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Rotifer enrichment with DHA did not improve growth and survival rate of yellowtail clownfish (*Amphiprion clarkii*) larvae

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Abstract
Docosahexaenoic acid (DHA) enrichment of live food is a common practice to improve growth and survival of larvae. The current research aimed to test the effect of rotifer enrichment with DHA on growth, survival, fatty acid profile, digestive enzymes, antioxidant parameters, and histology in yellowtail clownfish (*Amphiprion clarkii*) larvae. Four levels of enrichments emulsions with DHA were considered, including DHA6 (6% DHA), DHA12 (12% DHA), DHA24 (24% DHA), and DHA36 (36% DHA). No substantial differences in growth data were recorded, suggesting that even 6% DHA emulsion enrichment which resulted in 4.22% DHA in rotifers is enough to fulfil the DHA requirements of yellowtail clownfish larvae to maximise the growth rate. Excessive levels of DHA (36%) decreased the survival rate of larvae. This fatty acid accumulated in the larvae body at high levels (24.72%). The DHA12 group had the lowest growth, possibly due to lower digestive enzymes and antioxidant activities in this treatment. There was a positive correlation between final weight and lipase (59%), protease (73%), and lactate dehydrogenase (69%). Furthermore, amylase, lipase, protease, and alkaline phosphatase had significant positive correlations with catalase (72%, 84%, 74%, and 66%, respectively) and lactate dehydrogenase (64%, 85%, 79%, and 77%, respectively). Superoxidase dismutase and catalase also had positive correlations with arachidonic acid levels in the body. Increased DHA levels, based on histology data, caused lipid vacuoles in enterocytes and hepatocytes. In conclusion, yellowtail clownfish larvae can grow well with high survival even with feeding rotifers enriched with 4.22% DHA or less.

Keywords Highly unsaturated fatty acids · Larval feeding · Rotifer enrichment · Digestive enzymes · Growth

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Introduction

Understanding the larval nutritional, which is always challenging due to the vulnerability of fish larvae, would help to optimise diets and feeding protocols, thus improving larval and juvenile quality growth, survival, and development (Hamre et al. 2013; Izquierdo 1996). Nutritional imbalances in larval stages, such as deficiencies in vitamins, amino acids, or essential fatty acids, have decreased larval survival rate and, eventually, economic loss. Larval nutrient requirements differ qualitatively and quantitatively from juveniles or adults, as fish larvae experience huge morphological and physiological alterations (Holt 2011). Furthermore, fish larvae grow exponentially and feed continuously, even up to 100% daily (Conceição et al. 1998). Among all nutrients, lipids, especially n-3 highly unsaturated fatty acids (HUFA) such as docosahexaenoic acid (DHA), are crucial for larval development, growth performance, stress resistance, skeleton development, pigmentation, and survival rate, which was described in detail elsewhere (Hamre et al. 2013; Holt 2011).

Fish larvae require DHA for normal growth, optimal nerve tissue and visual system development, immune system, and antioxidant response (Koven 2003). The DHA deficiency can cause malformation, behavioural abnormalities (raptorial or schooling behaviour), and poor vision in larvae, eventually affecting the ability to catch prey such as rotifer and artemia (Benítez-Santana et al. 2007). The effect of DHA level on growth, muscle development, survival rate, immune response, and antioxidant system of fish larvae such as cobia (*Rachycentron canadum*) (Trushenski et al. 2012), California yellowtail (*Seriola dorsalis*) (Rombenso et al. 2016), blunt snout bream (*Megalobrama amblycephala*) (Wang et al. 2020), red porgy (Roo et al. 2009), and many more fish species was reported. Enriching live prey like rotifers with fatty acids DHA is an applicable and promising approach to providing DHA requirements in fish larvae (Kandathil Radhakrishnan et al. 2020). When rotifers were enriched with three levels of eicosapentaenoic acid (EPA) and DHA and then were fed to red porgy larvae, the best growth was recorded in those fed rotifers containing 7.82% DHA in total fatty acids (TFA) (Hernández-Cruz et al. 1999). For other species, such as red seabream (Izquierdo 1989), common dentex (*Dentex dentex*) (Mourente et al. 1999a; Mourente et al. 1999b), gilthead seabream (Izquierdo 2005), or striped trumpeter (*Latris lineate*) (Bransden et al. 2005), the minimum DHA requirement for optimum growth was found to be 1.2%, 2.3%, 0.8%, and 2.0% dry matter, respectively. In other studies, enriching rotifers (including DHA 7% TFA) and artemia (DHA 2% TFA) in red seabream (Izquierdo 1989) optimised the growth rate. Also, the optimum level of enriched artemia was DHA 1% TFA in Japanese flounder (*Paralichthys olivaceus*) (Izquierdo et al. 1992) and DHA 11.4% TFA in gilthead seabream (Salhi et al. 1994). Fish species, even within the same family, are varied in ontogeny, feeding physiology, and nutritional requirements (Hamre et al. 2013). Therefore, due to these variations, it is hard to translate their results into all species, such as yellowtail clownfish.

Among marine ornamental species, yellowtail clownfish (*Amphiprion clarkii*) is the most popular species in the aquarium trade. They are famous for their bright colours, small size, and remarkable tolerance. Clownfishes have important economic value in the Persian Gulf and are potential candidates for coastal and seawater aquaculture. Their farming has many advantages, such as a high growth rate and adaptability to salinity and temperature (Personal communication). However, few investigations discovered biology, nutritional requirements, or techniques for larval rearing of clownfish. The major problem with clownfish culture is the dependence on live prey during larval rearing. There are few studies on the effect of live feed enrichment on the

growth, survival, and health of clownfishes (Basford et al. 2020; Dhaneesh et al. 2017). Basford et al. (2020) found that DHA enrichment did not improve growth and survival rate of wide-band anemonefish (*Amphiprion latezonatus*). Furthermore, enriching live food with *Nannochloropsis salina* oil increased the survival rate and growth of orange clownfish (*Amphiprion percula*). However, there is no study that tested live food enrichment in yellowtail clownfish with docosahexaenoic acid (DHA). Therefore, the current study aims to evaluate the impact of live feed enrichment with essential fatty acids DHA in four levels (6, 12, 24, and 36%) on survival, growth performance, fatty acid profile, digestive enzymes, antioxidants, and histology of yellowtail clownfish larvae.

Materials and methods

Fish husbandry

This study adheres to current regulations regarding the use of animals in research (Ahmadi-Noorbakhsh et al. 2021). The larvae used in this study were obtained from broodstock kept in an aquarium at Tabsem Sahel Qeshm Aquaculture Company located in Persian Gulf Biotechnology Park (Qeshm Island, Iran). Yellowtail clownfish pairs (4 pairs, 6–8 cm) were kept in 200-L glass aquariums, and flower pots were supplied to the broodstock as a suitable substrate for egg laying. Photoperiod was maintained at 14-h light/10-h dark. The temperature was maintained at 28 °C, salinity at 30‰, and pH at 8–8.5 with aeration with air stone. Fish were fed twice daily with commercial frozen shrimp and squid for 2 months until spawning.

Following spawning, the flower pots (15-cm diameter) with eggs will hatch at the parental tanks (80-L glass aquarium). The egg clutch from separate batches was transferred to larval rearing tanks. The larval rearing experiment was carried out in the 80-L glass aquariums with black walls, where a gentle aeration of 6.5 ± 0.3 mg/L (dissolved oxygen) was provided. After an incubation period of 24 h at 28–30 °C, the hatched larvae in each tank were counted, and the density was recalculated. At a hatching rate of 70%, the initial stocking density (initial wet weight: 1.48 ± 0.05 mg and total length: 4.44 ± 0.55 mm) with five larvae L^{-1} (400 per replicate and 12 aquariums) was set up. The first 24 h after hatching was defined as day 0. Then, the light intensity at the water surface was progressively increased to 700 lux by adjusting the distance of the light source from the surface. The light was supplied by overhead fluorescent white light tubes. A 14-h light/10-h dark photoperiod was applied. Air stones were positioned at the bottom of the tanks to maintain suspended matter, including the food in suspension. During the yolk sac state (larvae start to feed after 8–10 h on the first day after hatch (dah)), the aeration was gentle to minimise physical shock. The average daily temperature ranged between 28 and 30 °C, and the salinity was 28–30 g/L. UV-filtered seawater, which had passed through sand filters, was used for larval rearing. Larvae were reared with no water exchange during the first days, and from day 2, the aquarium bottoms were cleaned, and 10–20% of the tank water was replaced on a daily basis without disturbing the larvae. The water in the larval tank was gently replaced once a day by a dripping system.

Experimental design, rotifer enrichment, and sampling

The experiment was carried out through a completely randomised design, including four treatments in triplicates and twelve tanks (80-L glass aquariums). EPA and DHA-rich oils (EPA 80% and DHA 80%, Shaanxi Pioneer Biotech Co., Ltd. (Shaanxi, China)) were used to prepare enrichment emulsions (Estévez and Giménez 2017). Rotifers were propagated in a batch culture system with *Nannochloropsis* sp (15–20 million cell/ml) and *Saccharomyces cerevisiae* (25 g/100 million rotifer). To enrich rotifers, organisms were stocked (500 organisms/mL) in 10-L plastic containers with mild aeration and enriched with 0.6 g/L with the experimental emulsions for 2 h at 20 °C (Estévez and Giménez 2017). The rotifer was enriched with four experimental emulsions containing four levels of DHA, including DHA6 (6% DHA), DHA12 (12% DHA), DHA24 (24% DHA), and DHA36 (36% DHA) (Table 1). Prior to use, the harvested rotifers were washed with fresh disinfected seawater and fresh water for 60 s to reduce bacterial load and salt residues. Then, excess water was removed by tissue paper. The 2–4 g rotifers were sampled for fatty acid analysis as well. After that, samples were stored in a −80 °C refrigerator until analysis (Eryalçın 2019).

