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Effects of n-3 HUFA-enriched *Artemia* on growth, biochemical response, skeletal morphology and stress resistance of Asian sea bass (*Lates calcarifer*) larvae reared at high temperature

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ABSTRACT

Improving the growth and health status of marine fish larvae to adapt to climate change, is crucial for commercial hatchery production in tropical regions. This study evaluated the effects of dietary essential fatty acids used for *Artemia* enrichment on growth, biochemical responses, skeletal morphology, and stress resistance of Asian sea bass larvae, reared at high temperatures of 30 °C and 34 °C. Starting 11 days after hatch, Asian sea bass larvae, were fed *Artemia*, enriched with a commercial emulsion of n-3 HUFA for 14 days, at four concentrations (0, 100, 300, and 500 ppm), at two rearing temperatures (30 °C and 34 °C). The results showed that enriching *Artemia* with n-3 HUFA significantly improved the final weight and specific growth rate of the larvae fed n-3 HUFA enrichment at 300 ppm, at 34 °C ($P < 0.05$). Moreover, n-3 HUFA enriched *Artemia* significantly increased the essential fatty acids of the larvae in parallel with the dose of enrichment. Fish fed unenriched HUFA *Artemia* resulted in significantly higher ratio of skeletal deformities and lower survival rate following an air exposure test than the larvae fed the n-3 HUFA enriched diets. Overall, the findings showed that n-3 HUFA-enriched *Artemia* diets had benefits on body weight, specific growth rate, and stress tolerance in Asian sea bass fed HUFA enriched diets, even at high temperatures.

1. Introduction

Nowadays, aquaculture industry is expanding due to improved and specialized production approaches as well as fish's inherent superior production characteristics. Although there are several difficulties associated with the production of fish in aquaculture systems, one of the fundamental problems is to maintain the temperature of the water, when fish are reared in open systems. Moreover, the water temperature has risen and is approaching the upper physiological threshold for many aquaculture species in tropical areas like in the Southeast Asian nations (Comte and Olden, 2017; Doan et al., 2019; Le et al., 2020; Sun and Chen, 2014). By 2050, global warming would cause up to 40% of aquaculture species to no longer be suitable for production (Oyinlola et al., 2020) because the temperature has a significant impact on the physiology, metabolism, and growth of many marine fish larvae. Extreme temperatures have been linked to adverse outcomes for some fish species, including diminished growth, reduced feed intake, and decreased survival rates (Kamiński et al., 2013; Le et al., 2020; Vagner et al., 2015). Furthermore, aquatic species raised at high temperatures were described as immunosuppressed (Altizer et al., 2013; Hooper et al., 2014; Le et al., 2021; Gupta et al., 2021), which subsequently was connected to reduced resistance to stressors and pathogens (Le et al., 2021). To uphold the standard physiological performances, fish need to modulate the fatty acid composition regarding highly unsaturated fatty acids (HUFA), in order to maintain membrane fluidity as water temperature changes (Ernst et al., 2016). A study by Hsieh et al. (2003) investigated the effects of temperature on fatty acid metabolism in milkfish (*Chanos chanos*) under cold stress and registered significant

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changes in proportions of monounsaturated fatty acids during acclimation from 25 to 15 °C. Additionally, white sturgeon (*Acipenser transmontanus*) larvae were found to have elevated levels of polyunsaturated fatty acids (PUFA) at 14 °C compared to low (8 °C) and high (20 °C) temperature, while freshly hatched larvae adjusted the ratio of saturated and unsaturated fatty acids in response to temperature changes (Buddington et al., 1993).

The Asian sea bass (*Lates calcarifer*) is a major commercially reared species due to its high stress tolerance, fast growth and production, and is of high demand in domestic and international markets as edible marine finfish (Gupta et al., 2020; Hender et al., 2021). However, there are still bottlenecks that affect Asian sea bass larvae during metamorphosis, including high mortality, aberrant skeletal development, and increased susceptibility to stress. These symptoms have been interconnected to HUFA deficiency, specifically docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (ARA) (Rimmer et al., 1994; Thépot et al., 2016). To produce 18:3 and 18:4 fatty acids, the juvenile Asian sea bass' HUFA metabolism showed some ability to chain extend and desaturate shorter-chain PUFA (Mohd-Yusof et al., 2010). However, the findings also indicated that Asian sea bass is unable to synthesize HUFA from shorter fatty acids *de novo* (Mohd-Yusof et al., 2010). The enrichment of live feeds like rotifers and *Artemia* with essential fatty acids (EFA) or microalgae, is crucial to improve the nutritional values of nutrient-deficient live prey, because HUFA play important roles in the development of the neutral and visual systems as well as the structure and function of the cell membrane of marine fish larvae (Fehér et al., 2013) and modulate the growth, survival, and stress resistance in the Asian sea bass larvae and other species (Rimmer et al., 1994; Thépot et al., 2016; Tocher, 2010).

There seems to be limited knowledge on the interactions between HUFA levels in live feeds and water temperature in marine fish larvae. In the current investigation, we sought to evaluate the combined effects of enriched HUFA *Artemia* and water temperature on growth and biochemical responses in Asian sea bass larvae. As indicators of larval health, enzymatic activity and skeletal development were also assessed.

2. Materials and methods

2.1. Artificial propagation and larvae collection

The brood-stock was cultured in a floating sea cage located in Nha Phu lagoon, Khanh Hoa, Vietnam. During the mature culture, the temperature was 28.5 to 30.5 °C with natural photoperiod. The matured fish were injected with a single dose of 600 IU human chorionic gonadotrophin and 20 μg luteinizing hormone-releasing hormone analogue, to induce spawning. The positively buoyant fertilized eggs were collected using a scoop net after spawning, washed with clean water, oxygen bagged, and transported to the laboratory for egg incubation. The water temperature was maintained at 29 °C during incubation. After hatching, newly hatched larvae were transferred into rearing cement tanks (5 m^3/tank) with same water temperature in green water using Nano 3600 paste (Reed Mariculture, USA) at a density of 80.000–100.000 cells/ml. Asian sea bass larvae were fed with enriched rotifer (*Brachionus plicatilis*) from 2 DAH (Day After Hatch) to 11 DAH.

