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Effects of feeding copepod and *Artemia* on early growth and behaviour of the self-fertilizing fish, *Rivulus marmoratus*, under laboratory conditions

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Abstract

Growth and survival have often been used as parameters to assess the effects of live feeds on marine finfish, however, behavioural effects, which entail energy cost and may have consequences on fish growth have been given less emphasis. Thus, a 20-day feeding experiment was conducted to determine the effects of copepod *Acartia tsuensis* (104-732 μm), unenriched, and docosahexaenoic acid, DHA-enriched, first instar *Artemia franciscana* nauplii (656-906 μm) on growth and behaviour of the mangrove killifish *Rivulus marmoratus*. Growth was significantly higher in *Acartia*-fed larvae compared with larvae fed *Artemia* (unenriched and DHA-enriched) until day 10. On day 20, *Acartia*-fed larvae had significantly lower growth than fish fed DHA-enriched *Artemia*. Feeding success was highest in larvae fed *Acartia* on day 1. Ingestion rate and satiation time did not differ among fish fed different types of feeds until day 20. Swimming activity before feeding was significantly lower in larvae fed *Acartia* compared with larvae fed *Artemia* (unenriched and DHA-enriched) until day 10. Higher growth in *Acartia*-fed fish on day 10 is probably due to the suitable size and high DHA content of *A. tsuensis*, and lower swimming activity of the larvae. However, on day 20, lower growth observed in *Acartia*-fed fish may be attributed to the shift in the food size preference of the fish. The present study was able to demonstrate the effects of copepods on growth and behavioural development of marine finfish using *R marmoratus* as a model animal.

Keywords: Feeding behaviour; Growth; Mangrove killifish; Swimming activity; n-3 HUFA
1. Introduction

Copepods have been recognized as the most suitable feed for early stages of fish larvae because of their nutritional advantage (high essential fatty acids) compared with other live feeds such as rotifers and *Artemia* (Nanton and Castell, 1998; Evjemo et al., 2003; Støttrup and McEvoy, 2003). Interest on copepod culture started as a result of the discovery that they have better nutritional value compared with commonly used rotifers and *Artemia* (Støttrup and McEvoy, 2003). Copepods have been mass-cultured as early as 1970s and 1980s in Japan and have been used as feed for Pacific cod larvae in rearing trials in fisheries stations (Hagiwara et al., 2001). In the early 1990s, intensive culture of different species of copepods expanded due to the increasing diversity in the marine finfish culture, particularly those with small larvae, such as grouper and red snapper, and the shortage of commonly used live feed *Artemia* (Doi et al., 1997; Støttrup and McEvoy, 2003). Since then, research on copepod culture (Barthel, 1983; Berggreen et al., 1988; Ohno and Okamura, 1988; Davis, 1993; Abu-Rezq et al., 1997; Hernandez Molejon and Alvarez-Lajonchere, 2003) for the main purpose of utilizing them as feed for commercially important marine fish species, led to their widespread use in larval rearing trials (Kraul et al., 1992; Nanton and Castell, 1998; Shields et al., 1999; Evjemo et al., 2003) as well as in European hatcheries (Støttrup, 2000). Although copepod culture in intensive indoor systems has been successful, its mass production at a commercial scale has not been attained due to technical constraints, and is still in progress (Støttrup, 2000; Hagiwara et al., 2001).

Positive nutritional effects of copepods on marine finfish have been reported such as increased growth and survival (Doi et al., 1997; Nanton and Castell, 1998; Støttrup et al., 1998; Copeman et al., 2002; Skalli and Robin, 2004), improved pigmentation (Bell...
et al., 2003), retinal morphology (Shields et al., 1999), broodstock reproductive performance, and egg and larval quality (Mazorra et al., 2003). However, less emphasis has been given on the effects of copepods on the behavioural development of fish, particularly on their feeding and swimming behaviour (Hunt Von Herbing and Gallager, 2000). Behavioural observations are useful in understanding patterns of prey selection and have important implications on metabolic energy costs. Increased efficiency in foraging has been shown to increase net energy gain and consequently growth and survival (Dill, 1983; Wahl et al., 1995). In this study, three types of diets using *Acartia tsuensis* and *Artemia franciscana*, with different nutritional composition were used to determine their dietary effects on growth and behavioural development using mangrove killfish, *Rivulus marmoratus*, as a model animal.

