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The effect of dietary n-3 HUFA levels and DHA/EPA ratios on growth, survival and osmotic stress tolerance of Chinese mitten crab *Eriocheir sinensis* larvae

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Abstract

The effect of varying levels of dietary n-3 highly unsaturated fatty acid (HUFA) and docosahexaenoic acid/eicosapentaenoic acid (DHA/EPA) ratios on growth, survival and osmotic stress tolerance of *Eriocheir sinensis* zoea larvae was studied in two separate experiments. In experiment I, larvae were fed rotifers and *Artemia* enriched with ICES emulsions with 0, 30 and 50% total n-3 HUFA levels but with the same DHA/EPA ratio of 0.6. In experiment II, larvae were fed different combinations of enriched rotifers and *Artemia*, in which, rotifers were enriched with emulsions containing 30% total n-3 HUFA, but different DHA/EPA ratio of 0.6, 2 and 4; while *Artemia* were enriched with the same emulsions, but DHA/EPA ratio of 0.6 and 4. In both experiments, un-enriched rotifers cultured on baker's yeast and newly-hatched *Artemia* nauplii were used as control diets. Larvae were fed rotifers at zoea 1 and zoea 2 stages; upon reaching zoea 3 stage, *Artemia* was introduced.

Experiment I revealed no significant effect of prey enrichment on the survival of megalopa among treatments, but higher total n-3 HUFA levels significantly enhanced larval development (larval stage index, LSI) and resulted in higher individual dry body weight of megalopa. Furthermore higher dietary n-3 HUFA levels also resulted in better tolerance to salinity stress. Experiment II indicated that at the same total n-3 HUFA level, larvae continuously receiving a low dietary DHA/EPA ratio had significantly lower survival at the megalopa stage and inferior individual body weight at the megalopa stage, but no negative effect was observed on larval development (LSI). The ability to endure salinity stress of zoea 3, zoea 5 and megalopa fed diets with higher DHA/EPA ratio was also improved.
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Keywords: *Eriocheir sinensis* larvae; n-3 HUFA; DHA/EPA ratio; Osmotic stress tolerance

1. Introduction

China is the only country in the world where Chinese mitten crab (*Eriocheir sinensis*) larval rearing and farming are practiced. In 2004, annual aquaculture production reached about 500,000 metric tons, valued at 4 billion US$

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(Yang and Zhang, 2005). A breakthrough in mass propagation in hatcheries of *E. sinensis* larvae was achieved in the early 1980s (Zhao, 1980). During the last two decades, substantial improvements of hatchery techniques have resulted in a rapid increase of larval availability. However, poor and variable survival of the larvae, particularly at molting of zoea 1 (Z1) to Z2 and metamorphosis of Z5 to megalopa stage, are still a major problem in *E. sinensis* larvae production (Zhou et al., 2000). Apart from ontogenetic and zootechnical factors, nutritional deficiencies or unbalances may contribute to the generally low larval survival.

As a catadromous species, *E. sinensis* spends most of its life time in freshwater and only migrates to the sea to reproduce. Before the benthic juveniles move upstream to freshwater, the pelagic larvae pass five zoea stages in seawater. In hatcheries, *E. sinensis* larvae are mostly cultured on a combination of microalgae, rotifers and *Artemia*. Rotifers and *Artemia* contain high levels of short chain saturated and mono-unsaturated fatty acids, but often lack n-3 highly unsaturated fatty acids (n-3 HUFA), such as eicosapentaenoic acid (EPA, 20:5 n-3) (in rotifers) and docosahexaenoic (DHA, 22:6 n-3) (in rotifers and *Artemia*) (Lavens and Sorgeloos, 1996). It is well known that marine crustaceans have a limited ability to elongate C18 n-3 poly unsaturated fatty acids (n-3 PUFA) to n-3 HUFA (Kanazawa and Teshima, 1977; Kanazawa et al., 1979; Suprayudi et al., 2004a), which therefore must be obtained from the feed, for example, through rotifer and *Artemia* enrichment. In contrast to marine species, freshwater species have a lower requirement for n-3 HUFA and a higher capacity to elongate and desaturate these from shorter chain fatty acids (Sargent et al., 1999). Because of the catadromous nature of *E. sinensis*, it is not clear whether it should be regarded as a freshwater or with marine species in aspects of lipid nutrition.

Dietary n-3 HUFA supplementation, mainly EPA and DHA have been reported to improve survival and/or growth of shrimp larvae and postlarvae, such as *Fenneropenaeus chinensis* (Xu et al., 1993), *Penaeus monodon* (Millamena et al., 1988; Rees et al., 1994), *Penaeus japonicus* (Kontara et al., 1997) and *Litopenaeus vannamei* (Leger and Sorgeloos, 1992; Lim et al., 1997; Wouters et al., 1997); as well as crab larvae, such as *Eurypanopeus depressus* (Levine and Sulkin, 1984), *Portunus trituberculatus* (Takeuchi et al., 1999) and *Scylla serrata* (Davis, 2003; Suprayudi et al., 2004b). Moreover, quantitative requirements for dietary n-3 HUFA have been extensively investigated. Kanazawa et al. (1979) suggested that a dietary provision of 1% (10 mg g^{-1} dw, dry weight) n-3 HUFA could be considered as a minimal value for postlarval penaeids. Rees et al. (1994) reported that 12.55 mg g^{-1} dw n-3 HUFA in the enriched *Artemia* enhanced survival of *P. monodon* postlarvae, while supplying very high n-3 HUFA levels (31.2 mg g^{-1} dw) to the postlarvae had no effect on the survival. Furthermore, dietary n-3 HUFA was also reported to have a positive effect on the ability of penaeid shrimp to resist stress conditions, such as osmotic shock (Tackaert et al., 1992; Rees et al., 1994; Palácios et al., 2004), temperature fluctuation (Chim et al., 2001) and ammonia (Martins et al., 2006). Although similar effects of dietary HUFA on the survival, growth and salinity stress tolerance of *E. sinensis* zoea larvae and juveniles were observed by Cheng et al. (1998), Xin et al. (1999) and Chen et al. (2000), no studies to date have investigated the quantitative requirements of mitten crab larvae for n-3 HUFA and more specifically the DHA/EPA ratio.

The aim of this study was to determine whether boosting rotifers and *Artemia* with different levels of n-3 HUFA and DHA/EPA ratios would affect growth, survival, metamorphosis and osmotic stress tolerance of *E. sinensis* zoea larvae.