2.2. Artemia enrichment

Protocols for *Artemia* incubation and enrichment followed the method described by Pham et al. (2022) (18). *Artemia* cysts (OSI 80, Ocean Star International, Utah, USA) were incubated in 300 L tanks at 30 °C and 31 ppt for 24 h. After hatching, six hour old *Artemia* was enriched with a commercial emulsion (A1 DHA Selco, INVE, Thailand) at 100, 300, and 500 ppm in 50 L enrichment tanks for 20 h at 28 °C with continuous aeration. As a basal diet, 100% unenriched *Artemia* was labelled as H0 while *Artemia* was emulsified with 100, 300, and 500 n-3 HUFA and labelled as H100, H300, and H500. Finally, the emulsified diets were subjected to low (30 °C) and high temperatures (34 °C), to examine the utilization of n-3HUFA enriched *Artemia* by Asia sea bass larvae and labelled as H0L, H0H, H100L, H100H, H300L, H300H, H500L, and H500H. Here, L stands for low temperature (30 °C) and H for high temperature (34 °C). For the biochemical study, a sample of each nauplius from each enrichment tank was kept at −80 °C and analysed for fatty acid composition (Table 1).

Table 1
Fatty acid profile (% of total fatty acid, TFA) of *Artemia* enriched with different n-3 HUFA levels.

Fatty acids	H0	H100	H300	H500
Lipids (g Kg^{-1})	131.15	211.19	246.56	249.53
C14:0	1.38	1.36	1.40	1.33
C16:0	14.68	14.39	14.05	14.64
C18:0	10.29	7.69	6.73	6.41
C20:0	0.79	0.62	0.51	0.43
C22:0	1.55	1.29	1.11	1.05
SFA	28.69	25.35	23.80	23.86
C16:1n-7	4.05	3.76	3.02	2.46
C18:1n-9	17.56	17.25	17.12	16.24
C18:1n-7	10.23	9.91	9.59	9.72
C20:1n-9	0.41	0.32	0.28	0.26
MUFA	32.25	31.24	30.01	28.68
C18:2n-6	8.49	8.62	8.74	8.49
C18:3n-3	21.55	20.55	20.11	19.05
C20:2n-6	0.75	0.69	0.56	0.46
C20:4n-6	1.05	1.38	1.94	2.27
C20:3n-3	0.81	0.78	0.68	0.64
C20:5n-3	2.34	3.27	5.79	6.55
C22:6n-3	0.00	4.11	7.25	9.75
PUFA	34.99	39.40	45.07	47.21
n-3HUFA	2.34	7.38	13.04	16.30
n-3/n-6	2.40	2.69	3.01	3.21
DHA/EPA	–	1.26	1.25	1.49
DHA/ARA	–	2.98	3.74	4.30
EPA/ARA	2.23	2.37	2.98	2.89

2.3. Larvae rearing and stress resistance

The 12 DAH Asian sea bass larvae were stocked at 15 larvae/L in eighteen 250 L tanks with central aeration. The larvae were fed *Artemia* enriched with three different n-3HUFA levels, at two temperatures (30 and 34 °C). The water temperature was adjusted 1 °C in six hours to designed temperature. Each treatment was assigned to three tanks. The enriched *Artemia* was administered to the experimental tanks three times per day at 08:00, 13:00 and 16:00, for 15 days. The uneaten *Artemia* was completely removed at night using a PVC pipe covered with a 250–500 μm net. Samples were taken on day 0 and 15 for the assessment of growth and the chemical analyses. An air exposure test was performed to assess the survival following the protocol described by Pham et al. (2022). Briefly, 100 larvae per tank were sampled and exposed to the air in a 500 μm net for 5 min and then returned to a 5 L aerated beaker. The exposure time was chosen based on the preliminary trials (2–10 min air exposure), in which after 4 min air exposure, about half of the unenriched *Artemia* fed larvae died.

2.4. Sample collection and biochemical analysis

At the commencement and termination of the feeding trial, Asian sea bass larvae from each enrichment level, were collected for biochemical examination. The fatty acids were subjected to a methylated ester method following the procedures described by O'Fallon et al., (2007). Amino acids were determined after acid hydrolysis by gas chromatography with GC 2010 Plus (Shimadzu, Kyoto, Japan).

2.5. Skeletal deformities

For the skeletal deformity evaluation, 50 larvae at 26 DAH per tank were randomly collected and fixed in a 4% formalin buffer. Fixed Asian sea bass samples were washed with distilled water and then stained with Alcian blue and alizarin red solutions, respectively following methods described by Darias et al. (2010) with slight revisions, as detailed by Pham et al. (2022). The stained samples were preserved in glycerol until use. The skeletal deformities was examined using a stereomicroscope (Amscope SM-2 T-EB, USA) with a 10MP camera. The criteria used for skeletal deformity in Asia sea bass larvae were described by Nguyen et al. (2008).

2.6. Statistical analysis

The specific growth rate (SGR) and survival rate were calculated as follows:

$$\text{Specific growth rate (SGR, \%/day)} = \left[\frac{ln\,(\text{final body weight}) - ln\,(\text{pooled initial body weight})}{\text{days}}\right] \times 100$$

$$\text{Survival (\%)} = \left[\frac{\text{number of final fish}}{\text{number of initial fish}}\right] \times 100$$

All data are presented as mean ± standard error (SE). All data were statistically analyzed using SPSS for Windows version 22 (IBM, New York, USA) unless otherwise specified. The effects of dietary n-3 HUFA inclusion, temperature, and their interactions on all Asian sea bass larvae parameters were analyzed using a two-way ANOVA. When a significant main effect was observed, data were analyzed to determine the differences among the dietary groups at different temperatures. When a significant interaction was found, one-way analysis (ANOVA) was used with *post hoc* Turkey's HSD multiple comparison tests to establish differences across treatments. The statistical significance was evaluated at $P < 0.05$.

In addition, the principal component analysis (PCA) was used on the datasets obtained from eight different dietary groups on growth, various fatty acids composition and essential and non-essential amino acids profiles, to assess the overall covariation of their respective variables. In PCA analysis, factor loading >0.30 is regarded as significant, >0.40 is regarded more significant, and >0.50 or above is regarded highly significant (Lombarte et al., 2012). The factors with loadings larger than 0.5 are considered significant ($P > 0.05$). The PCA analysis was conducted in the open-source environment R version 3.6.2 (R Core Team, Vienna, Austria).

