RESEARCH ARTICLE

Dietary Vitamin C and Lipid Feed Supplementations Influence the Expression of Immune-Related Genes in *Litopenaeus vannamei* (Boone 1931) Subjected to Thermal Stress

Eljenmer L. Sindo[1,2], Angel Queenee D. Dequito[2,3]*, Rex Ferdinand M. Traifalgar[2]

[1] *Institue of Fish Processing Technology, College of Fisheries and Ocean Sciences, University of the Philippines Visayas, Miagao 5023, Iloilo, Philippines*
[2] *Institute of Aquaculture, College of Fisheries and Ocean Sciences, University of the Philippines Visayas, Miagao 5023, Iloilo, Philippines*
[3] *Institute of Bioinformatics and Systems Biology, College of Engineering Bioscience, National Yang Ming Chiao Tung University, Hsinchu, Taiwan*

ABSTRACT

This study investigated the effects of dietary vitamin C (vitC) and lipid on the expression of immune-related genes in *Litopenaeus vannamei* postlarvae subjected to thermal stress, with water temperature fluctuating from 27.0 ± 1.1 to 34.0 ± 1.8 °C for 15 days. Three experimental diets were formulated containing two different levels of vitC (1 and 3 g kg $^{-1}$) and lipid (17 and 34 g kg $^{-1}$). Significantly higher total hemocyte count ($9.63 \times 10^5 \pm 3.28 \times 10^5$ cells ml $^{-1}$) was observed in shrimp fed 3 g kg $^{-1}$ vitC and 34 g kg $^{-1}$ lipid diets. Using real-time quantitative polymerase chain reaction and the comparative $2^{-\Delta\Delta Ct}$ method, the dietary vitC level of 3 g kg $^{-1}$ significantly upregulated heat shock protein 90 (HSP90), heat shock protein 70 (HSP70), anti-lipopolysaccharide factor (ALF), crustin, and the Prophenoloxidase (proPO) system. Meanwhile, the dietary lipid level of 34 g kg $^{-1}$ resulted in a significant upregulation of HSP90 and penaeidin 2 (PEN 2) genes. Regarding the growth and survival indices of the shrimps fed the formulated diets, no significant differences ($P > 0.05$) were observed. In conclusion, increased dietary supplementation of vitC (3 g kg $^{-1}$ diet) and lipid (34 g kg $^{-1}$ diet) upregulated the immune-related genes, enhancing the immunity of shrimps during thermal stress.

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1. INTRODUCTION

The Pacific white shrimp, *Litopenaeus vannamei* (Boone 1931), is among the widely cultivated fishery products and is a vital economic, food, and protein source (Fachri et al. 2021). From 1995 to 2019, it was the most significant contributor to global shrimp aquaculture production (FAO 2020), with an expected increase in inland culture due to its adaptability to low-salinity environments (Liao and Chien 2011).

Temperature is a crucial water parameter in shrimp culture, directly affecting shrimp's physiological performance, growth, survival, metabolic rate, and immune response (Zhang et al. 2019). Elevated water temperature stresses shrimp, resulting in poor immunity and resistance to infection, decreased phagocytosis activity, phenoloxidase activity, and respiration activity (Cheng et al. 2005). Fluctuations between optimal and high-water temperatures increase the infectivity of the white spot syndrome virus (WSSV) in juvenile *L. vannamei* (Rahman et al. 2007), while cyclic temperature fluctuations decrease growth rate, food utilization efficiency, and hemolymph glucose content (Guo et al. 2010). In other aquatic organisms, such as the Mozambique tilapia (*Oreochromis mossambicus*) exposed to temperatures as high as 35 °C, a significant reduction in the white blood cell count was observed, weakening the immune defenses of the organism

(Ndong et al. 2007). Ectothermic animals exposed to thermal stress suffer from oxidative stress and a shift in redox state (Nakano et al. 2014). Thermal stress results in adaptive responses in some aquatic animals, including crustaceans. However, these responses can be lost or dysfunctional when the animal is exposed to extreme and prolonged stress, resulting in growth inhibition, reproductive failure, and poor disease resistance (Wendelaar-Bonga 1997).

