Effects of L-tryptophan-supplemented dietary on growth performance and 5-HT and GABA levels in juvenile *Litopenaeus vannamei*

Yu-Ping Sun · Li-Zeng Guan · Jie-Hua Xiong · Qian-Yun Xi · Yong-Liang Zhang

Abstract   A 60-day feeding trial was conducted to evaluate the effects of dietary L-tryptophan level on the growth performance and neurotransmitter, including 5-hydroxytryptamine (5-HT) and γ-aminobutyric acid (GABA) of juvenile *Litopenaeus vannamei*. Six experimental diets were formulated to supplement with graded levels of tryptophan, resulting in different dietary tryptophan levels at 0 (control), 0.12, 0.24, 0.36, 0.48 and 0.60 %, respectively. A total of 960 shrimps (0.63 ± 0.01 g) were divided into six treatments and fed with the respective tryptophan diets in an indoor recirculating system. The results showed that the body weight, body length, live weight gain, specific growth rate, protein efficiency ratio and survival rate were significantly increased by dietary tryptophan levels, and the maximum were all obtained at 0.60 % tryptophan-supplemented diet. Feed conversion ratio was significantly decreased as dietary tryptophan level increased. No significant difference was found in hepatopancreas index and body proximate composition among all the treatments. High-performance liquid chromatography analysis showed that 5-HT level increased first and then decreased in all examined tissues except in thoracic ganglia with dietary tryptophan level increased, while GABA level first elevated and then decreased in X organ–sinus gland, brain, stomach and intestinal tract, and on the contrary, in thoracic ganglia, hepatopancreas, but declined in hemolymph with higher dietary tryptophan level. qRT-PCR results exhibited 5-HT_1 mRNA expression was similar to 5-HT level variation. Based on these results, the dietary tryptophan supplemented 0.36 % dry diet or 1.72 % protein diet is found to be optimum to improve *L. vannamei* growth

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Yu-Ping Sun and Li-Zeng Guan have contributed equally to this work.

Y.-P. Sun · J.-H. Xiong · Q.-Y. Xi · Y.-L. Zhang (✉)
College of Animal Science, SCAU-Alltech Research Joint Alliance, South China Agricultural University, 483 Wushan Road, Guangzhou 510642, China
e-mail: Zhangyl@scau.edu.cn

Y.-P. Sun
Institute of Animal Science, Guangdong Academy of Agriculture Sciences, Guangzhou 510640, China

L.-Z. Guan
Agriculture College, Yanbian University, Yanji 133000, China

performance. In conclusion, it suggested that diet supplemented with tryptophan was beneficial to improve growth performance possibly by mediating 5-HT as well as GABA pathway in *L. vannamei*. These data provide new insight into the physiological functions of tryptophan in shrimp.

Keywords Tryptophan · Growth performance · 5-HT · 5-HT$_1$ receptor · GABA · Juvenile *Litopenaeus vannamei*

Abbreviations

5-HT	5-Hydroxytryptamine, serotonin
GABA	γ-Aminobutyric acid
WG	Weight gain
SGR	Specific growth rate
FCR	Feed conversion ratio
PER	Protein efficiency ratio
HSI	Hepatosomatic index
XO–SG	X organ–sinus gland

Introduction

Litopenaeus vannamei has been becoming the most important shrimp cultured in the world, especially in China. According to financial analyses, survival and growth have the greatest impact on the economic performance of shrimp production. Tryptophan plays important and exceptional roles in animal nutrition and metabolism and gains more attention (Ahmed and Khan 2005; Ahmed 2012). Tryptophan becomes the third limiting amino acid when fed corn–soybean-based diets. So, tryptophan supplementation would be indispensable to improve protein utilization and reduce nitrogen excretion and thereby could improve animal growth performance. Papoutsoglou et al. (2005a) found dietary supplemented tryptophan could reduce body lipids, enhance food consumption and nutrient utilization for European sea bass *Dicentrarchus labrax*. It was reported that tryptophan could increase protein synthesis in livers of pigs (Zhang et al. 2007). Additionally, tryptophan-supplemented diet was beneficial to decrease size heterogeneity and increase survival of juvenile grouper, *Epinephelus coioides* (Hseu et al. 2003).

As a key precursor of the neurotransmitter serotonin (5-HT), tryptophan availability is reported to be the major factor determines 5-HT synthesis in brain (Johnston et al. 1990; Winberg et al. 2001). Hence, it is considered that tryptophan-induced 5-HTergic activity has been implicated in regulating many physiological and behavioral processes such as feed intake, susceptibility to stress and aggression (Leathwood 1987; Sève 1999; Laranja et al. 2010; Harlioğlu et al. 2014). In theory, deficiency of tryptophan decreases 5-HT content, and so, increased tryptophan intake would elevate 5-HTergic activity (Henry et al. 1992, 1996; Winberg et al. 2001; Koopmans et al. 2006). 5-HT widely occurs in tissues throughout the body of animal (Caamano-Tubio et al. 2007). The presence of different 5-HT receptor subtypes affinity for 5-HT, target actions and locations influence 5-HT roles (Tierney 2001; Sosa et al. 2004). Given that the presence of 5-HT in tissues is involved in tryptophan functional activity, however, except in the central nervous system and mostly in brain, there have been no studies of peripheral 5-HT or 5-HT receptor response to tryptophan. Furthermore, 5-HT is co-stored along with another important neurotransmitter γ-aminobutyric acid (GABA) (García et al. 1994; Kravitz 1962). Once synthesized, 5-HT

release would be possibly prohibited by the activation of GABA or even its respective auto-receptors (Gebauer et al. 1993). If tryptophan is the determinant for 5-HT synthesis, it is suggested that tryptophan supplemented would affect GABA synthesis through by regulating 5-HT metabolism.