3. Results

3.1. Growth performance

Dietary n-3HUFA significantly affected the survival rate of Asian sea bass after two weeks of feeding. However, no significant effects of temperature and interaction between n-3 HUFA and temperature on the survival of larvae were observed (Table 2). There were significant effects of dietary n-3 HUFA, temperature, and their interactions on final body weight (FBW), final body length (FBL), and specific growth rate (SGR) of Asian sea bass larvae. Without n-3 HUFA enrichment, the larvae reared at a high temperature exhibited significantly reduced FBW compared to those reared at a low temperature. The larvae fed n-3 HUFA enriched *Artemia* showed significantly improved FBL, FBW, and SGR compared to those fed unenriched *Artemia*, regardless of low or high temperature. At each enrichment level, there was no significant difference among the larvae reared at different temperatures. The highest FBW and SGR were achieved in the larvae fed 200 ppm n-3 HUFA enriched *Artemia*.

3.2. Fatty acid profile of Asian sea bass larvae

The fatty acid profile of Asian sea bass larvae is displayed in Table 3. Except for C18:1n-9 and C20:1, the n-3HUFA enrichment of *Artemia* had a substantial impact on Asian sea bass larvae. The temperature appear to have no substantial impact on MUFA, C17:1, C18:1n-9, C20:1, or C24:1 ($P > 0.05$). While there was no difference in the saturated fatty acid (SFA) and monounsaturated fatty acid (MUFA) content of the larvae fed the experimental diets, fish fed H300H exhibited considerably higher n-3 HUFA content than the fish in the other dietary groups. However, fish from the groups H300H, H300L, and H0L exhibited noticeably higher

Table 2
Growth performance of Asian sea bass larvae fed enriched *Artemia* at different temperatures.

Diet	FBL (cm)	FBW (mg/fish)	SGR (%/day)	Survival (%)
H0L	1.16 ± 0.02^{b}	35.46 ± 0.80^{b}	22.51 ± 0.15^{b}	83.22 ± 1.97
H0H	0.97 ± 0.01^{a}	25.67 ± 0.46^{a}	20.36 ± 0.12^{a}	79.99 ± 1.27
H100L	1.39 ± 0.04^{cd}	47.17 ± 0.80^{cd}	24.42 ± 0.11^{cd}	88.46 ± 0.41
H100H	1.35 ± 0.02^{c}	42.78 ± 0.72^{c}	23.77 ± 0.11^{c}	87.88 ± 2.07
H300L	1.46 ± 0.03^{d}	55.17 ± 2.79^{e}	25.45 ± 0.33^{e}	90.98 ± 0.70
H300H	1.46 ± 0.02^{d}	57.31 ± 2.17^{e}	25.71 ± 0.26^{e}	90.67 ± 0.89
H500L	1.45 ± 0.04^{d}	51.88 ± 2.15^{de}	25.04 ± 0.27^{de}	89.97 ± 0.55
H500H	1.36 ± 0.01^{cd}	45.50 ± 0.54^{cd}	24.18 ± 0.08^{c}	89.51 ± 1.48
Means of main effects of n-3HUFA levels				
H0	1.07 ± 0.02	30.57 ± 1.10	21.44 ± 1.41	81.60 ± 0.83^{A}
H100	1.38 ± 0.02	44.98 ± 1.11	24.09 ± 1.41	88.17 ± 0.93^{B}
H300	1.47 ± 0.02	56.24 ± 1.10	25.58 ± 1.41	90.82 ± 0.93^{B}
H500	1.41 ± 0.02	48.69 ± 1.10	24.61 ± 1.41	89.74 ± 0.93^{B}
Means of main effects of temperature levels				
30 °C	1.37 ± 0.01	47.42 ± 0.78	24.36 ± 0.10	88.16 ± 0.66
34 °C	1.29 ± 0.01	42.82 ± 0.78	23.51 ± 0.10	87.01 ± 0.66
Two-way ANOVA: *P* values				
n-3 HUFA	< 0.001	< 0.001	< 0.001	< 0.001
Temperature	< 0.001	0.001	< 0.001	0.234
n-3 HUFA x Temperature	0.004	0.001	< 0.001	0.647

Data are displayed as a mean of three replicates per treatment. FBL: Final Body Length (cm), FBW: Final body weight (mg/fish) and SGR: specific growth rate (%/day). Different lowercase subscripts (a, b, c, d, e) within a column indicate significant differences ($P < 0.05$) between the treatments.

Table 3
Fatty acid profile (% of total fatty acid, TFA) of Asian sea bass larvae fed enriched n-3HUFA *Artemia* at different temperatures.

Groups	C16:0	C17:0	C18:0	C16:1n-7	C17:1	C18:1n-9	C20:1	C24:1	SFA	MUFA
H0L	15.60	3.32^{abc}	13.08^{b}	2.15^{bc}	1.11	27.52	1.09	3.18	31.99	35.05
H0H	16.92	4.18^{d}	16.10^{c}	1.28^{a}	1.08	27.68	1.00	3.09	37.20	34.13
H100L	16.74	3.12^{ab}	13.45^{b}	1.85^{b}	0.95	26.57	1.01	1.89	33.32	32.27
H100H	17.47	3.81^{cd}	13.99^{b}	1.84^{b}	0.86	26.56	0.94	1.75	35.28	31.95
H300L	15.55	2.85^{a}	8.05^{a}	3.02^{d}	1.03	26.84	0.98	1.07	26.45	32.94
H300H	16.49	3.48^{bc}	9.59^{a}	2.29^{c}	1.07	26.18	1.05	1.13	29.56	31.72
H500L	14.90	3.09^{ab}	9.09^{a}	3.40^{e}	0.94	27.75	1.00	0.97	27.08	34.06
H500H	16.25	3.21^{ab}	9.43^{a}	2.41^{c}	0.97	27.80	0.98	0.98	28.90	33.13
Pooled SE	0.20	0.09	0.58	0.14	0.02	0.25	0.02	0.18	0.78	0.32
Means of main effects of n-3HUFA enrichment level										
H0	16.26^{AB}	3.75	15.59	1.72	1.09^{C}	27.60	1.05	3.13^{C}	34.59^{B}	34.59^{B}
H100	17.10^{B}	3.47	13.72	1.84	0.91^{A}	26.57	0.98	1.82^{B}	34.30^{B}	32.11^{A}
H300	16.02^{A}	3.16	8.82	2.66	1.05^{BC}	26.51	1.02	1.10^{A}	28.00^{A}	32.33^{A}
H500	15.58^{A}	3.15	9.26	2.91	0.95^{AB}	27.77	0.99	0.98^{A}	27.99^{A}	33.60^{AB}
Means of main effects of temperature level										
30 °C	15.70^{X}	3.09	10.92	2.61	1.01	27.17	1.02	1.78	29.71^{X}	33.58
34 °C	16.79^{Y}	3.67	12.28	1.96	0.99	27.05	0.99	1.74	32.73^{Y}	32.74
Two-way ANOVA: *P* values										
n-3HUFA	0.011	0.000	0.000	0.000	0.002	0.235	0.495	0.000	0.000	0.018
Temp	0.001	0.000	0.000	0.000	0.714	0.931	0.477	0.408	0.000	0.141
n-3HUFA x Temp	0.932	0.039	0.019	0.000	0.466	0.946	0.362	0.502	0.078	0.947

