Thesis for the Degree of Master of Science

Dietary Thiamine (Vitamin B_1) Requirement of Pacific White Shrimp (*Penaeus vannamei*)

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Department of Marine Life Science
The Graduate School
Jeju National University

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A Thesis submitted to the Graduate School of Jeju National University in partial fulfillment of the requirements for the Degree of Master of Science under the supervision of **Professor Kyeong-Jun Lee**

The Thesis for the degree of Master of Science by **Bopitige Udith Ravindu Wijemanna** has been approved by the dissertation committee

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TABLE OF CONTENT

Content **Page**

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ABSTRACT

Present two studies investigated the dietary thiamine (vitamin B_1) requirement of Pacific white shrimp (*Penaeus vannamei*) at two different stages of their life cycle. For both experiments, a fish meal based basal diet (T0) was formulated and five other test diets were formulated by incorporating thiamine hydrochloride at graded levels of 20, 40 60, 80 and 100 mg/kg of diet, at the expense of cellulose (designated as T0, T20, T40, T60, T80, and T100).

Chapter 1 investigated the dietary thiamine (vitamin B_1) requirement of juvenile *P. vannamei* and its effects on growth performance, feed utilization, innate immunity, and intestinal histomorphology. A Total of 360 shrimp (6.03 ± 0.03 g) were randomly assigned into 24 acryl tanks (240 L) to be quadruplicated per treatment, and feeding was done in six equal portions daily for 58 days. High-performance liquid chromatography of the test diets revealed that the thiamine concentration of T0, T20, T40, T60, T80, and T100 experimental diets at 3.32, 20.8, 38.7, 60.2, 80.7, and 108 mg/kg of diet respectively. The T20 diet fed group exhibited significantly higher growth performance compared to shrimp fed T0 and T100 diets. Feed conversion ratio (FCR) and protein efficiency ratio (PER) were not significantly different among experimental groups. A significantly higher phenoloxidase activity was exhibited in T20 dietary group than T0 and T100 groups. Lysozyme activity was significantly enhanced in

T20, T40, T60, and T80 groups than in T0 group and antiprotease activity was not significantly affected. Antioxidant parameters: superoxide dismutase activity (SOD) and glutathione peroxidase (GPx) activities were significantly elevated in T20 dietary treatment group. Significantly upregulated proPO gene expression was observed in T20 and T40 dietary treatment groups, although penaiedin 3a and insulin-like growth factor-1 (IGF-1) relative gene expressions were not significantly different. Glucose, total cholesterol, triglyceride, and total protein levels in hemolymph were not significantly different. Intestinal histology observations did not show significant differences in villi heights. A piecewise regression of weight gain percentage (WG%) estimates the optimal dietary thiamine requirement of *P. vannamei* at 19.7 mg/kg of diet.

Chapter 2 investigated the dietary thiamine (vitamin B_1) requirement of post larvae *P. vannamei* and its effects on growth performance, feed utilization, innate immunity, digestive enzyme activity and ammonia stress tolerance. High-performance liquid chromatography of the test diets revealed that the thiamine concentration of T0, T20, T40, T60, T80, and T100 experimental diets at 6.14, 28.5, 48.1, 73.3, 85.7 and 106 mg/kg of diet, respectively. A total of 1200 *P. vannamei* post larvae (5 mg) were randomly distributed into 24 acryl tanks (50 shrimp/tank, 10 L) with four replicates per treatment. Daily feed requirement was divided into six equal portions and the feeding was done at 08:00, 10:00, 12:00, 14:00, 16:00, and 18:00 h for 30 days. After the feeding trial, 60 shrimp from each dietary treatment were captured, pooled and redistributed into three replicate tanks (10 L) for the ammonia stress challenge test. Thiamine supplemented groups showed a significant improvement in growth performance and feed utilization. Final body weight of T40, T60, T80 and T100 dietary groups were significantly higher compared to the T0 group and both WG% and specific growth rate followed a similar trend. Significant enhancements in FCR were observed in T40, T60, T80 and T100 groups and PER was significantly elevated in thiamine supplemented groups (T60, T80, T100) compared to the thiamine deficient group (T0). Survival percentage was not

significantly different among the experimental groups. Thiamine supplementation significantly enhanced the activity of digestive enzymes (amylase, lipase, pepsin) and the significantly lowest activities were observed in T0 group. Compared to the thiamine deficient group, relative gene expression of IGF-1, insulin like growth factor binding protein (IGF-BP) gene expressions were significantly upregulated in thiamine supplemented groups. Antioxidative gene expressions (catalase, SOD, GPx) were significantly upregulated through thiamine supplementation. Similarly innate immune related genes (proPO, crustin) were significantly expressed in thiamine supplemented groups. In the challenge test, after 48 h, a significantly higher survival rate was observed in T80 dietary group while thiamine deficient group manifested the significantly lowest survival. A broken line regression of WG% estimated the dietary thiamine requirement of *P. vannamei* post larvae at 72.9 mg/kg of diet.

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LIST OF TABLES

Content **Page**

LIST OF FIGURES

Content **Page**

1.0 Introduction

1.1 Thiamine

In 1911, a Polish American biochemist, Dr. Casimir Funk was able to crystallize an amine compound from rice polishings, which was identified as the anti-beriberi factor, known as “vital amine” or “vitamin”. Thiamine, a highly water-soluble vitamin which is the first to be identified and isolated, therefore known as vitamin B_1 (Fattal-Valevski 2011). In 1926, Thiamine was successfully isolated by Barend Jansen and Willem Donath and introduced as “aneurine” (Carpenter 2012). Later, in 1936, Williams and Cline reported the first correct formula and synthesis for the vitamin. The structure of thiamine hydrochloride has a pyrimidine ring and a thiazole ring, which are linked through a methylene bridge, chemically known as $C_{12}H_{18}ON_4SCl_2$ (Fattal-Valevski 2011).

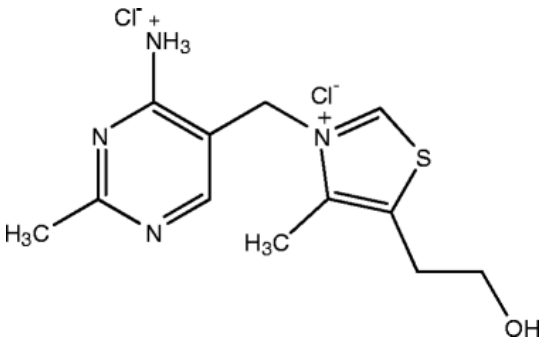

Figure 1. Molecular structure of thiamine hydrochloride (Pawar et al. 2013).

1.2 Biological importance of Thiamine

Thiamine is converted to its phosphorylated form, thiamine pyrophosphate (TPP) by the action of thiamine diphosphokinase enzyme. TPP functions as the bioactive class of thiamine, which is important in the enzymatic activities of carbohydrate, lipids, and branched-chain amino acid metabolism serving as a cofactor for enzymes: transketolase (TK), pyruvate

dehydrogenase complex (PDC) and α-ketoglutarate dehydrogenase complex (α-KGDC) (Jones et al. 2021; Martel et al. 2021) which are important in generating energy in the form of ATP. In the metabolism of carbohydrates, PDC catalyzes the conversion of pyruvate into acetyl-CoA, which is the end product of glycolysis and required to initiate TCA cycle (Zhou et al. 2022). It is evident that thiamine requirement changes with carbohydrate content in the diets. Diets which are rich in protein and fat may have a thiamine sparing effect, however, high carbohydrate diets may increase the thiamine requirement for the conversion glucose into energy through the TCA cycle (Markovich et al. 2013). Moreover, when increasing the amounts of plant-based materials with high starch levels in the diets of gilthead seabream (*Sparus aurata L.*), the thiamine requirement of also increased (Morris & Davies 1995). Furthermore, McDowell (1989) found that the thiamine requirement was decreased when carbohydrates in the diets were replaced by fat, which may be related to the energy metabolism in animals. Recent studies have found that thiamine requirement increases when the organism is under stress conditions due to intracellular disturbances, which may obstruct carbohydrate and energy metabolism (Zera et al. 2016; Zhou et al. 2022). Moreover, these thiamine-activated enzymes are essential for breaking down carbohydrates into other types of molecules, biosynthesizing brain chemicals such as neurotransmitters, synthesizing pentoses, production of fatty acids and steroids and production of oxidant stress defense equivalents (Martin 2001; Sun et al. 2022).

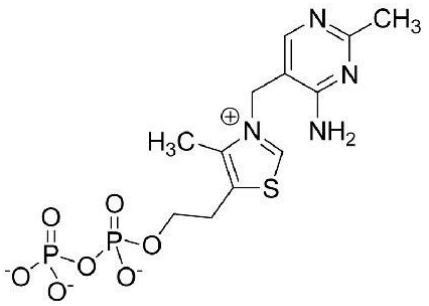

Figure 2. Molecular structure of thiamine pyrophosphate (TPP) (Demir et al. 2007).

Physiological processes may be hindered or inhibited if organisms do not receive adequate amount of thiamine in their diets. For example, a lack of thiamine can cause accumulation of pyruvate in mammals, which affects the biosynthesis of fatty acids and steroids (Martin et al. 2003) and may result in neurological interruptions (Huang et al. 2007). Thiamine deficiency leads to reduced activity of α-KGDC, which may result in reduced ATP synthesis and oxidative damage, followed by cell death (Whitfield et al. 2018). NRC (2011) reported that symptoms of thiamine deficiency in fish species include congested fin and skin, dark skin coloration and depigmentation, hemorrhaging, hyperirritability, loss of equilibrium and nervous disorders. High mortality levels were observed in Nile tilapia (*Oreochromis niloticus*) that were fed a thiamine-deficient diet (Lim et al. 2011). Naturally, plants, algae, and bacteria can synthesize thiamine, and are then consumed by aquatic animals to acquire their thiamine requirement. In aquaculture industry, the environment is artificially controlled, and moreover, the gut microorganisms can only synthesize limited quantities of thiamine (Harder et al. 2018; Sannino et al. 2018). Therefore, thiamine hydrochloride or thiamine mononitrate are often included in the diets to supplement the thiamine requirements of aquaculture organisms (Paul et al. 2010, Sun et al. 2022).

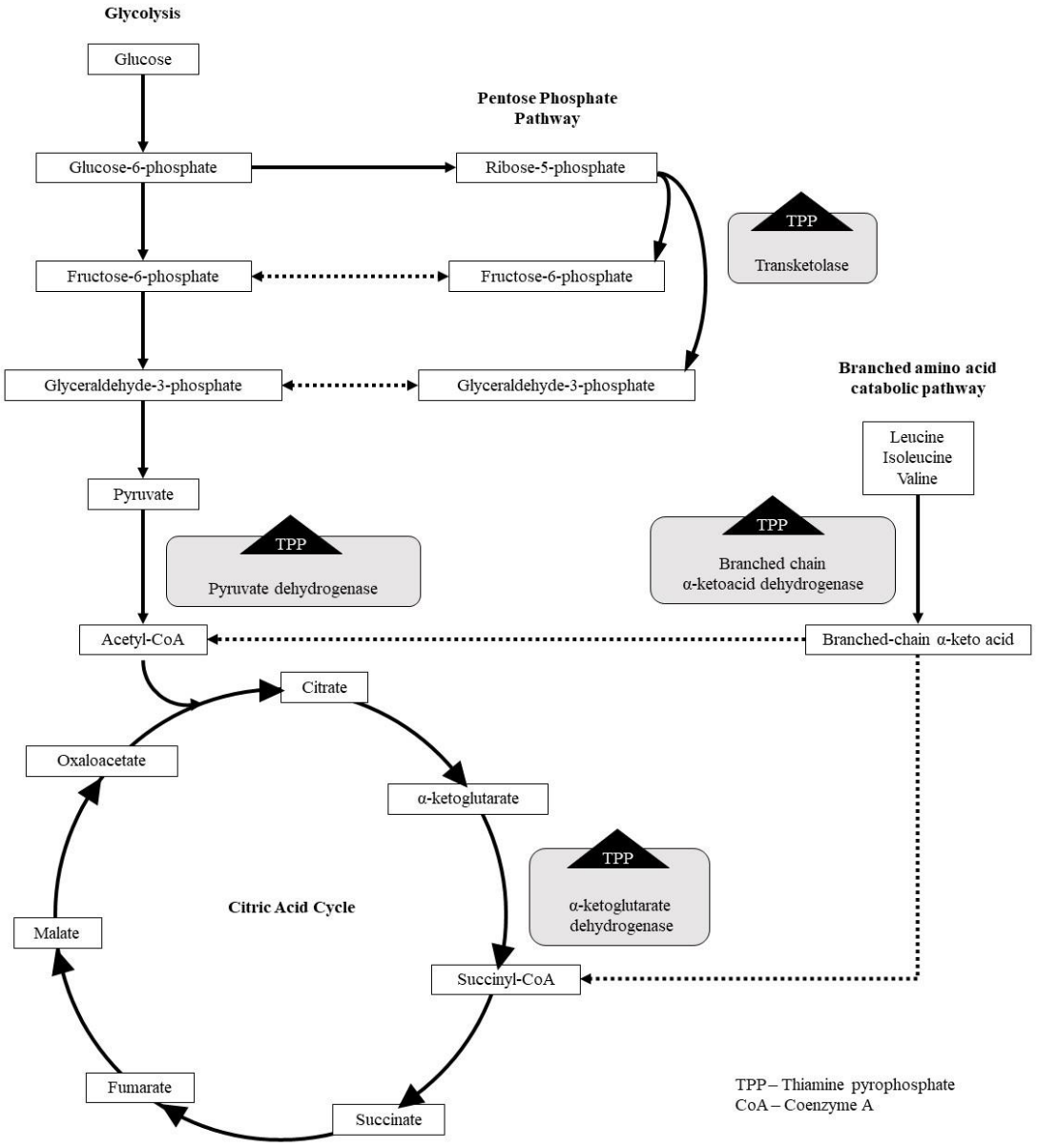

Figure 3. Metabolic processes requiring thiamine pyrophosphate (TPP).

1.3 Thiamine in aquaculture

At present, thiamine hydrochloride and thiamine mononitrate are successfully utilized as the dietary form of thiamine in aquaculture feed and nutrition trials (Halver 2003). Schneberger (1941) recorded the first study to use thiamine in fish nutrition. There, he observed that injecting crystalline thiamine cured disease conditions in rainbow trout. In aquatic environments, plants, algae, and bacteria can synthesize thiamine, and are then consumed by aquatic animals, to obtain sufficient levels of thiamine (Haas 1988; Harder et al. 2018; Sannino et al. 2018). However, it is suggested that thiamine must be supplemented in the dietary forms (thiamine hydrochloride or thiamine mononitrate), since gut microorganisms can only synthesize limited quantities (Mulholland 2006; Sun et al. 2022). An optimum level of thiamine reportedly enhanced the growth and feed utilization of aquatic animal species (Table 1), including, *Sclizothorax prenanti* (Xiang et al. 2016), grass carp (*Ctenopharyngodon Idella*) (Jiang et al. 2014), giant tiger shrimp (*Penaeus monodon*) (Chen et al. 1991), Oriental river prawn (*Macrobrachium nipponense*) (Sun et al. 2022), Kuruma shrimp (*Marsupenaeus japonicus*) (Deshimaru and Kuroki 1979), and abalone (*Haliotis discus hannai* Ino) (Zhu et al. 2002). In addition to the growth performance, thiamine supplementation improved the innate immunity and antioxidative capacity (Table 1) of spotted snakehead (*Channa punctata*) (Zehra and Khan 2018), *P. vannamei* (Huang et al. 2015), and Oriental river prawn (Zhou et al. 2022). According to NRC (2011), the optimum dietary thiamine requirement of fishes ranged between 0.5 to 15 mg/kg of diet, whereas shrimp species reported a higher requirement between 14 and 120 mg/kg of diet. Crustaceans are slow benthic feeders, with feed particles often suspended in water for long periods before consumption (Conklin 1989). Manipulation of feed particles using shrimp appendages during feeding may also increase nutrient leaching, thereby increasing their thiamine requirement compared to fish (Chen et al. 1991). Accordingly, it is obvious that thiamine is essential for growth and development of aquatic animals.

1.4 Pacific white shrimp

The Pacific white shrimp (*Penaeus vannamei*) is an important tropical marine species with a significant economic value, accounting for approximately 51.7% of the total crustacean production globally in 2020 (FAO 2022). It is a popular choice in aquaculture mainly due to higher growth rate, resistance to viral diseases such as IHHN and WSSV and adaptability to a broad range of temperature and salinity levels compared to other species of shrimp (Cuzon et al. 2004; Li et al. 2017; Li et al. 2018). However, there is only limited scientific data with regard to the thiamine requirement of this shrimp species. Therefore, these studies were conducted to investigate the dietary thiamine requirement and its effects in two different life stages of *P. vannamei*.

Table 1. Dietary thiamine requirement of selected aquatic animal species.