2. Materials and methods

2.1. Broodstock management and egg incubation

The study was carried out at the Tianjin Modern Fishery Technology and Engineering Center, Tianjin, China. The breeders were selected from crab farms in Chongming, Shanghai and Yangcheng Lake, Jiangsu, and were transported to the center in February 2004 and 2006, for experiments I and II, respectively. After acclimation to the indoor captive conditions for a week, the female and male crabs (in a ratio of 3:1) were then placed into mating tanks containing 20 g L^{-1} diluted seawater at 15 °C. After spawning, 10 berried females were selected and transferred into 1 × 1 × 1 m fiberglass incubation tanks equipped with shelters. Crabs were fed fresh clam *Ruditapes variegata* once a day at 10% of the total body weight. Different incubation temperatures were used in both experiments. This was done for practical reasons in order to be able to spread the hatching of the larvae in time. In experiment I, low temperature incubation was applied through the use of an aquarium cooling machine. Water temperature was decreased gradually from 15 °C to 8 °C at a rate of 1 °C per day. After 60 days incubation at 8 °C, water temperature was increased gradually by 0.5 °C per day until 21 °C. In experiment II, high temperature incubation was conducted by increasing water temperature immediately after spawning, from 15 °C to 21 °C with a daily increase of 0.5 °C. Once the incubation temperature reached 21 °C, embryogenesis was observed daily under a microscope. Water was renewed every 2 days during this process. One day before the eggs were expected to hatch, the crabs were disinfected in a 0.2 g L^{-1}

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formalin bath for 30 min and transferred individually into 160 L plastic hatching tanks containing 20 g L^{-1} diluted seawater at 22 °C. After the larvae were released, the spent crab was immediately removed from the hatching tank and the larvae were fed un-enriched rotifers *ad libitum*. One day after hatching (DAH 1), positively photo-tactic Z1 larvae from a single breeder were selected and distributed randomly into the experimental larval rearing set-up.

2.2. Larval rearing

From DAH 2 onwards, larvae were fed the different experimental diets twice a day (at 10 am and 22 pm). The prey density was kept as following: Z1: 10 rotifers mL^{-1}; Z2: 15 rotifers mL^{-1}; Z3: 10 rotifers+0.5 *Artemia* mL^{-1}; Z4: 1 *Artemia* mL^{-1}; Z5: 1.5 *Artemia* mL^{-1}.

Each treatment consisted of six replicate beakers containing 1.5 L diluted seawater stocked with 200 Z1 for the purpose of survival and larval stage index (LSI) determination; and four rectangular PVC tanks containing 20 L diluted seawater at an approximate stocking density of 130 Z1 L^{-1} for assessing body weight, osmotic stress tolerance and tissue HUFA composition.

During the larval rearing period, water temperature ranged between 22 °C and 23 °C. Temperature in both beakers and PVC tanks was maintained in a heated water bath. Salinity was controlled at 20 g L^{-1}. pH ranged between 8.1 and 8.4. Aeration was gentle at Z1 stage and gradually increased at each stage to become very strong when Z5 molted into megalopa in order to prevent cannibalism. Photoperiod was maintained at 12L:12D with white fluorescent tubes.

From Z3 onwards, part of the water in the PVC tanks was renewed every morning to remove dead larvae, faeces, uneaten rotifers and *Artemia*. The water renewal increased from 1/2 to 2/3 of the tank volume along with larval development. Each day the content of the plastic beakers was gently poured into a bowl and the surviving larvae were counted while being pipetted into new beakers filled with new culture water. These new containers had been incubated in the water bath beforehand to adjust the water to the same temperature.

2.3. Rotifer and Artemia culture and enrichment

Rotifers (*Brachionus rotundiformis*) were cultured in a 100 L cylindrical PVC tank and fed baker's yeast. Rotifer enrichment was performed over a 3 h period at 33 °C, at a density of 2000 rotifers mL^{-1}, with a dose of 0.1 g emulsion L^{-1} according to the treatments outlined below. *Artemia* cysts (EG® type, INVE Aquaculture NV, Belgium) were hatched and harvested as described by Van Stappen (1996). The newly-hatched nauplii were enriched for 24 h at 28 °C, at a density of 300 nauplii mL^{-1}, with doses of 0.3 g emulsion L^{-1} added at a 12 h interval. After enrichment, the animals were rinsed thoroughly with clean seawater to remove any remaining emulsion, and fed to the larvae. Rotifer enrichment was done twice a day (for each feeding). Both enriched metanauplii and newly-hatched *Artemia* which could not immediately be fed to the larvae were kept at ±7 °C to minimize fatty acid catabolism and hence keep the nutritional value constant (Merchie, 1996).

The lipid emulsions used for live feed enrichment in this study were ICES Standard Reference Emulsions (ICES, 1997). Over the two experiments, five different live feed enrichment emulsions were used: ICES 0/-/c emulsion containing mainly saturated fatty acids; ICES 30/0.6/c and ICES 50/0.6/c emulsions containing 30% and 50% total n-3 HUFA, respectively, but with the same DHA/EPA ratio of 0.6; ICES 30/2/c and ICES 30/4/c both containing the same level of 30% total n-3 HUFA, but with DHA/EPA ratios of 2 and 4, respectively.

In experiment I, the rotifers and *Artemia* nauplii were enriched with emulsions containing 0, 30% and 50% of total n-3 HUFA levels, respectively:

Treatment 1 (T1): Rotifers+*Artemia* nauplii enriched with emulsion ICES 0/-/c.
Treatment 2 (T2): Rotifers+*Artemia* nauplii enriched with emulsion ICES 30/0.6/c.
Treatment 3 (T3): Rotifers+*Artemia* nauplii enriched with emulsion ICES 50/0.6/c.

The control consisted of non-enriched rotifers and non-enriched newly-hatched *Artemia* nauplii.

In experiment II, the rotifers and *Artemia* nauplii were enriched with emulsions containing 30% of total n-3 HUFA, but varying DHA/EPA ratios: 0.6, 2 and 4, respectively:

Treatment 1 (T1): Rotifers enriched with 30/0.6/c+*Artemia* nauplii enriched with 30/0.6/c.
Treatment 2 (T2): Rotifers enriched with 30/0.6/c+*Artemia* nauplii enriched with 30/4/c.
Treatment 3 (T3): Rotifers enriched with 30/2/c+*Artemia* nauplii enriched with 30/0.6/c.
Treatment 4 (T4): Rotifers enriched with 30/2/c+*Artemia* nauplii enriched with 30/4/c.
Treatment 5 (T5): Rotifers enriched with 30/4/c+*Artemia* nauplii enriched with 30/0.6/c.
Treatment 6 (T6): Rotifers enriched with 30/4/c+*Artemia* nauplii enriched with 30/4/c.