Groups	C18:2n-6	C18:3n-3	C20:4n-6	C20:5n-3	C22:6n-3	PUFA	n-3HUFA	n-3	n-6	n-3/n-6
H0L	4.39^{b}	8.99^{d}	4.42	5.02	5.29^{a}	28.11^{a}	10.31^{a}	19.30	8.81^{a}	2.19^{a}
H0H	4.19^{b}	8.71^{d}	5.06	5.34	5.83^{a}	29.12^{a}	11.17^{a}	19.87	9.25^{ab}	2.16^{a}
H100L	3.76^{a}	7.37^{ab}	4.97	5.72	14.11^{b}	35.93^{b}	19.83^{b}	21.20	8.73^{a}	3.11^{c}
H100H	4.40^{b}	7.40^{ab}	5.35	5.96	15.04^{c}	38.14^{c}	20.99^{c}	28.39	9.75^{bc}	2.91^{bc}
H300L	5.13^{c}	8.58^{d}	5.42	6.04	15.42^{c}	40.60^{d}	21.47^{cd}	30.04	10.56^{cd}	2.85^{b}
H300H	4.19^{b}	7.23^{a}	5.90	6.81	16.52^{d}	40.65^{d}	23.30^{f}	30.56	10.09^{cd}	3.03^{bc}
H500L	5.11^{c}	8.51^{cd}	5.47	6.15	16.05^{d}	41.31^{d}	22.21^{de}	30.72	10.59^{cd}	2.90^{bc}
H500H	5.00^{c}	7.95^{bc}	5.77	6.47	16.16^{d}	41.35^{d}	22.63^{ef}	30.58	10.77^{d}	2.84^{b}
Pooled SE	0.10	0.14	0.10	0.12	0.92	1.07	1.02	0.94	0.17	0.07
Means of main effects of n-3HUFA enrichment level										
H0	4.29	8.85	4.74^{A}	5.18^{A}	5.56	28.61	10.74	19.59^{A}	9.03	2.18
H100	4.08	7.38	5.16^{B}	5.84^{B}	14.58	37.04	20.41	27.80^{B}	9.24	3.01
H300	4.66	7.90	5.66^{C}	6.43^{C}	15.97	40.63	22.40	30.30^{C}	10.33	2.94
H500	5.06	8.23	5.62^{C}	6.31^{C}	16.11	41.33	22.42	30.65^{C}	10.68	2.87
Means of main effects of temperature level										
30 °C	4.60	8.36	5.07^{X}	5.73^{X}	12.72	36.49	18.45	26.82^{X}	9.67	2.76
34 °C	4.45	7.82	5.52^{Y}	6.14^{Y}	13.39	37.32	19.53	27.35^{Y}	9.97	2.73
Two-way ANOVA: *P* values										
n-3HUFA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Temp	0.023	0.000	0.001	0.000	0.000	0.002	0.000	0.011	0.044	0.466
n-3HUFA x Temp	0.000	0.001	0.685	0.147	0.018	0.009	0.032	0.139	0.011	0.025

Data are displayed as a mean of three replicates per treatment. SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; n-3 HUFA: n-3 highly unsaturated fatty acids. Different lowercase subscripts (a,b,c,d,e,f) within a column indicate significant differences between the treatments. Different uppercase alphabets (A, B, C) within a column indicate significant differences ($P < 0.05$) among means of the main effects of n-3 HUFA enrichment level. Different uppercase alphabets (X, Y) within a column indicate the significant differences ($P < 0.05$) among means of the main effects of of temperature.

amounts of n-3 HUFA compared to fish fed the lower enrichment levels. Additionally, the n-3/n-6 ratio significantly improved in the larvae fed the enriched diets. There were significant interactions between enrichment level and temperature in relation to DHA, PUFA, n-3 HUFA and the n-3/n-6 ratio of the larvae ($P < 0.05$), while no interaction reported on ARA and EPA contents ($P > 0.05$). The n-3 HUFA in Asian sea bass larvae was significantly influenced by the increment of n-3 HUFA-enriched *Artemia* in the different dietary groups, whilst no variation was found in the Asian sea bass larvae reared at high and low temperature (Fig. 1).

3.3. The amino acid content of Asian sea bass larvae

The interaction between enrichment and temperature showed no significant effects on the glutamate, glycine, and tyrosine contents of Asian sea bass larvae, while differences were observed in the alanine, aspartic acid, proline, and serine contents (Table 4). On the other hand, among the total non-essential amino acids, TNEAAs only serine was not affected significantly by temperature ($P > 0.05$). The aspartic acid, proline, and TNEAA contents were significantly higher in the larvae fed H500L and H500H diets than those fed lower enrichment levels (Table 5). Furthermore, total essential amino acids, TEAAs were

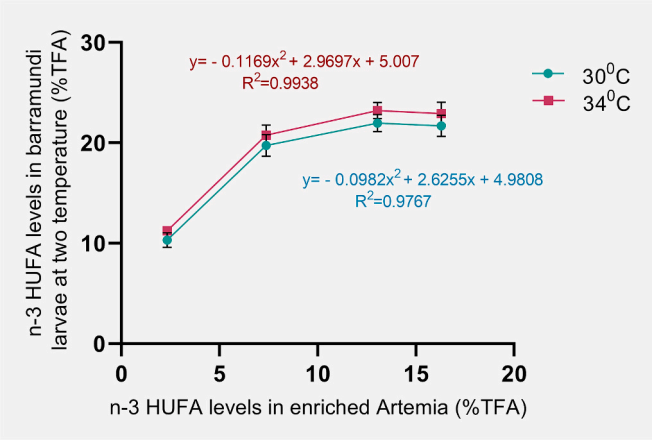

Fig. 1. The relationship between fatty acid profile (n-3 HUFA) in Asian seabass larvae and dietary n-3 HUFA-enriched *Artemia* levels in various dietary groups. Values are mean ± S.D. of triplicate groups of fish and n-3 HUFA-enriched *Artemia*. Significant difference considered at $P < 0.05$.

substantially affected by n3-HUFA enrichment at higher temperature and showed interaction between enrichment and temperature except lysin ($P < 0.05$). Overall, the larvae fed H500L and H500H diets improved EAAs compared to those fed lower enrichment levels, regardless of water temperature.

