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Effects of dietary eucommia ulmoides leaf extract on growth performance, expression of feeding-related genes, activities of digestive enzymes, antioxidant capacity, immunity and cytokines expression of large yellow croaker (*Larimichthys crocea*) larvae

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Abstract

A 30-d feeding trial was conducted to investigate effects of dietary eucommia ulmoides leaf extract (ELE) on growth performance, activities of digestive enzymes, antioxidant capacity, immunity, expression of inflammatory factors and feeding-related genes of large yellow croaker larvae. Five micro-diets were formulated with supplementation of 0 g kg^{-1} (the control), 5 g kg^{-1} (0·5 %), 10 g kg^{-1} (1·0 %) and 20 g kg^{-1} (2·0 %) of ELE, respectively. Results showed that the best growth performance was found in larvae fed the diet with 1·0 % ELE. Furthermore, ELE supplementation significantly increased the *npy* expression at 1·0 % dosage, while increased *ghrelin* in larvae at 0·5 % dosages. The activity of leucine aminopeptidase in larvae fed the diet with 1·0 % ELE was significantly higher than the control, while alkaline phosphatase was significantly upregulated in larvae fed the diet with 2·0 % ELE. A clear increase in total antioxidant capacity in larvae fed the diet with 1·0 % ELE was observed, whereas catalase activity was significantly higher in 1·0 % and 2·0 % ELE supplementation compared with the control. Larvae fed the diet with 1·0 % ELE had a significantly higher activities of lysozyme, total nitric oxide synthase and nitric oxide content than the control. Moreover, transcriptional levels of *cox-2*, *il-1β* and *il-6* were remarkably downregulated by the supplementation of 0·5–1·0 % ELE. This study demonstrated that the supplementation of 1·0 % ELE in diet could increase the growth performance of large yellow croaker larvae probably by promoting expression of feeding-related genes, enhancing antioxidant capacity and immunity and inhibiting expression of inflammatory factors.

Key words: Large yellow croaker larvae: *Eucommia ulmoides* leaf extract: Feeding-related genes: Digestive enzymes: Antioxidant capacity: Immunity: Inflammatory factors

Eucommia ulmoides (EU) is a traditional medicinal herb in eastern Asia[1–5]. It was said that more than forty compounds such as iridoids, phenolics and steroids exist in EU, among them a major bioactive compound called chlorogenic acid endows it with precious medicinal value[2,6]. Over the recent years, several researches reported that EU extract has the effects of anti-hypertensive[7,8], anti-obesity[9,10], anti-bacteria[11], anti-inflammation[12,13], antioxidation[14–16] and neuroprotection[16,17].

The leaf of EU showed a higher activity than its cortex, flower and fruit[18,19]. In particular, eucommia ulmoides leaves extract (ELE) can enhance the growth performance and improve meat quality in mammalian species[20–24]. In recent years, EU was used as a new feed addictive in the aquaculture industry to enhance the growth and immunological response. Supplementation of EU in turbot diets could strikingly improve the antioxidant activity and kept an active immune response[25]. Furthermore, Sun *et al.*[26] and Yang *et al.*[27] had revealed that chlorogenic acid supplementation (main active components in EU) could enhance the growth performance as well as improve flesh quality of grass carp. It is said that there are relatively few studies on the application of EU in fish larvae, but it is also valuable in marine carnivorous species.

Large yellow croaker (*Larimichthys crocea*) is a monetarily significant aquaculture species and broadly cultivated in

Abbreviations: ELE, eucommia ulmoides leaves extract; EU, *Eucommia ulmoides*; IS, intestinal segments; PS, pancreatic segments.

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southeastern China[28–32]. However, the sufficient development and survival are still the key limiting factors in marine fish larviculture[33,34]. The immature feeding and intestinal function of larvae limited to absorb the micro-diet, which is crucial for growth and survival of fish larvae[32,35]. Chinese herbs characterised by abundant bioactive substances, without side effects brought from chemicals, showed a promising potential for application and development in the aquaculture industry. Previous studies have found that supplementation of Chinese herbs at appropriate dosage in diets could enhance fish immunity, improve growth and regulate intestine development[36–38]. However, there were almost no reports about the regulatory effects of ELE supplementation on the fish larval nutrition and physiological states. Therefore, this study was to focus on the effects of ELE supplementation on the growth, survival, expression of feeding-related genes, activities of digestive enzymes, antioxidant capacity, immunity and cytokines expression of large yellow croaker larvae.