The control consisted of non-enriched rotifers and non-enriched newly-hatched *Artemia* nauplii.

2.4. Biological parameters

2.4.1. Larval stage index and individual body weight

Larval development was monitored daily by identifying the stage of each larva in the beakers and assigning it a value: Z1=1, Z2=2, etc. to megalopa M=6. To compare larval development among treatments, an average larval stage index (LSI) in each treatment was then calculated as described by Millamena and Bangcaya (2001). At different larval stages, respectively 200 Z1, 100 Z3, 50 Z5 and 20 megalopa from each replicate PVC tank were sampled and rinsed thoroughly

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with distilled water, and the individual dry body weight was determined (60 °C, 24 h).

2.4.2. Survival

In both trials, the main experiment was stopped when all Z5 had metamorphosed to megalopa. Survival at that time was calculated on the initial number of Z1 (200 individuals). In experiment II, newly-metamorphosed megalopa were however removed daily from the rearing containers to avoid cannibalism on the remaining Z5. These megalopa from each treatment were transferred to four plastic beakers containing 1500 mL 20 g L^{-1} diluted seawater and fed the different enriched *Artemia* as before for a follow-up experiment. The survival of these megalopa after 3 days (DAH 22) rearing was then determined.

2.4.3. Osmotic stress tolerance

In the different treatments, the osmotic stress tolerance of Z3, Z5 and megalopa was tested. Since resistance to osmotic stress is generally believed to increase with the age of crustacean larvae (Samocha et al., 1998), different salinities were used at Z3, Z5 and megalopa stages. The larvae were suddenly transferred from the normal rearing water (20 g L^{-1}) into water with a salinity of 45 g L^{-1}, 50 g L^{-1} and 60 g L^{-1}, respectively. A static system without aeration was applied. A group of 10 larvae from each replicate PVC tank was randomly sampled and transferred into plastic beakers containing 100 mL water. Four replicates were conducted for each treatment. Mortality was assessed at 10 min intervals. Larvae were considered dead when there was no movement of the appendages and when they did not respond to prodding with a glass pipette. Average cumulative mortalities of the different treatments at different time intervals are reported. In addition a cumulative stress index (CSI) was calculated on the accumulative number of dead larvae at different time intervals as described by Dhert et al. (1992).

2.5. Chemical analysis

Rotifers and *Artemia* (before and after enrichment) were sampled on three separate occasions and pooled for lipid analysis. Z1, Z3, Z5 and megalopa from each treatment were also randomly sampled from each replicate tank and pooled together. After rinsing with distilled water, the samples were stored at −20 °C for later analysis. The total lipid contained in the live food and larvae were extracted according to Folch et al. (1957) using chloroform and methanol (2:1, v/v). The fatty acid profiles were analyzed according to the modified procedure of Lepage and Roy (1984).

2.6. Statistical analysis

Data were subjected to statistical analysis using the software SPSS. Statistical significance of differences among treatments was determined using one-way ANOVA. Duncan multiple range test was applied to detect significant differences

Table 1
Experiment I: Fatty acid profile of rotifers and *Artemia* nauplii enriched with emulsion containing 0, 30 and 50% n-3 HUFA

Fatty acids (mg g^{-1} dw)	Rotifers				*Artemia* nauplii			
	T1	T2	T3	Control	T1	T2	T3	Control
	0% n-3 HUFA	30% n-3 HUFA	50% n-3 HUFA	(un-enriched)	0% n-3 HUFA	30% n-3 HUFA	50% n-3 HUFA	(un-enriched)
16:0	8.52	10.61	7.87	5.79	12.70	15.78	12.44	15.11
16:1 n-7	5.90	8.13	5.93	6.10	2.74	5.22	6.69	3.89
17:0	0.61	0.87	0.62	0.58	0.66	1.01	1.90	0.90
17:1 n-7	0.59	0.42	0.78	0.63	0.80	1.00	1.15	1.12
18:0	3.41	3.48	3.73	2.50	6.81	7.59	7.74	6.37
18:1 n-9+n-7	8.29	10.01	9.17	5.76	28.31	34.22	36.40	34.02
18:2 n-6	5.24	4.63	4.81	3.34	7.32	8.01	10.13	9.01
18:3 n-6	0.40	0.46	0.50	0.49	0.66	0.85	1.17	1.12
18:3 n-3	2.06	2.11	2.16	2.01	26.90	30.55	33.14	41.25
18:4 n-3	0.43	1.10	0.94	0.40	4.53	4.83	5.43	7.12
20:4 n-6	0.67	0.92	1.52	0.58	1.01	1.37	4.64	1.01
20:4 n-3	2.41	2.46	3.01	2.42	0.68	1.10	1.92	1.14
20:5 n-3	3.29	8.11	13.40	1.31	2.97	12.12	19.94	3.06
22:1 n-9+n-7	0.94	1.22	2.07	0.92	0.10	0.11	1.09	0.10
21:5 n-3	0.10	0.26	0.63	0.10	–	0.29	0.29	–
22:5 n-3	0.82	1.51	1.58	0.68	–	0.84	0.69	–
22:6 n-3	–	4.73	9.02	–	–	3.87	7.15	–
Total n-3 HUFA ($n \geq 20$)	6.22	17.07	27.64	3.01	3.65	18.22	29.99	4.20
Total n-6 HUFA ($n \geq 18$)	6.31	6.01	6.83	4.41	8.99	10.23	15.94	11.14
DHA/EPA	–	0.58	0.67	–	–	0.32	0.36	–
Total lipid (% dw)	15.21	15.49	18.90	4.83	16.40	18.72	21.71	14.37

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between means ($P<0.05$). Percentage data were arc-sin square root transformed prior to analysis.

3. Results

3.1. Experiment I

The enrichment notably affected the total n-3 HUFA content in the rotifers and *Artemia* (Table 1). The rotifers and *Artemia* enriched with ICES 0/-/c (T1) contained low total n-3 HUFA levels (6.22 mg g^{-1} dw in rotifers, 3.65 mg g^{-1} dw in *Artemia*), which was similar to the un-enriched ones (3.01 mg g^{-1} dw in rotifers, 4.20 mg g^{-1} dw in *Artemia*). On the other hand, the rotifers and *Artemia* enriched with ICES 30/0.6/c (T2) and ICES 50/0.6/c (T3) contained much higher levels of total n-3 HUFA (17.07 and 27.64 mg g^{-1} dw in rotifers, 18.22 and 29.99 mg g^{-1} dw in *Artemia*, respectively). Generally, when using the same emulsion, the DHA/EPA ratio in the enriched rotifers was higher than that in the enriched *Artemia*, (0.58 and 0.67 in T2 and T3 rotifers, 0.32 and 0.36 in T2 and T3 *Artemia*, respectively). Total n-6 HUFA content in rotifers and *Artemia* of the different treatments remained relatively stable.