The larvae were fed microalgae *Nannochloropsis* sp. (50000 cell/ ml) from 1 day up to 10 dah; from day 2 to 10 dah, they were fed rotifers with a density of 10 *Brachionus rotundiformis*, S-type, and *Brachionus plicatilis*, L-type, for 10 days. The feeding frequency was twice per day (09:00 and 16:00) in order to maintain a constant density of live prey in the rearing tanks.

Growth performance and survival

Standard length (measured to the nearest 0.1 mm), wet weight (measured to the nearest 0.1 mg), and % survival was calculated at the end of the experiment by counting alive larvae in each tank (after 10 days). Furthermore, twenty larvae from each tank were sacrificed at the end of the experiment to evaluate digestive enzymes, antioxidant enzymes, and fatty acid profiles. The samples were kept at −80 °C until further analysis.

Fatty acid analysis

Fatty acid profiles in larval, emulsion, and live food samples were performed using fatty acid methylation. The lipids were extracted from samples with a chloroform and methanol mixture (2:1 volume) according to the standard method (Folch et al. 1957). Fatty acid methyl esters were obtained by transmethylation with 1% sulphuric acid in methanol (Christie 1993) and were separated by gas chromatography (GC-2030; Shimadzu, Tokyo, Japan) in a Supercolvax-10-fused silica capillary column (constant pressure with 100 KPa, length 30 m; internal diameter 0.32 mm; 0.25 i.d (Ref.: 24080-U) Supelco, Bellefonte, PA, USA) using helium as a carrier gas. The column temperature was 180 °C for the first 10 min, increasing to 220 °C at a rate of 2 °C/min, and then held at 220 °C for 15 min. Fatty acid methyl esters were quantified and identified by comparison with external standards (EPA 28, Nippai, Tokyo, Japan).

Digestive enzymatic assay

To evaluate digestive enzymes (lipase, amylase, total protease, and alkaline phosphatase (ALP)), samples were rinsed twice with distilled water and were homogenated in 100

Table 1 Formulation of enrichment emulsions and their fatty acid profile

Treatments	DHA6	DHA12	DHA24	DHA36
EPA-enriched oil[1]	10	10	10	10
DHA-enriched oil[2]	6	12	24	36
ARA-enriched oil[3]	5	5	5	5
Olive oil	31.8	25.8	13.8	1.8
Soy lecithin	4	4	4	4
α-Tocopherol	1.2	1.2	1.2	12
Distilled water	42	42	42	42
Fatty acid profile (% of the lipid extracted)				
C14:0	0.95 ± 0.07	0.17 ± 0.01	0.50 ± 0.06	1.00 ± 0.12
C15:0	0.22 ± 0.01	0.22 ± 0.01	0.34 ± 0.03	0.32 ± 0.03
C16:0	12.01 ± 0.79	12.05 ± 0.93	19.83 ± 0.95	23.28 ± 1.20
C17:0	0.97 ± 0.08	0.94 ± 0.06	0.88 ± 0.08	1.04 ± 0.09
C18:0	0.59 ± 0.08	1.59 ± 0.13	1.78 ± 0.16	2.46 ± 0.18
C20:0	1.09 ± 0.11	1.06 ± 0.07	1.02 ± 0.09	1.09 ± 0.05
Total SFA	15.83 ± 1.53	14.93 ± 1.85	24.16 ± 1.90	32.79 ± 3.19
C18:1n-9 (OA)	55.76 ± 3.57	46.65 ± 3.94	27.97 ± 2.75	7.77 ± 1.50
C22:1n-9	0.18 ± 0.01	0.15 ± 0.01	0.40 ± 0.02	0.53 ± 0.05
Total MUFA	55.94 ± 2.77	46.80 ± 3.35	28.37 ± 2.85	8.51 ± 0.79
C18:2n-6 (LA)	5.15 ± 0.50	6.47 ± 0.59	5.87 ± 0.38	3.44 ± 0.19
C20:3n-6	0.09 ± 0.01	0.03 ± 0.00	0.14 ± 0.01	0.51 ± 0.03
C20:4n-6 (ARA)	3.98 ± 0.41	3.30 ± 0.36	2.94 ± 0.24	2.85 ± 0.22
C22:4n-6 (DTA)	1.57 ± 0.10	2.72 ± 0.17	5.20 ± 0.43	8.30 ± 0.74
Total n-6 PUFA	10.80 ± 1.04	12.51 ± 1.05	13.15 ± 1.06	15.13 ± 1.24
C18:3n-3 (LNA)	0.87 ± 0.08	0.62 ± 0.05	1.24 ± 0.09	0.51 ± 0.03
C20:5n3 (EPA)	8.33 ± 0.63	9.21 ± 0.66	8.87 ± 0.64	9.06 ± 0.59
C22:5n-3 (DPA)	0.17 ± 0.01	0.19 ± 0.02	0.18 ± 0.02	0.20 ± 0.02
C22:6n-3 (DHA)	6.46 ± 0.48	12.70 ± 0.45	21.56 ± 2.02	33.53 ± 2.27
Total n-3 PUFA	15.83 ± 1.00	22.72 ± 1.05	31.86 ± 1.63	43.06 ± 2.78
DHA/EPA	0.77	1.38	2.43	3.70
DHA/ARA	1.62	3.84	7.33	11.76

Data was reported mean ± standard deviation

[1]EPA 80%, Shaanxi Pioneer Biotech Co., Ltd. (Shaanxi, China)

[2]DHA 80%, Shaanxi Pioneer Biotech Co., Ltd. (Shaanxi, China)

[3]Vevodar™ (DSM IP Assets B.V., Heerlen, the Netherlands)

DHA-N very low level of DHA, *DHA-L* low level of DHA, *DHA-M* medium level of DHA, *DHA-H* high level of DHA, *OA* oleic acid, *LA* α-linoleic acid, *ARA* arachidonic acid, *LNA* linolenic acid, *EPA* eicosapentaenoic acid, *DPA* docosapentaenoic acid, *DHA* docosahexaenoic acid

mM Tris-HCl buffer prepared for digestive enzyme analyses (Furné et al. 2008). The lipase specific activity was measured using a commercial lipase kit (Bionik™, Canada), and amylase specific activity was determined using an amylase kit (Bionik™, Canada). The total protease activity was measured with haemoglobin (Anson 1938). The Bradford method (Bradford 1976) was used to measure total soluble protein using bovine

serum albumin as a standard. The specific activity of digestive enzymes was measured according to unit per milligrammes protein in samples.

Antioxidant parameters

For analysis of antioxidant enzyme activities, malondialdehyde content, and soluble protein levels, the samples of larvae were homogenised in ice-cold lysing buffer (1:10). The homogenates were centrifuged at 10,000×g for 10 min at 4 °C. The supernatants were collected and stored at −80 °C for further analysis. All assays were carried out using a microplate reader (BioTek™, Synergy HT, USA) and commercial kits (Navand Lab Kit™, Navand Salamat Co., Iran).

Histological studies

Yellowtail clownfish larvae ($n = 10$) were sampled at the beginning (initial sample) and also on day 10 from each tank and fixed in 10% neutral buffered formalin. All samples were dehydrated with graded series of ethanol, cleared in xylene, and embedded in paraffin wax, according to the standard histological procedures. Paraffin blocks were cut into serial longitudinal sections (4–5 µm) with a microtome (Leica RM2125, Germany) and stained with Mayer's haematoxylin and eosin (H&E). Slides were examined under an Olympus BX-51 light microscope equipped with an Olympus DP72 digital camera (Roberts et al. 2001). In this study, at least six sections were used for histological analysis. Histological changes (cellular structure of enterocyte and hepatocyte, accumulation of lipid vacuoles as reported by Castro-Ruiz et al. (2022)) that may occur in the liver, intestine, and pancreas were investigated in clownfish larvae.

Statistical analysis

Data were analysed using SPSS ver. 22.0 statistical software. All the data are presented as means ± SD determined from three replicates. Arcsine transformations were conducted on all data expressed as percentages to get homogeneity of variance before statistical analysis. One-way ANOVA was performed with a significance level of 0.05 following confirmation of normality and homogeneity of variance. Tukey's procedure was used for multiple comparisons.

Results and discussion

Fatty acid composition of rotifer and yellowtail clownfish larvae

Lipid and fatty acid composition of rotifer following DHA enrichment are presented in Table 2. Significant differences were found in the relative content of C16:0, C18:0, total saturated fatty acids (ΣSFAs), C16:1n-7, C18:1n-9, total monounsaturated fatty acids (ΣMUFA), C18:2n-6, C20:4n-6 (ARA), C22:4n-6, total n-6 polyunsaturated fatty acids (ΣPUFAs), C20:5n-3 (EPA), C22:6n-3 (DHA), and total n-3 PUFA. These fatty acids reflected the composition of the emulsions, and by adding DHA to that, MUFAs in live food decreased, but ΣSFAs, ARA, EPA, and DHA increased. DHA levels in rotifers enriched by DHA6, DHA12, DHA24, and DHA36 emulsions were found to be

Table 2 Fatty acid composition (%) of the extracted lipid from rotifer (*Brachionus plicatilis*) enriched with different levels of the experimental emulsions