Methods

Animal ethics

In the present study, those conventions for fish larvae were accordance with the Management Rule of Laboratory Animals strictly (Chinese Order No. 676 of the State Council, revised 1 March 2017).

Diets formulation

The experiment lasted 30 d and tested four diets in triplicate: the testing diets were formulated and supplementation with 0 g kg^{-1} (the control group), 5 g kg^{-1} (0·5 %), 10 g kg^{-1} (1·0 %) and 20 g kg^{-1} (2·0 %) of ELE, respectively (online Supplementary Table 1S). The testing diets were formulated in light of the nutritional requirements of large yellow croaker larvae based on Ai *et al.*[28] with slight adjustment. The ELE was purchased from Shaanxi Chen-xi Bio Co., Ltd in Shaanxi, China. The micro-diets were made according to the norm procedures in our laboratory and stored at −20°C prior to use in light of Yao *et al.*[39]

Fish and experimental procedures

Large yellow croaker larvae were acquired from Xiangshan Harbor Aquatic Seeds Company, Ningbo, China, and then reared at Marine and Fishery Science and Technology Innovation Base, Ningbo, China. Prior to the initial stocking, the fish larvae were acclimatised for 2–3 d in the laboratory condition by feeding live copepods and artificial experiment diets to adapt to the experimental feed. Larvae were weaned at 16 d of age (4·71 ± 0·27 g) and were randomly distributed into 220 L blue plastic tanks for a density of 3500 fish in each tank. During the trial, water temperature was kept in 21·0–23·0°C, salinity was kept in 25·0–28·0 g/l, dissolved oxygen content was maintained at 6·0 mg/l and maintain 100 % of the water change daily. Fish larvae were fed to the experiment micro-diets seven times a day (06.30, 08.30, 10.30, 13.30, 15.30, 17.30 and 22.30).

Sampling and dissection

A 24-h fasting experiment was conducted after feeding the last meal, and forty-five individuals (15/tank) were sampled at 1, 3, 6, 12 and 24 h, respectively, for the expression of feeding-related genes assay. The rest of fish larvae were sampled after 24 h of fasting, and the number and weight of fish larvae were sampled from each tank for calculating the survival rate and growth. Subsequently, the larval tissue (intestine segment-IS, pancreatic segments-PS, brain and visceral mass) was placed on the glass plate and then dissected under a microscope. The temperature was kept at 0°C for digestive enzyme activity assay as described by Cahu and Infante[40]. The final body weight of larvae in each tank was measured (forty-five individuals, fifteen/tank) with a microbalance. Initial body length and final body length of fish larvae in each tank were measured (forty-five individuals, fifteen/tank) with a Vernier caliper, and the rest of fish larvae were collected and stored at −20°C for body composition assay.

Proximate composition analysis

The feed samples and fish body were dried until a constant weight at 105°C for moisture assay. The crude protein and crude lipid of diets and fish larvae were determined following the standard methods (AOAC, 1995)[41].

Digestive enzyme activities assay

IS and PS of fish larvae were weighed (0·2–0·3 g) and then homogenised in 2 ml 0°C ultrapure water (from Milli-Qsystem) and centrifuged at 3300 ***g*** for 10 min. The supernatant had been utilised to digestive enzyme activities assay. The brush border membranes from fish larvae intestine were purified according to a method for intestinal scrapping[42] and adapted to intestinal segments[40]. Leucine-*p*-nitroanolide was used as substrate for leucine-aminopeptidase activity assay[43]. The enzyme activity was expressed by specific activity (mU/mg·protein). Protein was determined based on Bradford (1976) research that using bovine serum albumin (Sigma A-2153) as a criterion[44]. Activities of α-amylase and trypsin in PS and IS and alkaline phosphatase activity in the BBM were determined by commercial reagents and kits, purchased from Nanjing Jiancheng Bioengineering Insitute (Nanjing) and strictly refer to the operation method on the kit for determination.

Antioxidant and immune enzyme activity assay

The visceral mass of fish larvae was used to antioxidant and immune enzyme activity assay. Activities of catalase, total superoxide dismutase, total antioxidant capacity and the content of malondialdehyde were determined by commercial reagents and kits (Nanjing Jiancheng Bio-Engineering Institute). Besides, activities of lysozyme, total nitric oxide synthase, inducible nitric oxide synthase and the content of nitric oxide in visceral mass of larvae were determined by commercial reagents and kits (Nanjing Jiancheng Bio-Engineering Institute).