Species	Requirement (mg/kg)	Enhanced response criteria	Reference
Litopenaeus vannamei	44.66 - 152.83	WG, SGR, FE, PER, SOD, catalase and lysozyme activity	Huang et al. (2015)
	23.90 - 23.70	SGR, FCR, TKA, Survival rate	He (2010)
Macrobrachium nipponense	66.08 - 67.57	WG, SGR, PER, TKA, hemolymph thiamine and triglycerides concentrations	Sun et al. (2022)
	70.70	Carbohydrate metabolism	Zhou et al. (2022)
	143.17 - 161.20	Antioxidant capacity	
Penaeus monodon	14	WG	Chen et al. (1991)
Marsupenaeus japonicus	60 - 120	WG	Deshimaru and Kuroki (1979)
Fenneropenaeus indicus	100	WG	Boonyaratpalin (1998)
Channa punctatus	2.34 - 2.59	WG, PER, RNA/DNA ratio, FCR, TKA, SOD, catalase activity, liver thiamine concentration	Zehra and Khan (2018)
Sclizothorax prenanti	18.45 - 25.91	WG, SGR, FE, PER, TKA, liver thiamine concentration, serum triglyceride and total cholesterol content	Xiang et al. (2016)
Ctenopharyngodon idella	1.3 - 5.0	WG, SGR, FE, PER, serum triglycerides, total cholesterol, glucose, pyruvate contents, lactate dehydrogenase activity	Jiang et al. (2014)
Ictalurus punctatus	1.0	WG	Murai & Andrews (1978)
Haliotis discus hannai Ino	58 - 61	WG, TKA, TPP in viscera and muscle	Zhu et al. (2002)

WG, weight gain; SGR, specific growth rate; FE, feed efficiency; PER, protein efficiency ratio; SOD, superoxide dismutase; FCR, feed conversion ratio; TKA, transketolase activity; TPP, thiamine pyrophosphate

CHAPTER 1

Dietary thiamine requirement and its effects on growth performance, feed utilization, and innate immunity of Pacific white shrimp (*Penaeus vannamei*) juveniles

2.1 Materials and methods

2.1.1 Experimental diets

Formulation of the six experimental diets (crude protein: 32.9%, crude lipid: 8.38%) and the proximate compositions are provided in Table 2. A fish meal based basal diet (T0) with no thiamine added was formulated. Five other experimental diets were prepared by incorporating graded levels of thiamine hydrochloride (67-03-8, Sigma-Aldrich, St. Louis, MO, USA) into the basal diet at the levels of 20, 40, 60, 80 and 100 mg/kg of diet (designated as T20, T40, T60, T80 and T100, respectively), replacing cellulose. The main protein sources were fish meal, soybean meal and squid liver meal. Initially, all the dry ingredients of each experimental diet were weighed separately into a feed mixing bucket and were hand-mixed thoroughly. Then the mixture was transferred to a feed mixer (NVM-16, Gyeonggido, Korea), where fish oil (lipid source) and water (15%) was incorporated and mixed adequately. Then, the resultant moist dough was pelleted (2 mm) through a feed pelleting machine (SP-50, Kum Kang Engineering, Korea). Feed pellets were dried at 24-25°C for 8 h and stored at −20°C until used.

The proximate composition of the diets was analyzed following the protocols mentioned in AOAC (2005). Moisture content was measured by drying 2 g of samples in a heated oven at 125°C until obtained a constant weight. Crude ash content was determined by burning 2 g of samples in a muffle furnace at 550°C for four hours. According to Folch et al. (1957), crude lipid was quantified gravimetrically after extraction with chloroform-methanol

mixture. Crude protein levels were analyzed by the Kjeldahl method in a Kjeltec 2300 semi-automated system (FOSS, Hilleroed, Denmark). First, samples were acid hydrolyzed by concentrated sulfuric acid. Then distillation process was conducted using Kjeltec system with Sodium hydroxide. Released ammonia gas was captured by 1% boric acid and titrate with 0.1N HCl.

Thiamine concentrations of the diets were analyzed by high-performance liquid chromatography (HPLC) according to Zhu et al. (2002) with slight modifications and determined to be of 3.32, 20.8, 38.7, 60.2, 80.7 and 108 mg/kg (Table 2) for T0, T20, T40, T60, T80 and T100, respectively. Briefly, approximately 0.2 g of diet samples were homogenized in 9 mL of 0.01 M HCL, followed by the addition of 10% trichloroacetic acid (1 mL) and centrifuged at 10,000 × g for 10 min at 4°C for protein precipitation. Then, water-saturated diethyl ether was used to wash the resulting supernatant, allowed for evaporation, and filtered through a 0.45 μm syringe filter. Solutions were derivatized with 1M NaOH and freshly prepared 1% potassium ferricyanide prior injecting into the column (4.6 × 250 mm, Waters Atlantis). Mobile phase consisted of 25 mM potassium phosphate dibasic and 100% methanol in a 50:50 ratio, and the flow rate was set to 1 mL/min. Thiamine was detected using a fluorescence detector at excitation and emission wavelengths of 360 nm and 425 nm, respectively. Thiamine hydrochloride was dissolved in distilled water as standard, and the concentration was expressed as mg/kg.

Figure 4. Experimental diet preparation for Pacific white shrimp (*Penaeus vannamei*).

Table 2. Formulation and proximate composition of the experimental diets for Pacific white shrimp (*Penaeus vannamei*) (g/kg, dry matter basis).

Ingredients	Experimental Diets					
	T0	T20	T40	T60	T80	T100
Fish meal (sardine)	50.0	50.0	50.0	50.0	50.0	50.0
Fish meal (tuna)	50.0	50.0	50.0	50.0	50.0	50.0
Soybean meal	400	400	400	400	400	400
Squid liver meal	50.0	50.0	50.0	50.0	50.0	50.0
Wheat flour	180	180	180	180	180	180
Starch	140	140	140	140	140	140
Fish oil	50.0	50.0	50.0	50.0	50.0	50.0
Mineral Mix[1]	20.0	20.0	20.0	20.0	20.0	20.0
Vitamin Mix[2]	10.0	10.0	10.0	10.0	10.0	10.0
Cholesterol	2.00	2.00	2.00	2.00	2.00	2.00
Lecithin	10.0	10.0	10.0	10.0	10.0	10.0
Monocalcium phosphate	30.0	30.0	30.0	30.0	30.0	30.0
Thiamine hydrochloride[3]	0.00	0.02	0.04	0.06	0.08	0.10
Cellulose	8.00	7.98	7.96	7.94	7.92	7.90
Proximate composition (g/kg)						
Dry matter	939	938	939	940	939	937
Crude protein	326	325	322	323	324	325
Crude lipid	92.3	98.7	96.5	98.9	100	101
Crude ash	94.0	93.9	93.7	94.6	94.0	94.9
Thiamine content (mg/kg)	3.32	20.8	38.7	60.2	80.7	108

[1]$MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; $CuCl_2$, 0.2; $AlCl_3$. $6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0. [2]Beta-carotine, 0.57; riboflavin, 6.89; niacin, 18.0; pantothenate, 18.0; pyridoxine hydrochloride, 7.2; cyanocobalamin, 0.09; ascorbic acid 100.0; cholecalciferol, 144.0; alpha tocopheryl acetate, 20.0; menadione, 0.9; biotin, 0.9; folic acid, 7.2; inositol, 45.
[3]Xi'an Julong Bio-Tech Co., Ltd, Xi'an, China.

2.1.2 Shrimp selection and feeding trial

Five-day old post larvae (P.L. 5) were bought from Daedong Susan Inc., Muan, South Korea and reared in aquarium tanks until they reached the desired size by supplying a commercial diet (40% protein, 6% lipid, Woosung premium aqua feed, Korea), to their apparent satiation. Shrimp were then acclimated to experimental system and conditions for two weeks prior to the beginning of the trial, by providing the same commercial diet. After acclimation, shrimps (6.03 ± 0.03 g) were randomly allotted into 24 tanks (240 L) with a density of 15 shrimp per tank. Each experimental diet was assigned to four replicate tanks and the daily feed requirement was split into six equal feedings, implemented at 08:00, 10:00, 12:00, 14:00, 16:00, and 18:00 h, continued for 58 days. The shrimp in each experimental tank were weighed and counted fortnightly to determine the growth performance, and the feeding amount was adjusted to be 6-12% of the biomass, aimed at feeding to apparent satiation. To determine the actual feed intake, uneaten feed pellets were removed 30-40 minutes after feeding by siphoning, and dry weight was subtracted from the amount of diet provided. To maintain total ammonia nitrogen (0.04 ± 0.03 ppm) (Strickland and Parsons 1972) below toxic levels, experimental water (70%) in each tank was replaced with sand-filtered pre-heated seawater every third day throughout the experimental period. The water quality of the experimental setup was recorded and maintained as follows: temperature (29.18 ± 0.1 ºC) and dissolved oxygen (7.40 ± 0.12 ppm) levels (Pro20 Dissolved Oxygen Meter, YSI, Yellow springs, OH, USA) were monitored daily, and salinity (33.27 ± 0.40 ppt) (ATAGO™ Master-S28M Salinity Refractometer, Japan) and pH (6.49 ± 0.27) (SUNTEX TS-1 Classic Portable pH·mV Meter, Taiwan) levels were recorded weekly.

Figure 5. Experimental setting for the feeding trial of Pacific white shrimp (*Penaeus vannamei*).

2.1.3 Sample collection

After eight weeks of the experiment, feeding was ceased 12 h before the final sampling. The number of shrimps in each tank was counted, and individual weight was recorded to evaluate growth and feed utilization-related parameters. Three intermolt shrimps per tank (12 shrimps per dietary treatment) were captured arbitrarily, anesthetized by placing them in iced water for a few minutes and hemolymph was collected from the sinus located in the shrimp cephalothorax using a 1 mL syringe (25-gauge needle) containing 0.1 mL of Alsever's anticoagulant solution (A3551, Sigma-Aldrich, USA). After the collection, more anticoagulant solution was mixed to become a 1:1 ratio by hemolymph volume. Collected hemolymph samples were centrifuged (800 × g at 4°C for 20 min), and the supernatant was stored at −80°C for further analysis. Hemolymph was analyzed for immune parameters, antioxidative parameters, and other biochemical parameters: total protein, total cholesterol (TCH), triglyceride (TG), and glucose levels using a semi auto chemistry analyzer (SLIM, SEAC Inc, Florence, Italy) and relevant kit regents (Stanbio Laboratory, Texas, USA) following manufacturer's instructions.

$$\text{Total cholesterol (mg/dL)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \text{x200}$$

$$\text{Glucose (mg/dL)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \text{x100}$$

$$\text{Total protein (g/dL)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \text{x10}$$

$$\text{Triglyceride (mg/dL)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \text{x200}$$

Following the hemolymph collection, hepatopancreas and intestines were sampled separately by dissecting the same three individual shrimps of each tank, under sterile conditions. Hepatopancreas samples were immediately flash-frozen in liquid nitrogen and stored at −80°C for later use in relative gene expression and the intestine samples were stored in Davidson's fixative solution for histological observations.

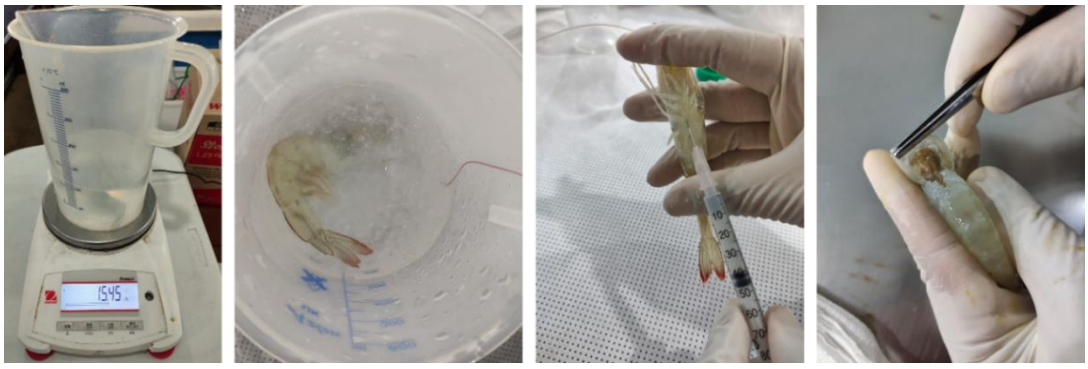

Figure 6. Weight measurement and sample collection from Pacific white shrimp (*Penaeus vannamei*) during final sampling.

2.1.4 Analysis of non-specific immune responses

2.1.4.1 Phenoloxidase activity

Phenoloxidase (PO) activity was determined spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA, Sigma-Aldrich) according to Hernández-López et al. (1996). In brief, 50 µL of hemolymph was incubated with trypsin solution in cacodylate buffer (CAC buffer, 10 mM sodium cacodylate, and 10 mM calcium chloride) at 25°C for 30 min. Afterward, 50 µL of L-DOPA (3 mg L-DOPA in 1 mL of CAC buffer) was added to the mixture and incubated for 30 min at 25°C, followed by optical density (absorbance value) measurement through a spectrophotometer (Multiskan^{TM} SkyHigh, Thermo Scientific, USA) at 492 nm wavelength. The optical density of the shrimp's phenoloxidase activity was expressed as dopachrome formation in 50 µl of cell-free hemolymph.

2.1.4.2 Lysozyme activity

A turbidimetric assay was carried out to assess the lysozyme activity, according to Hultmark (1980). In brief, 20 µL of hemolymph was incubated with 200 µL of bacterial suspension (0.75 mg of *Micrococcus lysodeikticus* in 1 mL of 0.1M sodium phosphate buffer, pH 6.4) at 37°C for 30 min. Decrease in the optical density was recorded using a spectrophotometer at 570 nm. Lysozyme from chicken egg white (12650-88-3, Sigma-Aldrich, USA) was used to design the standard curve. Lysozyme activity was measured by the reduction of bacteria due to enzyme activity during incubation period.

2.1.4.3 Anti-protease activity

Antiprotease activity was determined according to Ellis (1990) with slight modifications by Magnadóttir et al. (1999). Hemolymph (20 µL) was incubated with 20 µL of trypsin solution (10 mg trypsin dissolved in 2 mL of 50 mM Trizma® hydrochloride solution) at 22°C for 10 min. Then, the mixture was incubated at 22°C for 60 mins with phosphate buffer (200 µL) and 250 µL of azocasein protease substrate (102110-74-7, Sigma-Aldrich, USA) solution (2 g of azocasein dissolved in 100 mL of 0.1M NaOH solution). Afterward, the mixture was incubated for 30 mins at 22°C with trichloro acetic acid solution (10%, 550 µL), subsequently centrifuged at 6000 × g for 5 min. To conclude the reaction, 100 µL of 1M NaOH was added to 100 µL of the supernatant. The inhibition rate was calculated by reading the optical density at 430 nm using a spectrophotometer. The trypsin inhibition percentage was calculated using the following equation,

$$\text{Trypsin inhibition (\%)} = \frac{A1 - A2}{A1} \text{ x } 100$$

Where A1 = Control trypsin activity (without serum); A2 = Activity of trypsin remained after serum addition.

2.1.4.4 Superoxide dismutase activity

Serum superoxide dismutase (SOD) activity was measured by the percentage reaction inhibition rate of enzyme with WST-1 (Water Soluble Tetrazolium dye) substrate and xanthine oxidase using a SOD Assay Kit (Sigma-Aldrich, 19160) according to the manufacturer's instructions. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide) after 20 min of reaction time at 37°C. The percentage inhibition was normalized by mg protein and presented as SOD activity units.

2.1.4.5 Glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was assayed using kit (Biovision, Inc. California, USA). In this assay, cumene hydroperoxide was used as a peroxide substrate (ROOH), and glutathione reductase and NADPH (reduced nicotinamide adenine dinucleotide phosphate) were included in the reaction mixture. The change in 340 nm due to NADPH oxidation was monitored and was indicative of GPx activity. Briefly, 50 µl of serum was added to 40 µl reaction mixture and incubated for 15 min and then 10 µl cumene hydroperoxide was added and OD1 read at 340 nm. After 5 min of incubation OD2 read in 340 nm by a microplate reader. Activity of GPx was calculated as mU/mL.

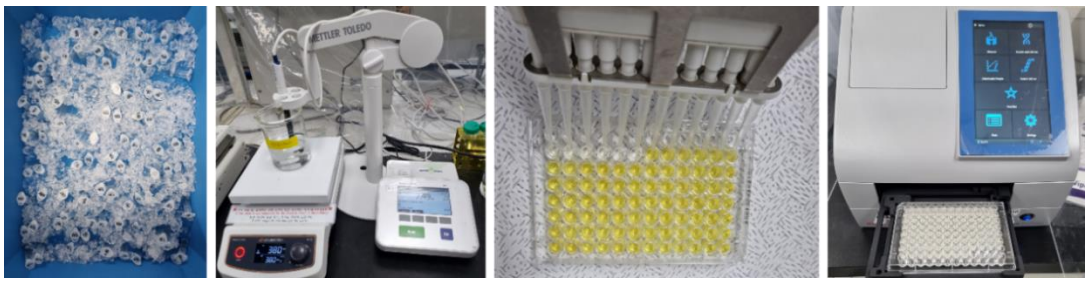

Figure 7. Hemolymph biochemical analysis procedures of Pacific white shrimp (*Penaeus vannamei*).

2.1.5 Real-Time qRT-PCR (Quantitative Reverse Transcription PCR)

Approximately 80 mg of hepatopancreas was homogenized in Trizol reagent (T9424, Sigma-Aldrich, USA) to extract total RNA. Complementary DNA synthesis was carried out from 2.5 µg DNase treated RNA using a PrimeScript™ 1st strand cDNA synthesis kit (Takara Bio, USA), according to Hasanthi and Lee (2023). Relative expression of target genes was performed by qRT-PCR device (Takara Bio, Shiga, Japan). Samples were analyzed following the PCR program mentioned in Hasanthi and Lee (2023). Initially, pre-heating was carried out

at 95 °C for 10 s, followed by 45 cycles, then 95 °C for 5 s, 58 °C for 20 s, and 60 °C for 30 s. Primer sequences of the corresponding genes were designed based on available cDNA sequences (GenBank® NIH genetic sequence database), are shown in Table 3 (reference gene: β-actin), and the Relative gene expression of insulin-like growth factor 1 (IGF-1), penaiedin 3a, and prophenoloxidase (proPO) was computed as reported by Pfaffl (2001).