Fatty acids	Initial	DHA6	DHA12	DHA24	DHA36
C14:0	2.12 ± 0.20	1.40 ± 0.16	1.32 ± 0.09	1.09 ± 0.08	1.12 ± 0.09
C15:0	2.55 ± 0.22	1.56 ± 0.09	1.32 ± 0.04	1.12 ± 0.06	1.31 ± 0.03
C16:0	17.28 ± 1.46	16.48 ± 1.80^{b}	18.16 ± 1.17^{b}	19.70 ± 1.48^{a}	21.77 ± 2.00^{a}
C17:0	1.22 ± 1.04	0.48 ± 0.02	0.80 ± 0.03	0.46 ± 0.04	0.67 ± 0.03
C18:0	5.42 ± 0.42	2.70 ± 0.10^{b}	2.43 ± 0.16^{b}	3.05 ± 0.21^{a}	3.27 ± 0.23^{b}
C20:0	1.73 ± 0.14	1.88 ± 0.14	1.65 ± 0.11	1.68 ± 0.10	1.29 ± 0.10
Total SFA	30.31 ±2.84	24.50 ± 2.87^{b}	25.66 ± 2.18^{a,b}	26.44 ± 2.25^{a}	28.96 ± 2.41^{a}
C16:1n-7	15.84 ± 1.34	5.45 ± 0.55^{a}	5.24 ± 0.41^{a}	1.63 ± 0.33 ^{b}	0.90 ± 0.52^{b}
C17:1n-7	0.31 ± 0.05	1.13 ± 0.26	1.82 ± 0.24	1.07 ± 0.27	1.09 ± 0.20
C18:1n-9 (OA)	15.96 ± 1.06	39.20 ± 2.55^{a}	35.68 ± 2.10^{a}	31.24 ± 2.11^{a}	18.96 ± 1.04^{b}
Total MUFA	32.11 ± 2.73	45.79 ± 3.64^{a}	43.83 ± 2.97^{a}	33.05 ± 3.10^{b}	19.24 ± 1.86^{c}
C18:2n-6 (LA)	9.21 ± 1.10	8.65 ± 1.13^{a}	5.84 ± 0.84^{b}	5.17 ± 0.79^{b}	1.98 ± 0.33^{c}
C20:3n-6	0.55 ± 0.06	0.23 ± 0.02	0.26 ± 0.02	0.26 ± 0.03	0.15 ± 0.01
C20:4n-6 (ARA)	3.60 ± 0.49	2.48 ± 0.30^{b}	2.87 ± 0.30^{ab}	3.12 ± 0.39^{a,b}	3.50 ± 0.33^{a}
C22:4n-6 (DTA)	0.71 ± 0.06	0.39 ± 0.02^{c}	1.09 ± 0.10^{c}	4.33 ± 0.44^{b}	8.50 ± 0.82^{a}
Total n-6 PUFA	14.12 ± 1.29	11.74 ± 1.42^{b}	10.06 ± 1.23^{b}	12.88 ± 1.77^{a,b}	14.13 ± 1.24^{a}
C18:3n-3 (LNA)	0.55 ± 0.03	1.90 ± 0.13	1.18 ± 0.14	1.47 ± 0.18	1.27 ± 0.20
C20:5n-3 (EPA)	10.55 ± 1.45	6.04 ± 0.70^{b}	6.23 ± 0.64^{b}	8.04 ± 0.72^{a}	8.94 ± 0.83^{a}
C22:5n-3 (DPA)	5.11 ± 0.44	3.03 ± 0.24	2.85 ± 0.13	2.84 ± 0.15	2.18 ± 0.12
C22:6n-3 (DHA)	3.23 ± 0.61	4.22 ± 0.67^{c}	7.01 ± 0.95^{bc}	14.34 ± 1.88^{b}	24.78 ± 2.95^{a}
Total n-3 PUFA	19.44 ± 2.05	15.18 ± 2.41^{b}	17.27 ± 2.20^{b}	26.69 ± 2.67^{a}	37.17 ± 3.50^{a}
DHA/EPA	0.30	0.69^{b}	1.12^{b}	1.78^{ab}	2.88^{a}
DHA/ARA	0.89	1.70^{b}	2.44^{b}	4.60^{a}	7.36^{a}

Data was reported mean ± standard deviation. The values less than 0.5 were not reported in the table, such as C22:1n-9

DHA-N very low level of DHA, *DHA-L* low level of DHA, *DHA-M* medium level of DHA, *DHA-H* high level of DHA, *n.d* not detected, *OA* oleic acid, *LA* α-linoleic acid, *ARA* arachidonic acid, *LNA* linolenic acid, *EPA* eicosapentaenoic acid, *DPA* docosapentaenoic acid, *DHA* docosahexaenoic acid

Letters a, b, and c indicated significant differences among groups based on the Tukey multiple range test at a significance level of 0.05

4.22, 7.01, 14.34, and 24.78, respectively. After 10 days of feeding larval yellowtail clownfish with the DHA-enriched live food, the fatty acid composition of the whole larvae changed, similar to dietary alterations (Table 3). In this regard, C18:1n-9, C20:1, ΣMUFAs, C18:2n-6, 20:4n-6 (ARA), 22:5n-3 (DPA), 22:6n-3 (DHA), Σn-3, and DHA/EPA were changed. With increasing DHA in emulsion, C18:1n-9, C20:1, ΣMUFAs, and C18:2n-6 in larvae decreased, and ARA, 22:5n-3 (DPA), DHA, Σn-3, and DHA/EPA levels increased in clownfish body. The relative DHA level in larvae fed enriched rotifer by DHA36 (24.72%) was significantly higher than DHA6 (11.12%) and DHA12 (16.93%) groups. The DHA/EPA ratio followed the same trend, so the DHA36 treatment (4.11) had a significantly higher level than others. The total SFAs in larvae did not follow the observed trend in rotifers, and no difference in whole yellowtail clownfish larvae was found (Table 3). It is well-known that enriching live foods with DHA can transfer DHA to larvae to provide optimum growth and health for numerous species

Table 3 Main fatty acids profiles (% total identified fatty acids) of yellowtail clownfish larvae (*Amphiprion clarkii*) fed with variable live foods enriched with the experimental emulsions after ten days ($n = 3$)

Fatty acid profile	DHA6	DHA12	DHA24	DHA36
C12:0	2.34 ± 0.18	2.41 ± 0.11	1.88 ± 0.14	2.28 ± 0.12
C14:0	1.95 ± 0.18	1.57 ± 0.07	1.72 ± 0.09	1.74 ± 0.10
C15:0	0.53 ± 0.04	0.45 ± 0.03	0.64 ± 0.03	0.52 ± 0.04
C16:0	21.79 ± 2.44	21.29 ± 2.48	22.13 ± 2.19	21.65 ± 1.98
C17:0	0.39 ± 0.03	0.35 ± 0.03	0.56 ± 0.05	0.48 ± 0.04
C18:0	7.13 ± 0.78	6.82 ± 0.66	6.05 ± 0.78	7.04 ± 0.69
C20:0	0.27 ± 0.02	0.23 ± 0.02	0.32 ± 0.01	0.25 ± 0.02
ΣSFAs	34.49 ± 3.52	33.46 ± 2.98	33.97 ± 2.99	34.39 ± 3.75
C16:1	3.53 ± 0.31	3.17 ± 0.36	3.71 ± 0.34	3.51 ± 0.29
C18:1n-9	29.26 ± 2.02^{a}	27.26 ± 2.17^{a,b}	20.95 ± 1.70^{b}	17.79 ± 1.51^{c}
C20:1	1.19 ± 0.08^{a}	1.01 ± 0.08^{a,b}	0.86 ± 0.05^{b}	0.5 ± 0.02^{c}
ΣMUFAs	35.13 ± 2.72^{a}	32.48 ± 2.63^{a}	26.47 ± 1.30^{b}	22.36 ± 1.45^{b}
C18:2n-6	8.05 ± 0.87^{a}	7.16 ± 0.83^{ab}	6.78 ± 0.61^{a,b}	6.32 ± 0.58^{b}
C20:3n-6	0.45 ± 0.04	0.49 ± 0.03	0.38 ± 0.02	0.51 ± 0.06
20:4n-6 (ARA)	2.21 ± 0.26^{b}	2.97 ± 0.28^{a,b}	3.43 ± 0.33^{a,b}	4.49 ± 0.38^{a}
Σ n-6	10.75 ± 1.08	10.87 ± 0.99	10.79 ± 0.99	11.52 ± 1.06
C18:3n-3	0.30 ± 0.02	0.46 ± 0.03	0.45 ± 0.03	0.27 ± 0.02
20:5n-3 (EPA)	5.09 ± 0.40	5.34 ± 0.33	5.50 ± 0.45	6.02 ± 0.46
22:5n-3 (DPA)	0.19 ± 0.01^{b}	0.74 ± 0.04^{a}	0.83 ± 0.05^{a}	0.64 ± 0.06^{a}
22:6n-3 (DHA)	11.12 ± 1.04^{c}	16.93 ± 1.08^{b}	20.42 ± 1.59^{a,b}	24.72 ± 1.65^{a}
Σ n-3	16.70 ± 1.02^{c}	23.47 ± 1.43^{b}	27.20 ± 1.81^{b}	31.65 ± 2.06^{a}
DHA/EPA	2.18^{c}	3.17^{b}	3.7^{b}	4.11^{a}
DHA/ARA	5.03	5.70	5.95	5.51

Data was reported mean± standard deviation. Different letters show significant differences among groups (*$p < 0.05$; Tukey's multiple range test). The values less than 0.5 were not reported in table such as C6:0, C8:0, C10:0, C21:0, C22:0, C23:0, C24:0, C24:1, C22:1n-9, C18:2n-6, and C18:3n-6

DHA-N very low level of DHA, *DHA-L* low level of DHA, *DHA-M* medium level of DHA, *DHA-H* high level of DHA, *OA* oleic acid, *LA* α-linoleic acid, *ARA* arachidonic acid, *LNA* linolenic acid, *EPA* eicosapentaenoic acid, *DPA* docosapentaenoic acid, *DHA* docosahexaenoic acid

reviewed elsewhere (Holt 2011). This study also confirms this point for yellowtail clownfish. However, the fewer levels (less than 6% DHA in the emulsion) should be tested to understand the optimum dosage for DHA enrichment in this species.