Total RNA extraction, cDNA synthesis and real-time quantitative polymerase chain reaction

Total RNA was extracted using TRIzol reagent (Takara). The quantity and concentration of extracted RNA was detected by a Nano Drop®2000 spectrophotometer (Thermo Scientific). Subsequently, reversed the extracted RNA into cDNA use the PrimeScript™ RT reagent Kit (Takara) following the manufacturer's instructions. The β-actin has been viewed as the housekeeping gene in the present study[45]. The RT-qPCR primers of the candidate genes were designed in light of the nucleotide sequences of large yellow croaker (online Supplementary Table 2S). The RT-qPCR was carried out in a quantitative thermal cycler (CFX96TM Real-Time System, BIO-RAD, USA). The volume of reaction system and the procedure of RT-qPCR program were conducted according to the methods of Huang *et al.*[46]. The PCR reaction mixtures with SYBR qPCR Mix (10 μl), forward and reverse primer (1 μl), cDNA (1 μl) and DEPC-treated water (7 μl). The reaction conditions were as follows 95°C for 2 min, followed by 39 cycles of 95°C for 10 s, anneal for 30 s and 72°C for 20 s. The level of gene expression was calculated with the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen[47].

Calculations and statistical analysis

Survival rate (SR, %) = $100 \times N_t/N_0$

Specific growth rate (SGR, % d^{-1}) = $100 \times (\text{Ln } W_t - \text{Ln } W_0)/d$ where N_t and N_0 were the fish larval final and initial numbers, respectively; W_t was the larval final wet body weight (g), W_0 was the larval initial wet body weight and d was the experimental duration, respectively.

In addition to feeding-related gene expression, all data were analysed using SPSS 23·0 (IBM, America) for one-way ANOVA. Expression of feeding-related genes were analysed by two-way ANOVA (diet × time) to analyse the effects of diet, fasting time and interaction. Statistics with $P < 0{\cdot}05$ were regarded to be significant, and the results were shown as means values with their standard errors.

Results

The effect of eucommia ulmoides leaves extract supplementation on growth performance of larvae

The SR of larvae was significantly higher than larvae fed diets with 0 % ELE (14·98 %), 0·5 % ELE (20·27 %) and 2·0 % ELE (14·78 %) after fed the diet with 1·0 % ELE (27·00 %) ($P < 0{\cdot}05$). Larvae fed diets with 0·5 % ELE (9·59 % d^{-1}) and 1·0 % ELE (9·52 % d^{-1}) showed significantly higher SGR than larvae fed the diet with 0 % ELE (8·38 % d^{-1}) and 2·0 % ELE (8·58 % d^{-1}) ($P < 0{\cdot}05$). The final body weight was significantly increased in larvae fed diets with 0·5 % ELE (84·00 mg) and 1·0 % ELE (81·80 mg) compared with larvae fed the diet with 0 % ELE (58·23 mg) and 2·0 % ELE (62·34 mg) ($P < 0{\cdot}05$). The final body length was significantly higher in larvae fed the diet with 1·0 % ELE (16·89 mm) than the control group (13·95 mm) ($P < 0{\cdot}05$) (Table 1). There were no significant differences in body composition (protein, lipid and moisture) of larvae among larvae fed different diets ($P > 0{\cdot}05$) (Table 2).

The effect of eucommia ulmoides leaves extract supplementation on feeding-related genes expression of larvae

Two-way ANOVA detected a strongly significant dependence of neuropeptide Y (*npy*) expression on diets and fasting time (diets: $P < 0{\cdot}001$; time: $P < 0{\cdot}001$), but not on the interaction of diets and fasting time (interaction: $P = 0{\cdot}169$). The transcriptional level of *npy* in larvae increased with the prolongation of fasting time and reached the highest level after a 24 h of fasting. At the specific time point, mRNA expression of *npy* was significantly increased in larvae fed diets with 0·5 % ELE and 1·0 % ELE compared with larvae fed the diet with 0 % ELE after fasting for 12 hours ($P < 0{\cdot}05$). Moreover, mRNA expression of *npy* significantly increased in larvae fed the diet with 1·0 % ELE compared with larvae fed the diet with 0 % ELE after fasting for 24 h ($P < 0{\cdot}05$) (Fig. 1).