Vitamin C (vitC) is essential for penaeid shrimps and aquatic animals. It improves stress resistance, growth performance, physiological function, and immunological response (Dawood and Koshio 2018). It helps in collagen synthesis by hydroxylating proline and lysine (Niu et al. 2009). Aside from its immunomodulatory properties, it is reported to have potent antioxidant properties that protect low-density lipoproteins (LDLs) from oxidation (Cheng et al. 2005). In *L. vannamei*, dietary vitC intake improves growth, survival, and antioxidant status (Ebadi et al. 2021). In genetically modified *O. niloticus*, increasing dietary vitC supplementation significantly increased weight gain, liver vitC content, muscle collagen, and total body lipid content (Huang et al. 2016).

Eicosapentaenoic (20:5n-3, EPA), docosahexaenoic (22:6n-3, DHA), and arachidonic (20:4n-6, ARA) acids are polyunsaturated fatty acids (PUFAs) essential for many physiological functions in shrimp, including survival, reproduction, growth, and immunological responses (Mercier et al. 2009). The n-3 PUFAs in dietary lipids, such as in fish oil, reportedly mediate the fluidity and restructuring of cellular membranes and alter the subcellular localization of proteins (Matesanz et al. 2010). EPA-rich fish oil is an alternative cyclooxygenase (COX) substrate and enhances lipid and lipoprotein metabolism. By increasing reduced glutathione (GSH) and HSP70, fish oil is also essential for reducing oxidative stress and minimizing cellular damage caused by heat stress (Millward 2012).

Recent studies have focused on examining immune-related genes and their expression levels as a secondary response to stress, including exposure to extreme temperatures. The family of Heat Shock Proteins (HSPs), such as HSP90 and HSP70, are known for their immediate response to stressors (Junprung et al. 2021). These immune genes aid in refolding, folding, translocating, assembling, and destroying proteins (Junprung et al. 2021) when animals are in stressful conditions (i.e., temperature stress, toxic stress, and infection). The Prophenoloxidase (proPO) system functions in tissue repair and defense against pathogens (Junprung et al. 2021). Penaeid shrimp also rely on antimicrobial peptides (AMPs) against invading pathogens (Destoumieux-Garzón et al. 2016). The AMPS found on shrimp hemocytes, such as penaeidin 3 (PEN 3) and penaeidin 2 (PEN 2), reportedly have antibacterial and antifungal activities in response to microbial infection (Destoumieux-Garzón et al. 2016). Crustin is a polypeptide with antiprotease properties, while anti-lipopolysaccharide factors (ALFs) have antifungal, antibacterial, and immunomodulatory abilities (de la Vega et al. 2008). Thermal stress weakens the pathogen resistance and immune response of shrimps (Rahman et al. 2007; Janewanthanakul et al. 2020). During these conditions, AMPs may provide immune defense due to their immunomodulatory and antibacterial properties. Pathogens can also cause tissue damage, and proPO may mediate these effects due to their role in repairing damaged tissue.

This study aimed to investigate the effects of increased dietary supplementation levels of vitC and lipid on *L. vannamei* postlarvae subjected to thermal stress, specifically on the expression levels of immune-related genes: HSP70, HSP90, ALF, PEN 3, PEN 2, Crustin, and proPO, with EF1a as the reference gene. The study also investigated its impacts on the total hemocyte count (THC), growth indices (specific growth rate, weight gain, and feed conversion ratio), and survival rate.

2. MATERIALS AND METHODS

2.1 Experimental animal and acclimation

Postlarvae (PL 12) specific pathogen-free (SPF) Pacific white shrimps, *L. vannamei* (Boone 1931), were purchased from HP Aquafarm Inc. Hatchery (Iloilo, Philippines) and transported to the Institute of Aquaculture Multi-Species Hatchery Complex, University of the Philippines Visayas. Prior to the experiment, randomly selected SPF shrimps were acclimated for seven (7) days in a 10-ton concrete tank. The seawater (35 ppt) was treated with 10 ppm chlorine. After 24 h, the seawater was neutralized with the same amount of thiosulfate prior to stocking, following the manufacturer's instructions (Rainbow™ OTO Solution Chlorine/Bromine Test). The rearing conditions were maintained at a temperature set at 27.5 ± 0.5 °C with moderate aeration and natural photoperiod. The acclimated shrimps were hand-fed with commercial feed (Vannamei #3 starter pellets, Oversea Aqua Feeds, Philippines, 37 % crude protein) at 5 % of their biomass daily at 0800, 1200, and 1600 h.