Growth performance of yellowtail clownfish larvae

Results obtained for growth, length, and survival are shown in Fig. 1. There was no significant difference in standard length among treatments showing that the skeleton growth was not affected by DHA enrichment. The larvae fed rotifer enriched with DHA6 emulsion (7.83 mg) were heavier than DHA12 ones (6.03 mg). DHA levels significantly affected the survival rate, and DHA36 (50.25%) had a lower value than others. These results showed that DHA6 and DHA24 performed better regarding the growth performance parameters. Accordingly, yellowtail clownfish larvae, even with rotifers enriched with 4% DHA and 0.7

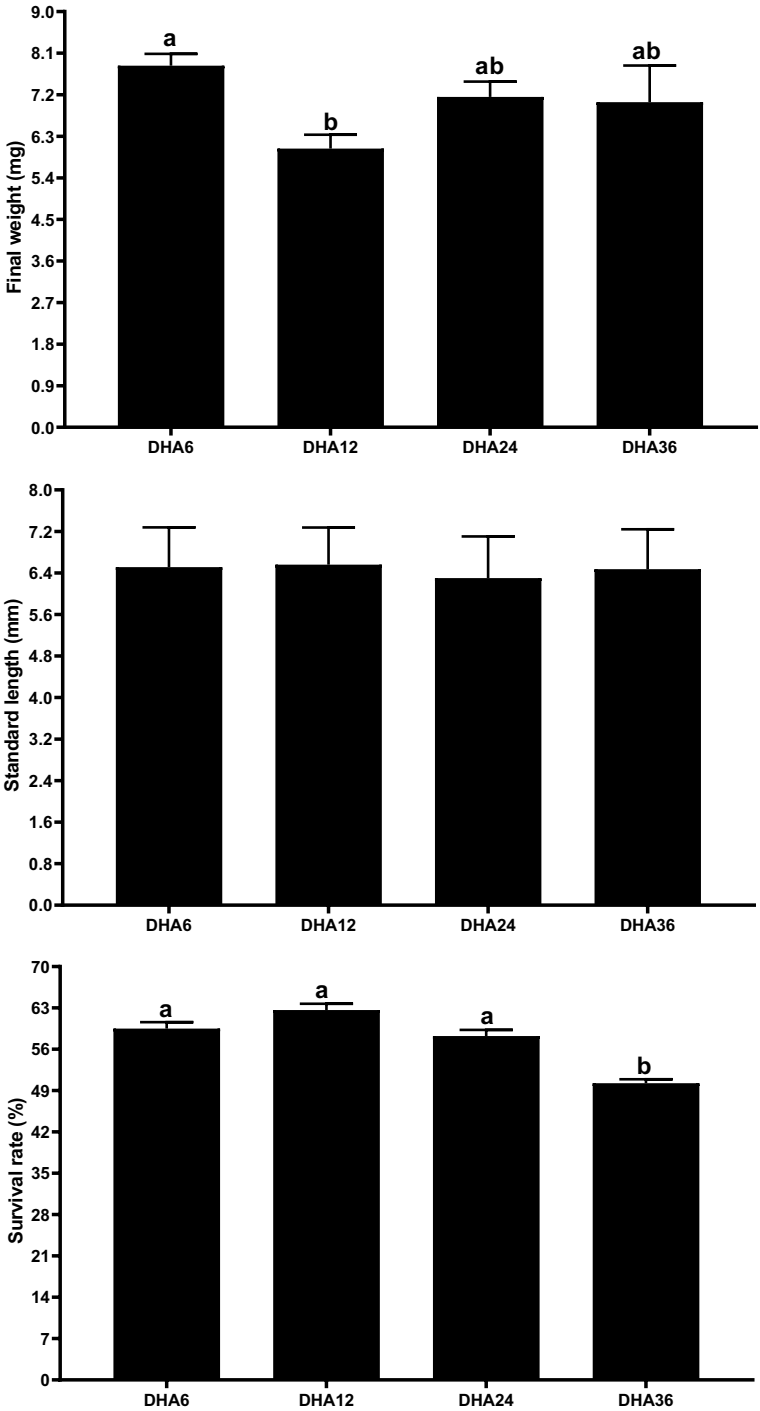

Fig. 1 Growth, standard length, and survival rate of yellowtail clownfish fed enriched rotifer with different DHA levels

DHA/EPA, can grow well. Commonly, usage of a ratio of 2:1 DHA/EPA is recommended to formulate in larvae diets as this ratio was observed in the body of marine species larvae (Sargent et al. 1997). Studies in other fish species showed that the DHA and DHA/EPA ratios play a key role in growth. For example, gilthead seabream with four different levels of n-3 HUFA either in rotifers demonstrated the best growth with adequate DHA/EPA ratios (> 1.3) and n-3 HUFA of 18% TFA (DHA 11.4% TFA and EPA 6.6% TFA; (Salhi et al. 1994)). Similarly, the best growth of gilthead seabream larvae was obtained with 7.8% ARA, DHA 11%, and EPA 6.3% of TFA (Bessonart et al. 1999). The positive effect of DHA enrichment in the incidence of skeleton deformities and improved survival rate in red porgy (about 50%, with increasing DHA from 9.68 to 20.52% TFA) was reported (Roo et al. 2009). A high DHA diet significantly increased the growth and survival rate of larvae by up to 44% in lake sturgeon (*Acipenser fulvescens*) compared to those fed freshly hatched *Artemia nauplii* (Yoon et al. 2022). Dietary DHA during the larval stage of spotted tiger shovelnose catfish (*Pseudoplatystoma punctifer*) promoted growth performance (Castro-Ruiz et al. 2022). However, DHA enrichment did not affect the growth and survival of yellowfin seabream (*Acanthopagrus latus*) larvae (Morshedi et al. 2022). In most cases, DHA improves the growth and survival of larvae, and it is crucial to find the optimum level of DHA in enrichment emulsions or rotifer. The question that can be raised is whether yellowtail clownfish larvae that eat a micro diet provided with ≈ 4% DHA can still grow and survive as same as those fed rotifer with around ≈ 4% DHA (the DHA level of rotifer in DHA6 treatment). It is likely that more DHA should be provided in a micro diet to reach the same amount of growth and survival rate. In our study, the ARA at 2.5% in rotifer with an EPA/DHA ratio of 2.4 was enough to provide maximum growth (Table 2). Similar investigations indicated at least 1.2% ARA for European seabass larvae with an EPA/ARA ratio of 4 was required to provide a maximum growth rate (Atalah et al. 2011a; Atalah et al. 2011b). It should be noted that an excessive level of DHA can result in negative consequences for larvae and oxidative stress. Betancor et al. (2012) also reported the negative impacts of excessive DHA contents in micro diets for seabass (*Dicentrarchus labrax*) that increase peroxidation risks. The possible reason could be related to the proliferation of free radicals derived from this fatty acid and the formation of toxic oxidised compounds and oxidative stress (Izquierdo et al. 2010). In the present study, we observed the negative impact of excessive DHA on the survival rate of larvae which can be due to oxidative stress. However, the growth and digestive enzymes, antioxidant abilities, and histology were not affected by excessive levels of DHA. In fish and other aquatic species, there is no direct relation between growth and survival rate in any stage of life. In the present study also, the same output was observed, and no relation between growth and survival rate under DHA treatments was observed (Table 4).

Digestive enzyme activity

Any effort to increase digestive enzyme activities in larvae, such as feeding with live foods, supplementing diets/live foods with bovine trypsin, porcine pancreatic extract, and digestive system neuropeptides, can eventually improve growth and survival rate (Kolkovski 2001). The result of this investigation indicated that the optimum level of DHA in live food can also increase the digestive enzyme activities of larvae. The result of digestive enzymes in whole yellowtail clownfish larvae was reported in Fig. 2. Accordingly, the DHA12 group had a significantly lower value of amylase (0.81), lipase (0.97), protease (1.89), and ALP (0.75) than others. This treatment also had the lowest growth as well. We can hypothesise that due to releasing not enough digestive enzymes, nutrient digestibility was decreased,

Table 4 Correlation between investigated factors in yellowtail clownfish larvae (*Amphiprion clarkii*) fed with variable live foods enriched with the experimental emulsions after 10 days

	DHA level	Final weight	Survival rate	Amylase	Lipase	Protease	ALP	SOD	Catalase	MDA	LDH	SFA	MUFA	ARA	EPA	n-3
DHA level	**1.00**	−0.27	**−0.72****	0.07	0.19	001	0.42	0.33	0.35	0.28	0.25	0.04	**-0.91****	**0.93****	0.44	**0.97****
Final weight		**1.00**	−0.21	0.54	**0.59***	**0.73****	0.52	0.30	0.28	−0.37	**0.69***	0.03	0.14	−0.31	−0.34	−0.37
Survival rate			**1.00**	−0.37	−0.68**	−0.43	**−0.78****	**−0.71****	**−0.72****	−0.29	**−0.74****	−0.27	**0.76****	**−0.76****	−0.51	**−0.67***
Amylase				**1.00**	**0.78****	**0.87****	**0.74****	0.54	**0.72***	−032	**0.64***	0.14	-0.38	0.18	0.01	0.12
Lipase					**1.00**	**0.89****	**0.84****	**0.76****	**0.84****	−0.14	**0.85****	0.04	-0.42	0.34	0.06	0.23
Protease						**1.00**	**0.74****	**0.65***	**074****	−0.25	**0.79****	0.17	-0.15	0.01	−0.19	−0.15
ALP							**1.00**	**0.66***	**0.77****	−0.18	**0.86****	0.23	**-0.61***	0.47	0.32	0.41
SOD								**1.00**	**0.95****	−0.24	**0.79****	0.17	**−0.62***	0.55	0.18	0.39
Catalase									**1.00**	−0.31	**0.80****	0.15	**−0.66***	**0.58***	0.19	0.49
MDA										**1.00**	−0.03	−0.32	−0.18	0.34	0.08	0.25
LDH											**1.00**	0.05	−0.47	0.32	−0.40	0.25
SFA												**1.00**	0.06	0.09	**0.83***	−0.34
MUFA													**1.00**	**−0.96****	−0.40	**−0.95****
ARA														**1.00**	0.48	**0.96****
EPA															**1.00**	0.40
n-3																**1.00**

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

ALP alkaline phosphatase, *SOD* superoxide dismutase, *MDA* malondialdehyde, *LDH* lactate dehydrogenase, *SFA* saturated fatty acids, *MUFA* monounsaturated fatty acids, *ARA* arachidonic acid, *EPA* eicosapentaenoic acid, *DHA* docosahexaenoic acid