The mRNA expression of *ghrelin* was related to diets, fasting time and the interactive effects of diets and fasting time (diets: $P < 0{\cdot}05$; time: $P < 0{\cdot}001$; interaction: $P < 0{\cdot}01$). Interestingly, the mRNA expression of *ghrelin* in larvae fed diets with 0·5 % ELE, 1·0 % ELE and 2·0 % ELE increased with the prolongation of fasting time. At the specific time point, the mRNA expression of *ghrelin* was significantly higher in larvae fed the diet with 0·5 % ELE than larvae fed diets with 0 % ELE and 2·0 % ELE after fasting for 24 h ($P < 0{\cdot}05$) (Fig. 2).

The mRNA expression of *leptin* was related to by diets and fasting time (diets: $P < 0{\cdot}001$; time: $P < 0{\cdot}001$), although no interactive effects between fasting time and diets were observed (interaction: $P = 0{\cdot}195$). At the specific time point, the mRNA expression of *leptin* was significantly higher in larvae fed the diet with 2·0 % ELE than the other three dietary treatments at time points of 1, 12 and 24 h during fasting ($P > 0{\cdot}05$) (Fig. 3).

The effects of eucommia ulmoides leaves extract supplementation on digestive enzyme activities of larvae

Both the activity of α-amylase and trypsin in larval PS and IS was independent of ELE supplementation ($P > 0{\cdot}05$). Similarly, the ratio of Try-IS/Try-(PS + IS) was independent of ELE supplementation ($P > 0{\cdot}05$). The activity of leucine-aminopeptidase was significantly higher in larvae fed the diet with 1·0 % ELE than larvae fed the diet with 0 % ELE ($P < 0{\cdot}05$), while alkaline phosphatase was significantly higher in larvae fed the diet with 2·0 % ELE than larvae fed the diet with 0 % ELE ($P < 0{\cdot}05$) (Table 3).

The effects of eucommia ulmoides leaves extract supplementation on antioxidation capacity of larvae

The antioxidant capacity of fish larvae visceral mass was assayed. Activity of total antioxidant capacity was significantly higher in larvae fed the diet with 1·0 % ELE than larvae fed the diet with 0 % ELE ($P < 0{\cdot}05$). Also, the activity of catalase was significantly higher in larvae fed diets with 1·0 % and 2·0 % ELE than larvae fed the diet with 0 % ELE and 0·05 % ELE ($P < 0{\cdot}05$). However, no significant differences were observed in the activity of total superoxide dismutase and malondialdehyde content in the visceral mass of larvae among four dietary treatments ($P > 0{\cdot}05$) (Fig. 4(a)–(d)).

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Table 1. Growth performance of large yellow croaker larvae fed graded level of eucommia ulmoides leaf extract (ELE) supplementation (means values with their standard errors)*

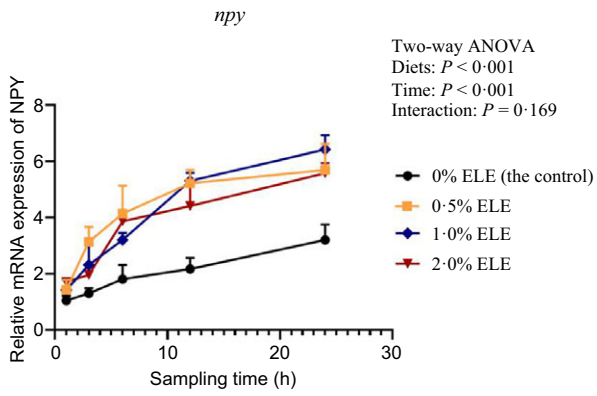
	Diets (ELE supplementation level %)							
	Diet 1 (0 %)		Diet 2 (0·5 %)		Diet 3 (1·0 %)		Diet 4 (2·0 %)	
Index	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Final body length (FBL, mm)	13·95^{b}	0·11	16·29^{ab}	0·78	16·89^{a}	0·18	16·20^{ab}	0·70
Final body weight (FBW, mg)	58·16^{b}	1·92	84·00^{a}	5·37	81·80^{a}	3·09	62·34^{b}	5·07
Specific growth rate (SGR, % d^{-1})	8·38^{b}	0·07	9·59^{a}	0·21	9·52^{a}	0·13	8·58^{b}	0·28
Survival rate (SR, %)	14·98^{b}	0·86	20·27^{b}	0·87	27·00^{a}	2·37	14·78^{b}	0·77

* Data are expressed as means values with their standard errors. Mean values with different superscripts are significantly as determined by Tukey's test ($P > 0·05$). SEM, standard error of means.

