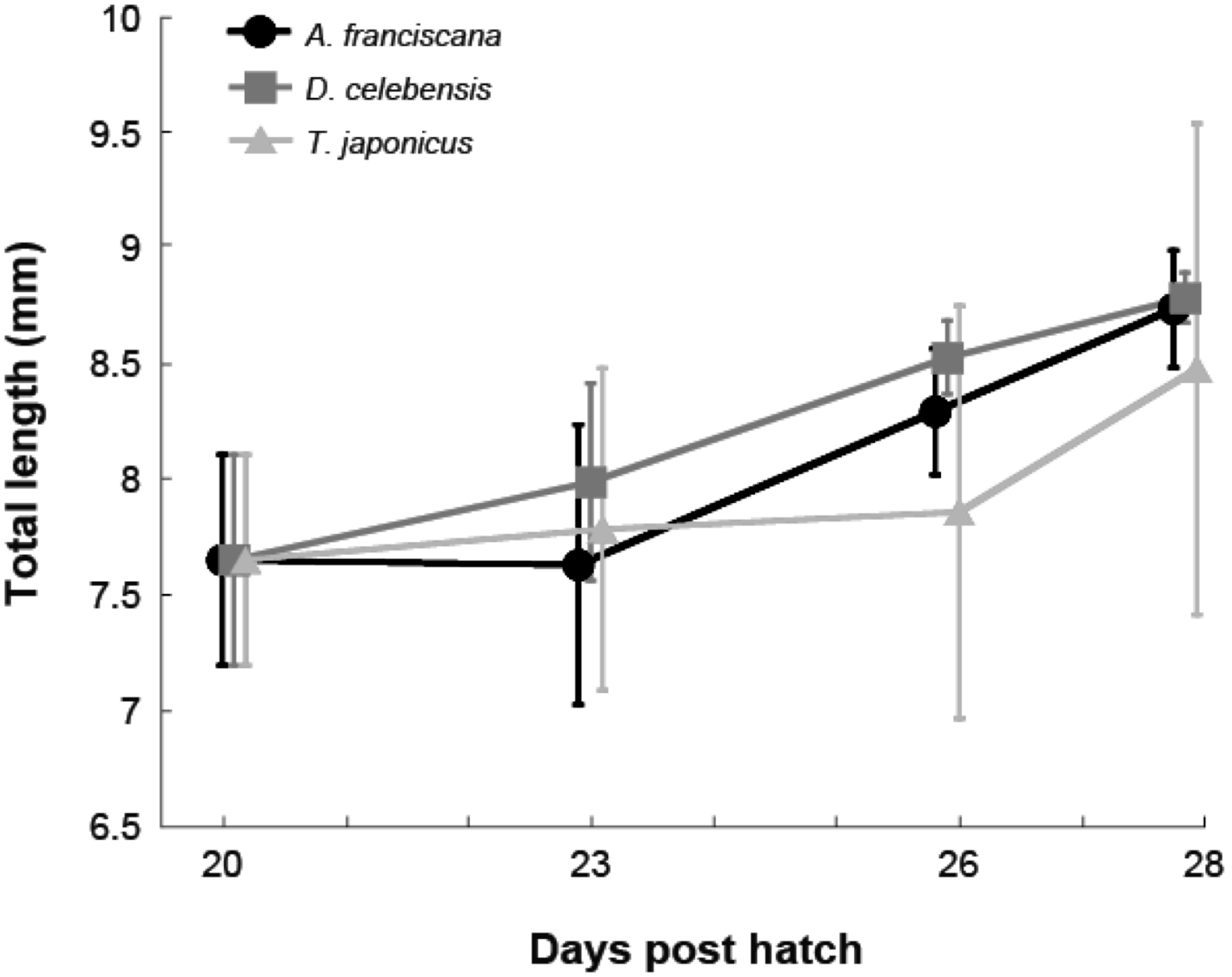
A. franciscana
D. celebensis
T. japonicus
Total length (mm)
10
9.5
9
8.5
8
7.5
7
6.5
20
23
26
28
Days post hatch

Fig. 4.