3.4. Air exposure test

The larvae fed unenriched *Artemia* registered the lowest survival rate after the air exposure challenge, regardless of water temperature (Fig. 2). At each enrichment level, the larvae reared at 34 °C exhibited no difference in the survival rate compared to those cultured at 30 °C. No differences were recorded between the larvae fed H300L, H300H, H500L, and H500H diets ($P > 0.05$), and significantly higher than the larvae fed H100L and H100H diets after the stress test ($P < 0.05$). Overall, dietary n-3HUFA enrichment significantly improved the stress resistance of Asian sea bass larvae reared at 30 and 34 °C.

3.5. Skeletal deformities

Typical skeletal deformities in Asian sea bass larvae were fusion of vertebral centrum (A), lower jaw increment (B), lordosis and kyphosis (C) and normal skeletal structure (D) (Fig. 3). The highest number of abnormal vertebrae were observed in the larvae fed unenriched *Artemia* at both low and high temperatures. The larvae fed HUFA enriched *Artemia* at H300L, H300H, H500L and H500H levels showed the lowest number of deformities compared to larvae fed the unenriched *Artemia*, regardless of the water temperature. In the group fed the diet with the lowest HUFA level (H100L and H100H level), appx. 18% of the larvae exhibited malformation, whereas only 5% of the larvae fed the diet with 300 to 500 HUFA levels exhibited malformations (Fig. 4).

3.6. PCA analysis

The principle component analysis (PCA) was used to examine how different dietary categories affected the nutritional and growth characteristics of Asian sea bass. The first two principal component axes (PC1 and PC2) revealed information about the predominate correlations between these data and explained >80.3% of the variation in the data. Fish fed diets containing H0H, H0L, and H100H were grouped together in the PCA biplot's negative site for SFA and exhibited negative correlations with FBW, SGR, PUFA, n3-HUFA, n3/n6, TNEAA, and TEAA (Fig. 5A, B). On the other hand, the PC1 positive zone contained the following compounds: FBW, SGR, PUFA, n3-HUFA, n3/n6, TNEAA, and TEAA, all of which were found to be strongly positively associated (Fig. 3b). Furthermore, the first principal component (PC1) forecasts a variance of 62.9%, followed by the second principal component (17.4%), the third principal component (7.8%), and so on. The eigenvalues depicted eight principal components in which SFA was found to be negative in PC1; SGR, TNEAA in PC2; FBW, PUFA, and TEAA in PC3; FBW, TEAA in PC4;

Table 4
Non-essential amino acid profile Asian sea bass larvae fed enriched n-3HUFA *Artemia* at different temperatures.

Diet	Ala	Asp	Glu	Gly	Pro	Ser	Tyr	TNEAA
H0L	0.81	0.93	1.43^{bc}	0.61^{c}	0.49	0.44	0.50^{bc}	5.21
H0H	0.91	0.77	1.31^{a}	0.44^{a}	0.45	0.42	0.37^{a}	4.66
H100L	0.81	0.96	1.44^{bc}	0.64^{cd}	0.52	0.44	0.54^{bc}	5.35
H100H	0.86	0.91	1.38^{b}	0.52^{b}	0.48	0.44	0.46^{ab}	5.05
H300L	0.84	1.03	1.59^{f}	0.72^{e}	0.53	0.46	0.54^{bc}	5.70
H300H	0.86	0.96	1.46^{cd}	0.68^{de}	0.50	0.45	0.59^{bc}	5.51
H500L	0.82	1.05	1.54^{ef}	0.73^{e}	0.62	0.45	0.61^{c}	5.81
H500H	0.88	0.97	1.52^{de}	0.70^{de}	0.51	0.46	0.59^{bc}	5.63
Pooled SE	0.01	0.02	0.02	0.02	0.01	0.01	0.02	0.08
Means of main effects of n-3HUFA levels								
H0	0.86	0.85^{A}	1.37	0.53	0.47^{A}	0.43	0.44	4.94^{A}
H100	0.83	0.94^{AB}	1.41	0.58	0.50^{AB}	0.44	0.50	5.20^{B}
H300	0.85	1.00^{B}	1.53	0.70	0.51^{AB}	0.46	0.56	5.61^{C}
H500	0.85	1.01^{B}	1.53	0.71	0.57^{B}	0.46	0.60	5.72^{C}
Means of main effects of temperature levels								
30 °C	$0.82^{\times}$	1.00^{Y}	1.50	0.67	0.54^{Y}	0.45	0.55	5.52^{Y}
34 °C	0.88^{Y}	$0.90^{\times}$	1.42	0.59	$0.49^{\times}$	0.44	0.50	$5.21^{\times}$
Two-way ANOVA: *P* values								
n-3 HUFA	0.570	0.001	0.000	0.000	0.017	0.021	0.000	0.000
Temperature	0.000	0.002	0.000	0.000	0.012	0.297	0.032	0.000
n-3 HUFA x Temp	0.303	0.429	0.008	0.003	0.433	0.411	0.021	0.086

Data are displayed as a mean of three replicates per treatment. TEAA, total essential amino acids, SE, standard error. Different lowercase subscripts (a, b, c, d, e, f) within a column indicate significant differences among the treatments. Different uppercase alphabets (A, B, C) within a column indicate significant differences ($P < 0.05$) among means of the main effects of n-3 HUFA levels. Different uppercase alphabets (X, Y) within a column indicate significant differences ($P < 0.05$) among means of the main effects of of temperature.

Table 5
Essential amino acid profile Asian sea bass larvae fed enriched n-3HUFA *Artemia* at different temperatures.