*R. marmoratus*, recently recognized as a synonym of *Kryptolebias marmoratus* (Costa, 2004), has been used as an experimental animal because it is capable of self-fertilization (produce clones) and it is easy to culture (Kallman and Harrington, 1964; Harrington and Kallman, 1968; Koenig and Chasar, 1984). *R. marmoratus* will be highly useful in investigating the effects of different types of diets since individuals are homozygous clones, thus, any observed variations in traits or characters can be attributed to nutritional effects and not to individual variations. Also, the early life history of this species has been studied in detail (Grageda et al., 2004) but the nutritional requirements during the early stages of its development have never been investigated.

With the aim to compare the effects of feeding different types of live feeds on early growth and behavioural development in *R. marmoratus*, a 20-day feeding
experiment was conducted using three types of diets namely: *A. tsuensis* (D1), unenriched (D2), and DHA-enriched (D3) first instar *A. franciscana* nauplii.

### 2. Materials and methods

#### 2.1 Culture and size measurement of live feeds

*A. tsuensis* were collected from the Yukinoura River of Ooseto in Nagasaki, Japan using a plankton net (45 μm mesh size). They were cultured in 5 l plastic containers with 4 l of 17 g l⁻¹ brackish water (prepared by mixing 2 l of distilled water and 2 l of natural seawater, filtered in 47 mm glass microfibre filter) with mild aeration, at a density of 2-10 ind. ml⁻¹. Copepods were fed daily with 1 x 10⁵ cells ml⁻¹ of *Chaetoceros* sp and 2 x 10⁵ cells ml⁻¹ of *Isochrysis* sp. The amount of feed left was checked daily and the amount of food fed was adjusted accordingly. Culture water was totally replaced every 2-3 days.

About 1 g of *A. franciscana* cyst was incubated in 3 l white plastic container with about 1.5 l of 17 g l⁻¹ brackish water with strong aeration. After 1 day, newly hatched nauplii were collected using a 100 μm sieve and distributed equally to two, 2 l plastic containers (for D2 and D3) half-filled with 17 g l⁻¹ artificial brackish water (Marine Art Hi, Tomita Pharmaceutical Co. Ltd., Naruto, Japan), provided with strong aeration and placed in a water bath maintained at 25 ± 1 °C. For D3, *A. franciscana* were enriched (0.3 g l⁻¹, Aquaran plus, BASF, Japan) for 12-16 h prior to feeding.

*A. tsuensis* (n = 95) and *A. franciscana* (n = 30) were randomly sampled and preserved in 5 % formaldehyde. Size (expressed in μm) was measured using a digital microscope (VH 6300, Keyence Corp., Japan). Body sizes of nauplii, copepodites and
adult copepods were measured as body length and prosome length (from the anterior end of the prosome to the posterior lateral end of the prosome), respectively (Mauchline, 1998).

2.2 Dietary treatments

Three types of diets (D1: *A. tsuensis*, D2: unenriched first instar *A. franciscana* nauplii, and D3: DHA-enriched first instar *A. franciscana* nauplii) were used in the feeding experiment. Details of the nutritional composition of each of the diet, with emphasis on their highly unsaturated fatty acid (HUFA) components are shown in Table 1.

2.3 Experimental fish and general rearing conditions

The killifish (*Rivulus marmoratus*) used in this study were the PAN-RS strain derived from reared broodstock, which originated from Bocas del Toro, Panama and obtained from W.P. Davis (U.S. Environmental Protection Agency, Florida). This strain was collected in 1994 and has been reared in our laboratory for over 5 generations since 1998.

Ten fish for each dietary treatment were individually reared from hatching (day 0) in 1 l aquarium filled with 700 ml of 17 g l\(^{-1}\) artificial brackish water, with mild aeration and under natural photoperiod for 20 days. Aquaria were arranged randomly in 150 l water bath maintained at 25 ± 1 °C using a cooling thermo pump (CTP 201, Eyela, Japan). The daily feed for each tank was as follows: 100-1500 individuals of mixed stages (nauplius, copepodite, and adult) of *A. tsuensis* for D1, and 12 to 365 individuals of 2-day old *A. franciscana* nauplii for D2 and D3, depending on the age of fish. In
order to feed the fish to satiation and to minimize the remaining feed in each tank, the amount of feed remaining was counted daily for each aquarium and the amount of feed fed was adjusted accordingly. *Chaetoceros* sp and *Isochrysis* sp were added at a density of $1 \times 10^5$ cells ml$^{-1}$ and $2 \times 10^5$ cells ml$^{-1}$, respectively to each aquarium every 2 days. The amount of microalgae left was checked and the amount fed was adjusted accordingly to maintain the same density. Culture water was not replaced throughout the experiment.