Tryptophan is an essential amino acid in crustaceans. The previous studies on the optimum dietary tryptophan level of some crustaceans have been produced contradictory results. It was found that the optimum tryptophan demand of shrimp *Penaeus monodon* was 0.2 % dry diet or 0.5 % protein diet (Millamena et al. 1999). Similar results have been observed the optimum tryptophan in mud crab, shrimp and crayfish diet were 0.72, 0.8 and 0.94 % protein diet, respectively (Akiyama et al. 1991; Laranja et al. 2010; Harlioğlu et al. 2014). The conflicting observations showed that tryptophan requirement of *P.monodon* was 0.78 % dry diet or 1.84 % diet protein (Pascual and Kanazawa 1986), yet the weight gain of *P.monodon* reached a maximum when tryptophan content was 0.46 % dry diet or 1.35 % diet protein (Han et al. 1995). The conflicting observations in decapod crustaceans showed that the effect of tryptophan may be influenced by species and dietary, especially the diets protein.

However, effect of tryptophan on *L. vannamei*, even the optimum dietary tryptophan is not clear until now. The objective of the present study is to investigate the effects of dietary tryptophan level on growth performance, tissue 5-HT, GABA levels as well as the expression level of 5-HT receptor of juvenile *L. vannamei*, providing the basis for its application in shrimp production.

Materials and methods

Experimental design and diets

Six experimental diets were formulated to supplement with graded levels of tryptophan, resulting in different dietary tryptophan levels at 0 (control), 0.12, 0.24, 0.36, 0.48 and 0.60 %, respectively. *L. vannamei* basal diet (control) formulated, and proximate composition is given in Table 1. ʟ-Tryptophan (>99 %, Sigma, USA) was first dissolved in sterile water, and graded level of tryptophan was added to feed as specified in Table 1. The ingredients were formulated and thoroughly mixed the minced dry ingredients with fish oil and then adding cold water until dough obtained. The stiff dough was fully blended for 5 min followed by vitamin–mineral mix was mixed. The stiff dough was then passed through an extruder with a diameter of 1.5 mm. After that, the string-like diets broken up and sieved into proper pellet size (about 3.0 mm in length) were oven dried at 45 °C. After drying, the granules were sieved through 60 meshes, a convenient pellet size and the feeds were stored in plastic bags at −20 °C until use. Proximate analyses (AOAC, 1990) of the diets showed about 46 % crude protein, 6.0 % crude lipid and 8.3 % moisture.

Experimental animals and culture conditions

Juveniles *L. vannamei* were acclimated in a circulating seawater system and fed with basal diet for 1 week. About 960 healthy and lively shrimps (0.63 ± 0.01 g) were equally divided into six treatments; each treatment was allotted to 4 tanks (as replicates) in a completely random design. As a replicate, group of 40 shrimps were randomly stocked in 350-L (80 cm diameter, 70 cm deep) cylindrical fiberglass tank with net cover. All experimental tanks were part of an indoor recirculating system, provided with passed

Table 1 Formulation, proximate composition and analyzed tryptophan levels of six experimental diets (%, dry diet)

Components	Dietary tryptophan levels					
	Control[a]	0.12 %	0.24 %	0.36 %	0.48 %	0.60 %
Fish meal	28	28	28	28	28	28
Soybean meal	15	15	15	15	15	15
Peanut meal	19	19	19	19	19	19
Cuttlefish meal	5	5	5	5	5	5
Corn starch	2	2	2	2	2	2
Shrimp shell meal	6	6	6	6	6	6
Flour	20	20	20	20	20	20
Fish oil	1	1	1	1	1	1
Lecithin	2	2	2	2	2	2
Calcium dihydrogen phosphate	1.05	1.05	1.05	1.05	1.05	1.05
Choline chloride	0.25	0.25	0.25	0.25	0.25	0.25
Vitamin premix[b]	0.2	0.2	0.2	0.2	0.2	0.2
Mineral premix[c]	0.5	0.5	0.5	0.5	0.5	0.5
Total	100	100	100	100	100	100
L-Tryptophan supplemented[d]	0	0.12	0.24	0.36	0.48	0.60
Glutamic acid supplemented[d]	0.60	0.48	0.36	0.24	0.12	0
Crude protein[e]	46.17	46.20	46.53	46.13	45.68	45.51
Crude lipid[e]	6.03	6.01	6.16	5.91	5.89	5.99
Ash[e]	7.89	8.16	7.98	8.24	8.45	8.07
Moisture[e]	6.4	6.3	6.2	6.3	6.5	6.4
Total tryptophan level[e]	0.43	0.55	0.67	0.79	0.91	1.03

[a] Contained as per kg control diet : tryptophan 43.42 g, and gross energy is 12.61 MJ

[b] Contained as g per kg vitamin premix: V_A 16, V_{D3} 1, V_E 40, V_{K3} 3.86, V_{B1} 6.15, V_{B2} 7.5, V_{B6} 10.1; calcium pantothenate 15.3, PTGA 2.1, V_H 10, VPP 30.15, V_{B12} 1; inositol 31.25, V_C 171.42; cellulose 654.17

[c] Contained as g per kg mineral premix: KCl 229.08, NaCl 118.39, $MgSO_4 \cdot 7H_2O$ 292.16, $FeSO_4 \cdot 7H_2O$ 9.9, $ZnSO_4 \cdot 7H_2O$ 7.34, $MnSO_4 \cdot H_2O$ 10.32, KI 0.0156, $CoCl_2 \cdot 6H_2O$ 0.1064, $NaSO_3$ 0.0132, $CuSO_4 \cdot 5H_2O$ 1.68, Zeolites 331

[d] Crystalline form

[e] Analysis value (% dry diet)

through sand-filtered seawater and equipped with vigorous aeration. The shrimps were manually fed to apparent satiation with respective diet three times per day at 08:00 a.m., 14:00 p.m. and 20:00 p.m. During the feeding time (1 h), the flow-through water and the vigorous aeration were both temporarily closed. Feed consumption was recorded daily. The uneaten feeds were collected 1 h after every meal by siphoning, dried in an oven at 45 °C until a constant weight was achieved and the final dry weight was then recorded. The dead shrimps were removed from the tanks, and the number and weight were also recorded before each feeding. The debris on the bottom of tanks was siphoned off so as to maintain the water quality. All shrimps were subjected to a natural photoperiod. During the feeding periods, water flow rate was kept 2.5–2.8 L/min and the dissolved oxygen maintained more than 4.5 mg/L. The salinity, temperature and pH were maintained 3–7 ‰, 23–30 °C and

7.8–8.2, respectively. Water was exchanged daily before the first feeding to keep the ammonia concentration 0.25–0.30 mg/L. The feeding trial lasted for 60 days. After feeding experiment, the remaining shrimps in each tank were collected and continued the following measurements.