Data in bold emphasis indicates a significant difference in correlation analysis

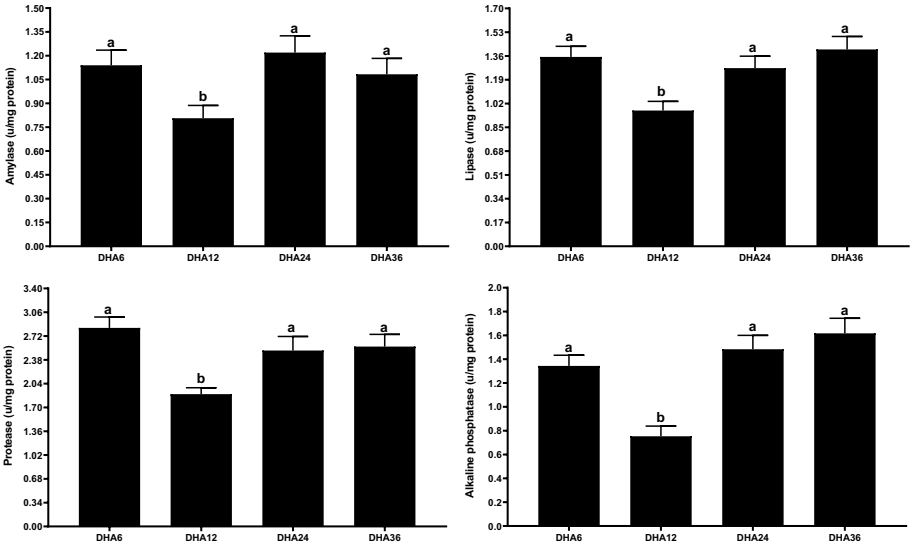

Fig. 2 Digestive enzyme activity of yellowtail clownfish fed enriched rotifer with different DHA levels

and eventually, fewer nutrients were available for growth. More studies are required, and this question can be raised why these results were observed in a middle dosage. Similar to our results, lipase increased in lake sturgeon at optimum DHA level that provided a maximum growth (Yoon et al. 2022). Different DHA enrichment levels of live food in yellowtail seabream increased the specific activity of digestive enzymes such as protease, lipase, amylase, and ALP but not the growth and survival rate (Morshedi et al. 2022). No significant differences were observed in trypsin and amylase activities in pancreatic and intestinal segments when shrimp larvae were fed the different DHA levels (Wang et al. 2017). In our study, with increasing DHA levels, ARA elevated as well, and the DHA36 group had a higher level of ARA compared to DHA6. However, the changed digestive enzymes cannot be connected to the ARA level as these groups did not differ significantly. It is well-known that ARA levels can affect digestive enzyme activities (Xu et al. 2022); however, it was not observed in the current study. When pikeperch (*Sander lucioperca*) larvae were fed different dietary DHA levels, intestinal brush border digestive enzymes and growth were not influenced, but the optimum level of ARA increased digestive enzymes (El Kertaoui et al. 2021). In general, in our study, the same trend between growth and digestive enzymes is further evidence that digestive enzymes are key parameters in larval stages to maximise growth (Holt 2011).

Antioxidant activities

Superoxide dismutase (SOD) and catalase (CAT) are the major antioxidant enzymes that can clear the internal reactive oxygen species to avoid the occurrence of fatty acid oxidation and subsequently protect organisms from oxidative damage. Therefore, SOD and CAT activities can show how aquatic animals' bodies can tackle oxidative stress and eventually indicate how their immune system is robust. The DHA levels significantly influenced four measured antioxidants in this study, including SOD, CAT, malondialdehyde (MDA), and lactate dehydrogenase (LDH) (Fig. 3). The larvae fed the DHA12 group had the lower

value of SOD, CAT, and LDH compared to others. Similarly, grass carp (*Ctenopharyngodon idellus*) (Ji et al. 2011) and yellow croaker (*Larimichthys crocea*) (Zuo et al. 2012) fed not optimum levels of DHA in terms of growth showed retarded antioxidant parameters such as SOD and MDA due to oxidative stress. Also, in ridgetail white prawn (*Exopalaemon carinicauda*), the DHA-enhanced artemia, at different concentrations, increased the enzyme activity of SOD compared to the control group (Wang et al. 2021), which is again compatible with our research. The suitable supplement of DHA in diets improved the activities of SOD and CAT in different tissues of sea cucumber (*Apostichopus japonicus*) (Yu et al. 2016). CAT and SOD in golden pompano (*Trachinotus ovatus*) were higher at the DHA/EPA ratio of 1.48 group, which growth was higher as well on this treatment (Zhang et al. 2019). However, the excessively high dietary DHA/EPA ratio adversely affected the growth and health of fish larvae (Zhang et al. 2019). It has been reported that increasing the level of DHA can cause the formation of reactive oxygen substances in fish larvae (Izquierdo et al. 2013), which was not observed in the yellowtail clownfish. The DHA/EPA also is an important index that can be connected to antioxidant activities. Japanese seabass (*Lateolabrax japonicus*) fed DHA/EPA 1.53 (DHA: 9.93%) showed the highest activity of serum SOD among experimental groups (Xu et al. 2016). In that study, the weight gain in DHA/EPA 1.53 was significantly higher than those fed DHA/EPA 0.55. In our study, the worse treatment (DHA12) ate a DHA/EPA of 1.12 (DHA: 7.01%) with the lowest growth and digestive enzymes as well. The results of these studies are comparable and show that the DHA levels with the appropriate DHA/EPA play a key role in oxidative stress and growth. The yellowtail clownfish in DHA12 was the worse treatment in growth, digestive enzymes, and antioxidant system which makes sense. It was a challenging result as even lower or higher levels did not result in such output and more studies are required. If it occurred in higher or lower dosages, we could say DHA deficiencies or too much DHA impaired the fish physiology but did not happen. When the body encounters oxidative stress, the energy will go toward getting the larvae back to normal haemostasis, and nutrient absorption and secretion of digestive enzymes will be the second priority. Therefore,

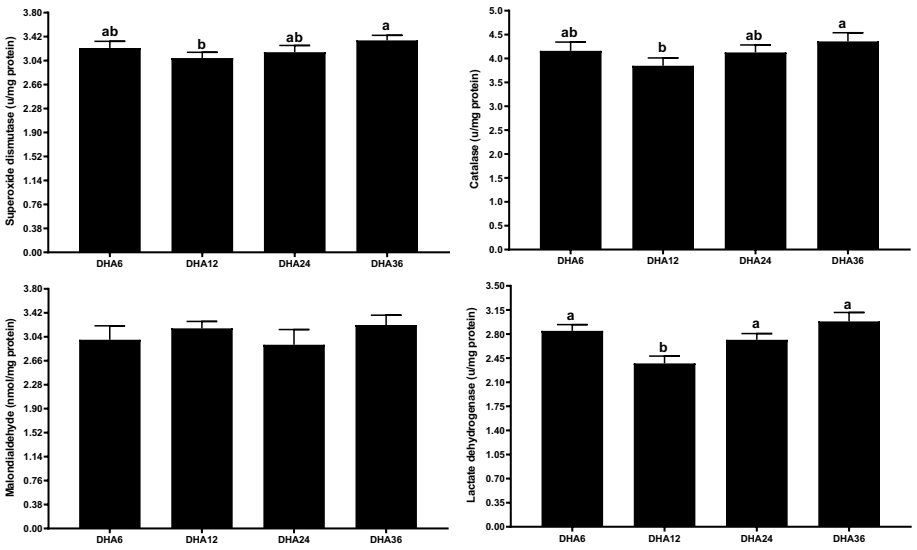

Fig. 3 Antioxidant activities of yellowtail clownfish fed enriched rotifer with different DHA levels

the nutrient digestibility and uptake will be reduced, and larvae cannot grow well. More studies are required to illustrate this phenomenon in yellowtail clownfish.

Histology

The main histological changes in lipid deposition in the liver, intestine, and pancreas of individuals reared under different rates of DHA (DHA6, DHA12, DHA24, DHA36) are shown in Figs. 4, 5 and 6. The results indicated the presence of pyknotic or necrotic hepatocytes in the liver in groups fed enriched rotifer (DHA6, DHA12, DHA24, DHA36) compared to those fed non-enriched rotifer (I). Erythrocyte infiltration was more common among the hepatocytes in-group fed enriched rotifers (DHA24). All groups showed lipid vacuoles in hepatic parenchyma. Therefore, the nuclei are located at the edge of the hepatocytes (nuclear migration in the hepatocytes). Groups fed enriched rotifers (DHA6, DHA12, DHA24, DHA36) presented high lipid accumulation with large lipid-containing vacuoles, whereas in the group fed non-enriched rotifers (I); the lipid vacuoles were smaller than other groups (Fig. 5). Lipids were deposited in the intestine of all groups, whereas small lipid deposits were also found at the base of the folds in groups fed non-enriched rotifers (I). In the DHA6 group, lipid deposits were concentrated in the apical zone of the intestinal folds. The lipid accumulation detected at the apical ends did not affect the intestinal structure or damage the enterocytes (Fig. 6). Histological analysis revealed that exocrine cells of the pancreas were pyramid shaped and showed acidophilic zymogen granules in all dietary groups.