The fatty acid composition of Z1, Z3, Z5 and megalopa is shown in Table 2. The total n-3 HUFA levels and DHA/EPA ratios of Z3, Z5 and megalopa reflected to some extent the levels in the enriched rotifers and *Artemia* with increasing total n-3 HUFA, DHA and EPA levels from T1 to T3. Both Z5 and megalopa generally contained higher levels of total n-3 HUFA (ranging from 4.92 to 20.50 mg g^{-1} dw for Z5, from 4.91 to 20.61 mg g^{-1} dw for megalopa) than Z3 (ranging from 7.10 to 12.39 mg g^{-1} dw), which mostly came from higher EPA levels in the body tissue.

No statistically significant differences were observed in megalopa survival among any of the treatments (Table 3). However, the megalopa receiving higher dietary n-3 HUFA levels (T2 and T3) had a higher LSI and individual body weight than those in T1 and the control group ($P<0.05$). When being abruptly transferred from 20 g L^{-1} culture water to 60 g L^{-1} brine water, the trend of cumulative mortality of megalopa was influenced by the dietary n-3 HUFA level. The cumulative stress index (CSI) at the megalopa stage was significantly affected by the dietary n-3 HUFA level, with increasing stress sensitivity in the order T3, T2, T1 and the control group ($P<0.05$) (Fig. 1 and Table 3).

Table 2
Experiment I: Fatty acid composition and total lipid content of *E. sinensis* Z3, Z5 and megalopa fed rotifers and *Artemia* containing different total n-3 HUFA levels

Fatty acids (mg g^{-1} dw)	Z1	Z3				Z5				Megalopa			
		T1 0% n-3 HUFA	T2 30% n-3 HUFA	T3 50% n-3 HUFA	Control (un-enriched)	T1 0% n-3 HUFA	T2 30% n-3 HUFA	T3 50% n-3 HUFA	Control (un-enriched)	T1 0% n-3 HUFA	T2 30% n-3 HUFA	T3 50% n-3 HUFA	Control (un-enriched)
16:0	4.42	4.85	5.19	5.07	3.41	5.81	7.20	7.32	5.10	7.39	8.37	6.80	4.64
16:1 n-7	1.93	1.40	1.58	1.57	0.89	2.87	5.35	3.88	2.91	4.37	6.00	3.83	2.21
17:0	–	–	–	–	–	–	–	–	–	–	–	–	–
17:1 n-7	–	–	–	–	–	–	–	–	–	–	–	–	–
18:0	2.08	3.69	3.62	3.69	2.92	3.38	4.64	4.72	3.58	4.07	4.25	4.22	0.00
18:1 n-9+ n-7	16.07	9.26	8.88	9.68	5.98	16.68	25.19	22.71	18.12	22.40	25.76	22.71	15.77
18:2 n-6	5.31	3.51	2.62	2.83	2.48	5.12	6.08	5.27	3.78	6.36	5.94	5.15	2.21
18:3 n-6	–	–	–	–	–	–	–	–	–	–	–	–	–
18:3 n-3	0.93	5.16	1.91	5.87	3.11	10.34	14.38	13.47	10.72	13.04	13.36	13.12	7.53
18:4 n-3	0.84	0.00	0.00	0.00	0.25	1.75	2.77	2.56	1.63	1.97	2.29	2.28	0.90
20:4 n-6	3.81	3.10	2.58	3.12	2.81	2.95	3.57	4.21	3.52	3.38	3.37	4.26	3.27
20:4 n-3	–	–	–	–	–	–	–	–	–	–	–	–	–
20:5 n-3	4.53	5.47	7.39	8.37	4.79	4.19	13.17	15.24	5.93	4.34	12.87	15.39	5.61
22:1 n-9+ n-7	0	0.95	0.65	0.84	0.91	0.51	0.65	0.75	0.49	0.56	0.76	0.86	0.22
21:5 n-3	–	–	–	–	–	–	–	–	–	–	–	–	–
22:5 n-3	–	–	–	–	–	–	–	–	–	–	–	–	–
22:6 n-3	3.26	1.63	3.43	4.01	1.50	0.74	4.73	5.26	1.00	0.57	4.09	5.22	0.71
Total n-3 HUFA ($n\geq20$)	7.80	7.10	10.82	12.39	6.28	4.92	17.90	20.50	6.93	4.91	16.96	20.61	6.32
Total n-6 HUFA ($n\geq18$)	9.12	6.61	5.20	5.96	5.29	8.06	9.65	9.48	7.30	9.74	9.31	9.42	5.48
DHA/EPA	0.72	0.30	0.46	0.48	0.31	0.18	0.36	0.35	0.17	0.13	0.32	0.34	0.13

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Table 3
Experiment I: Average cumulative stress index (CSI), larval stage index (LSI), individual dry body weight, survival of *E. sinensis* megalopa at DAH 16 fed rotifers and *Artemia* nauplii containing different total n-3 HUFA levels *

Treatments	Larval stage index (LSI)	Individual dry body weight (μg)	Survival (%)	Cumulative stress index (CSI)
T1 (0% n-3 HUFA)	5.83±0.12[bc]	956.67±89.63[b]	20.0±6.14[a]	69±3[b]
T2 (30% n-3 HUFA)	5.92±0.11[ab]	1203.33±70.95[a]	22.00±6.63[a]	62±6[ab]
T3 (50% n-3 HUFA)	5.99±0.02[a]	1203.33±89.63[a]	18.17±5.57[a]	57±5[a]
Control	5.74±0.09[c]	973.33±25.17[b]	24.67±7.94[a]	85±7[c]

* Values are means±S.D. Values in the same column showing the same superscript are not significantly different ($P>0.05$; $n=6$).