Relative expression ratio = $[(E_{Sample})^{\Delta Ct(control-sample)}]/[(E_{\beta\text{-}actin})^{\Delta Ct(control-sample)}]$

Figure 8. Real Time Quantitative Reverse Transcription PCR procedure of hepatopancreas samples of Pacific white shrimp (*Penaeus vannamei*).

Table 3. Sequences of primers used for real-time quantitative PCR.

Target Gene	Forward primer sequences (5’ to 3’)	Reverse primer sequences (5’ to 3’)	Accession number/reference
β-Actin	GAGCAACACGGAGTTCGTTGT	CATCACCAACTGGGACGACATGGA	AF300705.2
Penaidene 3a	CGGTTGATGGAGAACACGATGAAA	TCATTCATCGTGCATTCATGGAAA	Chen et al. (2015)
proPO	CGCAACGGTGACAAAGTTCCTCTT	TATGTTGTGCAGGTCGCCGTAGTA	AY723296.1
IGF-1	GGCTTCAGCGTCAGGTGTTCCC	ACCCTTCCCGCAGATGTAGCAG	HAAW0101594

β-Actin, beta actin; proPO, prophenoloxidase; IGF-1, insulin-like growth factor

2.1.6 Intestinal histomorphology observations

Collected intestine samples were cut into desired sizes (1 cm) and dehydrated by immersing them in a graded ethanol series. Then, dehydrated intestine samples were embedded in paraffin wax, sectioned using a rotary microtome to a thickness of 5 μm, and mounted on microscopic slides. Hematoxylin and eosin (H&E) staining was carried out for histological observations. Stained intestine sections were observed under a light microscope (Leica DM750, Leica microsystems, Korea) equipped with a digital camera (Leica ICC50 E, Leica microsystems, Korea). Villi heights were determined using image analyzing software (×40, Leica Application Suite, version 4.13.0, Switzerland).

Figure 9. Intestine histological specimen preparation and observation of Pacific white shrimp (*Penaeus vannamei*).

2.1.7 Statistical analysis

Experimental diets were assigned in a completely randomized design. All data sets were initially checked for normality (Kolmogorov-Smirnov test and Shapiro-Wilk test). Data analysis was performed by One way Analysis of Variance (ANOVA) with SPSS version 25 (SPSS Inc., Chicago, IL, USA), and the mean differences were compared by adopting Turkey's honestly significant difference test ($P<0.05$). Results were expressed as mean ± standard deviation (SD). Orthogonal polynomial comparisons determined whether the effect is linear and/or quadratic. Piecewise regression of weight gain percentage (WG%) was executed using SigmaPlot for Windows version 14.0 (Shearer 2000).

2.2 Results

2.2.1 Growth performance, feed utilization and survival

Thiamine supplementation significantly improved (P<0.05) final body weight (FBW) of shrimp fed T20 dietary treatment compared to those fed T0 diet with no thiamine supplementation (Table 4). Highest WG% was recorded by T20 dietary group, and the lowest WG% was observed in shrimp from T100 and T0 dietary treatment groups, respectively. Specific growth rate (SGR) was significantly highest (P<0.05) in T20 dietary treatment group, where the poorest overall growth performance was observed in T0 diet fed group. Feed conversion ratio (FCR), protein efficiency ratio (PER) and survival were not significantly different (P>0.05) among all the dietary groups. A piecewise regression graph of WG% against supplemented thiamine levels is depicted in Figure 11. According to the graph, it is estimated that the optimal dietary thiamine requirement of *P. vannamei* is at 19.7 mg/kg of diet.

Table 4. Growth performance, feed utilization efficiency and survival of Pacific white shrimp (*Penaeus vannamei*) fed experimental diets containing graded levels of thiamine for 58 days.

Dietary treatment	FBW (g)[1]	WG (%)[2]	SGR (%)[3]	FCR[4]	PER[5]	Survival (%)
T0	17.3±1.1^{b}	187±19^{b}	1.82±0.11^{b}	2.32±0.20	1.33±0.11	96.7±3.9
T20	20.9±0.6^{a}	248±10^{a}	2.15±0.05^{a}	1.90±0.11	1.62±0.09	96.7±3.9
T40	18.4±1.0^{ab}	205±16^{ab}	1.92±0.09^{ab}	2.14±0.14	1.45±0.10	100±0.0
T60	18.3±1.6^{ab}	204±28^{ab}	1.91±0.16^{ab}	2.16±0.21	1.45±0.14	96.7±3.9
T80	18.6±1.3^{ab}	208±22^{ab}	1.93±0.12^{ab}	2.12±0.20	1.47±0.13	100±0.0
T100	18.2±1.7^{ab}	200±27^{b}	1.89±0.15^{ab}	2.17±0.31	1.44±0.22	98.3±3.3
Pr>F*						
ANOVA	0.017	0.016	0.022	0.177	0.167	0.366
Linear	0.556	0.525	0.574	0.878	0.845	0.254
Quadratic	0.145	0.138	0.136	0.207	0.214	0.558

Values are mean of quadruplicated groups and presented as mean ± SD. Values with different superscript letters in the same column are significantly different (P<0.05). The lack of superscript letters indicates no significant differences among treatments.

[1]Final body weight (g)

[2]Weight gain (%) = [(final body weight - initial body weight) / initial body weight] x 100

[3]Specific Growth Rate (%) =100 x [(log final body weight - log initial body weight) / days]

[4]Feed conversion ratio = dry feed fed / wet weight gain

[5]Protein efficiency ratio = wet weight gain / total protein given

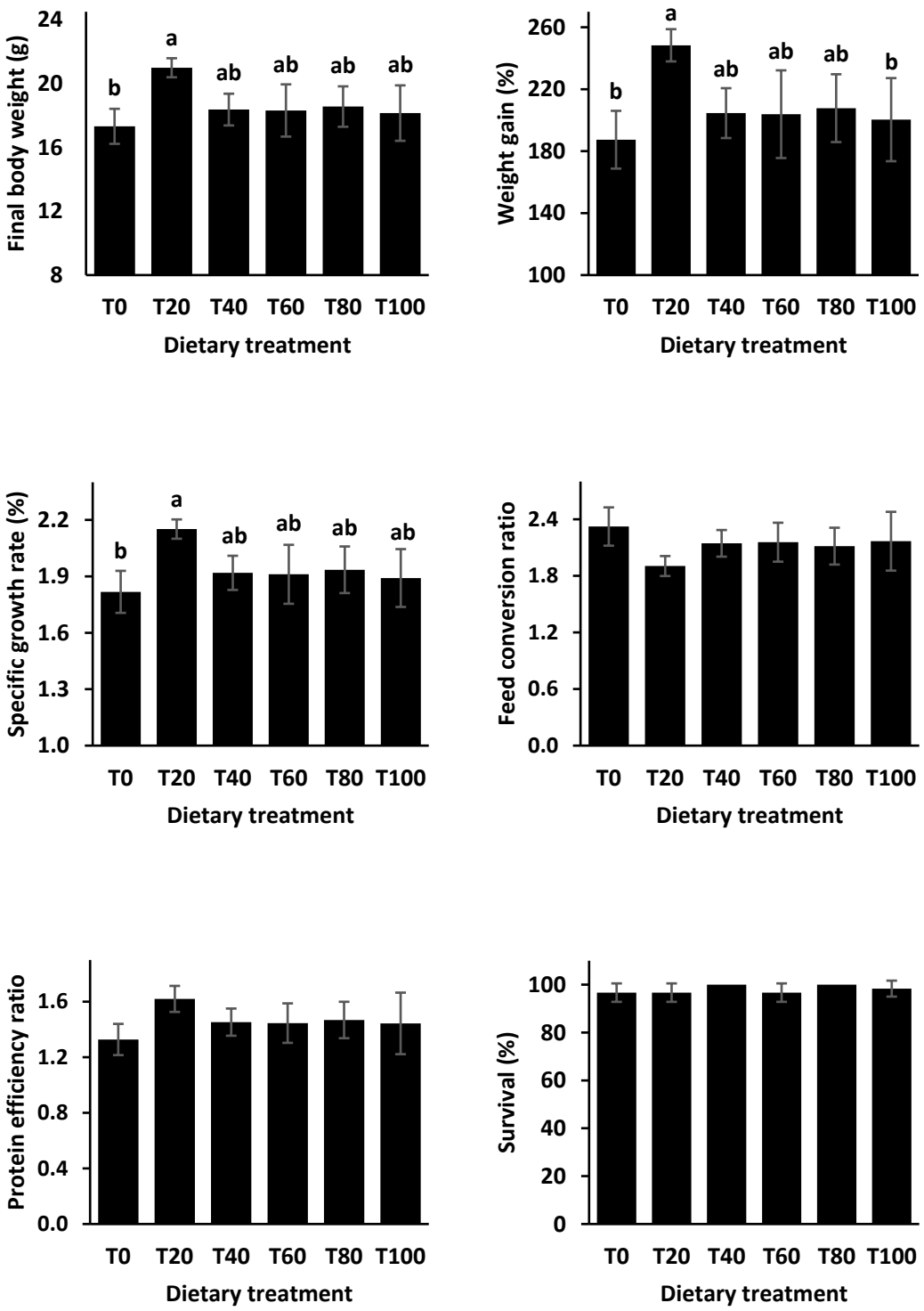

Figure 10. Growth performance, feed utilization efficiency and survival of Pacific white shrimp (*Penaeus vannamei*) fed experimental diets containing graded levels of thiamine for 58 days.

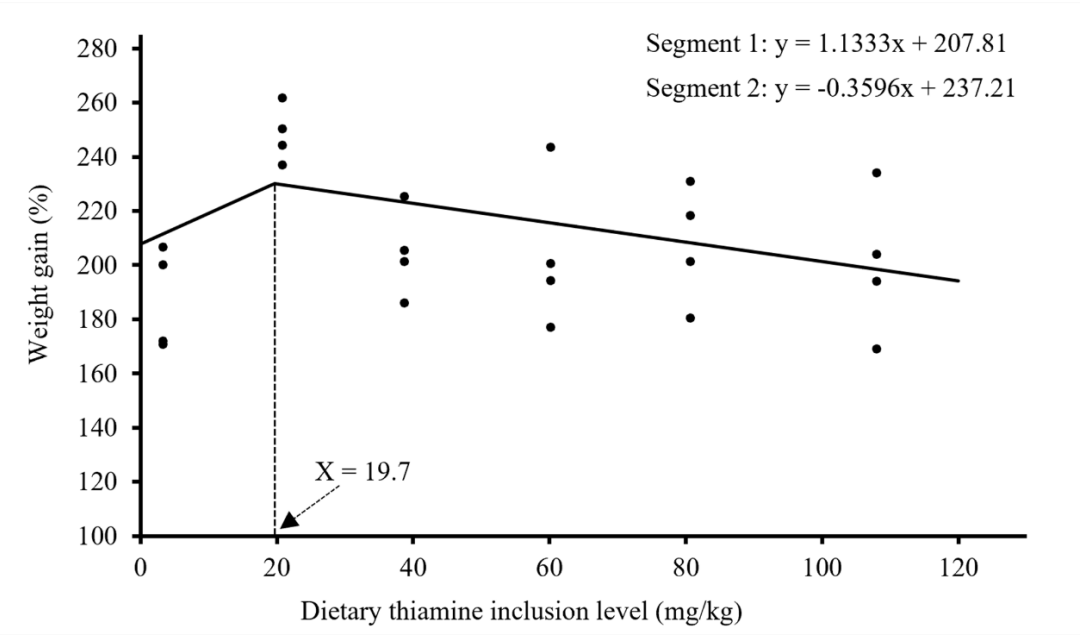

Figure 11. Piecewise regression graph showing the relationship between weight gain (%) and dietary thiamine levels. X represents the estimated optimum dietary thiamine requirement (19.7 mg/kg) of Pacific white shrimp (*Penaeus vannamei*). Some data points overlap (n = 4).

2.2.2 Non-specific immunity and antioxidant activity

Non-specific immune responses were significantly enhanced when thiamine was included in *P. vannamei* diets (Table 5). PO activity was significantly improved ($P<0.05$) in shrimp of T20 and T40 groups than in T0 group, and PO activity was significantly lower in the T100 group compared to T20 group. Lysozyme activity was significantly elevated in T20, T40, T60 and T80 dietary groups ($P<0.05$) than in T0 group. Both PO and lysozyme activities exhibited significant quadratic trends. Antiprotease activity was not significantly affected by the diets ($P>0.05$); however, a numerically higher activity was observed in T20 group. SOD and GPx activities were significantly enhanced ($P<0.05$) in T20 group compared to T0 dietary group, while showing significant quadratic trends.

Table 5. Non-specific immune parameters and antioxidant enzyme activities of Pacific white shrimp (*Penaeus vannamei*) fed the experimental diets containing graded levels of thiamine for 58 days.

Dietary treatment	PO[1]	Lysozyme[2]	Antiprotease[3]	SOD[4]	GPx[5]
T0	0.28±0.05^{c}	4.87±0.31^{b}	15.8±0.8	39.2±2.4^{b}	37.9±1.6^{b}
T20	0.44±0.04^{a}	6.53±0.17^{a}	16.3±2.3	51.6±5.3^{a}	61.1±9.1^{a}
T40	0.39±0.01^{ab}	6.34±0.51^{a}	15.8±3.0	49.0±8.2^{ab}	56.6±2.8^{ab}
T60	0.37±0.08^{abc}	6.29±0.91^{a}	16.1±2.4	49.6±4.8^{ab}	55.9±11.8^{ab}
T80	0.36±0.03^{abc}	6.38±0.78^{a}	14.7±2.9	45.6±1.1^{ab}	56.8±9.1^{ab}
T100	0.32±0.05^{bc}	5.97±0.66^{ab}	15.6±2.9	45.7±7.5^{ab}	52.2±11.7^{ab}
Pr>F*					
ANOVA	0.002	0.013	0.966	0.066	0.022
Linear	0.652	0.067	0.605	0.523	0.128
Quadratic	0.001	0.004	0.904	0.016	0.008

Values are mean of quadruplicated groups and presented as mean ± SD. Values with different superscript letters in the same column are significantly different (P<0.05). The lack of superscript letters indicates no significant differences among treatments.

[1]Phenoloxidase activity (absorbance); [2]Lysozyme activity (μg/mL); [3]Antiprotease activity (% inhibition); [4]Superoxide dismutase (% inhibition); [5]Glutathione peroxidase (mU/mL).

*Significance probability associate with the F-statistic

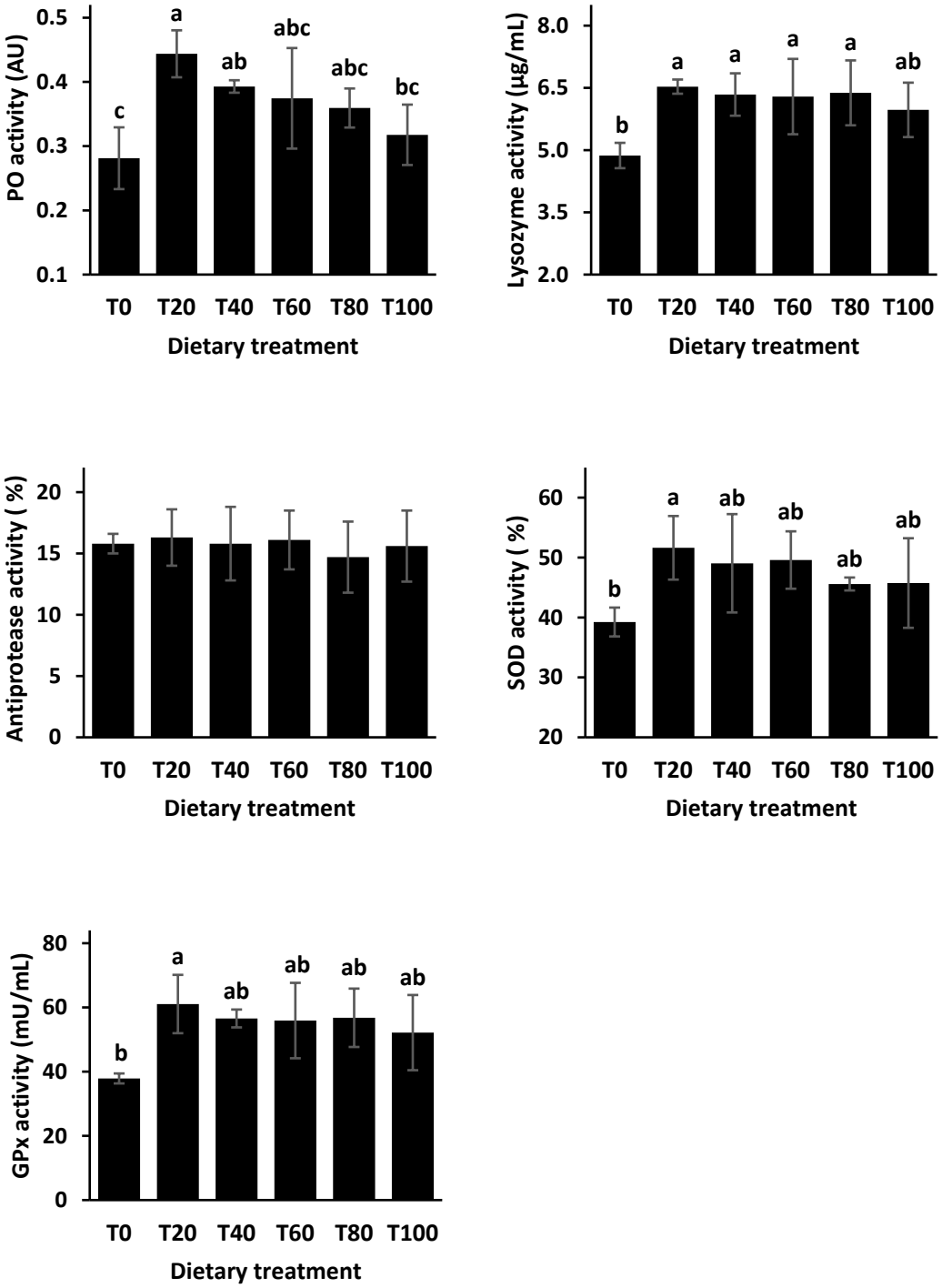

Figure 12. Non-specific immune parameters and antioxidant enzyme activities of Pacific white shrimp (*Penaeus vannamei*) fed the experimental diets containing graded levels of thiamine for 58 days.