Table 2. Body composition of large yellow croaker larvae fed graded level of eucommia ulmoides leaf extract (ELE) supplementation (means values with their standard errors)*

	Diets (ELE supplementation level %, dry matter)							
	Diet 1 (0 %)		Diet 2 (0·5 %)		Diet 3 (1·0 %)		Diet 4 (2·0 %)	
Whole-body (%)	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Protein	5·84	0·09	5·47	0·09	5·64	0·15	5·87	0·17
Lipid	2·40	0·11	2·25	0·12	2·24	0·11	2·42	0·06
Moisture	89·52	0·49	90·13	0·25	89·91	0·40	89·05	0·51

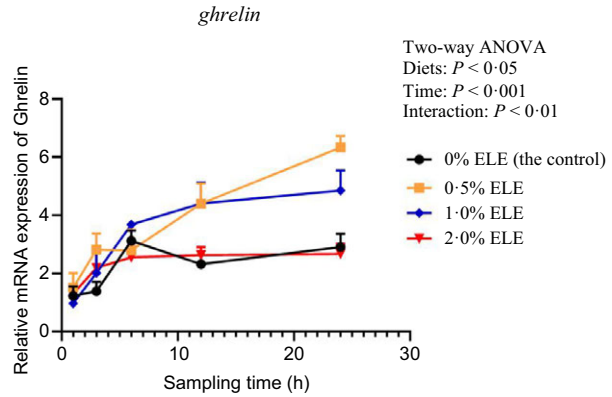
* Data are expressed as means values with their standard errors. Mean values with different superscripts are significantly as determined by Tukey's test ($P > 0·05$). SEM, standard error of means.



One-way ANOVA

	0%	0·5%	1·0%	2·0%
Fast-12h	b	a	a	ab
Fast-24 h	b	ab	a	ab

Fig. 1. The transcriptional levels of *npy* in the brain of large yellow croaker larvae. Two-way ANOVA to determine the effects of short-term fasting on diet, time and interaction. Different letters at the same time point indicate significant differences as determined by Tukey's test ($P < 0·05$). The results were shown as means values with their standard errors.



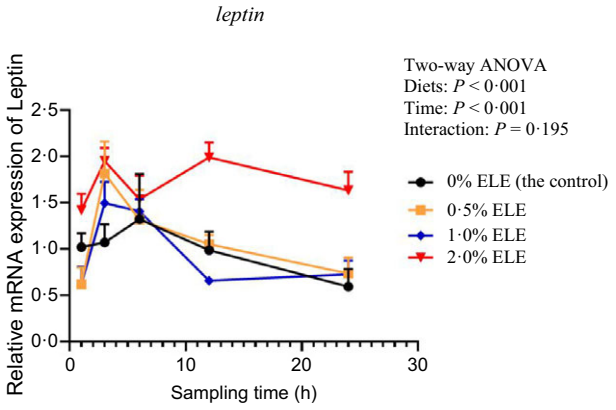
One-way ANOVA

	0%	0·5%	1·0%	2·0%
Fast-24 h	b	a	ab	b

Fig. 2. The transcriptional levels of *ghrelin* in the visceral mass of large yellow croaker larvae. Two-way ANOVA to determine the effects of short-term fasting on diet, time and interaction. Different letters at the same time point indicate significant differences as determined by Tukey's test ($P < 0·05$). The results were shown as means values with their standard errors.

The effects of eucommia ulmoides leaves extract supplementation on immunity capacity of larvae

The activity of lysozyme was significantly higher in larvae fed the diet with 1·0 % ELE than larvae fed the diet with 0 % ELE ($P < 0·05$). Similarly, the content of nitric oxide and the activity of total nitric oxide synthase were significantly higher in larvae fed the diet with 1·0 % ELE than larvae fed the diet with 0 % ELE ($P < 0·05$). Furthermore, the activity of inducible nitric oxide synthase in larvae fed diets with 0·5 % ELE, 1·0 % ELE, and 2·0 % ELE were significantly higher than larvae fed the diet with 0 % ELE ($P < 0·05$) (Fig. 4(e)–(h)). Besides the enzyme activity, the transcriptional levels of *tnfα* in larvae showed no significant differences among dietary treatments ($P > 0·05$). The transcriptional level of *ifnγ* was significantly lower in larvae fed the diet with 2·0 % ELE than larvae fed the diet with 0 % ELE ($P < 0·05$). The transcriptional level of *il-1β* was significantly lower in larvae fed diets with 0·05 % ELE and 1·0 % ELE than larvae fed the diet