3.2. Experiment II

The fatty acid profile of the un-enriched and enriched rotifers and *Artemia* is shown in Table 4. The total n-3 HUFA level in the enriched rotifers and *Artemia* (approximate 20 mg g^{-1} dw) were much higher than those of the un-enriched ones (less than 5 mg g^{-1} dw). The DHA/EPA ratios in the enriched rotifers and *Artemia* reflected those of the emulsions, ranging from 0.68 to 2.85 in rotifers and 0.28 to 0.96 in *Artemia*, respectively, and were much higher than in the un-enriched rotifers and *Artemia* (0.02 and 0.06, respectively). Similarly to the results of experiment I, the DHA/EPA ratio in rotifers was higher than in *Artemia* when enriched with the same emulsion.

The fatty acid composition of Z1, Z3, Z5 and megalopa is shown in Table 5. The total n-3 HUFA levels and DHA/EPA ratios of Z3 reflected the values of the enriched rotifers; while those of Z5 and megalopa reflected the values of the enriched *Artemia*, regardless of the type of rotifers they were fed on previously. Z5 fed with *Artemia* enriched with lower DHA/EPA ratio (0.6) emulsion, generally contained higher levels of total n-3 HUFA (ranging from 26.97 to 28.77 mg g^{-1} dw) than those receiving *Artemia* enriched with higher DHA/EPA ratio (4) emulsion (ranging from 18.48 to 22.47 mg g^{-1} dw), which were mostly contributed by higher EPA levels in the former treatments. However, similar n-3 HUFA levels (ranging from 21.06 to 22.81 mg g^{-1} dw) were observed at the megalopa stage. Metamorphosis from Z5 to megalopa resulted in all treatments in a slight drop of the DHA/EPA ratios and total n-3 HUFA levels. In the control group (un-enriched rotifers and newly-hatched *Artemia* nauplii), the total n-3 HUFA content of the larvae dropped substantially from 10.01 mg g^{-1} dw in Z1 to 5.21 mg g^{-1} dw in Z3, but tended to stabilize in Z5 and megalopa (5.78 and 6.10 mg g^{-1} dw, respectively).

A significantly lower LSI was observed for the control group ($P<0.05$), whilst the other treatments in which the larvae received enriched diets gave similar results (Table 6). On the other hand, megalopa in T6 had a significantly higher body weight than those in T1 and the control group ($P<0.05$). Larvae continuously fed prey enriched with a low dietary DHA/EPA ratio (T1) had significantly lower survival at the megalopa stage ($P<0.05$), similar to the control group. Ongrowing of the megalopa for another 3 days only showed a significant difference in survival between the enrichment groups and the control group ($P<0.05$). Survival in the latter was around 15% lower.

The osmotic stress tolerance of Z3, Z5 and megalopa is shown in Table 6 and Fig. 2a, b and c, respectively. When Z3 was abruptly transferred from 20 g L^{-1} culture water to 45 g L^{-1} brine water, there was a trend that a higher dietary DHA/EPA ratio resulted in better tolerance to salinity shock, especially for the DHA/EPA ratio of 4. The CSI was however not statistically different among the treatments. There was a clear tendency, however, that when Z5 was transferred directly from 20 g L^{-1} culture water to 50 g L^{-1} brine water, higher

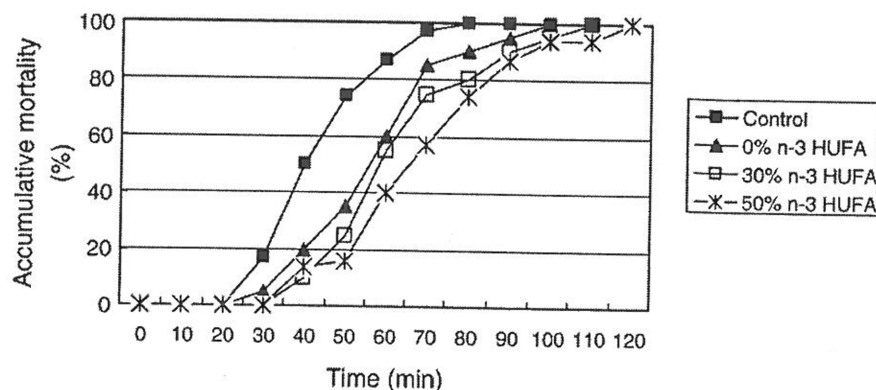

Fig. 1. Experiment I: Time–course of cumulative mortalities recorded when *E. sinensis* megalopa fed rotifers and *Artemia* nauplii containing different total n-3 HUFA levels were transferred from seawater with a salinity of 20 g L^{-1} to 60 g L^{-1} ($n=4$).

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Table 4
Experiment II: Fatty acid profile of rotifers and *Artemia* nauplii enriched with emulsions containing 30% total n-3 HUFA and with DHA/EPA ratio of 0.6, 2 and 4

Fatty acids (mg g^{-1} dw)	Rotifers				*Artemia* nauplii			
	DHA/EPA 0.6	DHA/EPA 2	DHA/EPA 4	Un-enriched	DHA/EPA 0.6	DHA/EPA 2	DHA/EPA 4	Un-enriched
16:0	15.48	15.23	14.98	4.91	17.93	17.15	16.22	14.44
16:1 n-7	17.89	17.09	14.01	16.16	9.85	8.40	8.43	7.72
17:0	1.24	1.44	2.17	–	2.44	2.34	2.66	1.85
17:1 n-7	0.87	0.91	0.73	0.47	1.40	1.25	1.42	1.35
18:0	7.14	6.74	4.09	2.91	10.26	10.09	8.77	7.07
18:1 n-9+n-7	31.23	30.95	29.61	22.10	46.91	43.50	41.67	35.53
18:2 n-6	8.54	8.21	8.69	3.81	12.10	11.47	10.67	10.35
18:3 n-6	–	–	–	0.78	0.82	0.88	0.95	1.19
18:3 n-3	1.05	1.00	1.05	0.45	36.76	34.09	32.91	40.41
18:4 n-3	1.98	2.10	3.18	2.01	5.29	5.34	5.65	7.45
20:4 n-6	1.23	1.42	2.01	0.78	3.06	4.56	5.33	2.99
20:4 n-3	1.32	1.32	1.10	1.20	0.88	1.10	1.37	1.17
20:5 n-3	9.02	6.98	4.05	0.20	12.81	11.09	8.93	2.14
22:1 n-9+n-7	0.21	0.20	1.05	0.19	0.17	0.50	1.10	0.09
21:5 n-3	1.45	1.25	1.70	0.49	1.25	1.89	0.63	0.66
22:5 n-3	1.63	1.73	0.84	0.48	1.82	1.39	0.67	0.41
22:6 n-3	6.09	8.17	11.52	0.23	3.56	6.23	8.25	0.13
Total n-3 HUFA ($n \geq 20$)	19.51	19.45	19.21	2.37	20.32	21.70	19.85	4.51
Total n-6 HUFA ($n \geq 18$)	9.77	9.62	10.71	5.60	15.97	16.91	16.94	14.54
DHA/EPA	0.68	1.17	2.85	0.02	0.28	0.56	0.92	0.06
Total lipid (% dw)	15.58	15.32	15.97	4.21	18.62	19.37	20.44	14.37