Diet	Arg	His	Iso	Leu	Lys	Met	Phe	Thr	Val	TEAA
H0L	0.61	0.22	0.54^{b}	0.78^{bc}	0.79	0.51^{b}	0.45^{b}	0.42^{b}	0.61^{bc}	4.93^{c}
H0H	0.52	0.21	0.42^{a}	0.66^{a}	0.70	0.33^{a}	0.36^{a}	0.31^{a}	0.54^{a}	4.05^{a}
H100L	0.63	0.22	0.58^{bc}	0.79^{bcd}	0.79	0.54^{b}	0.44^{b}	0.43^{b}	0.68^{d}	5.12^{bc}
H100H	0.60	0.23	0.44^{a}	0.73^{ab}	0.73	0.40^{a}	0.44^{b}	0.35^{a}	0.56^{ab}	4.48^{b}
H300L	0.67	0.24	0.62^{bc}	0.87^{de}	0.83	0.53^{b}	0.46^{b}	0.46^{b}	0.66^{cd}	5.34^{c}
H300H	0.61	0.22	0.63^{bc}	0.85^{cd}	0.77	0.60^{b}	0.45^{b}	0.43^{b}	0.68^{d}	5.25^{bc}
H500L	0.64	0.25	0.67^{c}	0.87^{de}	0.85	0.58^{b}	0.47^{b}	0.44^{b}	0.69^{d}	5.45^{c}
H500H	0.61	0.23	0.62^{bc}	0.88^{e}	0.84	0.57^{b}	0.47^{b}	0.44^{b}	0.66^{cd}	5.33^{c}
Pooled SE	0.01	0004	0.02	0.02	0.01	0.02	0.01	0.01	0.01	0.10
Means of main effects of n-3HUFA levels										
H0	0.57^{A}	0.21	0.48	0.72	0.75^{A}	0.42	0.40	0.36	0.58	4.49
H100	0.62^{B}	0.23	0.51	0.76	0.76^{A}	0.47	0.44	0.39	0.62	4.80
H300	0.64^{B}	0.23	0.63	0.86	0.80^{B}	0.57	0.46	0.45	0.67	5.29
H500	0.62^{B}	0.24	0.65	0.87	0.85^{C}	0.58	0.47	0.44	0.68	5.39
Means of main effects of temperature level										
30 °C	0.64^{Y}	0.23	0.60^{Y}	0.83	0.81^{Y}	0.54	0.46	0.44	0.66	5.21
34 °C	0.58^{X}	0.22	0.53^{X}	0.78	0.76^{X}	0.48	0.43	0.38	0.61	4.78
Two-way ANOVA: *P* values										
n-3HUFA	0.000	0.111	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Temp	0.000	0.162	0.000	0.002	0.000	0.001	0.010	0.000	0.000	0.000
n-3HUFA x Temp	0.053	0.626	0.005	0.023	0.062	0.000	0.003	0.001	0.001	0.000

Data are displayed as a mean of three replicates per treatment. TEAA, total essential amino acids, SE, standard error. Different lowercase subscripts (a, b, c, e) within a column indicate significant differences among the treatments. Different uppercase alphabets (A, B, C) within a column indicate significant differences ($P < 0.05$) among means of the main effects of of n-3 HUFA levels. Different uppercase alphabets (X, Y) within a column indicate significant differences ($P < 0.05$) among means of the main effects of temperature.

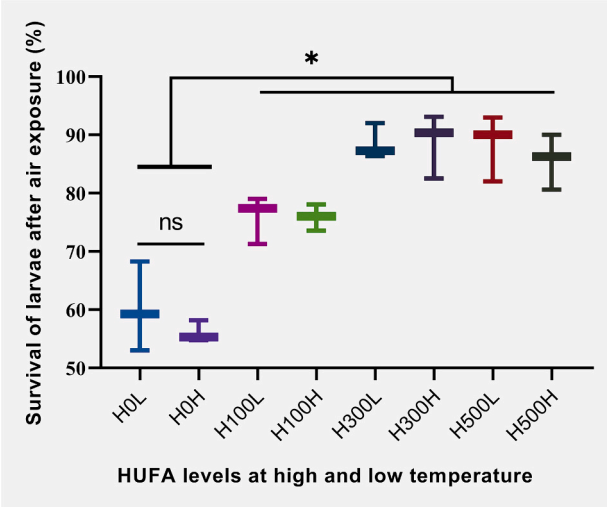

Fig. 2. Survival of Asian sea bass larvae after air exposure test. Results are expressed as means ± S.D. Means with asterisk (*) are significantly different at $P < 0.05$, ns; non-significant.

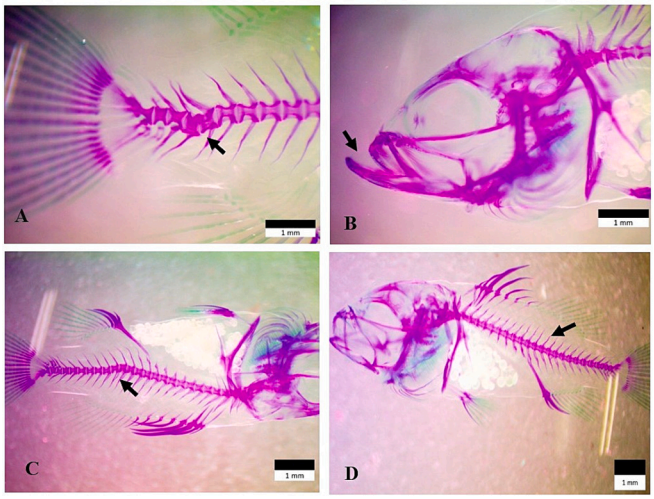

Fig. 3. Typical skeletal deformities in stained specimens of barramundi larvae at 26 days post-hatching. A: fusion of vertebral centrum; B: lower jaw deformity; C: lordosis and kyphosis; D: normal skeletal structure.

FBW, n-3/n-6, and TEAA in PC5; PUFA, TNEAA in PC6; n3-HUFA, n3/n6, TNEAA, TEAA in PC7; and FBW, PUFA, n3-HUFA, TEAA in PC8.

4. Discussion

This study, examined the effects of n-3 HUFA enriched *Artemia* on the performance of Asian sea bass larvae at high water temperatures. Our present findings indicate that *Artemia* enriched with 300 ppm n-3 HUFA had a significant impact on the FBL, FBW, and SGR of Asian sea bass larvae. These findings are similar to those that have been observed in other marine finfish species (Gapasin and Duray, 2001; Roo et al., 2019; Villalta et al., 2005). For instance, in grey mullet (*Liza ramada*), larvae and juveniles grew more rapidly when fed unenriched rotifers and *Artemia* at temperature higher than 24 °C compared to those maintained at 19 °C (Al-Dahar et al., 2013), something that supports our present findings. On the other hand, unenriched *Artemia* diet resulted in lower FBL, FBW, and SGR. This is because feeding a lower ratio, or insufficient micro diets, can delay or even prevent the start of organogenesis and subsequently the growth of larvae (Cahu and Zambonino Infante, 2001). The results also demonstrated that *Artemia* has very low quantities of DHA and EPA, which are not enough to satisfy the nutritional requirements of marine finfish larvae. As DHA plays important roles in the development of the visual and neural systems as well as the regulation of physiological processes in fish, deficiency in HUFA may affect the