2.2 Experimental diet

Three experimental diets in sinking pellet form were formulated to contain different amounts of vitC (ascorbic acid monophosphate) and dietary lipid (Danish fish oil). The Danish fish oil has around 1–7 % free fatty acids sourced from fresh fish (FF, Skagen). The diets were prepared following Genio et al. (2015) and Traifalgar et al. (2010). VitC and lipid are crucial inclusions in the practical and basal diets of shrimps for growth, development, and immune responses (Montayo and Molina 1995; González-Félix et al. 2002; Lee and Shiau 2002). The dietary supplementation level in Treatment 1 (control) for vitC was from Montayo and Molina (1995) and Ebadi et al. (2021). In the same treatment, the dietary supplementation level of lipids was within the practical range provided by Gong et al. (2001), Niu et al. (2011), and Maliwat et al. (2017). For 15 days, shrimp were fed the formulated experimental diets at 5 % of their biomass, subdivided into three feeding rations at 0800, 1200, and 1600 h. Treatment 1 (vitC+lipid) contained 1 g kg $^{-1}$ vitC and 17 g kg $^{-1}$ fish oil and was used as the control. Treatment 2 (*3vitC*+lipid) contained 3 g kg $^{-1}$ vitC and 17 g kg $^{-1}$ fish oil. Treatment 3 (vitC+*2lipid*) contained 1 g kg $^{-1}$ vitC and 34 g kg $^{-1}$ fish oil. The ingredients and the proximate composition of the formulated experimental feeds are presented in Table 1.

2.3 Experimental design

One hundred eighty (180) *L. vannamei* (4.0 ± 1.0 g) were randomly distributed among nine 120-L rectangular plastic tanks. Each treatment was in triplicate, resulting in nine tanks. Seawater was subjected to the same treatment method done during the acclimation process. Shrimps were subjected to thermal stress using an electric heater with a built-in thermometer turned on and off daily at 0700 and 1600 h, respectively, simulating fluctuating environmental

Table 1. Ingredients, formulation, and proximate composition of the experimental diets vitC+lipid, *3vitC*+lipid, and vitC+*2lipid*.

Ingredients	**Diets (g kg $^{-1}$)**		
	vitC+lipid	***3vitC*+lipid**	**vitC+*2lipid***
Soybean meal (48 solv)	230	230	230
Trace mineral premix[a]	15	14	15
Squid meal	80	80	80
Fish meal (70 % - Danish)	230	230	230
Rice bran	125	125	125
Corn/wheat starch	240	240	240
Fish oil	17	17	34
Soybean oil	17	17	0
Vitamin premix[b]	15	14	15
Lecithin - Soy (70 %)	10	10	10
Gluten (wheat)	20	20	20
Vitamin C[c]	1	3	1
Total:	1000	1000	1000
Proximate composition			
Crude Protein	37.5 ± 0.17	35.8 ± 0.08	37.1 ± 0.02
Crude lipid	6.4 ± 0.16	10.6 ± 0.12	7.6 ± 0.04
Ash	7.95 ± 0.04	7.63 ± 0.06	8.03 ± 0.06
Moisture	7.4 ± 0.01	7.2 ± 0.02	7.0 ± 0.02
Crude Fiber	2.9 ± 0.02	2.9 ± 0.05	2.9 ± 0.09
Nitrogen-Free Extract	37.8 ± 0.06	35.8 ± 0.03	37.5 ± 0.03

[a]Trace mineral premix (g kg $^{-1}$ in diet): $CaHPO_4 \cdot 2H_2O$, 8 g: $MgSO_4 \cdot 7H_2O$, 5 g: KH_2PO_4, 4 g: $Na_2H_2PO_4 \cdot 2H_2O$, 2 g: $MnSO_4 \cdot H_2O$, 0.6 g: $FeSO_4$, 0.6 g: $ZnSO_4 \cdot 7H_2O$, 0.6 g: $CO(NO_3)_3$, 0.1 g: $CuSO_4$, $5H_2O$, 0.1 g.