2.2.3 Biochemical parameters

Hemolymph biochemical parameters, namely, levels of glucose, TCH, TG and total protein, were not significantly affected by the diets (Table 6). However, numerically higher TCH and TG levels were observed in T20 dietary group.

Table 6. Hemolymph biochemical parameters of Pacific white shrimp (*Penaeus vannamei*) fed experimental diets containing graded levels of thiamine for 58 days.

Dietary treatment	Glucose[1]	Total Cholesterol[2]	Triglyceride[3]	Total protein[4]
T0	561±33	14.0±1.8	15.7±3.7	3.35±0.20
T20	567±15	15.6±1.4	17.1±1.5	3.28±0.37
T40	562±17	15.4±2.3	15.8±1.9	3.49±0.25
T60	575±41	14.7±2.6	13.7±2.6	3.40±0.15
T80	579±24	14.9±1.8	15.6±1.1	3.24±0.22
T100	562±24	14.8±1.4	13.1±3.0	3.39±0.23

Values are mean of quadruplicated groups and presented as mean ± SD. Values with different superscript letters in the same column are significantly different ($P<0.05$). The lack of superscript letters indicates no significant differences among treatments.

[1]Glucose level (mg/dL); [2]Total cholesterol level (mg/dL); [3]Triglyceride level (mg/dL); [4]Total protein level (g/dL)

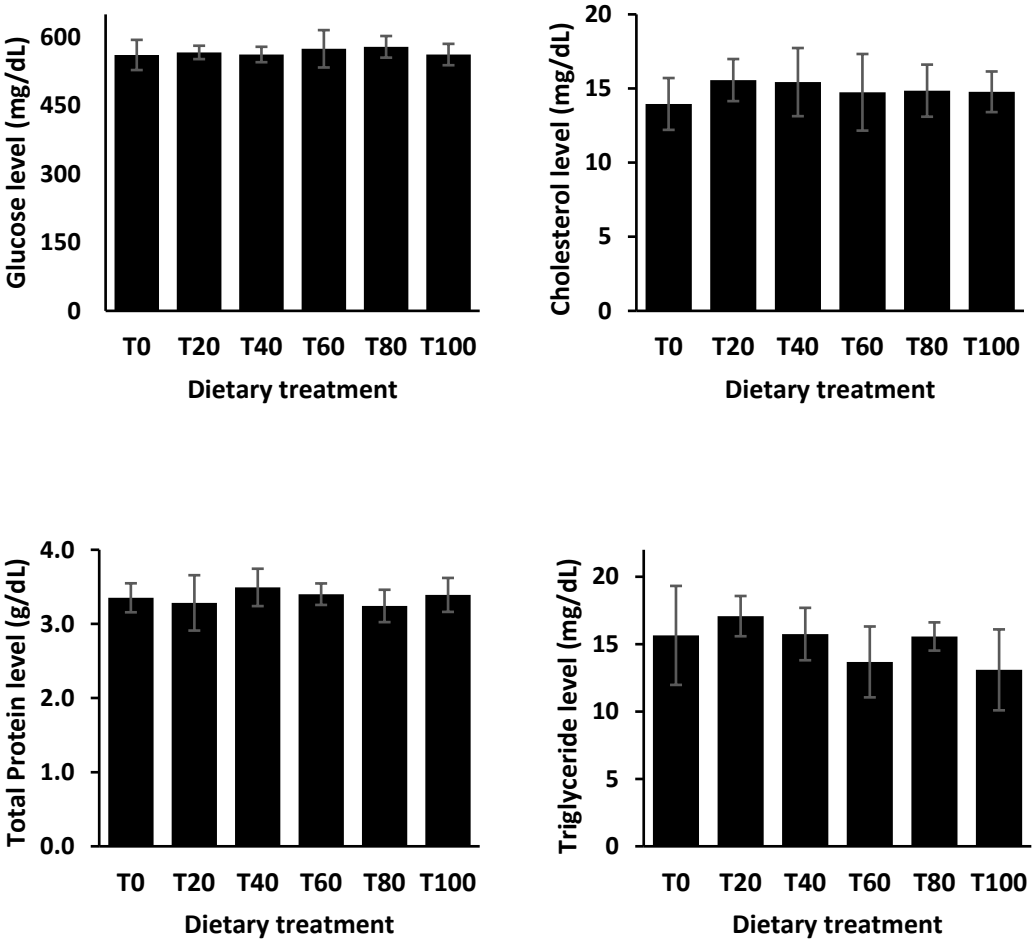

Figure 13. Hemolymph biochemical parameters of Pacific white shrimp (*Penaeus vannamei*) fed experimental diets containing graded levels of thiamine for 58 days.

2.2.4 Real-Time qRT-PCR (Quantitative Reverse Transcription PCR)

Relative expression of proPO gene was upregulated significantly (P<0.05) in both T20 and T40 groups (Figure 14) and showed a decline in the expression thereafter when further increasing dietary thiamine levels over 38.7 mg/kg. Relative expression of IGF-1 and Penaiedin 3a genes were not significantly affected (P>0.05) by the dietary treatments, however, numerically higher expressions were observed in thiamine supplemented groups than in T0 group.

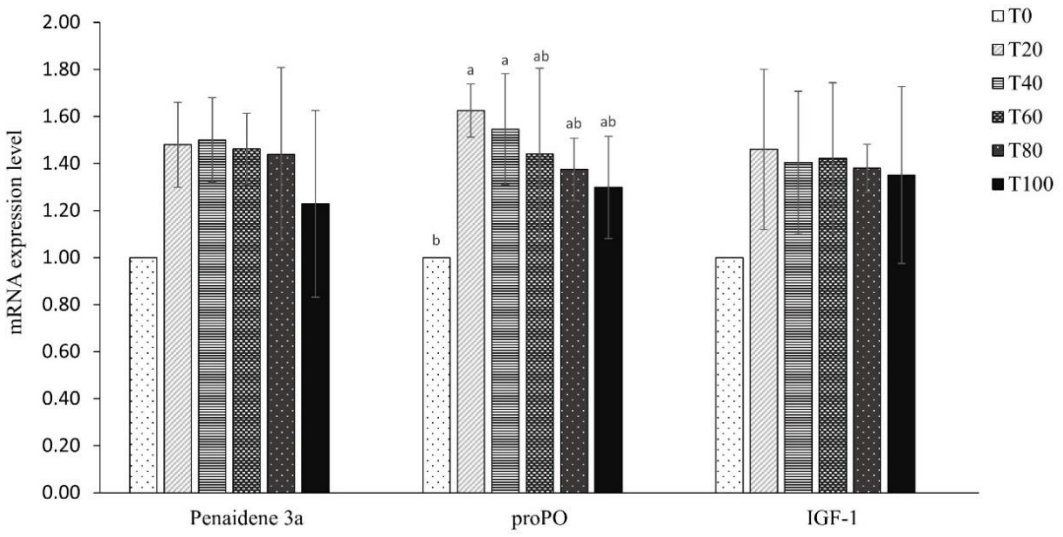

Figure 14. Relative mRNA expression of Penaidene 3a, proPO and IGF-1 in hepatopancreas of Pacific white shrimp (*Penaeus vannamei*) fed the experimental diets. The different letters on the bars represent significant differences (P<0.05). The lack of letters on the bars indicates no significant differences among treatments.

2.2.5 Intestinal histomorphology

Histological observations of the intestine (Figure 15) did not show any significant differences ($P>0.05$) in villi heights (Table 7) among the dietary treatments. However, numerically higher villi height was observed in T40 group.

Table 7. Histological parameters of Pacific white shrimp (*Penaeus vannamei*) fed the experimental diets for 58 days.

Dietary treatment	Villi height (μm)
T0	29.1±1.2
T20	29.9±1.5
T40	30.6±1.3
T60	30.1±0.7
T80	30.3±1.0
T100	29.5±0.3

Values are mean of quadruplicated groups and presented as mean ± SD. Values with different superscript letters in the same column are significantly different ($P<0.05$). The lack of superscript letters indicates no significant differences among treatments.

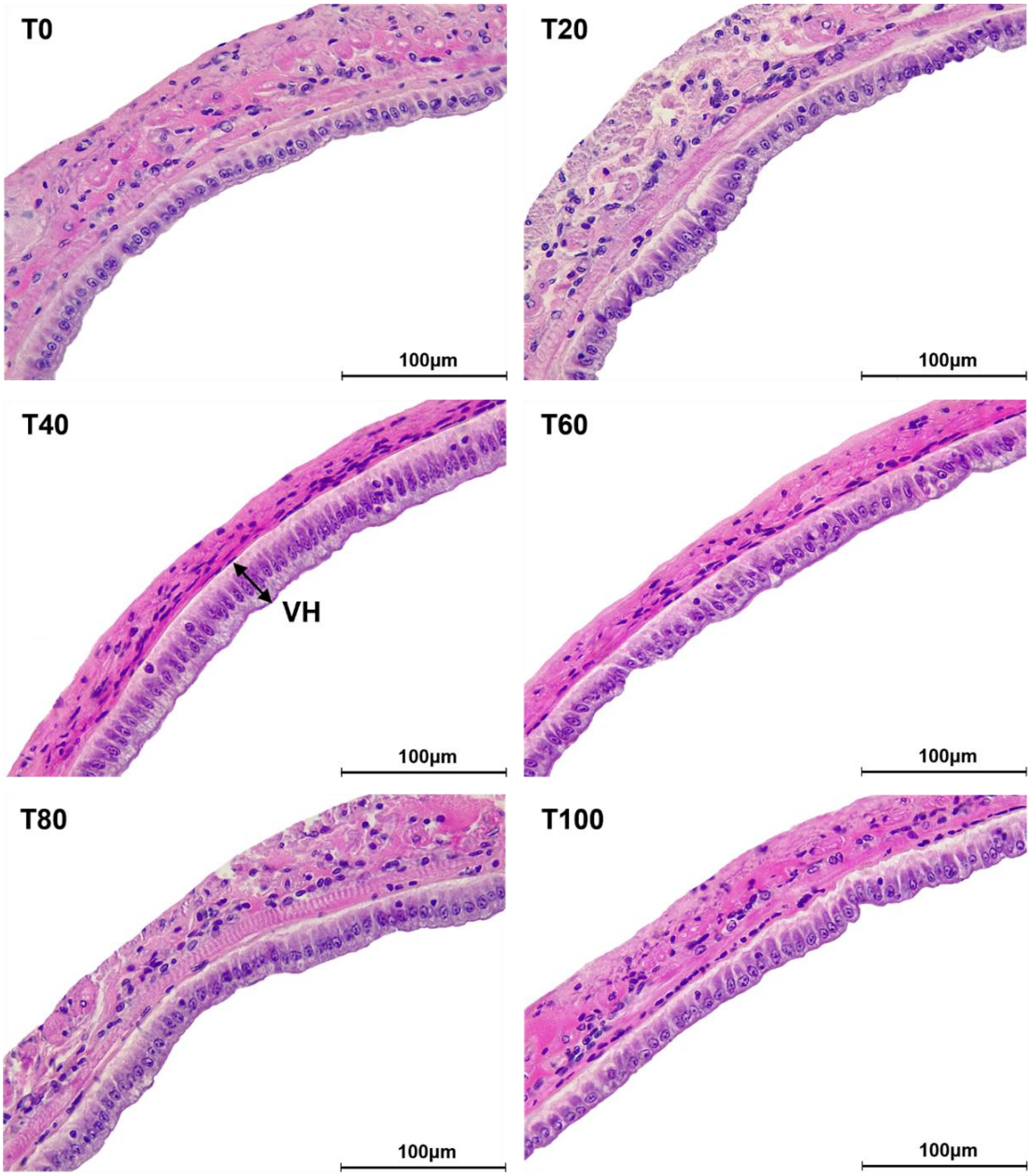

Figure 15. Representative intestinal morphology observations of Pacific white shrimp (*Penaeus vannamei*) fed six experimental diets for 58 days. (VH, villus height; magnification, ×40)

2.3 Discussion

The current study results show that an optimal thiamine level enhances the growth performance and innate immunity of *P. vannamei*. The improved growth performance demonstrated in this study may suggest that incorporating a suitable dose of thiamine to the diets of *P. vannamei* could promote the utilization of carbohydrates providing carbon skeletons for protein and lipid synthesis (Sun et al. 2022). Also, thiamine supplementation may improve carbohydrate metabolism and helps to maintain the balance in energy production and consumption (Zhou et al. 2022). In the present study, a piecewise regression model based on WG% depicted that the dietary thiamine requirement was 19.7 mg/kg of diet for the optimal growth performance, which was lower than the requirement concluded by Huang et al. (2015) for *P. vannamei*. Reasons behind this variation could be explained as follows; vitamin requirements of shrimp may vary by species, size, age, growth rate, environment, and nutrient interactions (NRC 2011). Most animals are primarily sensitive to nutrient requirements during their early stages due to rapid growth and development, immaturity and less capacity to store the nutrients (Hansen et al. 2015). Furthermore, maturity of the digestive system may have altered the gut microbiota composition, enhancing symbiotic thiamine production, likely to alter the thiamine requirement of the present study, which requires further investigations in the future.

The present study found no deficiency symptoms except for a reduction in growth performance and innate immune responses of shrimp groups fed the thiamine-deficient diet (T0). According to Sun et al. (2022), in their study, thiamine deficiency signs were not apparent in shrimp species, except for common symptoms such as inferior feed conversion, retarded growth, and poor survival. Huang et al. (2007) and Huang et al. (2011) also made similar observations in their respective studies. In the current study, several growth and immune related parameters showed a declining trend after reaching the optimal thiamine requirement, and this trend intensifies at thiamine levels over 80.7 mg/kg of diet.

Boonyaratpalin (1998) reported that an excessive thiamine supplementation may result in poor growth performance, reflecting a hypervitaminosis effect on metabolism of shrimp. Moreover, Huang et al. (2015) observed a reduction in WG% of shrimp after reaching the optimal thiamine content in the diets.

The active form of thiamine, TPP, performs a major role in carbohydrate, amino acid and lipid metabolism acting as a cofactor for enzymes involved in glycolysis, the citric acid cycle and the pentose phosphate pathway (PPP) (Depeint et al. 2006; Harder et al. 2018). More specifically, TPP is crucial in coordinating biochemical processes in the cytosol and glucose oxidation in mitochondria, enhancing glucose homeostasis. Moreover, thiamine, when serving as a cofactor, is vital in regulating respiratory chain complex enzymes enhancing overall mitochondrial activity, thereby playing a fundamental role in energy metabolism (Xu et al. 2022). The experimental diets in the present study contained a higher carbohydrate content (32%) compared to the diets of tiger shrimp (20%) (Chen et al. 1991), where the thiamine requirement was relatively lower (13-14 mg/kg) than in the current study. Huang et al. (2015) observed maximum WG% at a thiamine level of 44.66 mg/kg, where the carbohydrate content was 35.6% in diets for *P. vannamei*, which is higher than in the current study. A previous study showed that thiamine requirement increases when high carbohydrate diets are fed to terrestrial animals (Chen et al. 1991). Moreover, similar observations were made by Morris & Davies (1995), when increasing the amounts of plant-based materials with high starch levels in the diets of gilthead seabream (*Sparus aurata L.*). It was found that when dietary thiamine levels are inadequate, carbohydrate-rich diets deplete the body's reserves more rapidly than diets rich in fats and proteins (Zehra and Khan 2018). Furthermore, McDowell (1989) found that the thiamine requirement was decreased when carbohydrates in the diets were replaced by fat. These observations validate that dietary thiamine requirement increases in organisms when diets contain higher carbohydrate levels. Studies have shown that that thiamine supplementation significantly increases TCH and TG levels in serum, promoting TPP

production in the liver and facilitating oxidative decarboxylation of α-ketoacids, which may result in increased lipid synthesis (Jiang et al. 2014; Xiang et al. 2016). In the present study, the T20 group showed numerically higher TCH and TG levels than the T0 group.