One-way ANOVA

	0%	0·005%	0·01%	0·02%
Fast-1 h	**b**	b	b	a
Fast-12 h	b	b	b	a
Fast-24 h	b	b	b	a

Fig. 3. The transcriptional levels of *leptin* in the visceral mass of large yellow croaker larvae. Two-way ANOVA to determine the effects of short-term fasting on diet, time and interaction. Different letters at the same time point indicate significant differences as determined by Tukey's test ($P < 0·05$). The results were shown as means values with their standard errors.

with 0 % ELE ($P < 0·05$). Moreover, mRNA expression of *cox-2* and *il-6* decreased significantly with dietary ELE supplementation. Both cox-2 and *il-6* expressions in larvae fed diets with 0·5 % ELE, 1·0 % ELE and 2·0 % ELE were significantly lower than control ($P < 0·05$) (Fig. 5).

Discussion

In the present study, we demonstrated that dietary ELE at 1·0 % supplementation could improve survival rate of large yellow croaker larvae, which were probably linked to the immunity-promotion effects of active ingredients in EU[48–50]. Meanwhile, dietary ELE at 0·5–1·0 % supplementation could increase the specific growth rate of larvae, which agreed well with several previous studies investigations on weaned piglets[24], broiler chicks[51], turbot[25] and grass carp[26,27].

Orexigenic stimulation plays an important role in individual viability of the larval stage[52]. Ghrelin and leptin are key hormones in food intake regulation[53,54]. More specifically, the ghrelin would increase animal food intake via activating NPY/AgRP neurons or inhibiting POMC/CART neurons in the arcuate nucleus[55–57]. Conversely, leptin supposedly inhibits the NPY/AgRP neurons or activates the POMC/CART neurons[58]. In the present study, the mRNA expression of *npy* and *ghrelin* increased with the prolongation of fasting time, indicating that *npy* and *ghrelin* expression play a role in feeding stimulation. Similar results were also found in other fish species, such as *npy* expression in goldfish[59], coho salmon[60] and yellowtail[61], *ghrelin* expression in sea bass[62], zebrafish[63] and grass carp[64]. While the expression of *leptin* increased at first and then decreased, indicating that *leptin* expression was involved in feeding inhibition, which was consistent with northern snakehead[65]. Importantly, compared with the control, a significantly higher *npy* expression was observed in larvae fed the diet with 1·0 % ELE at 12 and 24 h of fasting, while a remarkably higher *ghrelin* expression was found in larvae fed the diet with 0·5 % ELE at 24 h of fasting. Additionally, larvae fed the diet with 2·0 % ELE showed a significantly higher *leptin* mRNA expression than the other three diets at time points of 1, 12 and 24 h during fasting, which suggested that excessive intake of ELE suppressed the appetite of fish larvae. Similarly, Zhang *et al.*[25] had elucidated that an overdose of EU Oliver (10·0–20 g kg^{-1}) supplementation in diets significantly suppressed the feed intake of turbot. Conclusively, these results indicate that ELE supplementation might enhance the appetite of large yellow croaker larvae at appropriate dosage but exert an inhibitory effect at a high dosage.

The imperfect intestinal function of fish larvae usually led to the hyposecretion of enzymes, which affected the growth and survival rate[32,35,66]. In particular, larval intestinal brush border membrane enzymes such as those with involvement of alkaline phosphatase and leucine-aminopeptidase can reflect the maturity of intestinal cells[67]. In the present study, dietary ELE supplementation improved the activities of alkaline phosphatase and leucine-aminopeptidase in brush border membranes, indicating that ELE played an important role in promoting larval intestinal development. Similar results were also observed in

Table 3. Activities of trypsin and α-amylase of large yellow croaker larvae fed graded level of eucommia ulmoides leaf extract (ELE) supplementation (means values with their standard errors)*