2.4 Growth and behavioural experiments

Growth measurements and behavioural observations were made on fish on days 1, 10, and 20. All fish were starved 24 h prior to observation. The observation container (7.5 cm x 10 cm) was placed in a water bath, which was maintained at $25 \pm 1 \, ^\circ$C. Fish were transferred to the observation container with a depth of 2 cm of 17 g l$^{-1}$ artificial brackish water. Fish were acclimated for 10 min prior to observation. Behaviour was recorded 10 min before and 10 min after feeding from above using a video camera (TRV 50, Sony Corp., Japan). At each observation period, the same amount of feed was fed to all individuals of the same age. For D1, fish were fed 100-185 individuals of *A. tsuensis*, and for D2 and D3, 11-35 individuals of 2-day old *A. franciscana*, depending on the age of fish. After each observation, fish were anaesthetized with 100 mg l$^{-1}$ of MS 222 (3-aminobenzoic acid ethyl ester, Sigma Chemical Co., St. Louis, MO) for a few seconds. Then, growth (standard length, SL) was measured to the nearest 0.01 mm using a digital microscope. Immediately after measurement, fish were allowed to recover in 1 l of aerated 17 g l$^{-1}$ artificial brackish water for 10 min, before being returned to the rearing aquaria.
The behaviours observed were as follows: focus (fish turns and orients toward the prey), attack (movement of fish towards the prey prior to ingestion), ingest (fish eats the prey), and fail (fish is unable to ingest prey). These data were used to calculate the following indices: feeding success (number of prey ingested over the number of attacks) and ingestion rate (number of prey ingested min\(^{-1}\)). Satiation time (min), the time the fish fed until satiation, was also recorded. The total time the fish spent at rest and swimming, 10 min before and 10 min after feeding were also observed (Almazan-Rueda et al., 2004).

Condition factor (CF), which is based on the final weight and length of fish fed the different diets was calculated using the formula: [wet weight (g)/standard length (cm)] X 10\(^3\).

2.5 Fatty acid analysis

Samples of *A. franciscana* (unenriched and enriched) and *A. tsuensis* were concentrated separately in a sieve (100 \(\mu\)m) and washed with distilled water. All fish fed the different live feeds were starved 24 h prior to sampling and were also washed with distilled water. All samples were weighed (mg), pooled for each treatment, immediately frozen and stored at –80 °C for fatty acid analysis. Samples were analyzed for fatty acid composition by a commercial laboratory (Chlorella Industry Co., Ltd, Fukuoka, Japan), and for total lipid content by the method of Folch et al. (1957).

2.6 Statistical analysis

Comparison of growth and behavioural parameters among fish fed the different diets at each age group was done using one-way ANOVA and further analyzed using Fisher’s PLSD posthoc test. Analysis was done using a statistical software program
(StatView ver. 5, SAS Inst. Inc.). Size of live feeds was compared using Student’s t-test
(Minitab, release 13.31, Minitab, Inc.). Final wet weight (mg) and CF of fish fed the
different diets were compared using one-way ANOVA and further analyzed using
Fisher’s PLSD posthoc test.

3. Results

The size frequency of mixed stages of *A. tsuensis* used in this study is shown in
Fig. 1. The sizes (mean ± S.D.) of *A. tsuensis* (454 ± 228 μm) and *A. franciscana* (762 ±
59 μm) differed significantly (Student’s t-test, \( t = -11.93, P < 0.001 \)). Size ranges of *A.
tsuensis* and *A. franciscana* were 104-732 μm and 656-906 μm, respectively.

Early growth in D1-fed larvae was significantly higher than D2- and D3-fed
larvae on day 10 (Fisher’s PLSD, \( P < 0.05 \)). On day 20, D3-fed larvae had a
significantly higher growth than the D1- and D2-fed larvae (Fisher’s PLSD, \( P < 0.0001 \);
Fig. 2). Similarly, final weight of D3-fed fish was significantly higher than fish fed D1
and D2 (\( P < 0.01 \)). However, condition factor did not differ among fish fed the different
diets.