[b]Vitamin premix (mg, mcg or IU kg $^{-1}$ in diet): vit. A, 1200000 IU; vit. D_3, 200000 IU; vit. E, 20000 IU; vit. B_1, 8000 mg; vit. B_2, 8000 mg; vit. B_6, 5000 mg, vit. B_{12} (1 %), 2000 mcg; Niacin, 40000 mg; Calcium Pathothenate, 20000 mg; Biotin, 40 mg, Folic Acid, 1800 mg; Ethoxyquin, 500 mg.

[c]35% min ascorbic acid monophosphate

temperatures. Shrimps were fed the experimental diets 1 h after being subjected to thermal stress. The recorded temperature in all tanks fluctuated from 27.0 ± 1.1 to 34.0 ± 1.8 °C. Shrimp exposed to temperatures above 34 °C are considered stressed (Allan et al. 2006), which adversely affects optimal shrimp growth performance and increases the occurrence of diseases in shrimp culture (Alonzo et al. 2017). In each tank, 80 % of the seawater volume was siphoned off and replaced with treated seawater weekly. The water temperature and salinity were measured at 0800 and 1400 h. Temperature was determined using an alcohol capillary laboratory thermometer, while salinity was measured using the REB-32ATC 0–32 % Brix refractometer.

2.4 Sampling

Random sampling was done on Day 0, 7, and 15 for growth indices, THC, and gene expression analysis. Ribonucleic acid (RNA) samples were extracted from the hemolymph of three shrimp per tank (9 shrimp per treatment) and pooled in one (1) tube as a sample. Average body weight (ABW) was determined randomly from five to ten individuals per tank. Weight gain rate (WGR), specific growth rate (SGR), feed conversion ratio (FCR), and survival rate (SR) were calculated following Genio et al. (2015).

2.5 Histological examination

Using Davidson's alcohol-formalin-acetic acid (AFA), hepatopancreas and gill tissues of *L. vannamei* were fixed for 48 h and transferred to 70 % ethanol (Bell and Lightner 1988). After dehydration in increasing ethanol concentrations, the tissue samples were cleared with xylene and embedded in paraffin. Microtome-cut sections (5 µm-thick) were stained using Hematoxylin and eosin (H&E). The histological slide sections were examined using light microscopy.

***2.6* Total hemocyte count (THC) determination**

Three shrimp individuals per treatment were collected for hemocyte count analysis during each sampling period. Precooled 0.4 mL anticoagulant composed of shrimp salt solution (SSS) containing 450 mM NaCl, 10 mM HEPES, 10 mM EDTA, and 10 mM KCl at pH 7.3 and 850 mOsm kg^{-1} was prepared in each syringe. A 1 mL sterile syringe with a 25-gauge needle was used to extract 0.2 mL of hemolymph from the first abdominal segment close to the genital pore of the shrimp. The hemolymph samples were then transferred to a 1.5 mL microcentrifuge tube containing a fixative solution (10 % formalin in 0.45 M NaCl) and kept at 4 °C. The fixed sample was added with a drop of Rose Bengal solution (1.2 % Rose Bengal in 50 % ethyl alcohol) and then stained for 20 minutes, following Genio et al. (2015). Hemocytes were counted using the Neubauer hemocytometer, and the total hemocyte count values were expressed as cells ml^{-1}, calculated following Joseph and Philip (2007).

2.7 RNA extraction

Hemolymph samples were extracted from the pleopod base at the first abdominal segment using a 1 mL syringe fitted with a 25-gauge needle. The extracted hemolymph was diluted with an anticoagulant medium prepared according to Hernández-López et al. (1996). The anticoagulant-hemolymph mixture was centrifuged at 4 °C at 10000 × g for 5 minutes, with the plasma drained after centrifugation. The cell pellet was added with 500 Trizol (Invitrogen) and stored at -20 °C.