		Diets (ELE supplementation level %)							
		Diet 1 (0 %)		Diet 2 (0·5 %)		Diet 3 (1·0 %)		Diet 4 (2·0 %)	
Parameters		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Trypsin†,‡	PS‡	404·88	27·63	396·91	23·33	379·34	36·18	349·76	36·72
	IS‡	391·59	39·97	535·58	40·77	488·84	51·98	524·37	77·28
	Try-IS/(PS + IS)	0·49	0·04	0·57	0·03	0·56	0·03	0·60	0·03
α-amylase†,‡	PS	0·48	0·04	0·39	0·02	0·47	0·03	0·32	0·04
	IS	0·33	0·01	0·36	0·01	0·32	0·01	0·31	0·01
LAP†,‡	BBM‡	1·30†	0·11	1·71^{ab}	0·08	1·96*	0·04	1·70^{ab}	0·14
AKP†,‡	BBM	945·18†	88·95	1363·62†	316·58	1030·56†	223·51	2707·70*	135·31

* Data are expressed as means values with their standard errors. Mean values with different superscripts are significantly as determined by Tukey's test ($P > 0·05$). SEM, standard error of means.
† The unit of enzyme activity is U/mg protein.
‡ PS, pancreatic segments; IS, intestinal segments; BBM, brush border membranes.

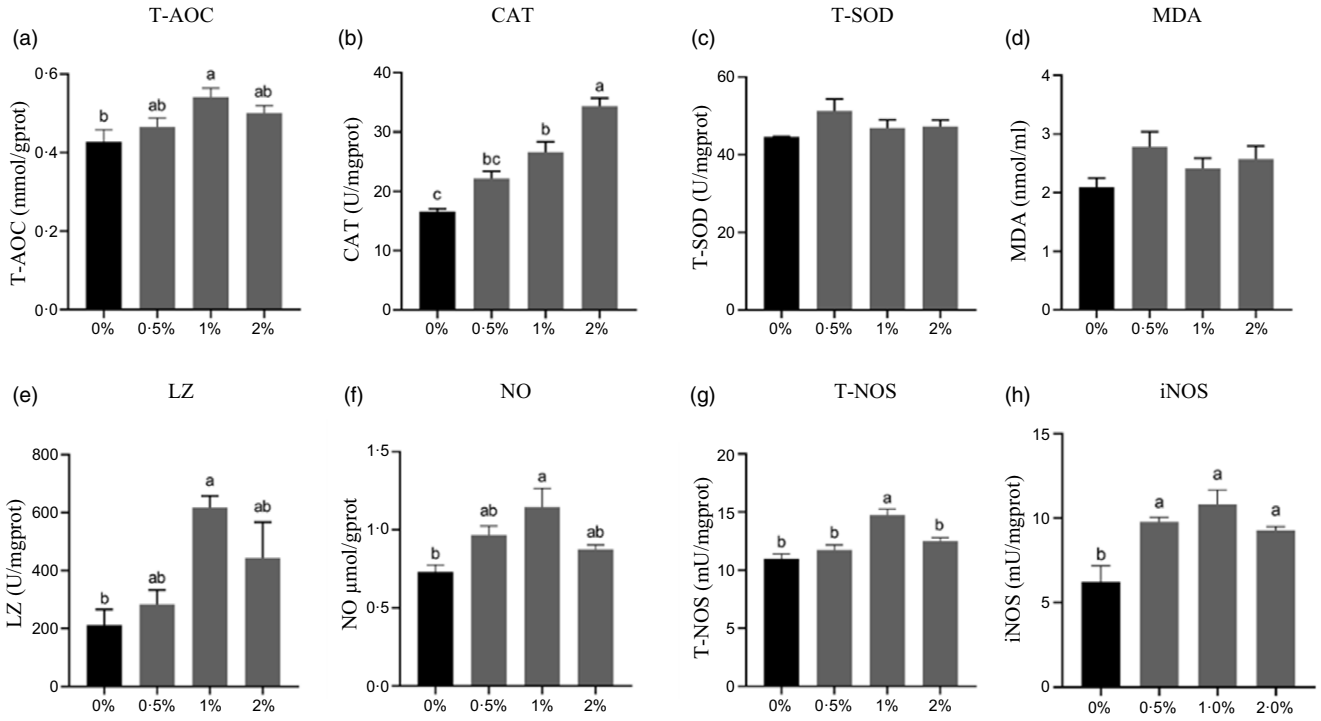


Fig. 4. Activities of T-AOC (A) and CAT (B), activities of T-SOD (C), concentration of MDA (D) and LZ (E), content of NO (F), activities of T-NOS (G) and iNOS (H) in the visceral mass of large yellow croaker larvae. T-AOC, total antioxidant capacity; CAT, catalase; MDA, malondialdehyde; T-SOD, total superoxide dismutase; LZ, lysozyme; NO, nitric oxide; T-NOS, total nitric oxide synthase; iNOS, inducible nitric oxide synthase. Columns sharing mean values with different superscripts are significantly as determined by Tukey's test ($P > 0{\cdot}05$).