One-day old larvae had significantly higher feeding success with D1 than D3
(Fisher’s PLSD, \( P < 0.05 \)). Feeding success among larvae fed the different diets did not
differ on days 10 and 20 (Fig. 3). Both ingestion rate and satiation time did not differ
among larvae fed the different diets at all age groups.

Swimming activity before feeding was significantly higher in larvae fed D2 and
D3 compared with D1 on day 10 (Fisher’s PLSD, \( P < 0.05 \)). However, swimming
activity among larvae fed the different diets was of the same level on day 20 (Fig. 4a).
With food present, swimming activity did not differ among larvae fed the different diets at all age groups (Fig. 4b).

Eicosapentaenoic acid, EPA (mg 100 g wet wt⁻¹) levels in fish fed all dietary treatments increased from 0.2 - 3 fold compared to the diets. Despite the absence of DHA in the diets, an increase of 120.9 mg 100 g wet wt⁻¹ was detected in fish. DHA (mg 100 g wet wt⁻¹) in D1- and D3-fed fish increased from 3 - 8 fold compared with the diet (Table 1 and 2).

4. Discussion

The present study was able to demonstrate the successful culture and use of copepods in a small-scale experiment to investigate their effects on early growth and behaviour of *R. marmoratus*. Most studies on the effect of copepods on marine finfish have reported improvement in larval growth and survival in yellowtail flounder, (Copeman et al., 2002) and red-spotted grouper (Doi et al., 1997), growth of European sea bass (Skalli and Robin, 2004), larval haddock and American plaice (Nanton and Castell, 1998), dorsal pigmentation of turbot and halibut (Bell et al., 2003), and broodstock reproductive performance and egg and larval quality of Atlantic halibut (Mazorra et al., 2003), which have been attributed to its nutritional effects. Copepods have been reported to contain higher amounts of highly unsaturated fatty acids (n-3 HUFA) content, particularly EPA (20:5n-3) and DHA (Watanabe et al., 1983; Evjemo et al., 2003; Hernandez Molejon and Alvarez-Lajonchere, 2003). The present study did not only confirm the positive effects of copepods on growth similar with previous reports, but also reports its effect on behavioural development in marine finfish using *R.*
marmoratus. It is also the first attempt to correlate growth with behavioural observations.

Higher growth observed in Acartia-fed fish on day 10 may be due to lower swimming activity of the larvae. Swimming activity is energy-costly especially for larvae with poor energy-saving mechanisms (Kamler, 1992). This activity involves consumption of high amounts of oxygen, ranging from 2 to 15 times above the resting level in some species of fish larvae, such as brown trout, Pacific sardine, whitefish, and in some cyprinids (Kamler, 1992).

Our results showed that the effects of the live feeds were mainly due to the type and size of live feed species rather than their nutritional composition. Despite the absence of DHA in the feed, DHA was detected in R. marmoratus indicating that they are capable of synthesizing DHA. Thus, higher growth in Acartia-fed fish on day 10 may be related to the suitable size (composed mainly of 55% of 500-600 µm copepodites and adults and 34% of 100-200 µm nauplii) of A. tsuensis rather than their DHA content. On the other hand, the positive nutritional effect of Acartia containing high amounts of DHA on larval growth still remains a possibility. Lower growth observed on day 20 may be due to the shift in food size preference of the fish. Also, it may be possible that EPA exerted a positive effect on the growth of fish fed DHA-enriched Artemia.