2.8 Immune-gene expression analysis by qRT-PCR

The concentration and quality of the extracted RNA were assessed using a spectrophotometer, 1 % agarose gel electrophoresis, and Qubit (Invitrogen). The SuperScriptTM First-Strand Synthesis System test kit from Thermo Fisher Scientific was used to synthesize cDNA following the manufacturer's procedure. The qRT-PCR gene analysis was performed in CFX Connect Real-Time PCR (Bio-Rad) and iQ SYBR Green Supermix (Bio-Rad). The forward and reverse sequences of the nucleotide primers are shown in Table 2. The qPCR analyzed data were interpreted using the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) to compute the relative mean fold expression of the target genes. Fold differences and cycle threshold (Ct) values were recorded using Cycling Fluorescence Excitation Manager software (Bio-Rad). Gene expression values are presented as geometric means.

2.9 Proximate analysis

The formulated feed was evaluated using the following methods and analytical tools: crude protein using the Kjeldahl method with block digestion and steam distillation (FOSS Tecator Digestion and FOSS Kjeltec 8200 Auto Distillation Unit), crude fat

Table 2. Nucleotide sequence of the primers (HSP70, HSP90, Crustin, PEN 3, Crustin, and ALF, PEN 2, proPO) with EF1 as the reference gene.

Primer	Sequence (5' – 3')	References
*Lv*HSP70-F	CTCCTGCGTGGGTGTGTT	Janewanthanakul et al. (2020)
*Lv*HSP70-R	GCGGCGTCACCAATCAGA	Janewanthanakul et al. (2020)
*Lv*HSP90-F	TGGGCTTCTACTCCGCCTACC	Junprung et al. (2017)
*Lv*HSP90-R	ACGGTGAAAGAGCCTCCAGCA	Junprung et al. (2017)
*Lv*PEN3-F	CACCCTTCGTGAGACCTTTG	Junprung (2019)
*Lv*PEN3-R	AATATCCCTTTCCCACGTGAC	Junprung (2019)
*Lv*ALF-F	GTTATCACGCCCTTTTCTAC	Junprung et al. (2017)
*Lv*ALF-R	GAAGAATGACCTGTCCAAC	Junprung et al. (2017)
*Lv*CrustinI-F	GCTGGCCTCGATAAGTGTTG	Janewanthanakul et al. (2020)
*Lv*CrustinI-R	CATCGGTCGTTCTTCAGATG	Janewanthanakul et al. (2020)
*Lv*PEN2-F	TCGTGGTCTGCCTGGTCTT	Junprung (2019)
*Lv*PEN2-R	CAGGTCTGAACGGTGGTCTTC	Junprung (2019)
*Lv*proPO1-F	AACTCCATTCCGTCCGTCTG	Junprung (2019)
*Lv*proPO1-R	GGCTTCGCTCTGGTTAGGAT	Junprung (2019)
*Lv*EF1a-F	CTTGATTGCCACACTGCTCAC	Janewanthanakul et al. (2020)
*Lv*EF1a-R	TCTCCACGCACATAGGCTTG	Janewanthanakul et al. (2020)

was determined using the Soxtec extraction method (FOSS Soxtec 2050 Automatic System), crude fiber determined using the AOAC Ceramic Fiber Filter method (FOSS Fibertec 2010 System), moisture content was determined using the Mettler Tornado Halogen Moisture Analyzer, ash was determined using the AOAC method (2010), while the Nitrogen-Free Extract was calculated by difference.

2.10 Data and statistical analysis

All analyzed data are presented as mean ± standard errors (SE; n = 3). The data were within normal distribution as verified by the Shapiro-Wilk test. The one-way analysis of variance (ANOVA) was used to examine significant differences, $P < 0.05$. When significant differences were detected, the nature of these differences was determined using Tukey's test.