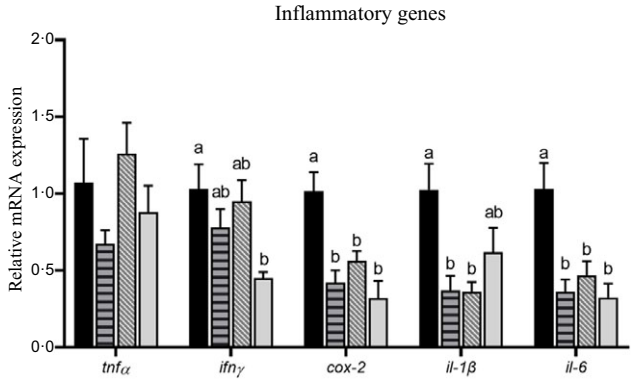


Fig. 5. The transcriptional levels of inflammatory genes in the visceral mass of large yellow croaker larvae. *Tnfα*, tumor necrosis factor *α*, *ifnγ*, interferon *γ*; *cox-2*, cyclooxygenase-2; *il-1β*, interleukin-1*β*; *il-6*, interleukin-6. Data are presented as means values with their standard errors. Columns sharing mean values with different superscripts are significantly as determined by Tukey's test ($P > 0{\cdot}05$). ■, 0 % ELE (the control); ▬, 0.5 % ELE; ▨, 1.0 % ELE; □, 2.0 % ELE

early-weaned piglets with incomplete digestive tract development[24,68]. The possible mechanism is that EU contain a variety of bioactive ingredients, which can stimulate the maturation process of the digestive function of animals[24]. Paradoxically, no significant effects were observed in *α*-amylase and trypsin activity on PS and IS, as well as the ratio of Try-IS/Try-(PS + IS), which was probably attribute to the allometric growth of fish biomass in the larval stage[40].

Marine fish larvae are vulnerable to oxidative stress due to the high energy consumption required for rapid growth[69]. Early pharmacological studies have shown that EU have a strong anti-oxidation capacity, which can scavenge free radicals to reduce oxidative damage to biomolecules[1,15,16]. In the present study, we found that appropriate dosage of ELE in micro-diets could improve larval antioxidant capacity of large yellow croaker larvae. Especially, total antioxidant capacity activity was significantly higher in larvae fed the diet with 1·0 % ELE than the control group. catalase activity significantly increased in larvae fed diets with 1·0 % ELE end 2·0 % ELE. These results were consistent with the research that ELE supplementation could increase activities of T-AOC and catalase in turbot[25].

High mortality is a key limiting factor during fish larviculture, and this project also considered the influence of dietary ELE on immunological enzymes and transcription factors thought to be pivotal in proinflammatory cytokine in particular. In the present study, activities of lysozyme, total nitric oxide synthase and the content of nitric oxide were significantly higher in larvae fed the diet with 1·0 % ELE than the control group, which was consistent with the highest survival rate in fish larvae fed the diet with 1·0 % ELE. Moreover, the activity of inducible nitric oxide synthase was significantly affected by dietary ELE. Purportedly, NO was produced via the NOS pathway and exerted protective roles of immuno-protection against pathogenic infection[70], and Lee *et al.*[71] had elucidated that ELE enhances NO production in ox-LDL treated human endothelial cells through NOS signalling pathways. Besides the nitric oxide, the inflammatory factors

were also an important indicator to evaluate the health status of fish[31]. In the study, the mRNA expression of *ifnγ*, *cox-2*, *il-1β* and *il-6* significantly decreased with ELE supplementation. Similarly, Kim *et al.*[12] had revealed that EU extracts had higher anti-inflammatory activities than the anti-inflammatory drugs. Additionally, the EU cortex also inhibited inflammatory cytokine production[13]. Overall, these results could be speculated that proper ELE supplementation could increase immunity in fish larvae by increasing lysozyme activity, regulating NOS signalling pathways and alleviating inflammation.

In summary, the micro-diet supplemented with 10 g kg^{-1} (1·0 %) ELE could improve the growth performance of large yellow croaker larvae via enhancing expression of feeding-related genes, promoting antioxidant capacity and immunity and inhibiting cytokines expression.

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Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S0007114521004621

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