Growth parameters analysis

At the termination of the feeding trial, the remaining number of shrimp from each tank was calculated, and then, the shrimp collected from each tank for measurement of body weight and total length. Feed amount was also calculated for each tank. The growth index of *L. vannamei,* including weight gain (WG), feed conversion ratio (FCR), survival rate (SR), protein efficiency ratio (PER), specific growth rate (SGR) and hepatosomatic index (HSI), were evaluated as following:

$$WG\% = 100 \times (final\ weight - initial\ weight)/initial\ weight$$
$$FCR = total\ feed\ given\ (dry\ weight)(g)/weight\ gain(wet\ weight)(g)$$
$$SR\% = 100 \times (final\ number\ of\ shrimp/initial\ number\ of\ shrimp)$$
$$PER\% = 100 \times total\ wet\ weight\ gain(g, wet\ weight\ basis)/crude\ protein\ fed(g, dry\ weight)$$
$$SGR\% = 100 \times (ln\ average\ final\ weight - ln\ average\ initial\ weight)/number\ of\ culture\ days$$
$$HSI\% = 100 \times hepatosomatic\ weight(g)/body\ weight(g)$$

Sampling procedure

After the above parameters were measured, five shrimps from each tank were randomly selected and rinsed with tap water and then stored at $-30\ °C$ for determination of body composition. The other shrimps were anesthetized on ice bath for 10 min, and hemolymph was drawn from the thorax using a syringe pretreated with heparin. Hemolymph samples were rapidly transferred to Eppendorf tubes and were centrifuged at $1,500g$ for 10 min at $4\ °C$ for separating the plasma. The samples of the brain, X organ–sinus gland (XO–SG), thoracic ganglion, hepatopancreas, stomach and intestine were rapidly isolated within 2 min and collected in sterilized Eppendorf tubes, respectively. All collected samples were immediately frozen in liquid nitrogen and stored at $-80\ °C$ for future analysis.

Chemical analysis

Samples were dried and then finely ground for proximate composition analysis. Crude protein was determined by the Kjeldahl method using an Auto Kjeldahl System after acid digestion (Kjeltec Analyzer unit 2300, Sweden; 1030-Auto-analyzer, Tecator, Sweden). Crude lipid content was determined by the ether-extraction method using Soxtec System HT (Soxtec System HT6, Tecator, Sweden). Moisture content was determined by oven drying at $105\ °C$ for 24 h.

5-HT and GABA levels determination

About 100 mg samples of the brain, XO–SG, thoracic ganglion, hepatopancreas, stomach and intestine were homogenized in 0.4 mol/L ice-cold perchloric acid (PCA) using an ultrasonic disintegrator and were centrifuged at $14,000g$ at $4\ °C$. Then, the

supernatants were collected and filtered through a 0.22-μm millipore filter for high-performance liquid chromatography (HPLC) analysis as described by (Tinikul et al. 2008).

Detection and quantification of total 5-HT and GABA in all samples were performed by HPLC using a C_{18} column (150 mm × 4.6 mm i.d., 5 μm; Hypersil, America Thermo) at a flow rate of 1 or 0.8 mL/min with a fluorescence detector (Waters 474, Germany). GABA levels were determined using precolumn *o*-phthaldehyde derivatization (Qujeq 1998). Average 5-HT and GABA levels were estimated for four replicates. Peaks corresponding to 5-HT and GABA were detected in the extracts at the same elution times as their corresponding standards. The identities of the peaks in each sample were further verified by spiking the tissue extracts with known amounts of 5-HT and GABA standards. Final contents were calculated relative to external standards (5-HT and GABA, >99.99 %, Sigma), and recovery rate was expressed as μg/g tissue.

5-HT$_1$ receptor mRNA expression analysis

Total RNA was isolated from samples using TRIZOL® reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA was quantified at 260 nm/280 nm. cDNA was produced by reverse transcription (RT), and reaction conditions used were those recommended by the manufacturer (RNA PCR Kit version 2 1, TaKaRa).

The quantitative PCR (qPCR) assay was carried out in the Mx3000P^{TM} Real-time PCR System (Stratagene, USA) using SYBR Green (TOYOBO, Japan). Primers of 5-HT$_1$ (5HT$_1$-F: 5′-GATCCTGATGGTGTGGCTGAC-3′ and 5HT$_1$-R: 5′-GACGTAGAAGGT GGCCATGGT-3′) designed according to the sequences published in GenBank (*Penaeus monodon*, accession AY661549; *Macrobrachium rosenbergii*, accession AY528822, AY528821 and EU363466) and synthesized by Sangon Technical Co. Ltd. (Shanghai, China) were used to perform RT-PCR. cDNA reverse transcription from different tissues was used as the template for analyzing the expression of 5-HT$_1$ receptor genes. The thermal protocol for the SYBR green real-time PCR was as follows: 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 40 s. After PCR, melting curve analysis was performed to confirm that there occured only one amplified product; then, the size of product was investigated on ethidium bromide-stained 2 % agarose gels in Tris acetate-EDTA buffer. RT-PCR data analysis was determined with the Mx3000P^{TM} Real-time PCR System Software (Stratagene, USA). The plasmid vector containing *L. vannamei*-specific cDNA fragment was prepared to be used as the RT-PCR standard curve of the gene. According to the formula $2^{-\Delta Ct\Delta Ct} = 2^{-(\Delta Ctsample - \Delta Ctcontrol)}$ (Livak and Schmittgen 2001), the relative mRNA expression of the target gene was calculated.