dietary DHA/EPA ratio resulted in markedly lower CSI ($P<0.05$). A similar trend was also observed when megalopa were transferred to 60 g L^{-1} brine water. Megalopa which received diets with higher DHA/EPA ratio in the *Artemia* had significantly lower CSI than those received diets with lower DHA/EPA ratio in the *Artemia*, despite of the DHA/EPA ratio in the rotifers that they received in early stages. This means that, even when larvae were fed rotifers enriched with a low DHA/EPA ratio in early stages, they could still recuperate when being fed with *Artemia* enriched with a high DHA/EPA ratio and giving improved results over the other treatments by the end of the culture period.

4. Discussion

As reported earlier by Dhert et al. (1993) and Sorgeloos et al. (2001), remarkable differences were observed in the possibility to boost the DHA/EPA ratio in rotifers and *Artemia*. Owing to the lower DHA and higher EPA retention in *Artemia*, lower DHA/EPA ratios were obtained after enrichment in *Artemia* compared with rotifers. Similar observations on DHA/EPA dynamics were noted by Wouters et al. (1997). This could be explained by the different life cycle of both species. Most of the rotifers are in adult stage where metabolic activities are not as pronounced as in the larval *Artemia* stage. As a consequence the fatty acid composition of the rotifers is more stable and does not change considerably during enrichment or starvation conditions. *Artemia* nauplii, on the other hand, do not synthesize DHA but metabolize HUFA very fast. Therefore the composition of *Artemia* nauplii is far more difficult to manipulate (Dhert et al., 1993). Nevertheless huge differences in the fatty acid composition were observed between the different enriched live prey and therefore the theoretical design of the experiment was achieved.

When conducting larval nutrition studies, it is necessary to minimize the "masking effect" of other zootechnical factors, such as larval density, feeding strategy, water quality parameters, etc. This is of particular importance for carnivorous species, as this could significantly influence the survival. Metamorphosis from Z5 to megalopa is indeed a crucial stage for *E. sinensis* because of the cannibalistic behavior of megalopa (Yu, X.Q., personnel comm.). In experiment I, cannibalism was evidently observed during metamorphosis from Z5 to megalopa, resulting in a drop in survival of about 20%. However, in experiment II, the influence of cannibalism on survival could be minimized by removing newly-metamorphosed megalopa from the rearing system. The survival obtained in experiment II should therefore be a better reflection of the n-3 HUFA requirement of *E. sinensis* larvae. The inferior survival obtained with low dietary n-3 HUFA in control group in

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Table 5
Experiment II: Fatty acid composition and total lipid content of *E .sinensis* Z3, Z5 and megalopa fed rotifers and *Artemia* nauplii containing different DHA/EPA ratios

Fatty acids (mg g^{-1} dw)	Z1	Z3				Z5						
		T1/T2 DHA/ EPA 0.6	T3/T4 DHA/ EPA 2	T5/T6 DHA/ EPA 4	Control	T1 DHA/ EPA 0.6/0.6	T2 DHA/ EPA 0.6/4	T3 DHA/ EPA 2/0.6	T4 DHA/ EPA 2/4	T5 DHA/ EPA 4/0.6	T6 DHA/ EPA 4/4	Control
16:0	5.39	6.41	6.52	6.31	4.27	11.49	7.10	10.81	7.66	10.12	8.07	8.35
16:1 n-7	3.26	3.50	3.45	3.49	3.79	6.27	2.80	5.70	3.33	5.32	3.10	3.52
17:0	0.45	0.57	0.76	1.05	–	1.64	1.08	1.38	1.17	1.28	1.26	0.97
17:1 n-7	0.22	–	0.22	0.16	–	0.84	0.49	0.81	0.58	0.81	0.63	0.53
18:0	2.87	4.74	4.55	4.29	3.98	6.18	4.23	5.89	4.35	5.55	4.67	4.53
18:1 n-9+n-7	9.75	11.67	12.09	11.70	13.13	31.45	17.40	28.37	20.02	27.07	20.08	22.60
18:2 n-6	3.28	3.68	3.60	3.61	3.35	7.73	3.99	6.99	4.43	6.59	4.47	5.62
18:3 n-6	–	–	–	–	0.16	–	–	–	–	–	–	0.33
18:3 n-3	0.61	0.89	0.53	0.59	0.65	17.51	9.25	15.38	9.46	14.58	9.25	19.64
18:4 n-3	0.60	1.95	1.11	1.28	1.15	3.26	1.38	2.95	0.94	2.90	1.04	2.60
20:4 n-6	2.35	2.30	2.39	2.28	1.83	4.64	3.71	4.27	3.92	4.02	4.52	3.70
20:4 n-3	0.02	0.85	0.40	0.27	–	1.15	0.50	1.07	0.49	1.01	0.69	0.93
20:5 n-3	5.54	8.96	7.73	6.95	3.37	18.00	8.80	17.19	9.07	16.18	9.79	3.83
22:1 n-9+n-7	0.92	0.50	0.40	0.12	–	0.75	0.45	0.57	0.33	1.02	0.96	0.93
21:5 n-3	0.47	0.79	0.51	0.15	–	0.57	0.57	0.27	0.73	0.90	2.04	0.34
22:5 n-3	0.25	0.86	0.63	0.73	–	1.40	0.55	1.24	0.54	1.23	0.74	0.13
22:6 n-3	3.73	6.04	6.85	6.41	1.84	7.29	8.06	7.87	8.06	7.65	9.20	0.54
Total n-3 HUFA ($n \geq 20$)	9.99	17.50	16.13	14.50	5.21	28.77	18.48	27.65	18.88	26.97	22.47	5.78
Total n-6 HUFA ($n \geq 18$)	5.64	5.98	6.00	5.89	5.34	12.37	7.70	11.27	8.35	10.61	8.99	9.66
DHA/EPA	0.67	0.67	0.89	0.92	0.55	0.41	0.92	0.46	0.89	0.47	0.94	0.14
Total lipid(% dw)	5.93	4.49	4.25	4.19	3.17	15.27	11.75	14.15	11.33	15.23	13.59	10.03