In living organisms, thiamine is synthesized as thiamine monophosphate (TMP) and is converted to free thiamine by dephosphorylation (Harder et al. 2018). According to Bettendorff et al. (2014), the biological functions of free thiamine and TMP in organism's body are currently unknown; however, free thiamine is known to have antioxidant properties (Lukienko et al. 2000). Moreover, in a study by Zhou et al. (2022), supplementing thiamine in the diets of Oriental river prawn significantly enhanced the total antioxidant capacity. In shrimps, the capacity to initiate oxidative responses is crucial for innate immune defense mechanisms. The generation of reactive oxygen intermediates (ROIs) during phagocytosis, as well a generation of hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl ion (OH^-) by the hemocytes are all modes of immune defense in invertebrates (Ren et al. 2009). Persistence of these ROIs for an extended period inside the animal body affects the metabolism and survival of the organism by damaging macromolecules in the cells; therefore, rapid elimination is required (Holmblad 1999; Campa-Córdova et al. 2002). Thiamine directly interacts with hydroperoxide radicals and oxidizes into thiochrome and thiamine disulfide by transferring $2H^+$ ions from the amine group of the pyrimidine ring to reactive substrates (Lukienko et al. 2000). Zhou et al. (2022), in their study concluded that, thiamine helps to alleviate hypoxia stress, thereby maintaining the balance between the production and the removal of ROIs. Thiamine also scavenges free radicals by disrupting heme oxygenase-1 enzyme activity and demoting the accumulation of advanced glycation end products (Li et al. 2014; Sandau et al. 1998).

The conversion process of O_2^- anions to H_2O_2 and molecular oxygen (O_2) is catalyzed by the SOD enzyme. In the current study, SOD activity was elevated in the T20 group, showing a significant quadratic trend, while T0 group reported the significantly lowest. Huang et al.

(2015) previously observed enhanced SOD activity when thiamine was supplemented in the diets of *P. vannamei*. Zehra and Khan (2018) and Li et al. (2014) observed lower SOD activity in thiamine-deficient dietary groups of spotted snakehead and Jian carp (*Cyprinus carpio* var. Jian), respectively. Furthermore, Oriental river prawns fed diets with highest levels of thiamine showed significantly enhanced SOD activity under hypoxia (Zhou et al. 2022). GPx is an enzyme that detoxifies H_2O_2 and lipid by-products generated during normal physiological metabolism and in respiratory bursts caused by microbial phagocytosis by activated macrophages (Liu et al. 2007). To catalyze the initial and final steps of the PPP, TPP-activated TK is required, as it is the most direct mode of NADPH synthesis from glucose (Lonsdale 2015), which generates several metabolites including glutathione (Depeint et al. 2006). A study on golden pompano (*Trachinotus ovatus*) fish showed that SOD and GPX activities were significantly enhanced in the thiamine-supplemented groups over the thiamine deficient groups (Xun et al. 2019). In the present study, a significant quadratic trend was observed in GPx activity, where the T20 group showed the highest activity and the T0 group showed the lowest.

In addition to hemocyte-mediated immunity, several proteolytic pathways including opsonization coagulation and melanin synthesis are involved in providing protective mechanisms against pathogens (Hellio et al. 2007). Melanotic encapsulation is a potent immune mechanism found in invertebrates. Studies have shown that PO in hemolymph is initially in an inactive state (proPO) and is activated through a series of reactions involving serine proteinases, in response to microbial cell wall components (Söderhäll and Cerenius 1998). Melanization, executed by PO and controlled by the proPO activation cascade, is vital for wound healing, the entrapment of parasites and the destruction of microbes (Amparyup et al. 2013). Moreover, PO promotes the production of ROIs, which are critical in innate immunity responses (Okumura et al. 2007). Results in the present study convey that optimum dietary thiamine levels significantly influenced the activity of PO. In a study by Thamizhvanan

et al. (2021), proPO gene-silenced freshwater prawn (*Macrobrachium rosenbergii*) showed 100% mortality in a challenge test exposing them to white spot syndrome virus (WSSV), while no mortality was observed in non-genetically modified shrimps injected with WSSV alone. It appears; therefore, the PO system is a crucial complementary response in the non-specific immune system of invertebrates. Lysozyme is an enzyme that is essential to the innate immunity of invertebrates (Zhao et al. 2010) by promoting the cleaving of the glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine in the cell wall (peptidoglycan) of bacteria resulting in cell lysis (Grunclová et al. 2003) and protection against gram-positive bacteria (Saurabh and Sahoo 2008). Huang et al. (2015), in their study for *P. vannamei*, significantly enhanced lysozyme activities were observed in thiamine supplemented shrimp groups. Ichikawa et al. (1997) observed that adding thiamine to the growth medium of *Saccharomyces cerevisiae* enhanced the production of various enzymes including lysozyme, suggesting that thiamine upregulates the expression levels of various genes. In the present study, increased lysozyme activity and the PO activity in thiamine-supplemented diet groups could be explained through the above mechanism.

The relative expression of genes related to growth and immunity was investigated to find the effect of dietary thiamine supplementation at the molecular level. Micronutrients, and particularly vitamins, can interact directly with transcription factors of specific genes through cis-regulatory components in promoter regions (De Caterina & Madonna 2004). The proPO system is regulated by complex systems, mainly through gene expression (Yang et al. 2012). The elevated proPO relative gene expression as well as subsequently enhanced PO activity in the present study, could be an effect of thiamine supplementation, which requires further investigations. IGFs, where the production is fine-regulated by the expression of IGF-1, are essential in the growth, development and differentiation of animal cells and tissues (Delafontaine et al. 2004). Even though growth performance was significantly enhanced in the present study, the relative expression of the IGF-1 gene was not significantly different among

the dietary groups. Penaeidins are a group of antimicrobial peptides with bactericidal and bacteriostatic properties against gram-positive bacteria and also have antifungal properties, reducing the growth and elongation of fungal hyphae (Bachère et al. 2000). However, in the present study, Penaeidin 3a relative gene expression was not affected by thiamine supplementation.

Previous studies reflect that thiamine influences intestinal health, function, and integrity. Mahmood et al. (1984) observed that the thinning of the intestinal tissues where villi were tall and narrow (tapering at tips) in thiamine-deficient rats was an adaptation to enhance nutrient absorption. In addition, the epithelial layer was shown to lift away from stroma and bleb formations on the villus surface. Furthermore, Huang et al. (2011) observed a significant increase in hepatopancreas and intestine weights, when thiamine was supplemented in the diets of Jian carp. Similarly, in the current study, intestinal villi heights were increased when increasing dietary thiamine levels up to 38.7 mg/kg, showing that thiamine influences on the structure and function of the shrimp digestive system. Moreover, increased growth performance and immune responses in thiamine supplemented groups, could be an effect of enhanced nutrient absorption and assimilation.

2.4 Conclusion

An optimum dietary thiamine level could positively influence growth performance, feed utilization, innate immunity, antioxidative activity, and immune related gene expression of *P. vannamei*. Based on the piecewise regression graph of WG%, the optimum dietary level of thiamine for *P. vannamei* would be 19.7 mg/kg of diet.

CHAPTER 2

Dietary thiamine requirement and its effects on growth, innate immunity, and digestive enzyme activity of Pacific white shrimp (*Penaeus vannamei*) post larvae

3.1 Materials and methods

3.1.1 Experimental diets

A fishmeal based basal diet (T0) (crude protein: 38.2%, crude lipid: 9.40%) was formulated with no thiamine added and the composition is given in Table 8. Five other experimental diets were formulated by incorporating graded levels of thiamine hydrochloride (67-03-8, Sigma-Aldrich, St. Louis, MO, USA) at 20, 40, 60, 80 and 100 mg/kg of diet (designated as T20, T40, T60, T80 and T100, respectively) to the basal diet at the expense of cellulose. Initially, all the dry ingredients were mixed well incorporating fish oil and water (15% by weight). Then the moist mixture of ingredients was sent through a pelleting machine and the resultant pellets (2 mm) were air dried at 24-25°C for 8 h. After air drying, feed pellets were crushed to get crumbles of sizes 500 µm and 800 µm, sieving through respective sieves. Then experimental feed was stored at −20°C refrigerator until used. The proximate composition (dry matter, crude protein, crude lipid, crude ash) of the diets was analyzed following the protocols mentioned in AOAC (2005).

Thiamine concentrations of the diets were analyzed by HPLC according to Wijemanna and Lee (2023) with slight modifications and determined to be of 6.14, 28.5, 48.1, 73.3, 85.7 and 106 mg/kg (Table 8) for T0, T20, T40, T60, T80 and T100, respectively. Briefly, approximately 1.5 g of diet samples were homogenized in 9 mL of 0.01 M HCL, followed by the addition of 10% trichloroacetic acid (3 mL) and centrifuged at 10,000 × g for 10 min at 4°C for protein precipitation and this step was repeated twice. Then, water-saturated diethyl

ether was used to wash the resulting supernatant, allowed for evaporation, and filtered through a 0.45 μm syringe filter. Finally, 80 μL of filtered sample was derivatized with 50 μL of freshly prepared 0.1% potassium ferricyanide in 15% NaOH prior injecting into the column (4.6 × 250 mm, Waters Atlantis). Mobile phase consisted of 25 mM potassium phosphate dibasic (adjusted to pH 8.4 with phosphoric acid) and 100% methanol in a 50:50 ratio, and the flow rate was set to 1.2 mL/min. Thiamine was detected using a fluorescence detector at excitation and emission wavelengths of 360 nm and 425 nm, respectively. Thiamine hydrochloride was dissolved in distilled water as standard, and the concentration was expressed as mg/kg.

Figure 16. Experimental diet preparation for Pacific white shrimp (*Penaeus vannamei*).

Table 8. Formulation and proximate composition of the experimental diets for Pacific white shrimp (*Penaeus vannamei*) (g/kg, dry matter basis).

Ingredients	Experimental Diets					
	T0	T20	T40	T60	T80	T100
Fish meal (sardine)	100	100	100	100	100	100
Fish meal (tuna)	100	100	100	100	100	100
Soybean meal	400	400	400	400	400	400
Squid liver meal	50.0	50.0	50.0	50.0	50.0	50.0
Wheat flour	80.0	80.0	80.0	80.0	80.0	80.0
Starch	147	147	147	147	147	147
Fish oil	50.0	50.0	50.0	50.0	50.0	50.0
Mineral Mix[1]	20.0	20.0	20.0	20.0	20.0	20.0
Vitamin Mix[2]	10.0	10.0	10.0	10.0	10.0	10.0
Cholesterol	2.00	2.00	2.00	2.00	2.00	2.00
Lecithin	10.0	10.0	10.0	10.0	10.0	10.0
Monocalcium phosphate	30.0	30.0	30.0	30.0	30.0	30.0
Thiamine hydrochloride[3]	0.00	0.02	0.04	0.06	0.08	0.10
Cellulose	1.00	0.98	0.96	0.94	0.92	0.90
Proximate composition (g/kg)						
Dry matter	945	947	950	950	951	951
Crude protein	389	395	392	392	389	390
Crude lipid	91.1	92.3	92.7	95.5	95.4	94.3
Crude ash	107	109	107	108	108	108
Thiamine content (mg/kg)	6.14	28.5	48.1	73.3	85.7	106

[1]$MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; $CuCl_2$, 0.2; $AlCl_3$. $6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0. [2]Beta-carotine, 0.57; riboflavin, 6.89; niacin, 18.0; pantothenate, 18.0; pyridoxine hydrochloride, 7.2; cyanocobalamin, 0.09; ascorbic acid 100.0; cholecalciferol, 144.0; alpha tocopheryl acetate, 20.0; menadione, 0.9; biotin, 0.9; folic acid, 7.2; inositol, 45.
[3]Xi'an Julong Bio-Tech Co., Ltd, Xi'an, China.

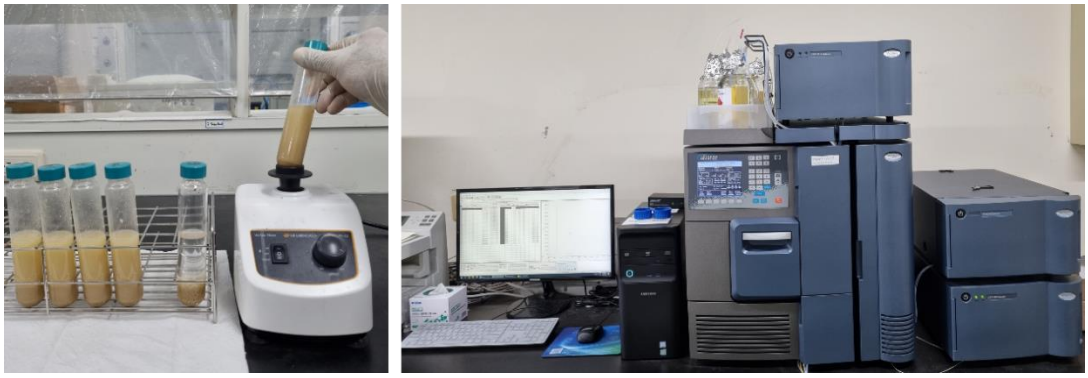

Figure 17. Thiamine extraction from six experimental diets and determining vitamin concentration through high performance liquid chromatography.

3.1.2 Shrimp selection and feeding trial

Five-day old post larvae (P.L. 5) were bought from Daedong Susan Inc., Muan, South Korea and reared in aquarium tanks until they reached the desired size by supplying a commercial diet (40% protein, 6% lipid, Woosung premium aqua feed, Korea), to their apparent satiation. Shrimp were then acclimated to experimental system and conditions for one week prior to the beginning of the trial, by providing the same commercial diet. After the acclimation, post larvae (5 mg) were randomly distributed into 24 tanks (10 L) with a density of 50 post larvae per tank. Each experimental diet was assigned to four replicate tanks and the daily feed requirement was split into six equal feedings, implemented at 08:00, 10:00, 12:00, 14:00, 16:00, and 18:00 h, continued for 30 days. The biomass of each tank was assessed two weeks after the initiation of the experiment. Feed requirement was calculated as a percentage (20-25%) of the predicted biomass for the next two weeks based on the latest available SGR data, aimed at feeding to apparent satiation. Uneaten feed at the bottom of tank was removed by siphoning and the dry weight was subtracted from the amount of diet provided to determine the actual feed intake. Experimental water (80%) was exchanged at every five days interval to maintain total ammonia nitrogen (0.04 ± 0.03 ppm) (Strickland and Parsons 1972) below toxic levels. The water quality of the experimental setup was recorded and maintained as follows:

temperature (30.31 ± 0.3 ºC) and dissolved oxygen (7.52 ± 0.22 ppm) levels (Pro20 Dissolved Oxygen Meter, YSI, Yellow springs, OH, USA) were monitored daily, and salinity (32.93 ± 0.39 ppt) (ATAGO™ Master-S28M Salinity Refractometer, Japan) and pH (7.28 ± 0.27) (SUNTEX TS-1 Classic Portable pH·mV Meter, Taiwan) levels were recorded weekly.

Figure 18. Experimental setting for the feeding trial of Pacific white shrimp (*Penaeus vannamei*).

3.1.3 Sample collection

After 30 days of feeding trial, feeding was terminated 12 hours before the final sampling. The number of shrimps in each tank was counted, and individual weight was recorded to evaluate growth and feed utilization-related parameters. 18 shrimps per tank (72 shrimps per dietary treatment) were captured randomly and anesthetized by placing them in iced water for few minutes and hepatopancreas samples were collected under sterile conditions; 9 shrimps for relative gene expression and another set of 9 shrimps for digestive enzyme analysis. Upon collection, hepatopancreas samples were flash frozen in liquid nitrogen and stored at −80°C for later use and the analysis was done in three replicate samples (3 hepatopancreas per replicate, homogenized) per experimental tank.

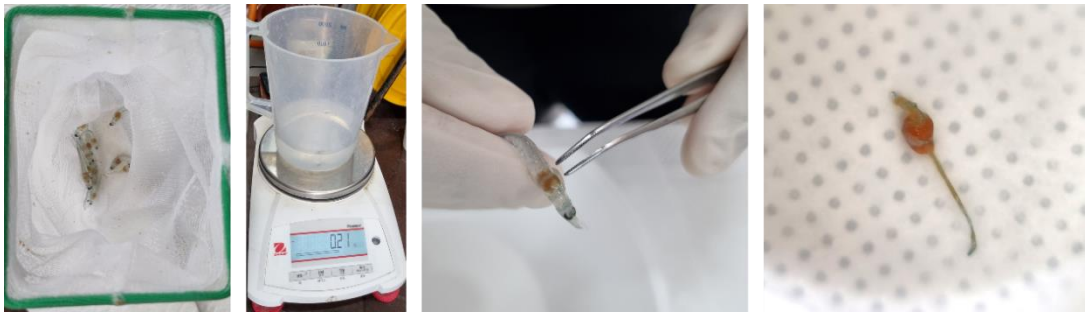

Figure 19. Weight measurement and sample collection from *Penaeus vannamei* during final sampling.