Lipid vacuoles are biomarkers used for the evaluation of lipid absorption and metabolism in nutritional studies. The high fatty acid content of compound diets causes the formation of lipid vacuoles in enterocytes (Deplano et al. 1991). Intestinal lipid vacuoles formed when fatty acid uptake exceeds the transport capacity of enterocytes are defined

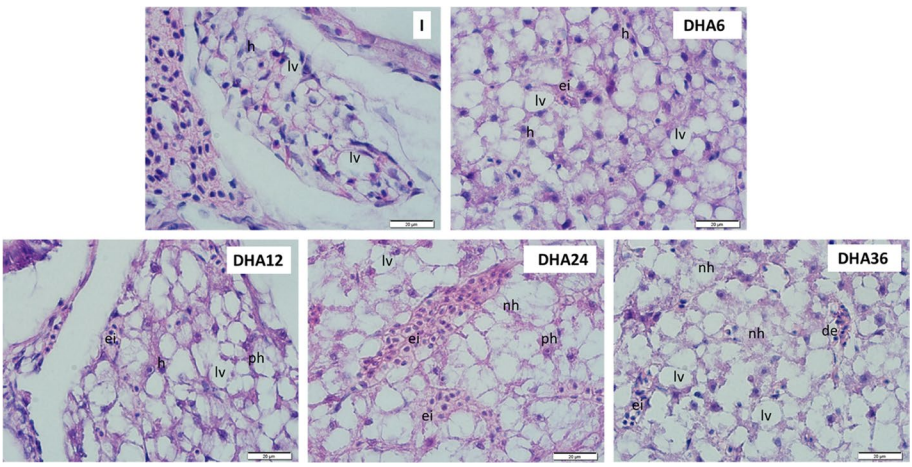

Fig. 4 Longitudinal paraffin sections of the liver of yellowtail clownfish fed enriched rotifers with different rates DHA (I): Initial. Lipid vacuoles in the liver are similar in all dietary groups, note that the lipid vacuoles are smaller in the group fed non-enriched rotifer (I); the presence of pyknotic or necrotic hepatocytes and erythrocyte infiltration in the liver in groups fed enriched rotifer (DHA24, DHA36) (ph, pyknotic hepatocyte; nh, necrotic hepatocyte; ei, erythrocyte infiltration; lv, lipid vacuole)

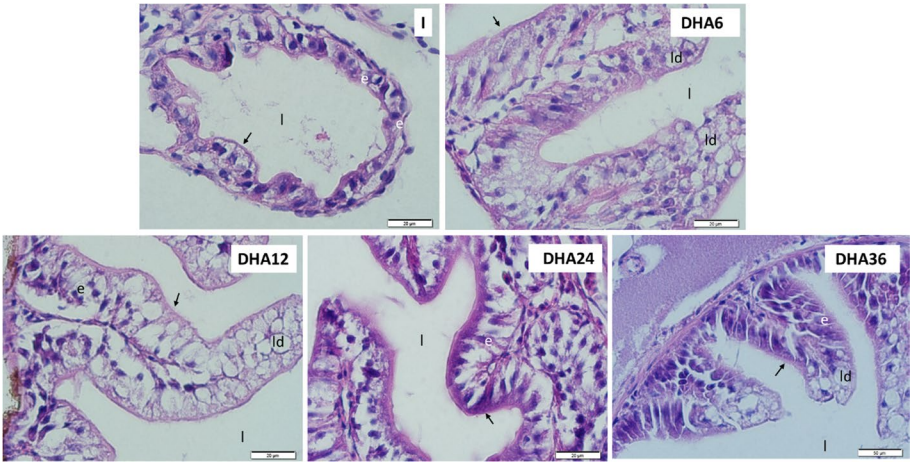

Fig. 5 Histological structure of intestine of yellowtail clownfish fed enriched rotifers with different rates of DHA (I): Initial, groups DHA6 and DHA36 showed lipid accumulation in the intestine was concentrated in the apical zone, whereas in groups L and M, lipids were accumulated along the intestinal folds (arrowed) (e, enterocyte; ld, lipid deposits; l, lumen)

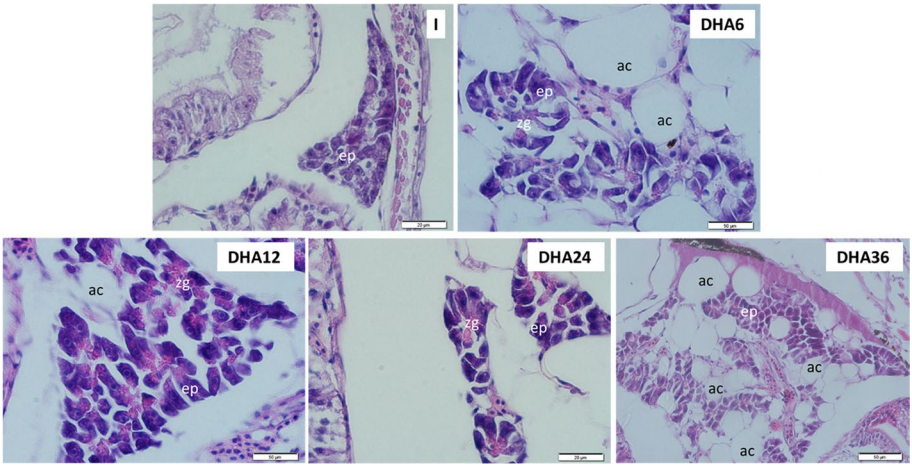

Fig. 6 Histological sections of the pancreas of yellowtail clownfish fed enriched rotifers with different rates DHA (I): Initial, exocrine cells of the pancreas are similarly shaped and include zymogen granules in all dietary groups; note that the lipid vacuoles are dense in the DHA36 group (ep, exocrine pancreas; ac, adipose cell; zg, zymogen granules)

as a temporary storage form of re-esterified fatty acids (Fontagne et al. 1998). The localisation and size of lipid vacuoles in the gut may depend on diets (Cahu et al. 2009). In general, the accumulation of lipid vacuoles in the basal layer of enterocyte cells indicates good digestion and absorption of dietary fats (Izquierdo et al. 2000; Lu et al. 2008). In this study, lipid vacuoles were only detected at the base of the folds in groups fed non-enriched rotifers (I). The accumulation of large lipid vacuoles in the intestinal tissue results from reduced transport of lipids from the intestinal mucosa to the circulatory system (Fontagne

et al. 1998). Similarly, this study detected large lipid vacuoles in the intestinal folds of all groups (DHA6, DHA12, DHA24, and DHA36) and also pancreatic tissue. This may be due to the decrease in the DHA added to the feed and, eventually, a decline in its levels in the circulatory system. A large accumulation of lipids in enterocytes, called steatosis, may cause pathological damage to intestinal tissue. This damage causes cellular necrosis and an inflammatory reaction as a result of structural deterioration in the enterocyte membrane in the intestinal mucosa (Cahu et al. 2009; Kowalska et al. 2011). In the present study, the addition of DHA at different rates in all groups (DHA6, DHA12, DHA24, and DHA36) did not cause structural deterioration in enterocyte membranes. Similar to the present study, Castro-Ruiz et al. (2022) detected dense lipid vacuoles in intestinal tissue and reported that these vacuoles did not affect the shape or organisation of intestinal folds, and no signs of epithelial damage were observed.

In recent studies, aquatic nutritionists have focused on the effects of various feed ingredients on fish liver health since the liver has an important function in processes such as lipid storage, digestive, and detoxification. The most common histological change in fish feeding studies is the formation of lipid vacuoles in the liver. It has been reported that lipid vacuoles are formed in the liver of Russian sturgeon (*Acipenser gueldenstaedtii*) (Kamaszewski et al. 2014), barbel larvae (*Barbus barbus*) (Prusińska et al. 2020), spotted tiger shovelnose catfish (Castro-Ruiz et al. 2022) and African catfish, *Clarias gariepinus* (Verreth et al. 1994), fed on live food enriched with different fatty acids. Similarly, lipid vacuoles were detected in liver tissue of all groups (DHA6, DHA12, DHA24, and DHA36) in the present study. Accordingly, lipid vacuoles can cause accumulation in the liver as a result of not meeting the need for dietary lipids (Watanabe et al. 1989), but lipid accumulation in the liver has been interpreted as a disturbance in the hepatocellular lipid transfer and metabolism (Segner and Witt 1990). In addition to the lipid vacuoles observed in the liver tissue of all groups, pycnosis was noted in the nuclei of hepatocyte cells, as well as larger lipid vacuoles in the DHA12, DHA24, and DHA36 groups. It has been reported that hepatic pathological changes can be seen as a result of feeding with high levels of long-chain polyunsaturated fatty acids in different fish species such as Atlantic bluefin tuna (*Thunnus thynnus*) (Betancor et al. 2019), zebrafish (*Danio rerio*) (Ding et al. 2022), and grass carp (Du et al. 2008).

Correlation between measured parameters in larvae

The correlation results are reported in Table 4, and many parameters had correlations with each other. The only significant ones were reported in this table, and also only important FAs were reported. The DHA levels in the larvae body were correlated with other parameters. There was a negative correlation between DHA levels and survival rate (−72%) and MUFA (−91%). These results show that the DHA requirement of this larvae species is low and high DHA negatively affects the survival rate. There were key parameters that can be introduced as markers of growth in yellowtail clownfish larvae. Accordingly, there were positive correlations between final weight and lipase (59%), protease (73%), and LDH (69%). These outputs indicated the positive correlation between growth and digestive enzymes reported earlier in other fish species (Esmaeili et al. 2017; Magouz et al. 2020; Sotoudeh and Esmaeili 2022; Zemheri-Navruz et al. 2020). Digestive enzymes also had positive correlations with each other and also antioxidant parameters. For example, amylase, lipase, protease, and ALP had significant positive correlations with CAT (72%, 84%, 74%, and 66%, respectively) and LDH (64%,

85%, 79%, and 77%, respectively). These results indicated that antioxidant activities were in an optimum condition that caused higher digestive enzyme activities and eventually ended up with improved growth. Other studies show that if the antioxidant system haemostasis is optimum, the digestive system secretes more digestive enzymes (Abdel-Tawwab and Monier 2018; Jiang et al. 2016; Liu et al. 2019). SOD and CAT also had positive correlations with ARA, which for CAT (58%) was significant. These results show that the key role of ARA is antioxidant activities which were earlier observed in goby (*Synechogobius hasta*) (Luo et al. 2012) and yellow catfish (*Pelteobagrus fulvidraco*) (Ma et al. 2018).

Conclusion

Conclusively, the enrichment process was successfully translated into rotifer and larvae bodies. DHA enrichment of rotifers did not increase the growth and survival rate of yellowtail clownfish, and a DHA level of 4.2% in rotifers is enough to provide maximum growth and survival rate. While it was a middle level, the DHA12 group, although was a middle dosage, showed the lowest weight, digestive enzymes, and antioxidant parameters that required more studies to find a possible reasons. However, these impairments were not observed in upper levels such as DHA24 and DHA36. There was a positive correlation between final weight and digestive enzymes. Digestive enzymes also had significant positive correlations with CAT and LDH, showing that measured parameters changed with each other in the same direction. SOD and CAT also had positive correlations with ARA levels and illustrated the key role of this fatty acid in the antioxidant system. Measuring more parameters, such as the development of bone deformities, is required to elucidate the importance of these fatty acids on yellowtail clownfish metabolism. Furthermore, as this species requires few DHA levels in their diet, after carefully considering the optimum fish meal and oil in their diet, they can be a sustainable option for marine aquaculture. More lipid studies are recommended to illustrate this issue.