Statistical analyses

All data are presented as means with standard deviations (mean ± SD), and all statistical analyses were performed using SPSS 17.0 statistical software (SPSS 17.0, Chicago, IL, USA). A multiple comparison (Duncan's) test was used to examine the significant difference among treatments tested by one-way ANOVA. Results were considered statistical significance difference at $P < 0.05$ level.

Results

Effects of tryptophan on growth performance and body proximate composition

The results of the present study indicated that *L. vannamei* growth performance and body proximate composition were significantly affected by graded tryptophan supplemented (Table 2). On day 60, the average body length was 7.29 ± 0.22 cm in the 0.60 % treatment and significantly higher than that in the control (6.20 ± 0.46 cm) ($P < 0.05$). Moreover, the average body length in the other treatment was also greater than that in the control ($P > 0.05$). Shrimp feed diets administrated 0.60 % tryptophan obtained the maximum live weight gain (925.01 %) ($P < 0.05$). The specific growth rate (SGR) showed the similar patterns to the final weight and the live weight gain. Tryptophan supplemented significantly altered the feed conversion rate (FCR) ($P < 0.05$), and the minimum (1.86) was achieved at 0.60 % tryptophan administrated diets. Protein efficiency ratio (PER) was also the highest in the 0.60 % treatment, which was significantly higher than that in the control and 0.12 % treatment ($P < 0.05$). The maximum survival rate (88.13 %) was recorded in the 0.60 % treatment, which was significantly higher than that in the control and 0.12 % treatment ($P < 0.05$).

Compared with the control, the higher dietary tryptophan level resulted in higher hepatosomatic index (HSI) and body protein content, but lower crude lipid content. However, no significant differences were found for HSI and proximate composition ($P > 0.05$; Table 2).

5-HT level changes in tissues

We detected 5-HT level variation in shrimp after tryptophan supplementation. During HPLC, the elution time of 5-HT peaks in the collected sample extracts is about 2.4 min, which was agreement with the external standards. The results showed that 5-HT level in organs of the nervous system, such as the XO–SG, brain and thoracic ganglia, was dramatically affected by dietary tryptophan level (Fig. 1a). 5-HT levels in XO–SG and the brain elevated with higher dietary tryptophan level and peaked in 0.48 and 0.36 % treatment, respectively, then showed a decreasing trend. On the contrary, 5-HT levels in the thoracic ganglia declined first and then returned to normal level with tryptophan increased in diets, and reached the minimum in 0.36 % treatment ($P < 0.05$). Dietary tryptophan level also significantly affected 5-HT levels in the hepatopancreas and intestinal tract ($P < 0.05$, Fig. 1b). It peaked in 0.36 and 0.48 % treatment, respectively, and then decreased, reaching the minimum level in 0.60 % treatment. In contrast, 5-HT level in the stomach showed no significant difference among all the treatments ($P > 0.05$, Fig. 1b). 5-HT level increased significantly in the hemolymph, and it peaked in 0.48 % treatment and then decreased with further tryptophan increasing ($P < 0.05$, Fig. 1c).

5-HT$_1$ receptor mRNA expression levels in tissues

To confirm 5-HT$_1$ mRNA receptor expression in shrimp, we first cloned a full-length (160 nucleotides) cDNA encoding 5-HT$_1$ receptor of *L. vannamei* and assessed 5-HT$_1$ expression using qRT-PCR. Remarkably, dietary tryptophan level affected 5-HT$_1$ mRNA receptor expression in tissues of the XO–SG, brain, thoracic ganglia, hepatopancreas, stomach and intestinal tract (Fig. 2). Relative to the control, 5-HT$_1$ mRNA receptor

Table 2 Growth, feed conversion ratio, body proximate composition, hepatosomatic index and survival rate of *L. vannamei* fed dietary tryptophan level for 60 days

Parameters	Dietary tryptophan levels (%, dry diet)[1]					
	Control	0.12 %	0.24 %	0.36 %	0.48 %	0.60 %
Mean initial weight (g/shrimp)[2]	0.63 ± 0.00	0.63 ± 0.00	0.63 ± 0.00	0.63 ± 0.00	0.63 ± 0.00	0.63 ± 0.01
Mean final weight (g/shrimp)[2]	6.20 ± 0.46[b]	6.56 ± 0.48[ab]	6.90 ± 0.19[ab]	6.94 ± 0.28[ab]	6.73 ± 0.20[ab]	7.29 ± 0.22[a]
Mean final length (cm/shrimp)[2]	8.25 ± 0.23[b]	8.41 ± 0.19[ab]	8.45 ± 0.09[ab]	8.52 ± 0.14[ab]	8.54 ± 0.11[ab]	8.76 ± 0.10[a]
Live weight gain (%)[3]	720.29 ± 78.42[b]	777.07 ± 58.27[b]	828.11 ± 45.53[ab]	803.55 ± 71.13[ab]	837.06 ± 39.51[ab]	925.01 ± 20.94[a]
Specific growth rate[3]	3.93 ± 0.12[b]	4.02 ± 0.13[ab]	4.13 ± 0. 03[ab]	4.14 ± 0.08[ab]	4.18 ± 0.04[ab]	4.22 ± 0.04[a]
Feed intake (g DM/shrimp)[2]	9.93 ± 0.38	9.94 ± 0.31	9.79 ± 0.30	9.79 ± 0.30	10.03 ± 0.43	10.04 ± 0.44
Feed conversion ratio[3]	2.03 ± 0.036[a]	2.02 ± 0.05[a]	1.91 ± 0.08[ab]	1.88 ± 0.07[b]	1.87 ± 0.05[b]	1.86 ± 0.01[b]
Protein efficiency ratio[3]	1.07 ± 0.03[b]	1.07 ± 0.03[b]	1.12 ± 0.04[ab]	1.13 ± 0.05[ab]	1.15 ± 0.04[ab]	1.19 ± 0.01[a]
Hepatopancreas index[4]	5.50 ± 0.28	5.72 ± 0.13	5.71 ± 0.14	5.56 ± 0.05	5.53 ± 0.22	5.52 ± 0.21
Body crude protein (%)[3]	71.61 ± 0.33	72.06 ± 0.62	72.29 ± 0.68	72.41 ± 0.55	72.52 ± 0.30	72.62 ± 0.18
Body crude lipid (%)[3]	6.53 ± 0.36	6.25 ± 0.29	6.10 ± 0.36	5.98 ± 0.28	5.99 ± 0.27	5.87 ± 0.21
Body moisture (%)[3]	73.38 ± 0.30	73.76 ± 0.40	74.19 ± 0.61	74.22 ± 0.32	74.35 ± 0.79	74.10 ± 0.20
Survival rate (%)[3]	76.25 ± 3.3[b]	76.75 ± 3.61[b]	78.13 ± 3.13[ab]	80.63 ± 4.00[ab]	85.63 ± 5.98[ab]	88.13 ± 2.36[a]