3.1.4 Real-Time qRT-PCR (Quantitative Reverse Transcription PCR)

Approximately 80 mg of hepatopancreas was homogenized in Trizol reagent (T9424, Sigma-Aldrich, USA) to extract total RNA. Complementary DNA synthesis was carried out from 2.5 µg DNase treated RNA using a PrimeScript™ 1st strand cDNA synthesis kit (Takara Bio, USA), according to Wijemanna and Lee (2023). Relative expression of target genes was performed by qRT-PCR device (Takara Bio, Shiga, Japan). Samples were analyzed following the PCR program mentioned in Wijemanna and Lee (2023). Initially, pre-heating was carried out at 95 °C for 10 s, followed by 45 cycles, then 95 °C for 5 s, 58 °C for 20 s, and 60 °C for 30 s. Primer sequences of the corresponding genes were designed based on available cDNA sequences (GenBank® NIH genetic sequence database), are shown in Table 9 (reference gene: β-actin), and the relative gene expressions of IGF-1, Insulin like growth factor binding protein (IGF-BP), target of rapamycin (TOR), Penaiedin 3a, proPO, crustin, SOD, GPx and catalase (CAT) were computed as reported by Pfaffl (2001).

Relative expression ratio = $[(E_{Sample})^{\Delta Ct(control-sample)}]/[(E_{\beta\text{-}actin})^{\Delta Ct(control-sample)}]$

Table 9. Sequences of primers used for real-time quantitative PCR.

Target Gene	Forward primer sequences (5’ to 3’)	Reverse primer sequences (5’ to 3’)	Accession number/reference
β-Actin	GAGCAACACGGAGTTCGTTGT	CATCACCAACTGGGACGACATGGA	AF300705.2
IGF-1	GGCTTCAGCGTCAGGTGTTCCC	ACCCTTCCCGCAGATGTAGCAG	HAAW0101594
IGF-BP	GTGGGCAGGGACCAAATC	TCAGTTACCACCAGCGATT	Wijemanna and Lee (2023)
TOR	TGCCAACGGGTGGTAGA	GGGTGTTTGTGGACGGA	XM_027372359.1
Penaiedin 3a	CGGTTGATGGAGAACACGATGAAA	TCATTCATCGTGCATTCATGGAAA	Wijemanna and Lee (2023)
proPO	CGCAACGGTGACAAAGTTCCTCTT	TATGTTGTGCAGGTCGCCGTAGTA	AY723296.1
Crustin	CTTGCACACGTGTTCTCCCAAACA	ACCAAGATACTCGACTGCCCACAA	AY486426.1
SOD	GGGCTTCATTAACAACCTAATTGC	ATGTTGGTCCAGAAGATGGTGT	AB108065
GPx	AGGGACTTCCACCAGATG	CAACAACTCCCCTTCGGTA	AY973252
Catalase	TCAGCGTTTGGTGGAGAA	GCCTGGCTCATCTTTATC	AY518322

β-Actin, beta actin; IGF-1, insulin-like growth factor; IGF-BP, insulin like growth factor binding protein; TOR, target of rapamycin; proPO, prophenoloxidase; SOD, superoxide dismutase; GPx, glutathione peroxidase

Figure 20. Real Time Quantitative Reverse Transcription PCR procedure of hepatopancreas samples of Pacific white shrimp (*Penaeus vannamei*).

3.1.5 Hepatopancreas digestive enzyme analysis

For the digestive enzyme analysis, intestine samples were weighted and homogenized separately in distilled water (1 g of tissue in 1 mL of distilled water) using a homogenizer (Daihan Scientific Co, Ltd. Wonju, Republic of Korea). The homogenate was centrifuged at 10,000× g for 15 min at 4 °C and the supernatant was collected. Then the crude enzyme extract was stored at −20 °C until used. The BioRad assay kit (Bio-Rad Laboratories, Inc., Seoul, Republic of Korea) was used to assess the total protein level of the supernatant and standard solutions were prepared using bovine serum (Bradford 1976).

3.1.5.1 Pepsin activity

Worthington's digestive enzyme analysis method was used to determine pepsin activity. Hemoglobin (2%) in 0.06 N HCl was used as the substrate (500 µL) and incubated with crude enzyme extract (100 µL) at 37 °C for 10 minutes (Worthington 1993). The reaction was terminated by adding 1 mL of 5% TCA and left for 5 min.

$$\text{Pepsin activity (U/mg protein)} = \frac{\text{Absorbance at 280 nm (Supernatant - Blank)}}{(10\ \text{min} \times \text{mg protein in the assay})} \text{x1000}$$

3.1.5.2 Amylase activity

Amylase activity was measured following the method proposed by Worthington (1993). Briefly, 0.5 mL of enzyme extract was mixed with 0.5 mL of 1% starch solution in 20 nM sodium phosphate buffer (pH 6.9) containing 6.0 nM NaCl and the mixture was incubated at 37 °C for 3 min. The released maltose was reacted with 0.5 mL dinitro salicylic acid (609-99-4. Sigma-Aldrch, St. Louis, MO, USA) and incubated for 5 min in a boiling water bath. The absorbance was measured at 540 nm and the amount of maltose released during 3 min incubation was evaluated using a standard.

$$\text{Amylase activity (U)} = \frac{\mu\text{mol maltose released}}{(\text{mg enzyme in reaction mixture} \times 3\ \text{min})}$$

3.1.5.3 Lipase activity

Lipase activity was determined following the Borlongan (1990) method. Briefly 1 mL of crude enzyme extract was incubated with 1 mL of stabilized olive oil substrate in 1.5 mL of 0.1 M Tris-HCl buffer at pH 8.0 for 6 h at 37 °C. The reaction was terminated by adding 3 mL of 95% ethanol. Then the mixture was titrated with 0.01 N NaOH using 0.9% (w/v) thymolphthalein in ethanol as indicator. values were expressed as 0.01 N NaOH volume required to neutralize the fatty acid released due to the hydrolysis of triglycerides in the stabilized standard olive oil emulsion (1.5 mL) by 1 mL of crude enzyme extract.

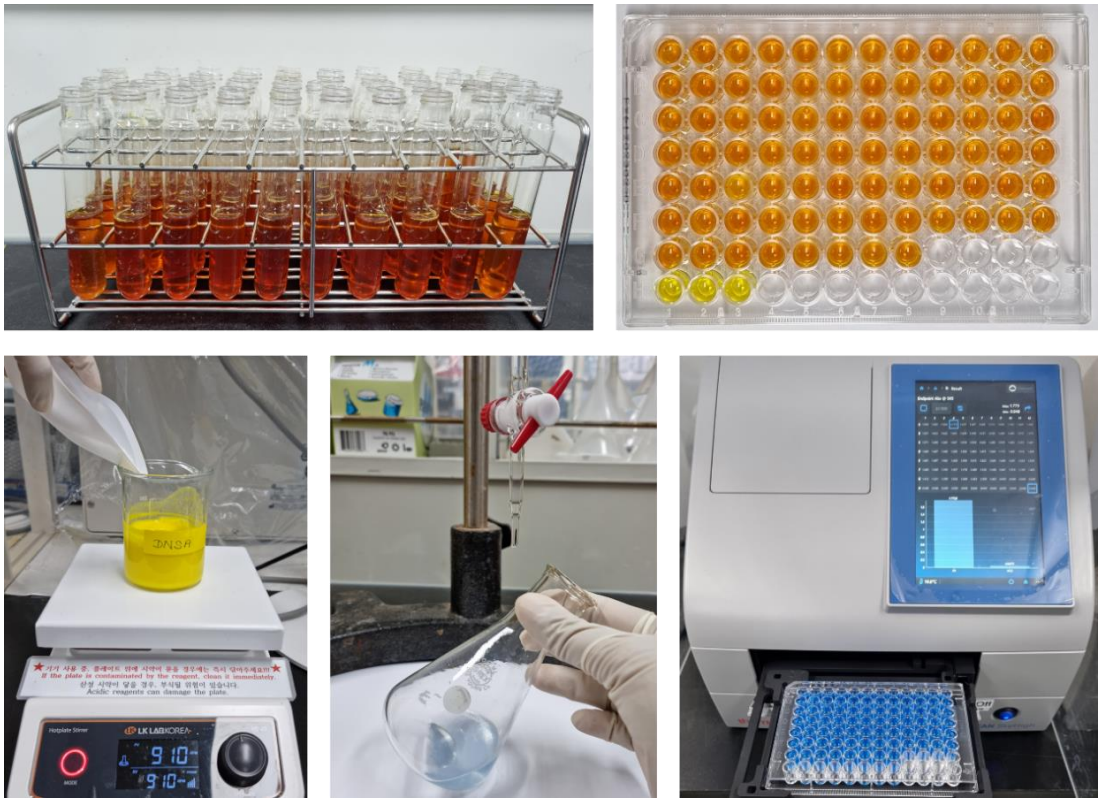

Figure 21. Digestive enzyme analysis procedures of Pacific white shrimp (*Penaeus vannamei*).

3.1.6 Ammonia stress challenge test

After the feeding trial, 60 shrimp from each dietary treatment (15 shrimp from each tank) were randomly captured, pooled and redistributed into three replicate tanks (10 L) with 20 shrimp per tank for the ammonia challenge test. A lethal dose of 150 mg/L ammonium chloride was added to each tank and the mortality was recorded for 48 h at 1 h intervals. LD_{50} concentration of ammonium chloride for *P. vannamei* post larvae was determined through a previously conducted preliminary test according to Liu and Chen (2004). The concentration of non-ionized ammonia (NH_3) at 30.5 °C and pH 7.48 was calculated according to Armstrong et al. (1978) and was around 3.68 mg/L. Moderate aeration was supplied to the tanks and shrimp were starved throughout the 48h exposure period.

$$\text{Non-ionized ammonia concentration} = \frac{\text{Total ammonia concentration}}{(1 + 10\text{^}(\text{pKa - pH})}$$

$$\text{pKa} = 0.09018 + \frac{2729.92}{\text{Temperature (K)}}$$

3.1.7 Statistical analysis

Experimental diets were assigned in a completely randomized design. All data sets were initially checked for normality (Kolmogorov-Smirnov test and Shapiro-Wilk test). Data analysis was performed by One way Analysis of Variance (ANOVA) with SPSS version 25 (SPSS Inc., Chicago, IL, USA), and the mean differences were compared by adopting Turkey's honestly significant difference test ($P<0.05$). Results were expressed as mean ± standard deviation (SD). Orthogonal polynomial comparisons determined whether the effect is linear and/or quadratic. A broken-line regression analysis quantified the optimum dietary thiamine requirement (Robbins, 1986).

3.2 Results

3.2.1 Growth performance, feed utilization and survival

Thiamine supplementation significantly enhanced (P<0.05) the growth performance and the feed utilization of *P. vannamei* (Table 10). A significantly higher (P<0.05) FBW was observed in T60 group, whereas thiamine deficient group (T0) showed the significantly lowest FBW. Similarly, significantly highest WG% was recorded by T60 dietary treatment group and the lowest was shown by the T0 group. Thiamine supplementation significantly enhanced (P<0.05) the SGR in T60 group and poor SGR was observed in T0 group. The FCR was elevated in thiamine deficient group, depicting a poor feed efficiency and thiamine supplementation significantly enhanced (P<0.05) the FCR and PER in T60 and T80 dietary treatment groups. Dietary thiamine supplementation did not affect the survival among dietary treatment groups (P>0.05). All the growth performance and feed utilization parameters followed significant quadratic trends. A broken line regression of WG% (Figure 23) estimated the dietary thiamine requirement of *P. vannamei* post larvae at 72.9 mg/kg of diet.

Table 10. Growth performance, feed utilization efficiency and survival of Pacific white shrimp (*Penaeus vannamei*) fed the experimental diets containing graded levels of thiamine for 30 days.

Dietary treatment	FBW (g)[1]	WG (%)[2]	SGR (%)[3]	FCR[4]	PER[5]	Survival (%)
T0	0.158±0.007^{d}	3199±151^{d}	11.7±0.2^{d}	1.15±0.03^{a}	2.23±0.05^{c}	90.0±1.6
T20	0.171±0.004^{cd}	3455±86^{cd}	11.9±0.1^{cd}	1.11±0.05^{ab}	2.25±0.10^{c}	90.5±1.0
T40	0.181±0.008^{bc}	3670±163^{bc}	12.1±0.1^{bc}	1.07±0.04^{bc}	2.41±0.09^{bc}	91.0±2.6
T60	0.199±0.010^{a}	4049±216^{a}	12.4±0.2^{a}	0.98±0.04^{d}	2.61±0.11^{a}	90.5±1.0
T80	0.188±0.006^{ab}	3825±120^{ab}	12.2±0.1^{ab}	0.99±0.02^{d}	2.60±0.04^{a}	90.5±3.0
T100	0.182±0.008^{bc}	3689±161^{bc}	12.1±0.1^{abc}	1.01±0.03^{cd}	2.53±0.09^{ab}	91.5±1.9
Pr>F*						
ANOVA	0.000	0.000	0.000	0.000	0.000	0.925
Linear	0.000	0.000	0.000	0.000	0.000	0.414
Quadratic	0.000	0.000	0.000	0.006	0.010	0.957

Values are mean of quadruplicated groups and presented as mean ± SD. Values with different superscript letters in the same column are significantly different (P<0.05). The lack of superscript letters indicates no significant differences among treatments.

[1]Final body weight (g)

[2]Weight gain (%) = [(final body weight - initial body weight) / initial body weight] x 100

[3]Specific Growth Rate (%) =100 x [(log final body weight - log initial body weight) / days]

[4]Feed conversion ratio = dry feed fed / wet weight gain

[5]Protein efficiency ratio = wet weight gain / total protein given

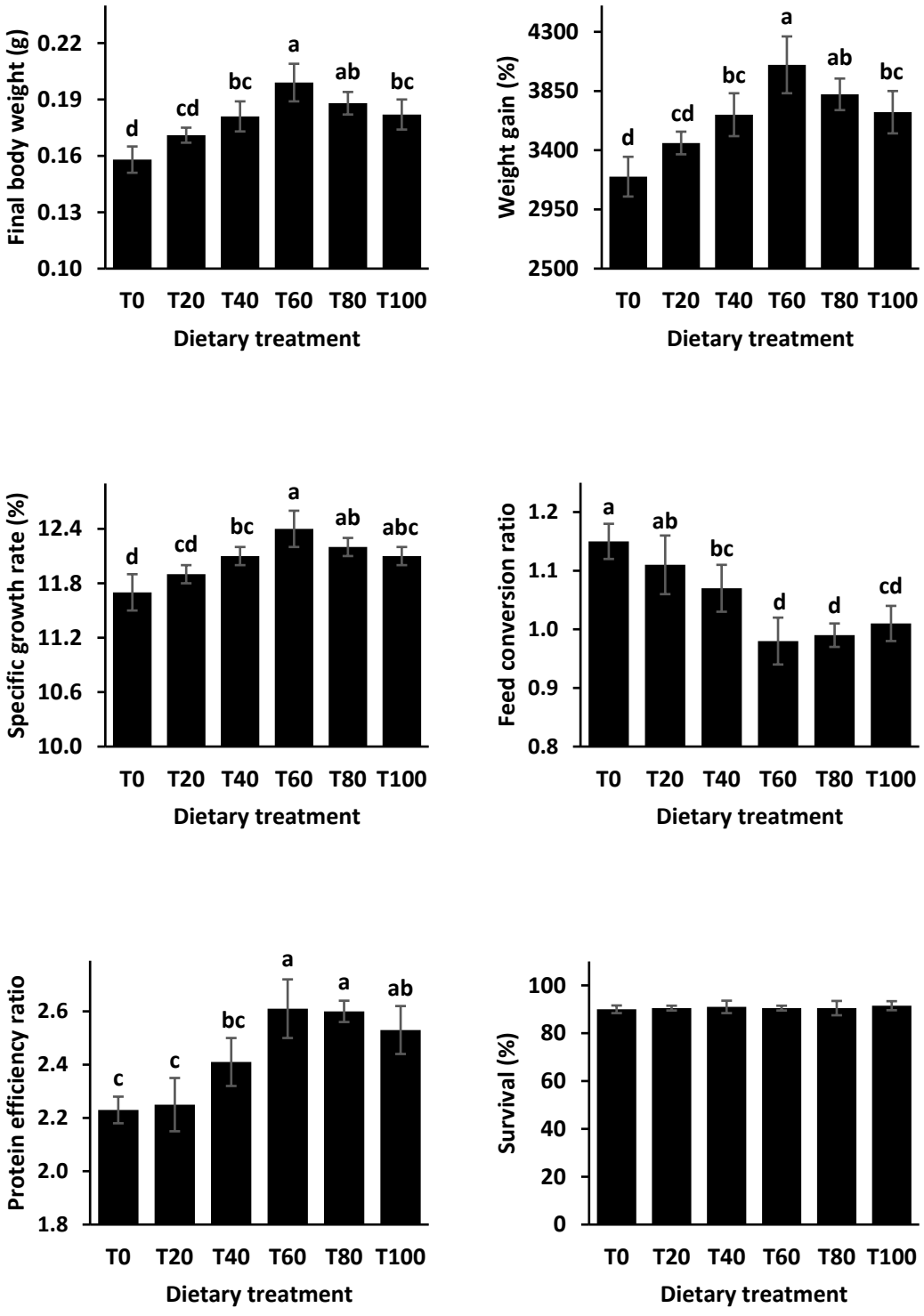

Figure 22. Growth performance, feed utilization efficiency and survival of Pacific white shrimp (*Penaeus vannamei*) fed the experimental diets containing graded levels of thiamine for 30 days.