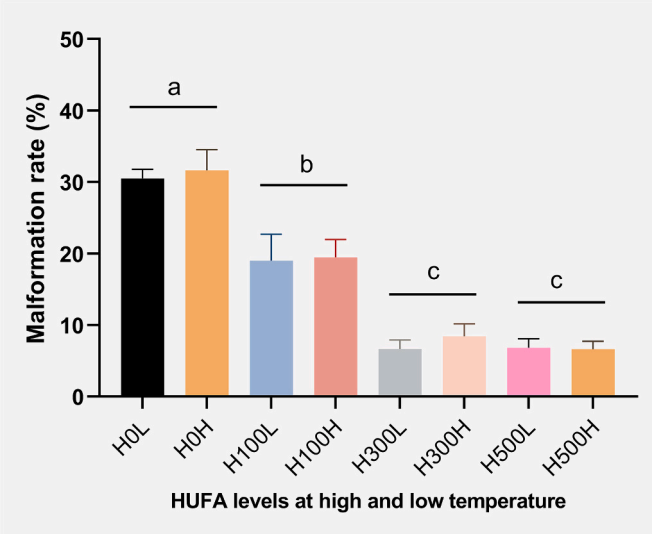

Fig. 4. Percentage of skeletal malformation observed in 26 day-old sea bass fed different n-3 HUFA-enriched *Artemia*. Results are expressed as means ± S.D. Means with different superscript are significantly different ($P < 0.05$).

development of the prey capture behaviour and abilities in marine fish larvae (Izquierdo et al., 2013; Roo et al., 2019; Thépot et al., 2016).

There is relatively little information on HUFA requirements of Asian sea bass during the larval phase. Although EPA was indispensable to boost the survival rate of Asian sea bass during metamorphosis, high DHA content was not required (Rimmer et al., 1994). This is in line with the findings of Villalta et al. (2005) in Senegalese sole (*Solea senegalensis*), in which dietary DHA requirements could be compensated with sufficient supplementation of EPA in the diet. Similarly, Thépot et al. (2016) also indicated that Asian sea bass larvae fed dietary DHA and EPA at 11.5 and 8.8% of TFA respectively, had higher final length and weight than those fed rotifers containing 20% DHA and 5% EPA. Pham et al. (2022) also found that high contents of DHA and EPA (10 and 6.7% of TFA) in the enriched *Artemia* did not improve the growth performance of Asian sea bass larvae compared to those fed the medium levels of DHA and EPA (7.7 and 6.2% of TFA), which suggests that the dietary DHA and EPA levels should be in range of 7.7–11.5% and 6.2–8.8% of TFA, respectively to meet the requirements of barramundi during larval phase. In the present study, the enrichment of *Artemia* with n-3 HUFA showed that H300H treatment (34 °C) did improve the growth performance, whithout affecting the survival rate, compared to other dietary groups. Interestingly, Al-Dahar et al. (2013), observed that enriched rotifers and higher temperatures insignificantly increased fish larvae survival ($P > 0.05$) in grey mullet, which differs from the present findings. The present findings suggest that fatty acids of the H300H group have already satisfied the nutritional requirement of Asian sea bass larvae during *Artemia* feeding even at high temperatures (34 °C). Although extreme temperatures have been linked to adverse outcomes for some fish species, such as diminished growth reduced feed intake, and decreased survival rates (Kamiński et al., 2013; Le et al., 2020; Vagner et al., 2015), in the present experiment, we found that H300H treatment (34 °C) had higher growth compared to the other treatments at 30 °C. This may be because fish were provided with n-3 HUFA to preserve membrane fluidity as the water temperature fluctuates and maintain the fish's normal physiological functions (Ernst et al., 2016). However, neither temperature nor the interaction of n-3 HUFA and temperature had a discernible effect, but n-3 HUFA enrichment had substantial effects on larval survival even at high temperatures.

In the current study, the n-3 HUFA enrichment of *Artemia* showed a significant effect on fatty acid profiles of Asian sea bass larvae, except for C18:1n-9 and C20:1. Moreover, the temperature showed no discernible effect on MUFA ($P > 0.05$). The amount of PUFA in the larvae fed the H300H diet was much higher than that in other dietary groups. A similar trend was observed in another study, when Asian sea bass fed enriched rotifers (Thépot et al., 2016). In the case of Pacific cod (*Gadus macrocephalus*) larvae had elevated n-3 HUFA and DHA levels following *Artemia* supplementation with n-3 HUFA and DHA levels, however, dietary EPA had little effect on these larvae's EPA concentrations, which highly corroborates our present findings (Choi et al., 2021; Roo et al., 2019). In addition, the n-3/n-6 ratio significantly increased in the larvae fed the H300H-enriched diet. A similar finding has been reported earlier for white-leg shrimp (*Litopenaeus vannamei*) (Ahmadi et al., 2019). According to another research, the dietary fatty acid profile is often reflected in the composition of the larval fatty acids (Evjemo et al., 2003; Hamre et al., 2002), which is in line with the present findings. Interactions between n-3HUFA and temperature had a substantial impact ($P < 0.05$) except for the larvae's C18:0 and C16:1n-7 levels. This might be the result of fish being given an n-3 HUFA enriched *Artemia* diet to maintain normal physiological processes and maintain membrane fluidity in higher water temperatures.