The decrease in growth from day 10 to 20 in Acartia-fed fish and conversely, the increase in growth in Artemia-fed fish may be attributed to the shift in food size preference. The shift in size preference can be related to morphological, physiological, and behavioural changes occurring at these phases, which were previously described (Grageda et al., 2004; unpublished observations). Based on the size, larvae fed Acartia
on day 10 can be classified under the shift to exogenous feeding phase while the fish fed enriched *Artemia* on day 20, under the juvenile phase. During the shift to exogenous feeding, higher growth was observed in larvae feeding on smaller-sized prey (*A. tsuensis*; 104-732 μm), however, as it approached the juvenile phase, higher growth was observed in fish feeding on larger-sized prey (*A. franciscana*; 656-906 μm). During the shift to exogenous feeding, *R. marmoratus* have been reported to possess complete fin-ray counts in the majority of the fins, and to undergo increased ossification in the skull, vertebrae and fin rays (Grageda et al., 2004), which coincided with increased swimming activity similar with observations in sea breams (Faustino and Power, 2004). These features may have contributed to increased efficiency in catching *A. tsuensis*, which has been reported to swim in an irregular and zigzag motion (Shuvayev, 1978 as cited in Govoni et al., 1986). However, digestive enzyme activities in the digestive tract (such as esterase and alkaline phosphatase) at this phase are still low (Kolkovski, 2001), indicating that the larva has limited absorptive capacity, thus, it prefers smaller-sized and easily digestible prey. As the fish approached the juvenile phase, it shifted to a prey with a more regular swimming movement, complementing with the unchanged swimming activity of the fish at this phase. Since a positive effect in growth was observed among fish feeding on *A. franciscana*, larger-sized prey may be preferred at the juvenile phase. This indicates that the fish is physiologically capable of digesting and absorbing larger prey, as evidenced by efficient transformation of food to somatic growth. This could be attributed to increased digestive and absorptive efficiency, as evidenced by a significant increase in digestive enzyme activities such as alkaline phosphatase and esterase, and increased mucosal folds and goblet cells in the digestive tract at the juvenile phase, as observed in developing seabream larvae (Moyano et al.,
1996). Also, zymogen granules, known precursors of proteolytic enzymes (Gisbert et al., 2004), are distinctly visible at this phase (unpublished observations), indicating active pancreatic secretions. Positive correlation between gape size and body size of fish and the size of prey has been reported (De Vries, 1998). In *R. marmoratus*, gape size increases with age, however, gape size relative to standard length decreases with age at the early stage of development (Grageda et al., 2004). A similar observation has been reported in both field-caught and laboratory-reared red drum larvae and juveniles, showing that the size of prey consumed was not constrained by gape size (Krebs and Turingan, 2003). This suggests that other prey-capture mechanisms such as the development of feeding apparatus (such as hyoid apparatus) may influence a shift in prey size preference (Krebs and Turingan, 2003). Moreover, this observed increase in consumption of larger prey with growth is consistent with previous findings on greenback flounder, long-snouted flounder and red drum (Jenkins, 1987; Krebs and Turingan, 2003). Apart from prey size, characteristics of prey have also been identified as an important factor in prey selectivity (Checkely, 1982). Other factors affecting prey selection have been identified related to the characteristics of the larvae. Meng and Orsi (1991) demonstrated that learning and swimming behaviour of striped bass larvae and their interaction with their prey strongly affects prey selectivity. Moreover, the importance of learning behaviour and innate preference by the percid and flounder larvae has been suggested (Jenkins, 1987; Wahl et al., 1995). Similarly, a positive preference for familiar prey has been observed in greenback flounder larvae (Cox and Pankhurst, 2000).

The importance of copepods in the early larval nutrition of *R. marmoratus* was also demonstrated in this study. This species performed better when fed with *A. tsuensis*
during the early larval stage as evidenced by higher feeding success compared with *A. franciscana* on day 1, and a positive effect on larval growth on day 10. Higher density of *A. tsuensis* compared with *A. franciscana*, may have contributed to this effect, however, these densities had to be maintained to be able to feed the fish to satiation and to reduce the remaining feed in each tank. Higher feeding preference for *Acartia* may be indicative of the innate preference of the larvae for copepod prey as previously suggested by Jenkins (1987). Also, our study confirmed previous observations regarding food preference of *R. marmoratus*, revealing the presence of an unidentified harpacticoid copepod based on indirect observation through gut analysis of specimens collected from the field (Taylor, 1992). Copepods are commonly present in mangrove estuaries, thus, calanoids, another order of copepods to which *Acartia* belongs, may play an important role in early larval feeding of *R. marmoratus*. Previous studies on food habits of this species have reported gastropods, insects, amphipods, isopods, crustacean parts, fragments of annelid worms, and fish scales as their food (Harrington and Rivas, 1958; Huehner et al., 1985; Taylor, 1992). It is possible that calanoids would constitute a significant part of their diet during the larval stage in their natural habitat, although this needs further confirmation in the field. *A. tsuensis* belongs to the family Acartiidae, a group composed of species found in estuarine and neritic environments throughout the world (Mauchline, 1998).