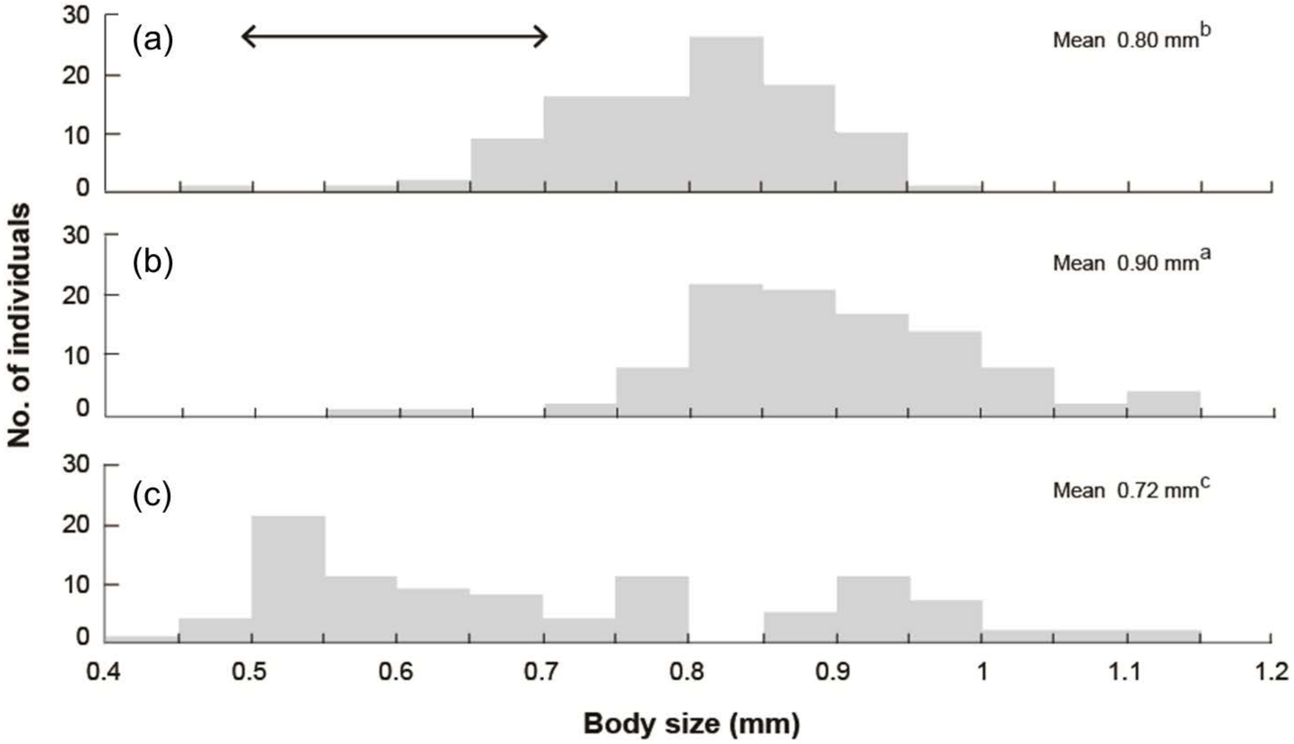
No. of individuals
30
20
10
0
(a)
Mean 0.80 mm^b
(b)
Mean 0.90 mm^a
(c)
Mean 0.72 mm^c
0.4
0.5
0.6
0.7
0.8
0.9
1
1.1
1.2
Body size (mm)

Fig. 5.

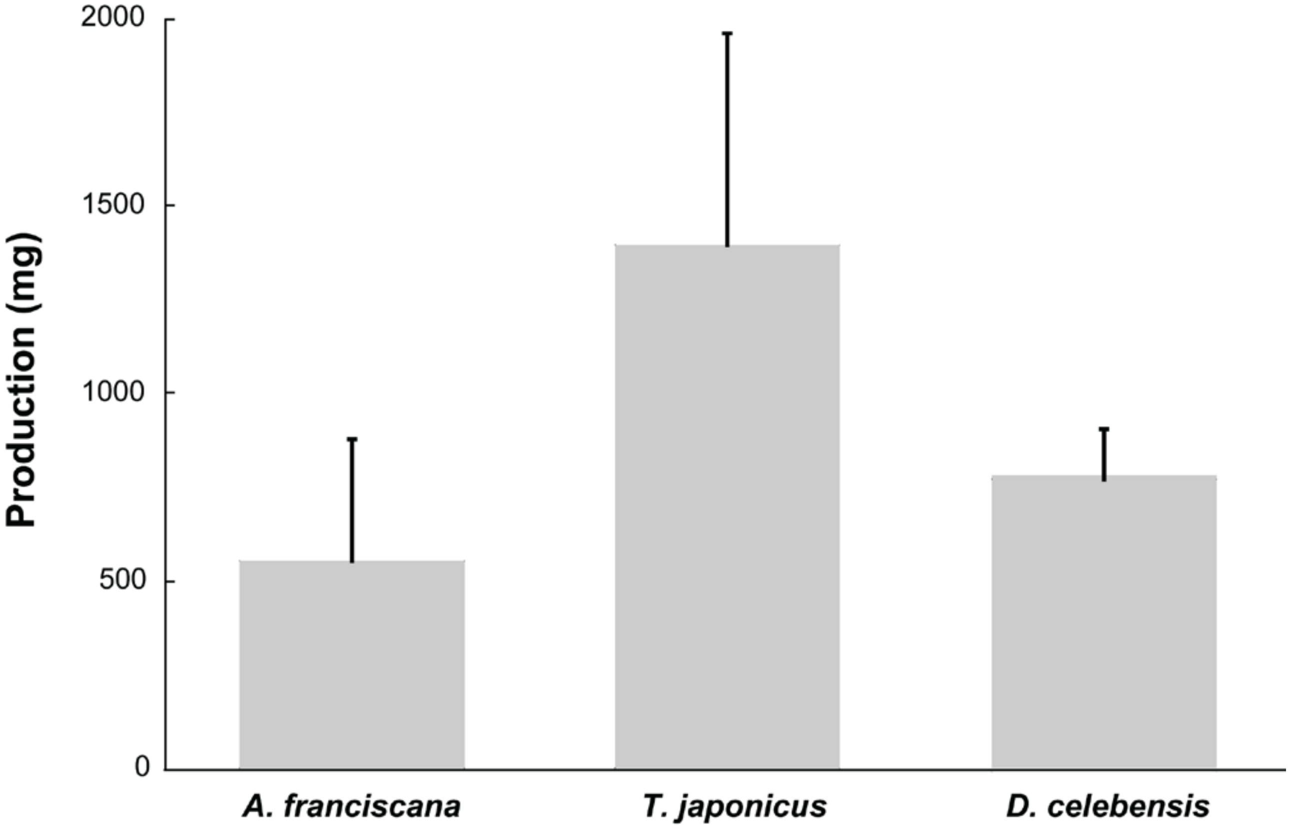
2000
1500
1000
500
0
Production (mg)
A. franciscana
T. japonicus
D. celebensis

Fig. 6.