[1] Mean ± SD (N = 4); treatment mean values in a column having the same superscript letter are not significantly different at $P > 0.05$

[2] Initial and final mean weight (n ≥ 30)

[3] Values are means of four duplicated tanks

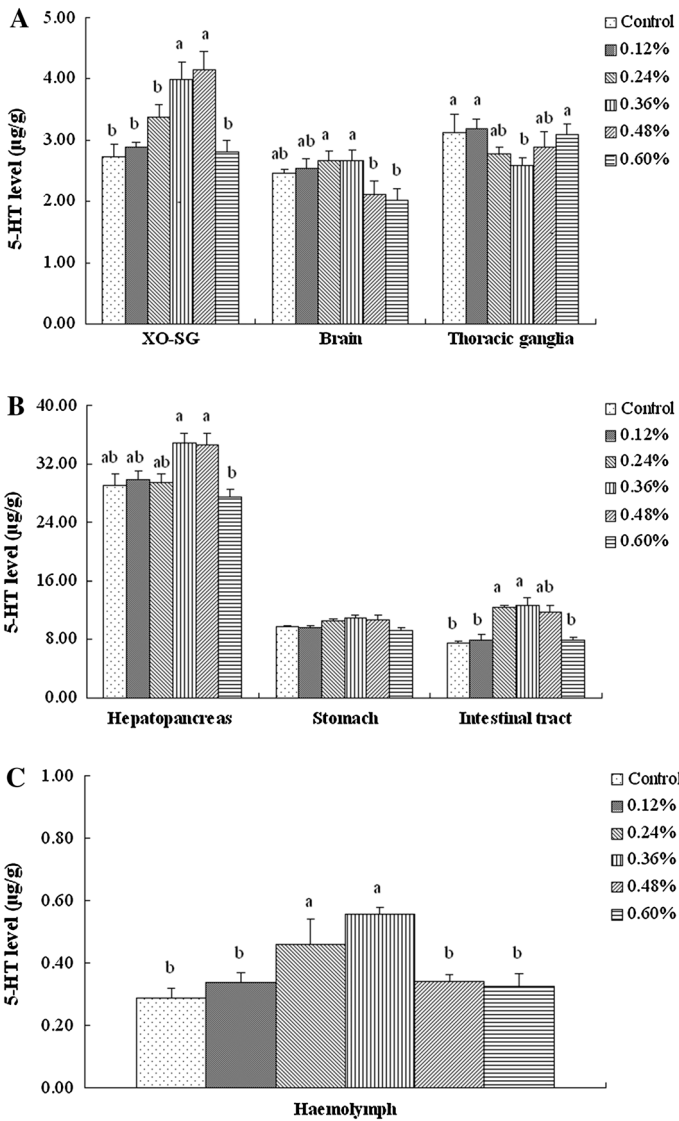[4] Hepatopancreas index (n = 20)

Fig. 1 **a** Effects of dietary tryptophan level on 5-HT in XO–SG, brain and thoracic ganglia by RP-HPLC after 60 days of feeding. **b** Effects of dietary tryptophan level on 5-HT in hepatopancreas, stomach and intestinal tract by RP-HPLC after 60 days of feeding. **c** Effects of dietary tryptophan level on 5-HT in hemolymph by RP-HPLC after 60 days of feeding The data shown represent the mean ± SD. [a,b]Values with *different letters* indicate significantly different ($P < 0.05$) by one-way ANOVA test

expression of 0.24, 0.36 and 0.48 % treatment was significantly enhanced in the XO–SG and brain ($P < 0.05$). On the contrary, in thoracic ganglia, it was significantly decreased in 0.24, 0.36 and 0.48 % treatment ($P < 0.05$, Fig. 2a). A similar pattern was observed in the hepatopancreas, stomach and intestinal tract. 5-HT$_1$ mRNA receptor expression of 0.48 % treatment was maximum in the hepatopancreas, while it returned to normal level in 0.60 %

treatment. In the stomach and intestinal tract, this receptor expression increased first and then returned to normal level. The maximum 5-HT$_1$ mRNA receptor expression occurred in 0.24 and 0.36 % treatment, respectively (Fig. 2b, c). Finally, from the results above, it can be seen that the variation in 5-HT$_1$ mRNA receptor expression was consistent with the variation in 5-HT level.