Fatty acids (mg g^{-1} dw)	Megalopa						
	T1 DHA/ EPA 0.6/0.6	T2 DHA/ EPA 0.6/4	T3 DHA/ EPA 2/0.6	T4 DHA/ EPA 2/4	T5 DHA/ EPA 4/0.6	T6 DHA/ EPA 4/4	Control
16:0	9.48	10.05	9.15	9.88	9.63	10.31	7.69
16:1 n-7	5.32	4.91	5.34	4.76	5.60	5.09	3.07
17:0	1.21	1.54	1.19	1.53	1.29	1.56	0.79
17:1 n-7	0.83	0.90	0.86	0.93	0.85	1.02	0.46
18:0	4.73	4.73	4.54	4.80	4.66	5.13	4.04
18:1 n-9+n-7	27.87	29.14	27.22	29.36	28.73	30.82	20.31
18:2 n-6	6.13	6.34	6.30	6.38	6.77	6.53	4.94
18:3 n-6	0.09	–	–	–	–	–	0.08
18:3 n-3	15.86	15.84	15.18	15.77	16.59	16.60	17.67
18:4 n-3	2.58	2.38	2.60	2.30	3.10	2.03	2.33
20:4 n-6	3.61	4.53	3.63	5.64	3.59	4.74	3.95
20:4 n-3	0.95	0.63	0.97	0.79	0.91	1.26	1.07
20:5 n-3	13.55	10.32	13.48	10.49	14.16	11.16	3.95
22:1 n-9+n-7	0.98	0.53	0.55	0.70	0.91	0.59	0.12
21:5 n-3	0.26	1.18	1.13	0.85	0.62	0.98	0.06
22:5 n-3	1.07	0.71	1.15	0.63	1.16	0.73	0.60
22:6 n-3	5.24	9.00	5.82	8.55	5.96	8.68	0.32
Total n-3 HUFA ($n \geq 20$)	21.06	21.84	22.54	21.30	22.81	22.80	6.10
Total n-6 HUFA ($n \geq 18$)	9.83	10.87	9.94	12.01	10.36	11.26	8.97
DHA/EPA	0.39	0.87	0.43	0.82	0.42	0.78	0.08
Total lipid(% dw)	16.68	17.57	16.71	17.43	15.38	17.64	11.28

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Table 6
Experiment II: Average cumulative stress index (LSI) at Z3, Z5 and megalopa stages, larval stage index (LSI), individual dry body weight and survival at DAH 19 and ongrown at DAH 22 of *E. sinensis* megalopa fed rotifers and *Artemia* nauplii containing different DHA/EPA ratios *

Treatments	Larval stage index (LSI)	Individual dry body weight (μg)	Survival % (DAH 19)	Survival (%) (ongrown DAH 22)	Cumulative stress index (CSI)		
					Z3	Z5	Megalopa
T1 (DHA/EPA 0.6/0.6)	5.93 ± 0.07^{a}	487 ± 40^{bc}	52.33 ± 6.15^{b}	73.30 ± 3.98^{a}	41 ± 4^{a}	80 ± 6^{c}	31 ± 1^{b}
T2 (DHA/EPA 0.6/4)	5.99 ± 0.02^{a}	563 ± 12^{ab}	61.92 ± 6.01^{a}	76.18 ± 2.69^{a}		55 ± 3^{b}	26 ± 2^{a}
T3 (DHA/EPA 2/0.6)	5.93 ± 0.10^{a}	533 ± 42^{ab}	67.50 ± 3.79^{a}	73.24 ± 4.04^{a}	38 ± 4^{a}	56 ± 7^{b}	27 ± 3^{ab}
T4 (DHA/EPA 2/4)	5.94 ± 0.06^{a}	553 ± 40^{ab}	65.00 ± 6.82^{a}	72.33 ± 5.16^{a}		55 ± 7^{b}	26 ± 1^{a}
T5 (DHA/EPA 4/0.6)	5.94 ± 0.08^{a}	577 ± 64^{ab}	65.20 ± 7.29^{a}	77.21 ± 2.57^{a}	35 ± 6^{a}	53 ± 9^{b}	31 ± 5^{b}
T6 (DHA/EPA 4/4)	5.93 ± 0.07^{a}	613 ± 78^{a}	66.50 ± 4.92^{a}	75.44 ± 7.10^{a}		38 ± 7^{a}	25 ± 3^{a}
Control	5.80 ± 0.14^{b}	423 ± 21^{c}	50.00 ± 5.90^{b}	58.38 ± 4.22^{b}	41 ± 2^{a}	91 ± 6^{d}	50^{c}

* Values are means±S.D. Values in the same column showing the same superscript are not significantly different ($P > 0.05$; $n = 6$).

experiment II might indicate the importance of n-3 HUFA on the survival of *E. sinensis* larvae.

The results from experiment I suggest that the low dietary n-3 HUFA level in T1 and the control group (6.22 and 3.01 mg g^{-1} dw in rotifers, 3.65 and 4.20 mg g^{-1} dw in *Artemia*), resulted in DHA depletion in the body tissues of the larvae, an inferior growth in terms of individual body weight and larval development, compared to T2 in which the live prey were boosted with n-3 HUFA (17.07 mg g^{-1} dw in rotifers and 18.22 mg g^{-1} dw in *Artemia*). A further increase in the dietary n-3 HUFA levels in T3 (27.64 mg g^{-1} dw in rotifers and 29.99 mg g^{-1} dw in *Artemia*) did not improve growth. Therefore we might suggest that 17 to 18 mg g^{-1} dw n-3 HUFA in the live food might be optimal for *E. sinensis* larval growth, and no deleterious effects of excess dietary n-3 HUFA were observed within the test range. These data are in accordance with a similar study in the swimming crab *P. trituberculatus*, for which the suitable range of n-3 HUFA in rotifers was between 9 and 17 mg g^{-1} dw (Takeuchi et al., 1999).