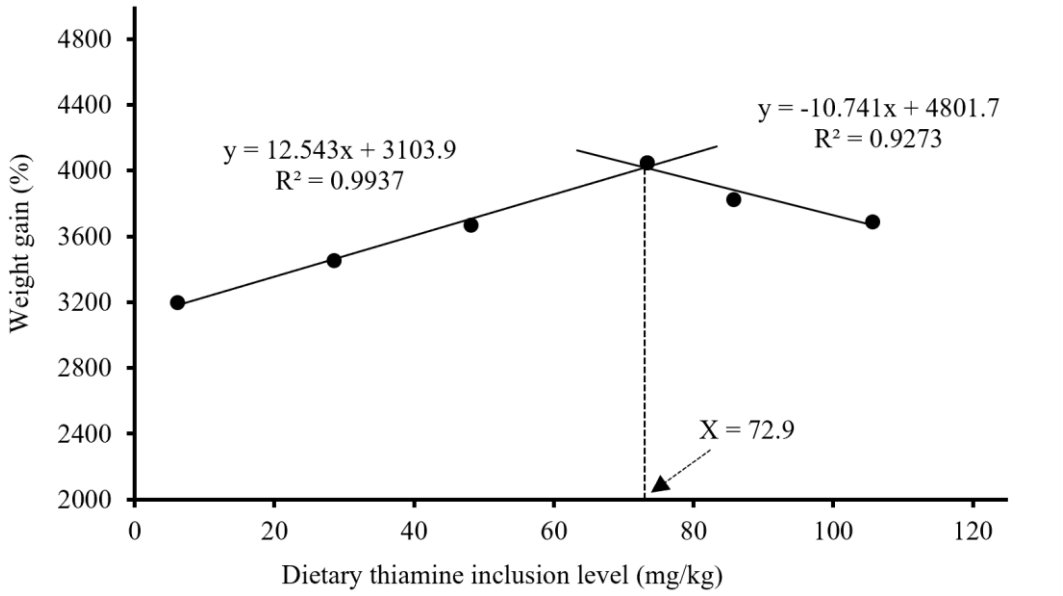

Figure 23. Broken line graph showing the relationship between weight gain (%) and dietary thiamine levels. X represents the estimated optimum dietary thiamine requirement (72.9 mg/kg) of Pacific white shrimp (*Penaeus vannamei*).

3.2.2 Real-Time qRT-PCR (Quantitative Reverse Transcription PCR)

Relative expression of growth, antioxidant and immune related genes were significantly enhanced when increasing the dietary thiamine levels in the diets of *P. vannamei*. IGF-1 relative gene expression was significantly elevated in the T60 dietary group whereas the lowest expression was recorded by T0 group (Figure 24). Relative expression of IGF-BP gene was significantly upregulated in T60 and T80 dietary groups and the significantly lowest values were observed in thiamine deficient group. TOR gene expression was not significantly different among experimental groups. When increasing dietary thiamine levels, mRNA expression of proPO gene was gradually upregulated and significantly higher expression levels were achieved by T80 and T100 dietary groups (Figure 25). Crustin gene expression was significantly elevated at the highest dietary thiamine level (T100) and the significantly lowest value was recorded by T0 group. Irrespective of thiamine supplementation, relative mRNA expression of Penaidine 3a gene was not significantly different. Thiamine supplementation significantly upregulated the relative expressions of genes related to antioxidative properties (Figure 26). CAT gene expression was significantly upregulated in the T80 group compared to the control group. SOD gene expression was significantly enhanced in T80 and T100 groups while GPx relative gene expression was significantly upregulated in T60, T80, T100 dietary treatment groups.

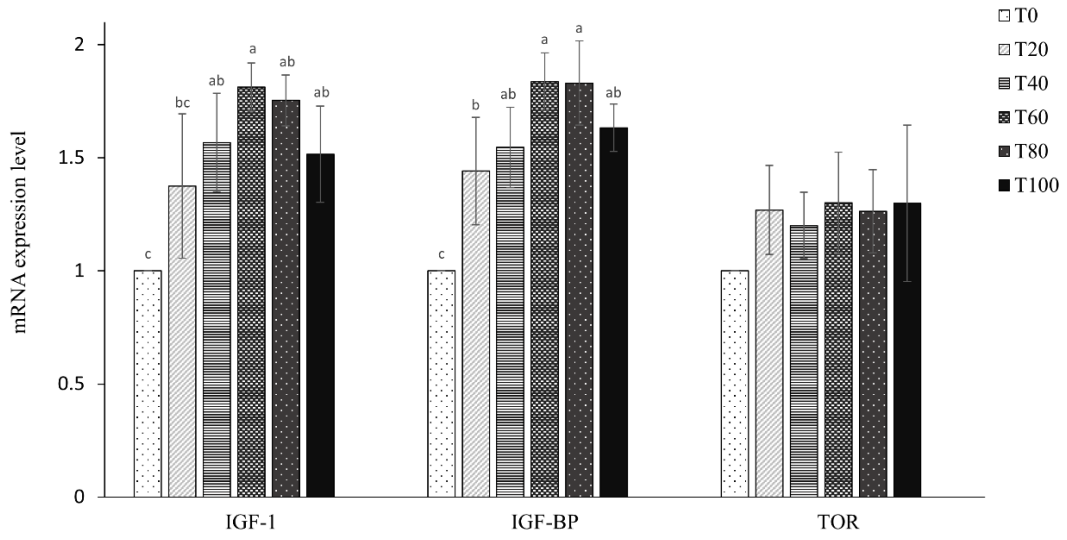

Figure 24. Relative mRNA expression of IGF-1, IGF-BP and TOR in hepatopancreas of Pacific white shrimp (*Penaeus vannamei*) fed the experimental diets. The different letters on the bars represent significant differences (P<0.05). The lack of letters on the bars indicates no significant differences among treatments.

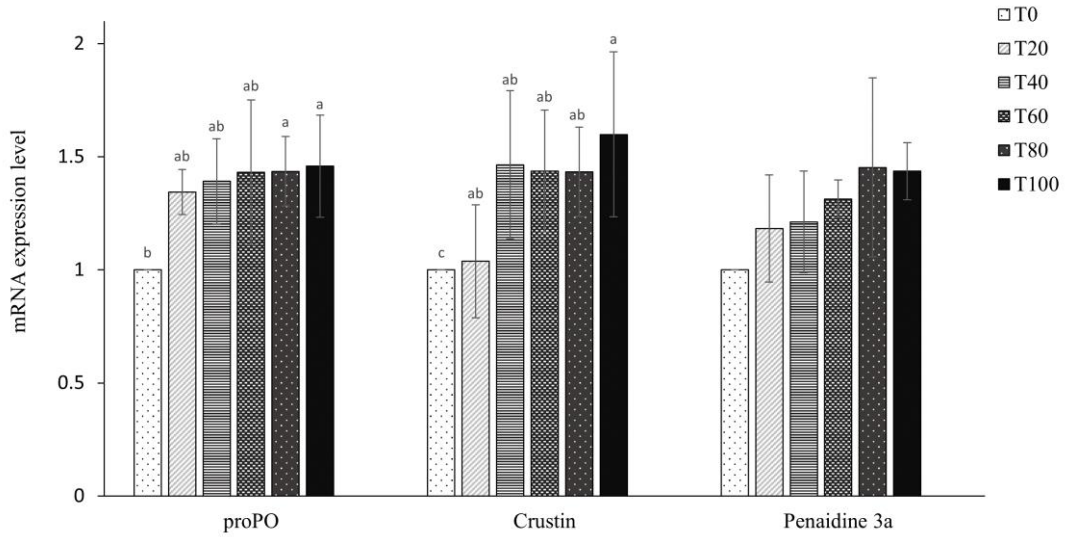

Figure 25. Relative mRNA expression of proPO, Crustin and Penaidine 3a in hepatopancreas of Pacific white shrimp (*Penaeus vannamei*) fed the experimental diets. The different letters on the bars represent significant differences (P<0.05). The lack of letters on the bars indicates no significant differences among treatments.

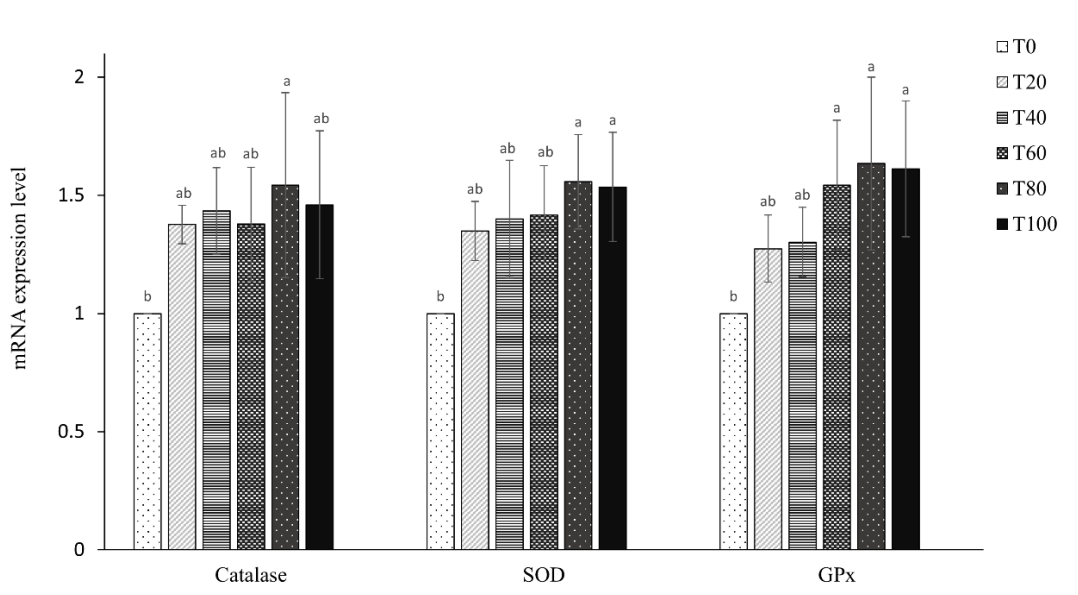

Figure 26. Relative mRNA expression of Catalase, SOD, and GPx in hepatopancreas of Pacific white shrimp (*Penaeus vannamei*) fed the experimental diets. The different letters on the bars represent significant differences ($P<0.05$). The lack of letters on the bars indicates no significant differences among treatments.

3.2.3 Digestive enzyme activity

Present study results show that thiamine supplementation significantly enhanced the digestive enzyme activities of *P. vannamei* (Table 11). Amylase activity was significantly elevated in T80 and T100 dietary treatment groups and T0 group showed the significantly lowest. Similarly, when increasing the dietary thiamine content, the pepsin activity was significantly enhanced, while thiamine deficient shrimp group showed poor pepsin activity. Lipase activity showed a significant quadratic trend when increasing dietary thiamine level and T0 dietary treatment group showed the significantly lowest lipase activity while T20, T40, T80, and T100 dietary groups resulted in the significantly highest lipase activity.

Table 11. Digestive enzyme activities of Pacific white shrimp (*Penaeus vannamei*) fed experimental diets containing graded levels of thiamine for 30 days.

Dietary treatment	Amylase activity[1]	Lipase activity[2]	Pepsin activity[3]
T0	13.7±0.7^{b}	2.15±0.18^{b}	1.31±0.23^{c}
T20	14.7±0.5^{ab}	2.79±0.24^{a}	1.75±0.33^{bc}
T40	14.6±1.4^{ab}	2.95±0.33^{a}	2.40±0.20^{ab}
T60	15.7±0.6^{ab}	2.70±0.19^{ab}	2.86±0.51^{a}
T80	16.5±1.0^{a}	3.17±0.43^{a}	3.10±0.62^{a}
T100	16.3±1.1^{a}	2.94±0.23^{a}	3.21±0.67^{a}
Pr>F*			
ANOVA	0.004	0.001	0.000
Linear	0.000	0.001	0.000
Quadratic	0.654	0.024	0.134

Values are mean of quadruplicated groups and presented as mean ± SD. Values with different superscript letters in the same column are significantly different (P<0.05). The lack of superscript letters indicates no significant differences among treatments.

[1]Amylase activity (U).

[2]Lipase activity (U/mg protein).

[3]Pepsin activity (U/mg protein).

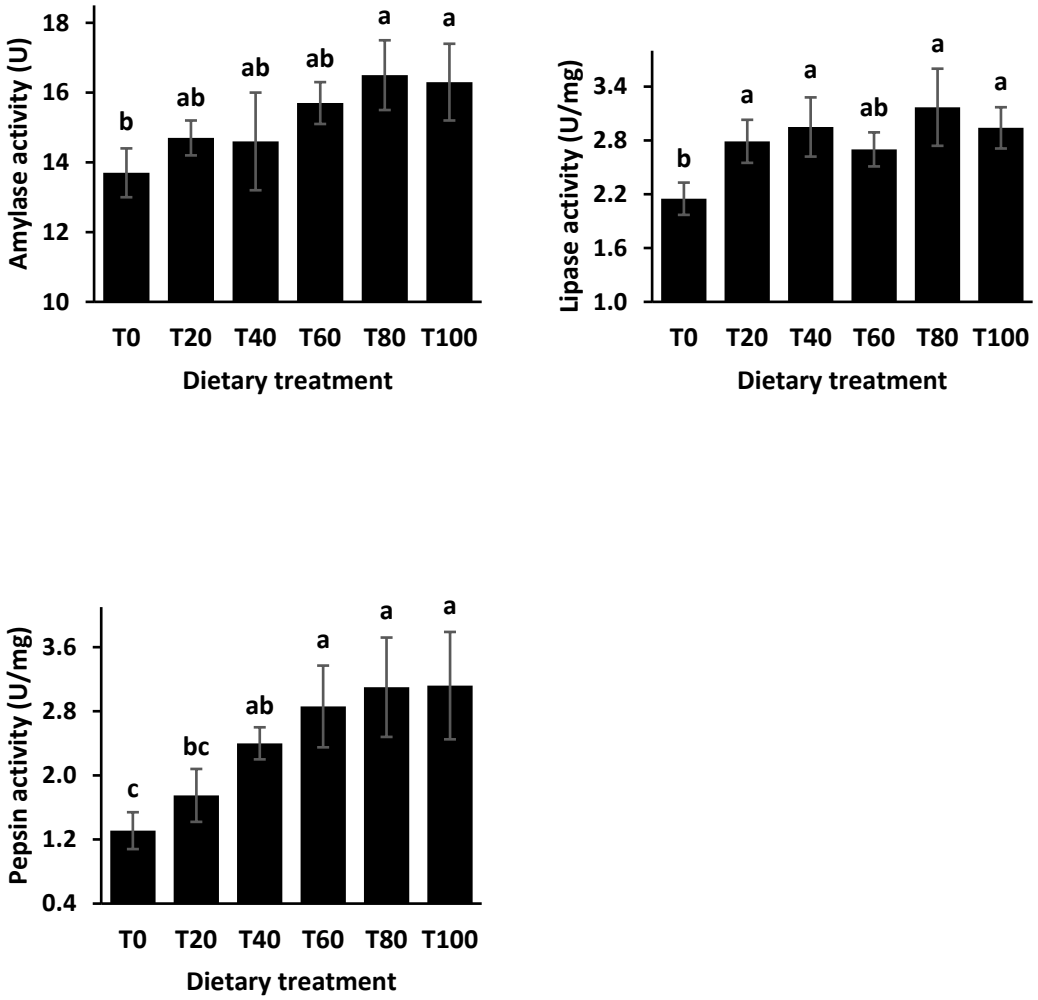

Figure 27. Digestive enzyme activities of Pacific white shrimp (*Penaeus vannamei*) fed experimental diets containing graded levels of thiamine for 30 days.

3.2.4 Ammonia stress challenge test

The survival of shrimp under ammonia stress is shown in Figure 28. Notably, mortality was initially observed after 5 h of exposure to a lethal dose of ammonium chloride. After 48 h, significantly higher survival (P<0.05) was observed in T80 dietary treatment group (Table 12), whereas thiamine deficient group (T0) recorded the significantly lowest survival (P>0.05).

Table 12. Survival (%) of Pacific white shrimp (*Penaeus vannamei*) during the ammonia stress challenge test.

Dietary treatment	Survival (%)
T0	40 ± 10^{a}
T20	53 ± 7.6^{ab}
T40	58 ± 7.6^{ab}
T60	55 ± 5.0^{ab}
T80	65 ± 8.7^{a}
T100	60 ± 8.7^{ab}

Values are mean of quadruplicated groups and presented as mean ± SD. Values with different superscript letters in the same column are significantly different (P<0.05). The lack of superscript letters indicates no significant differences among treatments.