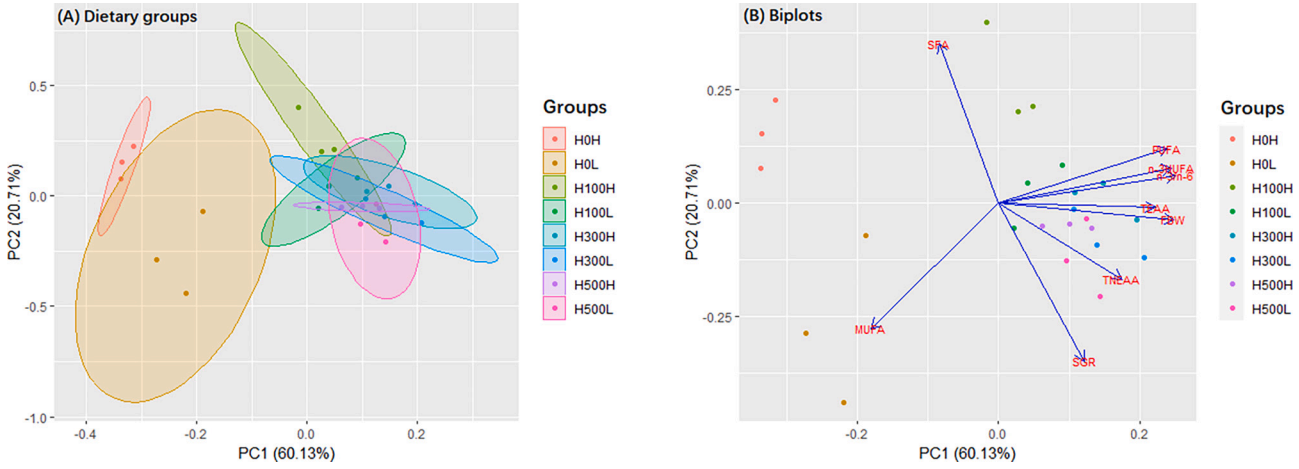

Fig. 5. PCA score and biplot with various dietary groups and some measured parameters at the end of the feeding trial. The graph shows positive and negative association between various dietary groups (A) and variables loading (B). Different markers with confidence eclipse indicate samples from various dietary groups utilized in the investigation, and loadings show how the variable contributed to the development of PC1 and PC2. FBW, final body weight; SGR, specific growth rate; SFA, Saturated Fatty Acid; PUFA, Polyunsaturated Fatty Acid; n3-HUFA, n-3 Highly Unsaturated Fatty Acid; TNEAA, Total Non-essential Amino acid; and TEAA, Total Essential Amino Acid.

Almost all TNEAAs, except for alanine and serine, were strongly impacted by n-3 HUFA enrichment. On the other hand, except for lysin, n-3HUFA enrichment at higher temperatures had a substantial impact on all of the essential amino acids (EAAs). In our study, we have found that the TNEAAs and EAAs of the larvae fed the H500L and H500H diets were generally better than those of the lower enrichment levels regardless of the water temperature. It may be due to the provision of a higher n-3 HUFA enriched *Artemia*. Furthermore, live food organisms stimulate larval ingestion activity (Cahu and Zambonino Infante, 2001), which might increase the rate of ingestion by the Asian sea bass larvae. In addition, *Artemia* is a significant source of protein, making up an average of 51.24% of the total weight basis (Hamsah et al., 2017). In the present experiment, the greater protein content, chiefly essential and non-essential amino acids in higher n-3HUFA enriched *Artemia*, at a lower temperature diet, have undoubtedly improved the amino acid content of the Asian sea bass larvae. Therefore, the provision of n-3 HUFA-enriched *Artemia* at low temperature (30 °C) rather than at high temperature (34 °C) should be employed to improve the amino acid contents in Asian sea bass larvae.

Regardless of the water temperature, the larvae fed unenriched *Artemia* exhibited the lowest survival rate when challenged with air exposure. This might be attributed to a deficiency of n3-HUFA, which did not satisfy the larvae's minimal HUFA requirements. Fish with low HUFA levels have been reported to be more vulnerable to various stressful conditions (Roo et al., 2014). But in the present study, dietary n-3HUFA supplementation markedly increased the ability of Asian sea bass larvae reared at 30 and 34 °C to withstand stress. This is might be due to the fatty acids which are able to regulate the fluidity of cell membranes, improving oxygen transfer efficiency and the properties of the gill membrane to restore normal breathing after a stressful event (Roo et al., 2019). Our experiment showed that even at higher temperatures, n-3 HUFA-enriched *Artemia* diets can be used to improve Asian sea bass stress tolerance.

The PCA analysis applied for investigating the effect of n-3 HUFA-enriched *Artemia* dietary groups on various growth, and nutritional parameters in Asian sea bass. Previously, PCA was also used to assess data from the growth and hematological parameters in Asian sea bass in order to determine their dietary preferences and enhancement in antioxidant response, immunity, and disease resistance (Siddik et al., 2022). In the present experiment, the position of the dietary groups and growth, immunity and health parameters in the plot showed that H300H diet had a favorable influence on fish growth and H0H, H0L, and H100H had a negative association with fish performance. From the PCA analysis, it can be suggested that, HUFA emulsification up to 300 ppm at high temperature may improves physiological functioning through growth, survival as well as all nutritional parameters.

In the present investigation, abnormal vertebrae were observed in the larvae fed n-3 HUFA unenriched *Artemia* at both low and high temperatures. Similar observations have been made about the relationship between inadequate HUFA diets and an increase in lordosis and kyphosis in gilthead seabream (*Sparus aurata*) larvae (Izquierdo et al., 2013). Conversely, the larvae fed enriched *Artemia* did not show any abnormal skeleton, regardless of the water temperature. Red seabream fed enriched *Artemia*, supplemented with Zn and Mn, showed a considerably decreased rate of overall skeletal abnormalities (Nguyen et al., 2008) which corroborates with the present findings. However, the skeletal abnormalities of great amberjack (*Seriola dumerili*) larvae fed with a range of dietary n-3HUFA concentrations did not show any variation (Roo et al., 2019). These skeletal abnormalities in marine fish are caused by a variety of reasons, including the environment, nutrient imbalances, raising conditions, and particular species, which may have an impact on the larvae's osteological development (Boglione et al., 2013).

5. Conclusion

Asian sea bass larvae fed n-3 HUFA-enriched *Artemia* outperformed those fed a non-n-3 HUFA-enriched diet in terms of growth and stress resistance, suggesting that n-3 HUFA levels in *Artemia* may be sufficient to meet the nutritional needs of Asian sea bass larvae at 13.04% of TFA. Therefore, it is advised to use n-3 HUFA-enriched *Artemia* at a level of H300 ppm to enhance the culture performance of this species during larval rearing. This study will help to identify the nutritional requirement of Asian seabass at early life and also help to improve diets would sustain the production of constant high quality fingerlings in the hatchery.

Ethics statement

The animal care was fully compliant with the Vietnamese Code of Practice for the care of animals for scientific purposes. The design was approved by the animal ethics committee of Nha Trang University (NTU), Khanh Hoa, Vietnam.

Author contributions

HDP and MABS conceptualized and designed the study. HDP and LTH performed the experiment. MAR, MABS and HDP wrote the manuscript, analyzed data and prepared the figs. AN revised the manuscript. INV edited and revised the manuscript. All authors read and approved the manuscript.

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Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

The original contributions presented in this study are included in the article, further inquiries can be directed to the corresponding author.

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