Apart from the quantitative requirements for dietary n-3 HUFA, the proportion of specific fatty acids, e.g. DHA and EPA, is known to be a critical nutritional factor. It has been observed that developing eggs and larvae of marine fish preferentially consume DHA over EPA as a more efficient energy source or as precursors for prostaglandins (Watanabe, 1978), and DHA was superior to EPA in larval development of mud crab (*S. serrata*) (Suprayudi et al., 2004a). Although *E. sinensis* spend most of their life time in freshwater, larval development does take place in a marine/brackish environment; hence one could expect their requirements to be close to marine organisms in that they require a high DHA/EPA ratio in the diet. In experiment II, a low DHA/EPA ratio in the T1 diet (0.68 in rotifers and 0.28 in *Artemia*, respectively) resulted in significantly inferior larval development (LSI), and lower megalopa survival rate than treatments with higher dietary DHA/EPA ratios (T2 to T6, ranging from 1.17 and 2.85 in rotifers, and 0.56 to 0.92 in *Artemia*). However, no significant differences were obtained in between the latter treatments. Thus we speculate that the DHA/EPA ratio of 1.17 in rotifers and 0.56 in *Artemia* is sufficient for optimal growth, survival and metamorphosis of *E. sinensis* larvae. This is somehow lower than the optimum ratio for marine fish species (in the range of 2:1) reported by Sargent et al. (1999). Meanwhile no negative effects of elevated dietary DHA/EPA ratios were observed within the range tested in this study.

The current study confirms earlier findings that crustacean larvae fed nutritionally balanced n-3 HUFA rich diets, display a better resistance to osmotic shock (Tackaert et al., 1992; Rees et al., 1994; Martins et al., 2006). In experiment I, with the same DHA/EPA ratio, a higher dietary n-3 HUFA resulted in better resistance to osmotic shock at megalopa stage. Whereas in experiment II, with the same total n-3 HUFA level, a higher DHA/EPA ratio resulted in better resistance to osmotic shock at the Z3, Z5 and megalopa stages. It is known that n-3 HUFA (especially DHA) are mainly incorporated in the cell membranes, and increase the permeability of membranes and hence their fluidity (Watanabe, 1993). Moreover the effect on osmoregulation may also be partially related to the modification of fatty acid composition of the gills as higher n-3 HUFA levels result in larger gill area, which in turn enhances osmoregulatory capacity, and thus the survival upon salinity stress (Palácios et al., 2004). It should be mentioned, on the other hand, that the capacity of osmoregulation for mitten crab increases along with larval development (Zhao et al., 1998). Therefore the better tolerance to salinity stress in experiment I might also be linked to the faster development of the larvae. This was however not confirmed in experiment II, where higher DHA/EPA ratio resulted in better tolerance

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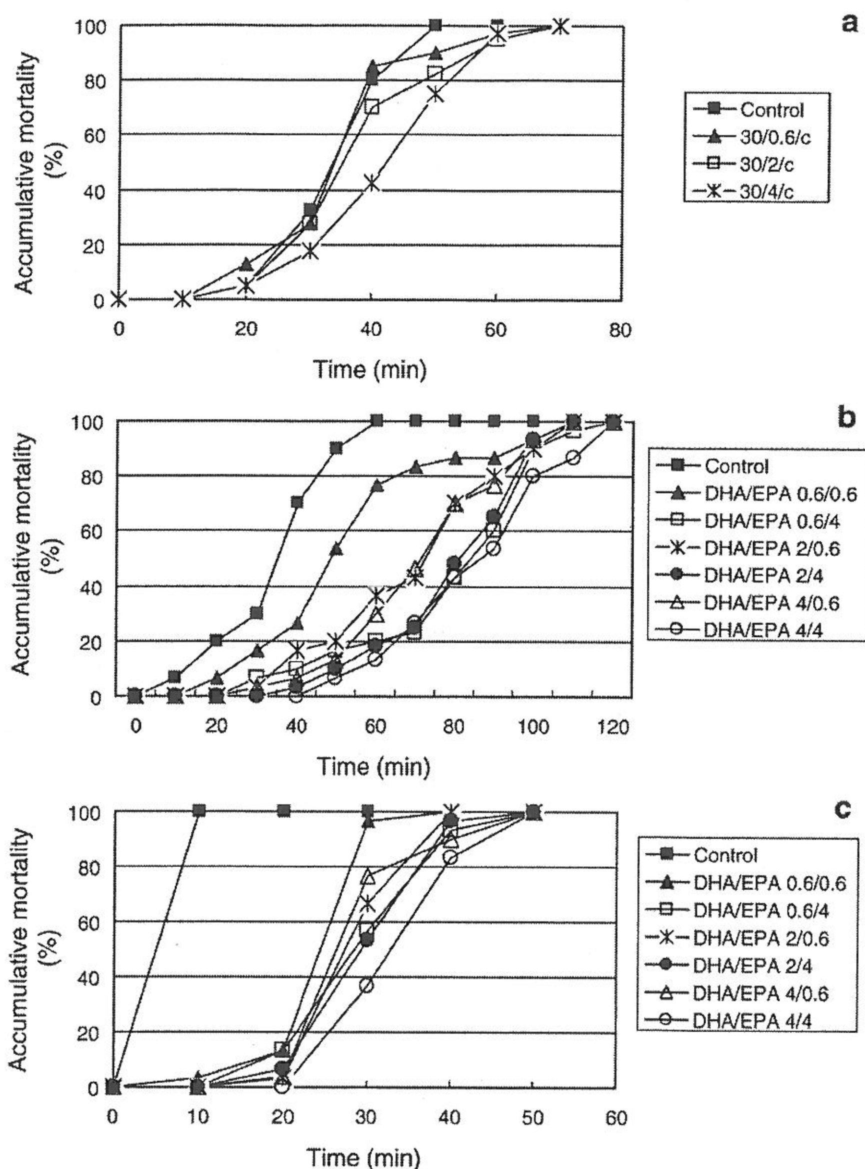

Fig. 2. Experiment II: Time–course of cumulative mortalities recorded when *E. sinensis* larvae fed rotifers and *Artemia* nauplii containing different DHA/EPA ratios were transferred from seawater to different water medium (n=4). a) Z3: from 20 g L^{-1} to 45 g L^{-1}; b) Z5: from 20 g L^{-1} to 50 g L^{-1}; c) megalopa: from 20 g L^{-1} to 60 g L^{-1}.

without showing significant growth difference of the zoeal larvae. Overall the result of the current study proved that not only higher levels of total n-3 HUFA but also the correct balance of DHA and EPA enhances larval response to stress conditions.

This study suggests that, due to its catadromous nature, *E. sinensis* larvae require the provision of preformed n-3 HUFA, with a suitable ratio of DHA to EPA in their diet. Total n-3 HUFA levels of 17 to 18 mg g^{-1} dw in both rotifers and *Artemia*, and a DHA/EPA ratio of 1.17 in rotifers and 0.56 in *Artemia* seem sufficient for optimal growth and survival. Furthermore an elevated dietary n-3 HUFA level and DHA/EPA ratio moreover enhanced the tolerance of larvae to salinity shock.

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