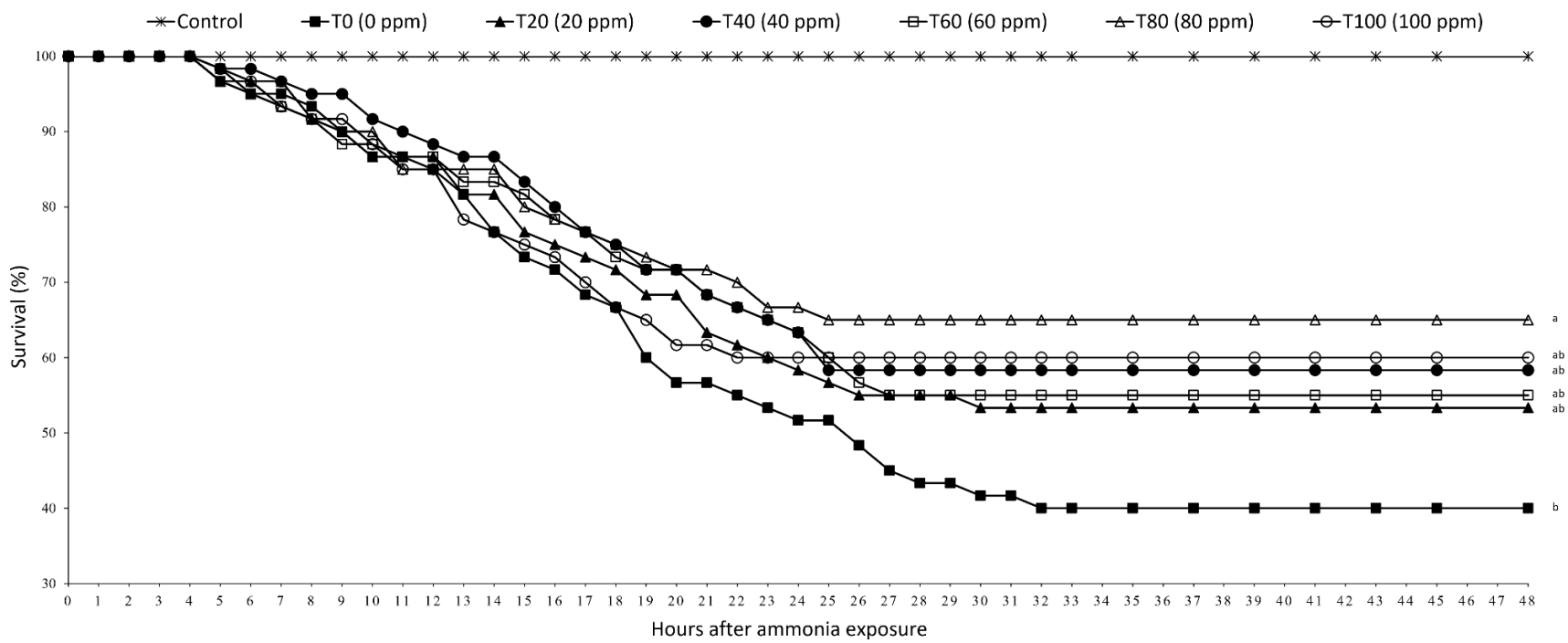

Figure 28. Survival (%) of Pacific white shrimp (*Penaeus vannamei*) during the ammonia stress challenge test. Shrimp were fed six experimental diets with graded thiamine levels for 30 days before the challenge test.

3.3 Discussion

Present study results depict that incorporating an optimum dose of thiamine to the aquaculture diets enhanced the growth performance, feed utilization, digestive enzyme activities and immune related relative gene expressions of *P. vannamei* post larvae. A broken line regression model showed that the optimum WG% was achieved at the thiamine level of 72.9 mg/kg of diet. As per previous studies, it is perceivable that an optimum level of thiamine enhances the growth and feed utilization of aquatic animals, including, *Sclizothorax prenanti*; 21.49 mg/kg (Xiang et al. 2016), grass carp; 1.3 mg/kg (Jiang et al. 2014), giant tiger shrimp (Chen et al. 1991), Oriental river prawn; 66.80 mg/kg (Sun et al. 2022), and abalone; 51 mg/kg (Zhu et al. 2002). In the current study, these observations were further validated through the relative expression of growth-related genes, IGF-1 and its binding protein (IGF-BP), which showed significant enhancements in thiamine supplemented groups compared to the thiamine deficient groups. Additionally, thiamine deficiency can cause poor activity of α-KGDC which negatively affects the functioning of mitochondria, disrupting energy production required for normal functioning of tissues and organs. Moreover, thiamine deficiency may lead to endoplasmic reticulum stress that results in overexpression of growth arrest and DNA damage-inducible protein 153, responsible for apoptosis and inducing cell cycle arrest (Igase et al. 2001; Liu et al. 2017). According to NRC (2011), vitamin requirements of shrimp may be altered by species, size, age, growth rate, environment, and nutrient interactions. Evidently, in a previous study conducted by Wijemanna and Lee (2023) for juvenile *P. vannamei* (initial weight, 6 g), recorded an optimum level of thiamine at 19.7 mg/kg of diet, which is lower than that of current study for *P. vannamei* post larvae. Moreover, in another study for juvenile *P. vannamei* (initial weight, 0.5 g), recorded an optimum level of 44.66 mg/kg (Huang et al 2015) and the requirement was higher than in the study by Wijemanna and Lee (2023). These observations suggest that, for the same shrimp species, dietary thiamine requirement varies

with the growth stage, where the requirement increased at early life stages, mainly due to rapid growth rate, immaturity and limited ability to store nutrients in the body (Hansen et al. 2015).

Reactive oxygen intermediates (ROIs), namely, hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl ion (OH^-) play a crucial role during immune defense mechanisms in invertebrates, such as phagocytosis (Ren et al. 2009). Excess levels of ROIs may lead to oxidative stress in organisms, which may damage proteins, lipids and DNA, eventually leading to cell injury and cell death (Chauhan et al. 2018). In organisms, SOD enzyme converts O_2^- into H_2O_2, and GPx and CAT metabolize H_2O_2 into H_2O, where these enzymes play a crucial role in antioxidant activity in organisms (Reiter et al. 2006). Moreover, Chauhan et al. (2018) and Sharma et al. (2013) observed significantly declined SOD, GPx and CAT activities in the brain mitochondria of house mouse (*Mus musculus*) fed a thiamine deficient diet. In the present study, thiamine supplementation significantly enhanced the relative expression of genes which are responsible for encoding the SOD and CAT enzyme, compared to the thiamine deficient group. Previous studies show beneficial effects on SOD activity by supplementing an appropriate dose of thiamine. Wijemanna and Lee (2023) and Huang et al. (2015) in their respective studies for *P. vannamei*, observed an improvement in the SOD activity of shrimp supplemented with thiamine compared to the thiamine deficient groups. Similarly, SOD activities of spotted snakehead (Zehra and Khan 2018) and Jian carp (Li et al. 2014) were elevated along with the supplementation of thiamine. Moreover, Zhou et al. (2022) observed that thiamine supplemented groups showed a higher SOD activity when Oriental River prawns were subjected to hypoxia stress.

In the present study, relative expression of the GPx gene was significantly elevated in the thiamine supplemented group over the deficient groups. Furthermore, in a study by Xun et al. (2019), they observed significantly enhanced GPx and SOD activities in golden pompano fed with thiamine supplemented diets. The phosphorylated form thiamine diphosphate, also known as TPP, serves as a cofactor for enzymes responsible for energy metabolism,

specifically TK enzyme (Gioda et al. 2010). TK is involved in the first and the final steps of the pentose phosphate pathway (PPP) which plays a crucial role in cellular functions in the production of NADPH for maintaining glutathione levels (Depeint et al. 2006). Glutathione serves as an electron donor in the redox process of GPx and its recycling and regeneration is regulated by GPx (Shao et al. 2020). Moreover, PPP is crucial in supplying ribose for nucleic acid synthesis (Depeint et al. 2006). Deficiency in thiamine may reduce the DNA synthesis as a result of disrupted transketolase activity, poor ribose synthesis and impaired neurotransmitter synthesis caused by demyelination of nerve fibers (Mrowicka et al. 2023). Therefore, possible causes for the enhanced relative gene expressions could be explained as follows. Metabolic pathways provide precursor molecules and more importantly ATP that supplies energy required for gene expressions and is highly variable on nutrient uptake. Thus, cells regularly calibrate expression of genes based on changes in metabolites and energy availability (Carthew 2021).

Shrimp's natural immune system acts as an efficient defense mechanism against pathogens, through various modes, including phagocytosis, antimicrobial peptides, lysosomal enzymes prophenoloxidase sytems. In the present study, immune related gene expressions of proPO and crustin were significantly elevated through the dietary supplementation of thiamine. Bacterial and fungal cell walls contain different antigens which stimulates the conversion of proPO into PO, which initiates a series of immune responses including melanotic encapsulation and subsequent destruction of pathogenic agents (Söderhäll & Cerenius 1998; Yeh et al. 2009). In a previous study by Wijemanna and Lee (2023), they observed that thiamine supplementation significantly enhances the PO activity as well as the relative expression of proPO gene in *P. vannamei,* however penaidine 3a gene expression was not significantly different among the experimental groups and similar results were observed in the present study. Both crustin and penaidine have antimicrobial and anti-fungal properties that plays a crucial role in the innate immunity of crustaceans (Bachère et al. 2000; Wang et al.

2021). De Caterina & Madonna (2004) concluded that micronutrients, and specifically vitamins, can interact directly with transcription factors of specific genes through cis-regulatory components in promoter regions, which could be a possible reason to enhance immune related gene expressions which are compatible with the patterns of growth and feed utilization parameters.

Nutrients are digested by digestive enzymes, namely amylase, lipase, trypsin and pepsin, and it has been observed that dietary manipulations can affect the activity of these enzymes (Mohseni et al. 2023). It was observed that nutritional physiology is affected by the activity of digestive enzymes and may directly or indirectly regulate the growth of crustaceans (Wang et al. 2019). Present study results showed a significant improvement in the enzymatic activities of amylase, pepsin and lipase in thiamine supplemented groups, whereas thiamine deficient groups showed poor activities. In a previous study by Zhao et al. (2020) for juvenile yellow catfish (*Pelteobagrus fulvidraco*), they observed significant improvements in amylase activity of thiamine supplemented groups, however the lipase activity did not differ significantly among the dietary groups. Moreover, thiamine supplementation significantly enhanced the digestive enzyme activities of juvenile Jian carp (Huang et al. 2011). Furthermore, in a study by Mohseni et al. (2023) for juvenile beluga (*Huso huso*), they observed that when increasing dietary thiamine levels, amylase, lipase and pepsin activities were also significantly improved. Hakim et al. (2006) concluded that digestion ability and the absorption function correlate with the feed utilization and directly affects the growth performance of animals, where in the present study shrimp groups that showed a significantly higher growth performance and feed utilization, also exhibited enhanced digestive enzyme activities. However, the reasons behind the improvement of digestive enzymes activities in thiamine supplemented shrimp groups of the present study are still unclear and empirical data to confirm these observations are almost nonexistent.

To the best of our knowledge, there have been no previous studies that investigated the effect of dietary thiamine supplementation on the ammonia stress of *P. vannamei*. In shrimp aquaculture, ammonia is a major water pollutant as well as result in vast mortalities in shrimp causing huge economic losses (Zhao et al. 2020b). Furthermore, it results in poor growth, decreased osmoregulatory capacity, increased molting frequency and increased susceptibility to pathogens in shrimp (Xia et al. 2016). In a study by Zhang et al. (2018), they concluded that ammonia exposure could induce the generation of ROIs and result in oxidative stress juvenile yellow catfish. Moreover, in a previous study, it was observed that higher ability of ammonia detoxification and accelerated energy metabolism to supply energy might be possible adaptations to tolerate ammonia stress (Xiao et al. 2019). As previously mentioned, SOD, GPX and CAT enzymes contribute to mitigate the over development of ROIs and provide protection from oxidative stress. Thiamine has the ability to interact and oxidize ROIs into thiamine disulfide and thiochrome by transferring H^+ from the thiazole ring and $2H^+$ from the amine group of pyrimidine ring, respectively (Lukienko et al. 2000) and helps to maintain the equilibrium in the production and removal of ROIs (Zhou et al. 2022). Moreover, it was observed that thiamine has a potential to mitigate iron-catalyzed oxidative stress in hepatocytes of Male Sprague–Dawley rats by lessening the mitochondrial damage, lipid peroxidation, and DNA oxidation (Mehta et al. 2011). Furthermore, thiamine inhibits production of advanced glycation end-products (AGE) which cause oxidative damage and inflammation to tissues and organs by modifying proteins and causing cellular malfunction and apoptosis, suggesting that thiamine is a potent antioxidant in organisms' body (Beltramo et al. 2008; Li et al. 2014; Mrowicka et al. 2023). When focusing on ammonia stress challenge of the present study, it is obvious that thiamine supplemented groups had a higher survival rate compared to thiamine deficient groups, that may result due to antioxidant properties of thiamine, however, requires further investigations to validate the role of thiamine in ammonia stress tolerance.

3.4 Conclusion

An optimum dietary thiamine level could positively influence growth performance, feed utilization, innate immunity, antioxidative activity, immune related gene expression, digestive enzyme activity, and ammonia stress tolerance of *P. vannamei*. Based on the broken line graph of WG%, the optimum dietary level of thiamine for *P. vannamei* would be 72.9 mg/kg of diet.

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Dietary Thiamine (Vitamin B_1) Requirement of Pacific White Shrimp (*Penaeus vannamei*)

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요약문

흰다리새우(*Penaeus vannamei*)의 juvenile 및 post-larvae 성장 단계에서 사료 내 thiamine (비타민 B1)의 요구량을 평가하였다. 두 실험 모두, 어분을 주 단백질원으로 사용한 대조사료(T0)를 사용하였으며 cellulose 를 20, 40, 80, 100 mg/kg 씩 thiamine hydrochloride 로 대체하여 5 가지 실험사료를 제작하였다(T20, T40, T60, T80 및 T100).

Chapter 1 에서는 juvenile *P. vannamei* 의 성장률, 사료 이용성 및 비특이적 면역력에 미치는 영향을 바탕으로 사료 내 thiamine 요구량을 평가하였다. 총 360 마리의 새우 (6.03 ± 0.03g)를 24 개의 아크릴 수조 (240L)에 실험구 당 4 반복으로 무작위 배치하여 58 일 동안 1 일 6 회(08:00, 10:00, 12:00, 14:00, 16:00 및 18:00) 사료를 공급하였다. 고성능 액체 크로마토그래피로 분석한 실험사료의 thiamine 농도는 T0, T20, T40, T40, T60, T80 및 T100 에서 각각 3.2, 20.8, 38.7, 60.2, 80.7 및 108 mg/kg 으로 나타났다. T20 사료를 공급한 새우는 T0 와 T100 사료를 공급한 새우에 비해 유의적으로 높은 성장률을 보였다. Feed conversion ratio (FCR)과 protein efficiency ratio (PER)는 모든 실험구 간에 유의적인 차이가 없었다. T20 구는 T0 구 및 T100 구보다 유의적으로 높은 phenoloxidase (PO) 활성도를 보였다. Lysozyme 활성도는 T20, T40, T60, T80 구에서 T0 구에 비해

유의적으로 향상되었다. Anti-protease 활성도는 사료 내 thiamine 농도에 영향을 받지 않았다. Superoxide dismutase (SOD)와 glutathione peroxidase (GPX) 활성도는 T20 구에서 T0 에 비해 유의적으로 향상되었다. Penaiedin 3a 와 insulin-like growth factor-1 (IGF-1)의 유전자 발현량은 사료 내 thiamine 농도에 영향을 받지 않았다. T20 및 T40 사료를 공급한 새우는 T0 사료를 공급한 새우보다 유의적으로 높은 prophenoloxidase (proPO) 발현량을 보였다. Hemolymph 내 glucose, total cholesterol, triglycerides 및 total protein 수준은 모든 실험구에서 유의적인 차이가 없었다. 장 내 융모 길이는 사료 내 thiamine 농도에 영향을 받지 않았다. Juvenile *P. vannamei* 의 사료 내 최적 thiamine 농도는 weight gain (WG)을 바탕으로 한 piecewise regression 분석 결과, 19.7 mg/kg 일 것으로 판단된다.

Chapter 2 에서는 post-larvae *P. vannamei* 의 성장률, 사료 이용성, 비특이적 면역력, 소화효소 활성도 및 암모니아 스트레스 내성을 바탕으로 사료 내 thiamine 요구량을 평가하였다. 총 1,200 마리의 새우(5 mg)를 24 개의 아크릴 수조(10L)에 실험구 당 4 반복으로 무작위 배치하여 30 일 동안 1 일 6 회 사료를 공급하였다(08:00, 10:00, 12:00, 14:00, 16:00 및 18:00). 고성능 액체 크로마토그래피로 분석한 실험사료의 thiamine 농도는 T0, T20, T40, T60, T80 및 T100 에서 각각 6.1, 28.5, 48.1, 73.3, 85.7 및 106 mg/kg 으로 나타났다. 사육실험 후, 실험구 당 60 마리(수조 당 15 마리)의 새우를 무작위로 포획하여 암모니아 공격실험을 위해 3 반복으로 배치하였다. 모든 thiamine 첨가구는 T0 구에 비해 성장률과 사료 이용성이 유의적으로 높았다. T40, T60, T80 및 T100 구의 final body weight 은 T0 구에 비해 유의적으로 높았고 WG 와 specific growth rate 도 유사한 경향을 보였다. Feed conversion ratio 는 T40, T60, T80 및 T100 구에서 T0 구에 비해 유의적으로 향상되었으며 protein efficiency ratio 는 모든 thiamine 첨가구에서 T0 구에 비해 유의적으로 높았다. 생존율은 모든 실험구 간에 유의적인 차이가 없었다. 새우의 소화 효소(amylase, lipase 및 pepsin) 활성도는 사료 내 thiamine

농도가 높을수록 향상되었으며 T0 구에 비해 유의적으로 높았다. 모든 thiamine 첨가구에서 T0 구에 비해 유의적으로 높은 IGF-1, insulin-like growth factor binding protein (IGF-BP), proPO 및 crustin 발현량이 나타났다. 암모니아 침지 48 시간 후, 새우의 생존율은 T80 구에서 T0 구에 비해 유의적으로 높았다. Post-larvae *P. vannamei* 의 사료 내 최적 thiamine 농도는 WG 를 바탕으로 한 piecewise regression 분석 결과, 72.9 mg/kg 일 것으로 판단된다.