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Author contribution Vahid Morshedi: Statistical analysis, Resources, Research, Investigation, Ideas, revision; Kamil Mert Eryalcin: Research, Lab works, Investigation, Validation, revision; Noah Esmaeili: Writing, Validation, Supervision, Ideas; Mohamad Niromand: Research, Investigation, Resources; Reza Gamori: Research, Investigation, Resources: Cigdem Urku: Validation, Investigation, revision; Omid Safari: Investigation, Resources.

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Data availability Data available on request due to privacy/ethical restrictions (the data that support the findings of this study are available on request from the corresponding authors. The data are not publicly available due to privacy or ethical restrictions).

Declarations

Ethical approval This study adheres to current regulations regarding the use of animals in research in Iran (Ahmadi-Noorbakhsh et al. 2021).

Competing interests The authors declare no competing interests.

References

Abdel-Tawwab M, Monier MN (2018) Stimulatory effect of dietary taurine on growth performance, digestive enzymes activity, antioxidant capacity, and tolerance of common carp, *Cyprinus carpio* L., fry to salinity stress. Fish Physiol Biochem 44:639–649

Ahmadi-Noorbakhsh S, Mirabzadeh Ardakani E, Sadighi J, Aldavood SJ, Farajli Abbasi M, Farzad-Mohajeri S, Ghasemi A, Sharif-Paghaleh E, Hatami Z, Nikravanfard N (2021) Guideline for the care and use of laboratory Animals in Iran. Lab Anim 50:303–305

Anson ML (1938) The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. J Gen Physiol 22:79

Atalah E, Hernández-Cruz CM, Benítez-Santana T, Ganga R, Roo J, Izquierdo M (2011a) Importance of the relative levels of dietary arachidonic acid and eicosapentaenoic acid for culture performance of gilthead seabream (*Sparus aurata*) larvae. Aquac Res 42:1279–1288

Atalah E, Hernández-Cruz CM, Ganuza E, Benítez-Santana T, Ganga R, Roo J, Montero D, Izquierdo M (2011b) Importance of dietary arachidonic acid for the growth, survival and stress resistance of larval European sea bass (*Dicentrarchus labrax*) fed high dietary docosahexaenoic and eicosapentaenoic acids. Aquac Res 42:1261–1268

Basford AJ, Mos B, Francis DS, Turchini GM, White CA, Dworjanyn S (2020) A microalga is better than a commercial lipid emulsion at enhancing live feeds for an ornamental marine fish larva. Aquaculture 523:735203

Benítez-Santana T, Masuda R, Carrillo EJ, Ganuza E, Valencia A, Hernández-Cruz C, Izquierdo M (2007) Dietary n-3 HUFA deficiency induces a reduced visual response in gilthead seabream *Sparus aurata* larvae. Aquaculture 264:408–417

Bessonart M, Izquierdo M, Salhi M, Hernandez-Cruz C, Gonzalez M, Fernandez-Palacios H (1999) Effect of dietary arachidonic acid levels on growth and survival of gilthead sea bream (*Sparus aurata* L.) larvae. Aquaculture 179:265–275

Betancor MB, Caballero M, Terova G, Saleh R, Atalah E, Benítez-Santana T, Bell JG, Izquierdo M (2012) Selenium inclusion decreases oxidative stress indicators and muscle injuries in sea bass larvae fed high-DHA microdiets. Br J Nutr 108:2115–2128

Betancor MB, Ortega A, De La Gándara F, Tocher DR, Mourente G (2019) Performance, feed utilisation, and hepatic metabolic response of weaned juvenile Atlantic bluefin tuna (*Thunnus thynnus* L.): effects of dietary lipid level and source. Fish Physiol Biochem 45:697–718

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Anal Biochem 72:248–254

Bransden M, Cobcroft JM, Battaglene S, Morehead D, Dunstan G, Nichols P, Kolkovski S (2005) Dietary 22: 6n-3 alters gut and liver structure and behaviour in larval striped trumpeter (*Latris lineata*). Aquaculture 248:275–285

Cahu CL, Gisbert E, Villeneuve LA, Morais S, Hamza N, Wold PA, Zambonino Infante JL (2009) Influence of dietary phospholipids on early ontogenesis of fish. Aquac Res 40:989–999

Castro-Ruiz D, Andree KB, Magris J, Fernández-Méndez C, García-Dávila C, Gisbert E, Darias MJ (2022) DHA-enrichment of live and compound feeds influences the incidence of cannibalism, digestive function, and growth in the neotropical catfish *Pseudoplatystoma punctifer* (Castelnau, 1855) during early life stages. Aquaculture 561:738667

Christie WW (1993) Preparation of ester derivatives of fatty acids for chromatographic analysis. Adv Lipid Methodol 2:e111

Conceição L, Dersjant-Li Y, Verreth J (1998) Cost of growth in larval and juvenile African catfish (*Clarias gariepinus*) in relation to growth rate, food intake and oxygen consumption. Aquaculture 161:95–106

Deplano M, Diaz J, Connes R, Kentouri-Divanach M, Cavalier F (1991) Appearance of lipid-absorption capacities in larvae of the sea bass *Dicentrarchus labrax* during transition to the exotrophic phase. Mar Biol 108:361–371

Dhaneesh K, Kumar TA (2017) Oil extraction from microalgae for live prey enrichment and larviculture of clownfish Amphiprion percula. J Mar Biol Assoc United Kingdom 97:43–58

Ding Q, Hao Q, Zhang Q, Yang Y, Olsen RE, Ringø E, Ran C, Zhang Z, Zhou Z (2022) Excess DHA induces liver injury via lipid peroxidation and gut microbiota-derived lipopolysaccharide in zebrafish. Front Nutr 9. https://doi.org/10.3389/fnut.2022.870343

Du Z, Clouet P, Huang L, Degrace P, Zheng W, He J, Tian L, Liu Y (2008) Utilisation of different dietary lipid sources at high level in herbivorous grass carp (*Ctenopharyngodon idella*): mechanism related to hepatic fatty acid oxidation. Aquac Nutr 14:77–92

El Kertaoui N, Lund I, Betancor MB, Carpentier C, Montero D, Kestemont P (2021) Dietary DHA and ARA level and ratio affect the occurrence of skeletal anomalies in pikeperch larvae (*Sander*

lucioperca) through a regulation of immunity and stress related gene expression. Aquaculture 544:737060
Eryalçın KM (2019) Nutritional value and production performance of the rotifer *Brachionus plicatilis* Müller, 1786 cultured with different feeds at commercial scale. Aquac Int 27:875–890
Esmaeili M, Abedian Kenari A, Rombenso A (2017) Effects of fish meal replacement with meat and bone meal using garlic (*Allium sativum*) powder on growth, feeding, digestive enzymes and apparent digestibility of nutrients and fatty acids in juvenile rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792). Aquac Nutr 23:1225–1234
Estévez A, Giménez G (2017) Optimisation of emulsion properties and enrichment conditions used in live prey enrichment. Aquac Nutr 23:1264–1273
Folch J, Lees M, Sloane-Stanley G (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497–509
Fontagne S, Geurden I, Escaffre A-M, Bergot P (1998) Histological changes induced by dietary phospholipids in intestine and liver of common carp (*Cyprinus carpio* L.) larvae. Aquaculture 161:213–223
Furné M, García-Gallego M, Hidalgo MC, Morales AE, Domezain A, Domezain J, Sanz A (2008) Effect of starvation and refeeding on digestive enzyme activities in sturgeon (*Acipenser naccarii*) and trout (*Oncorhynchus mykiss*). Comp Biochem Physiol A Mol Integr Physiol 149:420–425
Hamre K, Yufera M, Rønnestad I, Boglione C, Conceição LE, Izquierdo M (2013) Fish larval nutrition and feed formulation: knowledge gaps and bottlenecks for advances in larval rearing. Rev Aquac 5:S26–S58
Hernández-Cruz C, Salhi M, Bessonart M, Izquierdo M, González M, Fernández-Palacios H (1999) Rearing techniques for red porgy (*Pagrus pagrus*) during larval development. Aquaculture 179:489–497
Holt GJ (2011) Larval fish nutrition. John Wiley & Sons
Izquierdo M (1996) Essential fatty acid requirements of cultured marine fish larvae. Aquac Nutr 2:183–191
Izquierdo M (2005) Essential fatty acid requirements in Mediterranean fish species. Cahiers Options Méditerranéennes 63:91–102
Izquierdo M, Arakawa T, Takeuchi T, Haroun R, Watanabe T (1992) Effect of n-3 HUFA levels in Artemia on growth of larval Japanese flounder (*Paralichthys olivaceus*). Aquaculture 105:73–82
Izquierdo M, Scolamacchia M, Betancor M, Roo J, Caballero M, Terova G, Witten PE (2013) Effects of dietary DHA and α-tocopherol on bone development, early mineralisation and oxidative stress in *Sparus aurata* (Linnaeus, 1758) larvae. Br J Nutr 109:1796–1805
Izquierdo M, Socorro J, Arantzamendi L, Hernández-Cruz C (2000) Recent advances in lipid nutrition in fish larvae. Fish Physiol Biochem 22:97
Izquierdo M, Socorro J, Roo J (2010) Studies on the appearance of skeletal anomalies in red porgy: effect of culture intensiveness, feeding habits and nutritional quality of live preys. J Appl Ichthyol 26:320–326
Izquierdo MS (1989) Requirement of larval red seabream *Pagrus major* for essential fatty acids. Nippon Suisan Gakkaishi 55:859–867
Ji H, Li J, Liu P (2011) Regulation of growth performance and lipid metabolism by dietary n-3 highly unsaturated fatty acids in juvenile grass carp, *Ctenopharyngodon idellus*. Comp Biochem Physiol B: Biochem Mol Biol 159:49–56
Jiang J, Wu X-Y, Zhou X-Q, Feng L, Liu Y, Jiang W-D, Wu P, Zhao Y (2016) Effects of dietary curcumin supplementation on growth performance, intestinal digestive enzyme activities and antioxidant capacity of crucian carp *Carassius auratus*. Aquaculture 463:174–180
Kamaszewski M, Ostaszewska T, Prusińska M, Kolman R, Chojnacki M, Zabytyvskij J, Jankowska B, Kasprzak R (2014) Effects of Artemia sp. enrichment with essential fatty acids on functional and morphological aspects of the digestive system in *Acipenser gueldenstaedtii* larvae. Turk J Fish Aquat Sci 14:929–938
Kandathil Radhakrishnan D, Akbarali I, Schmidt BV, John EM, Sivanpillai S, Thazhakot Vasunambesan S (2020) Improvement of nutritional quality of live feed for aquaculture: an overview. Aquac Res 51:1–17
Kolkovski S (2001) Digestive enzymes in fish larvae and juveniles—implications and applications to formulated diets. Aquaculture 200:181–201
Koven W (2003) Key factors influencing juvenile quality in mariculture: a review. Isr J Aquac 55:283–297
Kowalska A, Zakęś Z, Jankowska B, Demska-Zakęś K (2011) Effect of different dietary lipid levels on growth performance, slaughter yield, chemical composition, and histology of liver and intestine of pikeperch, *Sander lucioperca*. Czech J Anim Sci 56:136–149