GABA levels in tissues

5-HT level variation was detected by HPLC in shrimp of different dietary tryptophan level. GABA peaks in the collected sample extracts, and the corresponding external standards showed the same elution times (about 8.1 min). HPLC analysis showed that GABA level in the XO–SG significantly increased in 0.48 % treatment ($P < 0.05$, Fig. 3a). However, GABA level in the thoracic ganglia and brain showed no significant difference responding to dietary tryptophan level ($P > 0.05$, Fig. 3a). GABA level decreased in the hepatopancreas, but it increased in the stomach and intestinal tract after feeding 0.24 % and 0.36 % dietary tryptophan level ($P < 0.05$, Fig. 3b). Moreover, GABA level in the hemolymph dramatically decreased with dietary tryptophan level, and the maximum was in 0.36 % treatment ($P < 0.05$, Fig. 3c).

Discussion

Tryptophan is a nutritionally important essential amino acid and as a precursor of neurotransmitter 5-HT in animal. It has been reported that dietary tryptophan supplementation played a key role in physiological process regulation, such as feed intake, growth, aggressive behavior and hormonal secretion in some animals (Walton et al. 1984; Henry et al. 1992; Papoutsoglou et al. 2005a, b; Höglund et al. 2007; Tejpal et al. 2009; Laranja et al. 2010; Harlioğlu et al. 2014), but there are no direct data on the effects of dietary tryptophan on *L. vannamei* growth performance and neurotransmitter. The present study demonstrates for the first time the effect of dietary tryptophan on growth performance, tissue 5-HT, GABA levels as well as the expression level of 5-HT receptor of juvenile *L. vannamei*.

The growth performance results showed that dietary tryptophan supplemental was contributed to *L. vannamei* growth. The control showed the poorest growth compared with the tryptophan treatments, and the best growth was observed in 0.60 % tryptophan supplementation diets. It suggested that fed much higher levels of tryptophan might better improve the shrimp growth in this diet. Our results were consistent with those observations that had been previously reported. For example, dietary supplementation of tryptophan showed a significant effect on the weight gain and specific growth rate in *Cirrhinus mrigala* fingerlings (Tejpal et al. 2009). In high-density groups, dietary tryptophan supplementation improved the growth parameters of fish (Höglund et al. 2007). Walton et al. (1984) found better growth and survival in rainbow trout fed tryptophan-supplemented diets than the control. The similar result showed that supplemental dietary tryptophan caused a significant increase in the growth and survival rate of freshwater crayifh *Astacus leptodactylus* Eschscholtz (Harlioğlu et al. 2014). However, a decrease in body weight and total length was observed in tryptophan-supplemented groups than in the control group in mud crab and grouper (Kim et al. 1987; Laranja et al. 2010), but the phenomenon did not occur in this study. It was thought that tryptophan supplementation could evoked the amino acid imbalance may result in inhibition of food intake and the impaired growth
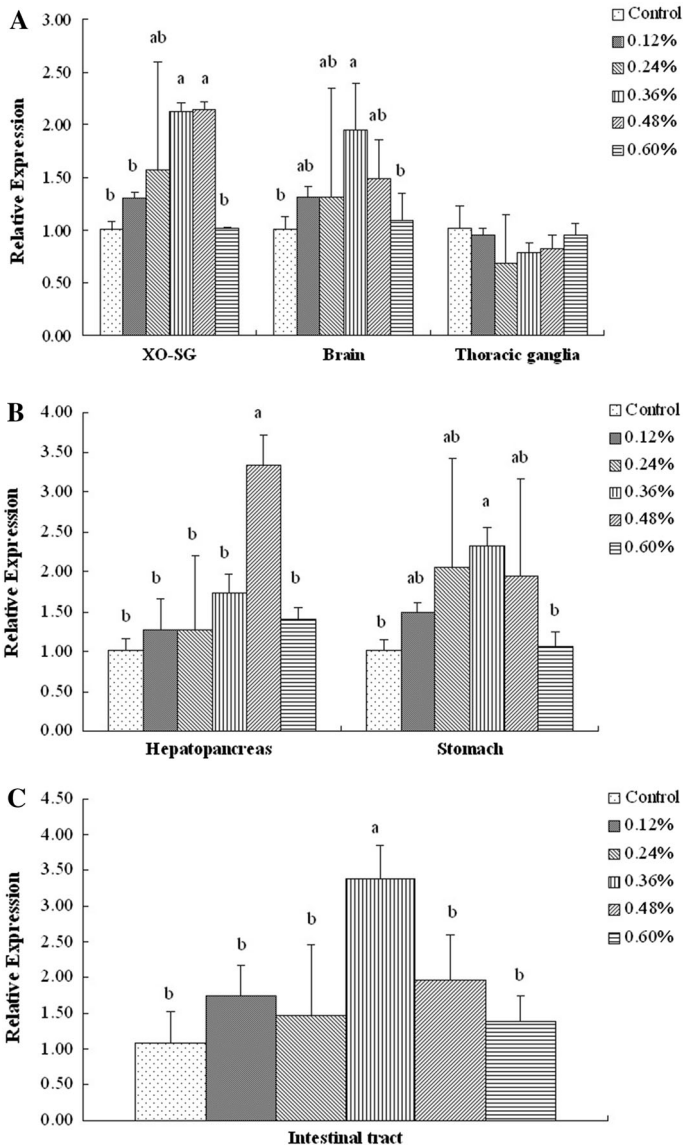
Fig. 2 **a** Effects of dietary tryptophan level on 5-HT$_1$ receptor expression in XO–SG, brain and thoracic ganglia after 60 days of feeding. **b** Effects of dietary tryptophan level on 5-HT$_1$ receptor expression in hepatopancreas and stomach after 60 days of feeding. **c** Effects of dietary tryptophan level on 5-HT$_1$ receptor expression in intestinal tract after 60 days of feeding The data shown represent the mean ± SD. ^{a,b}Values with *different letters* indicate significantly different ($P < 0.05$) by one-way ANOVA test