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References


Table 1. Highly unsaturated fatty acid (HUFA) composition (mg 100g wet wt⁻¹) of different diets (D1: *Acartia tsuensis*; D2: unenriched *Artemia franciscana* and D3: DHA-enriched *A. franciscana*).

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<th>HUFA</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
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<tr>
<td>Eicosapentaenoic acid</td>
<td>14.2 (4.7)</td>
<td>17.3 (2.1)</td>
<td>71.4 (5.3)</td>
</tr>
<tr>
<td>Docosahexaenoic acid</td>
<td>26.1 (8.6)</td>
<td>0 (0)</td>
<td>57.9 (4.3)</td>
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<tr>
<td>Arachidonic acid</td>
<td>1.5 (0.5)</td>
<td>5.8 (0.7)</td>
<td>14.8 (1.1)</td>
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<tr>
<td>DHA/EPA</td>
<td>1.8</td>
<td>-</td>
<td>0.8</td>
</tr>
<tr>
<td>Σ n-3 HUFA</td>
<td>41.8 (13.8)</td>
<td>23.1 (2.8)</td>
<td>144.1(10.7)</td>
</tr>
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</table>

Number in parenthesis indicates % of total fatty acids.
Table 2. Highly unsaturated fatty acid (HUFA) composition (mg 100g wet wt\(^{-1}\)) of mangrove killifish fed different diets (D1: *Acartia tsuensis*; D2: unenriched *Artemia franciscana* and D3: DHA- enriched *A. franciscana*) at 22 days after hatching.

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<th>HUFA</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
</tr>
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<tr>
<td>Eicosapentaenoic acid</td>
<td>20.4 (1.7)</td>
<td>69.5 (2.3)</td>
<td>87.7 (2.1)</td>
</tr>
<tr>
<td>Docosahexaenoic acid</td>
<td>233.2 (19.4)</td>
<td>120.9 (4.0)</td>
<td>213.0 (5.1)</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>19.2 (1.6)</td>
<td>33.2 (1.1)</td>
<td>66.8 (1.6)</td>
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<tr>
<td>DHA/EPA</td>
<td>11.4</td>
<td>1.7</td>
<td>2.4</td>
</tr>
<tr>
<td>Σ n-3 HUFA</td>
<td>272.8 (22.7)</td>
<td>223.6 (7.4)</td>
<td>367.5 (8.8)</td>
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Number in parenthesis indicates % of total fatty acids.
Fig. 1. Size frequency (%) of mixed stages (nauplius, copepodite, and adult) of *Acartia tsuensis* used in the feeding experiment.

Fig. 2. Growth expressed as standard length (mean mm ± S.D.) of mangrove killifish fed different diets (D1: *Acartia tsuensis*, triangle with short broken lines; D2: unenriched first instar *Artemia franciscana* nauplii, circle with solid line; D3: DHA-enriched first instar *A. franciscana* nauplii, square with long broken lines) for 20 days. Different letters indicate significant difference among fish fed different diets at each age group (Fisher’s PLSD, P < 0.05, a > b).

Fig. 3. Feeding success (mean % ± S.D.) of mangrove killifish fed different diets (D1: *Acartia tsuensis*, solid bars; D2: unenriched first instar *Artemia franciscana* nauplii, open bars; D3: DHA-enriched first instar *A. franciscana* nauplii, bars with diagonal lines) for 20 days. Different letters indicate significant difference among fish fed different diets at each age group (Fisher’s PLSD, P < 0.05, a > b).

Fig. 4. Swimming activity (mean % ± S.D.) of mangrove killifish fed different diets (D1: *Acartia tsuensis*, solid bars; D2: unenriched first instar *Artemia franciscana* nauplii, open bars; D3: DHA-enriched first instar *A. franciscana* nauplii, bars with diagonal lines) for 20 days at (a) 10 min before and (b) 10 min after feeding. Different letters indicate significant difference among fish fed different diets at each age group (Fisher’s PLSD, P < 0.05, a > b).
Fig. 1.
Fig. 2.
Fig. 3.
Fig. 4.