Liu HP, Wen B, Chen ZZ, Gao JZ, Liu Y, Zhang YC, Wang ZX, Peng Y (2019) Effects of dietary vitamin C and vitamin E on the growth, antioxidant defence and digestive enzyme activities of juvenile discus fish (*Symphysodon haraldi*). Aquac Nutr 25:176–183

Lu S, Zhao N, Zhao A, He R (2008) Effect of soybean phospholipid supplementation in formulated microdiets and live food on foregut and liver histological changes of *Pelteobagrus fulvidraco* larvae. Aquaculture 278:119–127

Luo Z, Tan XY, Li XD, Yin GJ (2012) Effect of dietary arachidonic acid levels on growth performance, hepatic fatty acid profile, intermediary metabolism and antioxidant responses for juvenile *Synechogobius hasta*. Aquac Nutr 18:340–348

Ma H-N, Jin M, Zhu T-T, Li C-C, Lu Y, Yuan Y, Xiong J, Zhou Q-C (2018) Effect of dietary arachidonic acid levels on growth performance, fatty acid profiles and lipid metabolism of juvenile yellow catfish (*Pelteobagrus fulvidraco*). Aquaculture 486:31–41

Magouz FI, Dawood MA, Salem MF, Mohamed AA (2020) The effects of fish feed supplemented with Azolla meal on the growth performance, digestive enzyme activity, and health condition of genetically-improved farmed tilapia (*Oreochromis niloticus*). Ann Anim Sci 20:1029–1045

Morshedi V, Mozanzadeh MT, Hamedi S, Naserifard I, Ebrahimi H, Agh N, Nafisi M, Azodi M, Rashidian G (2022) Enrichment of livefeed with very low level of docosahexaenoic acid (DHA) is enough for yellowtail sea bream (*Acanthopagrus latus*) larvae. Aquac Rep 26:101310

Mourente G, Rodrýguez A, Grau A, Pastor E (1999a) Utilisation of lipids by *Dentex dentex* L.(Osteichthyes, Sparidae) larvae during lecitotrophia and subsequent starvation. Fish Physiol Biochem 21:45

Mourente G, Tocher DR, Diaz-Salvago E, Grau A, Pastor E (1999b) Study of the n− 3 highly unsaturated fatty acids requirement and antioxidant status of *Dentex dentex* larvae at the Artemia feeding stage. Aquaculture 179:291–307

Prusińska M, Nowosad J, Jarmołowicz S, Mikiewicz M, Duda A, Wiszniewski G, Sikora M, Biegaj M, Samselska A, Arciuch-Rutkowska M (2020) Effect of feeding barbel larvae (*Barbus barbus* (L, 1758)) Artemia sp. nauplii enriched with PUFAs on their growth and survival rate, blood composition, alimentary tract histological structure and body chemical composition. Aquac Rep 18:100492

Roberts R, Smail D, Munro E (2001) Laboratory methods. In: Fish Pathology. WB Saunders, UK, pp 380–412

Rombenso AN, Trushenski JT, Jirsa D, Drawbridge M (2016) Docosahexaenoic acid (DHA) and arachidonic acid (ARA) are essential to meet LC-PUFA requirements of juvenile California yellowtail (*Seriola dorsalis*). Aquaculture 463:123–134

Roo FJ, Hernández-Cruz CM, Socorro JA, Fernández-Palacios H, Montero D, Izquierdo MS (2009) Effect of DHA content in rotifers on the occurrence of skeletal deformities in red porgy *Pagrus pagrus* (Linnaeus, 1758). Aquaculture 287:84–93

Salhi M, Izquierdo M, Hernández-Cruz C, Gonzalez M, Fernández-Palacios H (1994) Effect of lipid and n− 3 HUFA levels in microdiets on growth, survival and fatty acid composition of larval gilthead seabream (*Sparus aurata*). Aquaculture 124:275–282

Sargent J, Mcevoy L, Bell J (1997) Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. Aquaculture 155:117–127

Segner H, Witt U (1990) Weaning experiments with turbot (*Scophthalmus maximus*): electron microscopic study of liver. Mar Biol 105:353–361

Sotoudeh E, Esmaeili M (2022) Effects of Biotronic® Top3, a feed additive containing organic acids, cinnamaldehyde and a permeabilising complex on growth, digestive enzyme activities, immunity, antioxidant system and gene expression of barramundi (*Lates calcarifer*). Aquac Rep 24:101152

Trushenski J, Schwarz M, Bergman A, Rombenso A, Delbos B (2012) DHA is essential, EPA appears largely expendable, in meeting the n− 3 long-chain polyunsaturated fatty acid requirements of juvenile cobia *Rachycentron canadum*. Aquaculture 326:81–89

Verreth J, Coppoolse J, Segner H (1994) The effect of low HUFA-and high HUFA-enriched Artemia, fed at different feeding levels, on growth, survival, tissue fatty acids and liver histology of *Clarias gariepinus* larvae. Aquaculture 126:137–150

Wang C-C, Liu W-B, Huang Y-Y, Wang X, Li X-F, Zhang D-D, Jiang G-Z (2020) Dietary DHA affects muscle fiber development by activating AMPK/Sirt1 pathway in blunt snout bream (*Megalobrama amblycephala*). Aquaculture 518:734835

Wang Y, Li G, Han Z, Hu G, Yan B, Zhang Q, Gao H (2021) Effect of concentration of DHA nutrient-enhanced Artemia on the growth and physiology of the ridgetail white prawn, *Exopalaemon carinicauda* (Decapoda, Caridea, Palaemonidae). Crustaceana 94:29–43

Wang Y, Li M, Filer K, Xue Y, Ai Q, Mai K (2017) Replacement of fish oil with a DHA-rich *Schizochytrium* meal on growth performance, activities of digestive enzyme and fatty acid profile of Pacific white shrimp (*Litopenaeus vannamei*) larvae. Aquac Nutr 23:1113–1120

Watanabe T, Thongrod S, Takeuchi T, Satoh S, Kubota SS, Fujimaki Y, Cho CY (1989) Effect of dietary n-6 and n-3 fatty acids on growth, fatty acid composition and histological changes of white fish *Coregonus lavaretus* maraena. Nippon Suisan Gakkaishi 55:1977–1982

Xu H, Meng X, Wei Y, Ma Q, Liang M, Turchini GM (2022) Arachidonic acid matters. Rev Aquac 14:1912–1944

Xu H, Wang J, Mai K, Xu W, Zhang W, Zhang Y, Ai Q (2016) Dietary docosahexaenoic acid to eicosapentaenoic acid (DHA/EPA) ratio influenced growth performance, immune response, stress resistance and tissue fatty acid composition of juvenile Japanese seabass, *Lateolabrax japonicus* (Cuvier). Aquac Res 47:741–757

Yoon GR, Amjad H, Weinrauch AM, Laluk A, Suh M, Anderson WG (2022) Long-term effects of EPA and DHA enriched diets on digestive enzyme activity, aerobic scope, growth and survival in age-0 lake sturgeon (*Acipenser fulvescens*). Aquaculture 552:737972

Yu H, Gao Q, Dong S, Zhou J, Ye Z, Lan Y (2016) Effects of dietary n-3 highly unsaturated fatty acids (HUFAs) on growth, fatty acid profiles, antioxidant capacity and immunity of sea cucumber *Apostichopus japonicus* (Selenka). Fish Shellfish Immunol 54:211–219

Zemheri-Navruz F, Acar Ü, Yılmaz S (2020) Dietary supplementation of olive leaf extract enhances growth performance, digestive enzyme activity and growth related genes expression in common carp *Cyprinus carpio*. Gen Comp Endocrinol 296:113541

Zhang M, Chen C, You C, Chen B, Wang S, Li Y (2019) Effects of different dietary ratios of docosahexaenoic to eicosapentaenoic acid (DHA/EPA) on the growth, non-specific immune indices, tissue fatty acid compositions and expression of genes related to LC-PUFA biosynthesis in juvenile golden pompano *Trachinotus ovatus*. Aquaculture 505:488–495

Zuo R, Ai Q, Mai K, Xu W, Wang J, Xu H, Liufu Z, Zhang Y (2012) Effects of dietary n-3 highly unsaturated fatty acids on growth, nonspecific immunity, expression of some immune related genes and disease resistance of large yellow croaker (*Larmichthys crocea*) following natural infestation of parasites (*Cryptocaryon irritans*). Fish Shellfish Immunol 32:249–258

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