(Papoutsoglou et al. 2005b). Also, it may be attributed to serotonin-mediated inhibition of normal feeding behavior (de Pedro et al. 1998). These inconsistent results indicate that tryptophan exerts different effects in different animals under different experimental conditions. Tryptophan is also involved in protein and lipid metabolism in animals (Sainio et al. 1996). In the present study, tryptophan could enhance the whole body crude protein
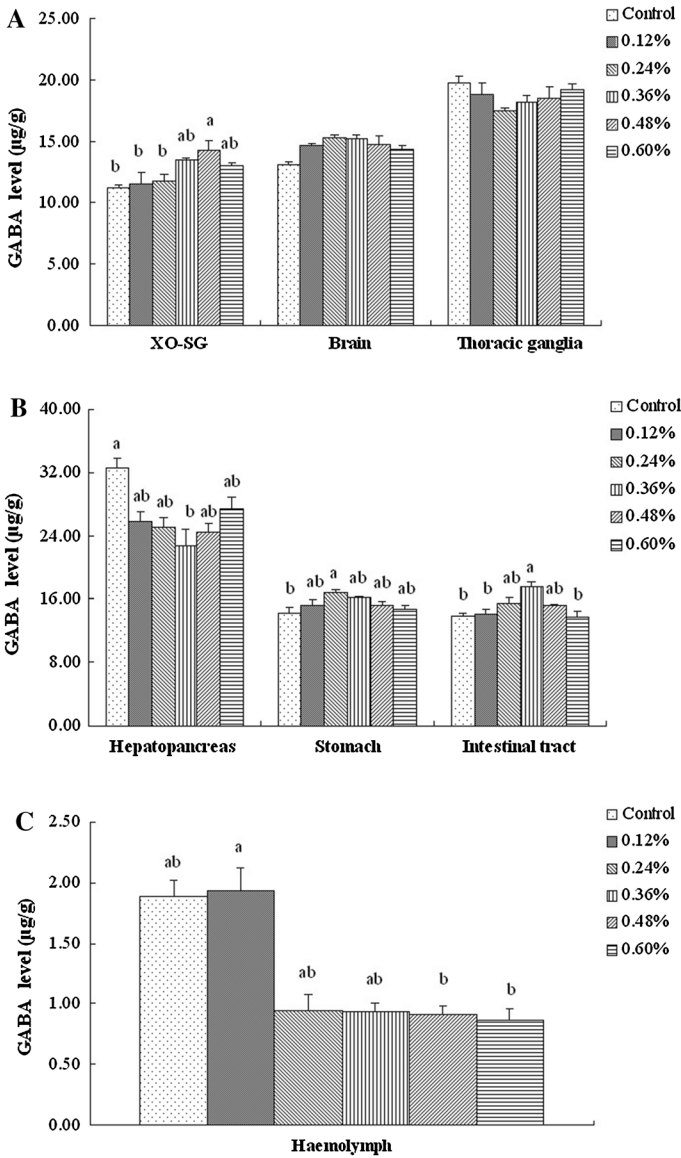
Fig. 3 **a** Effects of dietary tryptophan level on GABA in XO–SG, brain and thoracic ganglia by RP-HPLC after 60 days of feeding. **b** Effects of dietary tryptophan level on GABA in hepatopancreas, stomach and intestinal tract by RP-HPLC after 60 days of feeding. **c** Effects of dietary tryptophan level on GABA in hemolymph by RP-HPLC after 60 days of feeding The data shown represent the mean ± SD. [a,b]Values with *different letters* indicate significantly different ($P < 0.05$) by one-way ANOVA test

and moisture proportions ($P > 0.05$) and reduce the crude lipid levels ($P > 0.05$). The similar results were obtained in groupers (Kim et al. 1987). This might be associated with the effect of tryptophan on 5-HT. 5-HT acts as a sensor that detects and determines the proportions of energy produced from protein and carbohydrate (Leathwood 1987; Le

Floc'h and Seve 2007). Tryptophan together with elevated 5-HT levels could speed up body crude lipid peroxidation. However, a reduction in body protein, increased lipid content was observed in juvenile rainbow trout when fed higher levels of tryptophan (Papoutsoglou et al. 2005b), and this inconsistent in different animals still needs to be explained.

The present study showed that tryptophan-supplemented diets of *L. vannamei* caused significantly lower FCR. The FCR of *L. vannamei* fed control was 2.03, significantly higher than that of shrimp fed 0.36 % dietary tryptophan level 1.88. Henry et al. (1992) also obtained lower FCR in tryptophan-supplemented diets of pigs. Nevertheless, the FCR in the present trial were a bit higher than those reported by some authors (Davis and Arnold 2000; Ye et al. 2012), but similar to those examined by others (Suárez et al. 2009; Molina-Poveda et al. 2013). The possible reasons for the higher FCR may be due to the recirculating seawater system provided with passed through sand-filtered water and equipped with vigorous aeration. During the feeding period, the water flow rate continuously kept at 2.5–2.8 L/min only closed the feeding time. Under this conditions, the feed is the mainly food to sustain shrimp growth. In addition, the apparent satiation fed style would lead to the higher FCR.

Given the above, the possible reasons for improved growth performance and FCR observed for *L. vannamei* seems to be related to dietary tryptophan level. At present, the optimum tryptophan of *L. vannamei* has not been reported but some other crustaceans like P.monodon, crayfish, mud crab (Pascual and Kanazawa 1986; Akiyama et al.1991; Han et al. 1995; Millamena et al. 1999; Laranja et al. 2010; Harlioğlu et al. 2014). In the present study, basal diet supplemented tryptophan more than 0.36 in % dry diet or 1.72 in % protein gained better growth, and it was close to the suggested tryptophan levels for shrimps by Pascual and Kanazawa (1986), but higher than the others recommended (Akiyama et al. 1991; Han et al. 1995; Millamena et al. 1999; Laranja et al. 2010; Harlioğlu et al. 2014). From the economic standpoint, dietary with cheaper tryptophan in a practical diet for shrimp can alleviate the problem of low survival rate and high cost. Tryptophan can improve shrimp growth performance while the present cost of most commercial tryptophan is US$ (17–20)/kg. Therefore, further research will be needed to determine optimum shrimp dietary tryptophan in different protein feed.

5-HT is a neurotransmitter occurs in the brain, and also in platelets and the gut (Elofsson et al. 1982; Laxmyr 1984). As the precursor of 5-HT, the relationship between tryptophan and 5-HT is usually believed to the mechanism involved in the effect of tryptophan-supplemented diet on growth, aggression and survival (Winberg et al. 2001; Hseu et al. 2003; Lepage et al. 2005; Papoutsoglou et al. 2005b; Tejpal et al. 2009; Wolkers et al. 2012; Harlioğlu et al. 2014). So 5-HT levels in shrimp after tryptophan supplement were detected in this study. In this study, tryptophan supplemental increased 5-HT levels in several tissues except thoracic ganglia (Fig. 1a). Higher tryptophan supplemented in diets increases its availability for uptake, resulting in elevating body tryptophan levels, which may enhance 5-HT synthesis (Johnston et al. 1990). However, a greater decrease in 5-HT concentration was observed above 0.48 % than below 0.36 % tryptophan administrated dietary, as reported in previous (Henry et al. 1992). Tryptophan supplementation has also been shown to have a lagging effect on elevating 5-HT levels in different tissues. It is possible due to endocrine cells in the gill epithelium of different tissues have the capacity for uptake, storage and synthesis of 5-HT (Olson 1998). Tryptophan is 10 times more likely to enter kynurenine pathways than 5-HT pathways (Sainio et al. 1996). The proportion of tryptophan metabolized into kynurenine increased when dietary tryptophan supply increased, and this resulted in the increase of 5-HT level may not be associated with the

increase in tryptophan supply. Additionally, 5-HT changes in the thoracic ganglia were not in agreement with other tissues. Crustacean thoracic ganglia have been considered to be the source of a vitellogenesis-stimulating hormone (Alfaro and Vega 2010). Moreover, 5-HT is noted to stimulate ovarian maturation in crustaceans (Meeratana et al. 2006, Tinikul et al. 2008). With higher tryptophan supplementation, it is possible that the tryptophan requirement in shrimps for body protein deposition is greater than its requirement for 5-HT synthesis, leads to reduce 5-HT in the thoracic ganglia and promotes body nutrient deposition. On the other hand, enough nutrient deposition would facilitate gonadal development and result in 5-HT elevation in the thoracic ganglia. Therefore, reduction in 5-HT level in thoracic ganglia contributes to inhibition of gonadal development and body nutrient deposition. However, the mechanism still needs to be further studied.

Diverse physiological effects of 5-HT are probably mediated through various types of serotonergic receptors, and 5-HT level is controlled by down-regulation of its auto-receptor sensitivity and in turn results in up-regulation of 5-HT neurotransmission, especially since tryptophan availability is augmented (Tiu et al. 2005). $5-HT_1$ receptor is potentially involved in many physiological processes such as growth, muscle contraction, immune response, reproduction and escape behavior (Ongvarrasopone et al. 2006; Martin Marti et al. 2010). Hence, it was chosen to analyze response to tryptophan supplementation. $5-HT_1$ receptor expression corresponded to 5-HT levels (Fig. 1). These findings suggested that 5-HT may mediate the physical roles through an interaction with the putative $5-HT_1$ receptors in this study. Nevertheless, it is believed that the interaction between 5-HT and its receptors needed further investigation.

GABA is also an important neurotransmitter and coexist with 5-HT in the paracrine/hormone systems (Santhoshi et al. 2008). GABA is synthesized principally from glutamate in a single enzymatic step catalyzed by glutamate decarboxylase and that is reported to play a role in tryptophan-induced serotonergic activity (Ciranna 2006). Since supplemental tryptophan generally increased the 5-HT content, the decrease in the treatment with higher tryptophan dietary supplementation may be interpreted as an inhibitory effect of GABA on 5-HT synthesis, or stimulatory effect of GABA on 5-HT degradation. In the present study, HPLC analysis showed that GABA level in different tissues changed with tryptophan supplemented, and corresponded to variations in 5-HT, except in hepatopancreas and hemolymph (Fig. 3). This demonstrated a possible role of GABA in the regulation of 5-HT synthesis. The similar results reported in previous studies; for example, 5-HT release was considered to be inhibited by activation of GABA or their respective auto-receptors (Gebauer et al. 1993). However, it was discrepancy at present study. Some reasons could explain this phenomenon. First, GABA probably reduced the toxicity caused by excessive tryptophan. Furthermore, high brain glutamine concentrations are known to stimulate blood–brain barrier transport of large neutral amino acids such as tryptophan, which in turn can increase 5-HT turnover (Papoutsoglou et al. 2005a). However, the glutamate produced through GABA pathway is not available to pass through the blood–brain barrier into brain. In addition, 5-HT regulates the synthesis and secretion of GABA by acting on its receptors (Tao et al. 1996). So far, it remains to be determined whether a similar glutamine-stimulated increase in brain tryptophan uptake or a different mechanism is responsible for 5-HT and GABA variation.

In conclusion, dietary tryptophan supplementation is beneficial to shrimp growth and survival and affects 5-HT, $5-HT_1$ receptor mRNA expression, as well as GABA levels in different tissues. It suggested that diet supplemented with tryptophan was beneficial to improve growth performance possibly by mediating 5-HT as well as GABA pathway in *L.vannamei*. The current experiment indicates that dietary (analysis protein 46 %,

tryptophan 0.43 %) supplemented tryptophan above 0.36 % dry diet or 1.72 % protein diet may gain better growth performance in *L. vannamei*. It is promising that tryptophan will play an important role in *L. vannamei* production. These data provide new insight into the physiological functions of tryptophan in shrimp.

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