3. RESULTS

3.1 Growth indices and survival rate

The growth and survival indices of the shrimp subjected to thermal stress and fed three distinct formulated diets are presented in Table 3. The formulated diets contained 1 g kg $^{-1}$ vitC and 17 g kg $^{-1}$ lipid (vitC+lipid), 3 g kg $^{-1}$ vitC and 17 g kg $^{-1}$ lipid (*3vitC*+lipid), and 1 g kg $^{-1}$ vitC and 34 g kg $^{-1}$ lipid (vitC+*2lipid*).

When subjected to thermal stress, shrimp fed vitC+*2lipid* resulted in the highest survival rate of 81.67 ± 7.26 and the lowest FCR of 1.41 ± 0.11. Meanwhile, *3vitC*+lipid resulted in the highest SGR of 4.90 ± 0.26 and WGR of 219.32 ± 9.30. Shrimp fed vitC+lipid resulted in the lowest survival rate of 71.67 ± 4.41 and the highest FCR of 2.08 ± 0.4. No

Table 3. Growth indices and survival rate of *L. vannamei* (n = 20) subjected to thermal stress.

Parameters	vitC+lipid	*3vitC*+lipid	vitC+*2lipid*
Initial weight (g)	4.07 ± 0.05	4.08 ± 0.05	4.10 ± 0.19
Final weight (g)	8.52 ± 0.24	8.96 ± 0.50	8.55 ± 1.12
Weight gain rate (%)	209.49 ± 3.39	219.32 ± 9.30	208.47 ± 24.89
Specific growth rate (%)	4.60 ± 0.10	4.90 ± 0.26	4.50 ± 0.79
Survival rate (%)	71.67 ± 4.41	76.67 ± 4.41	81.67 ± 7.26
Feed conversion ratio (FCR)	2.08 ± 0.44	1.60 ± 0.33	1.41 ± 0.11

Shrimp fed diets with 1 g kg $^{-1}$ vitC and 17 g kg $^{-1}$ lipid (vitC+lipid), 3 g kg $^{-1}$ vitC and 17 g kg $^{-1}$ lipid (*3vitC*+lipid), and 1 g kg $^{-1}$ vitC and 34 g kg $^{-1}$ lipid (vitC+*2lipid*). Data are mean ± SE. No significant differences were observed ($P > 0.05$).

significant differences were observed between diets ($P > 0.05$).

3.2 Nonspecific immune response, THC

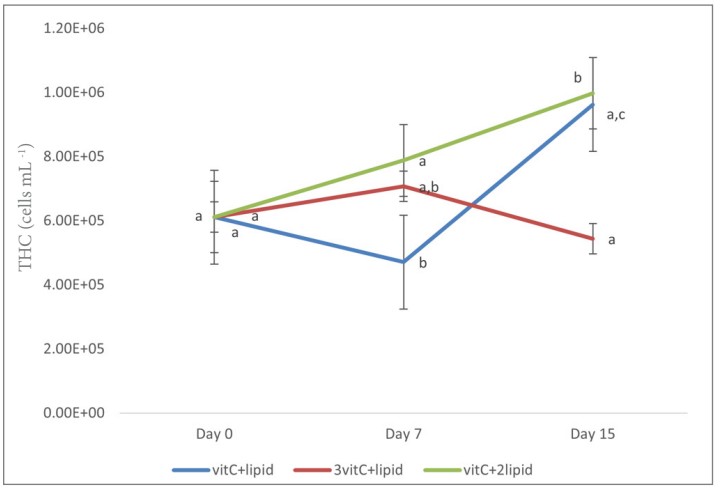

Figure 1. THC of *L. vannamei* (n = 20) subjected to thermal stress fed diets with 1 g kg $^{-1}$ vitC and 17 g kg $^{-1}$ lipid (vitC+lipid), 3 g kg $^{-1}$ vitC and 17 g kg $^{-1}$ lipid (*3vitC*+lipid), and 1 g kg $^{-1}$ vitC and 34 g kg $^{-1}$ lipid (vitC+*2lipid*). Data are mean ± SE. Different superscript on Day 7 and 15 sampling period indicates significant differences among groups ($P < 0.05$).

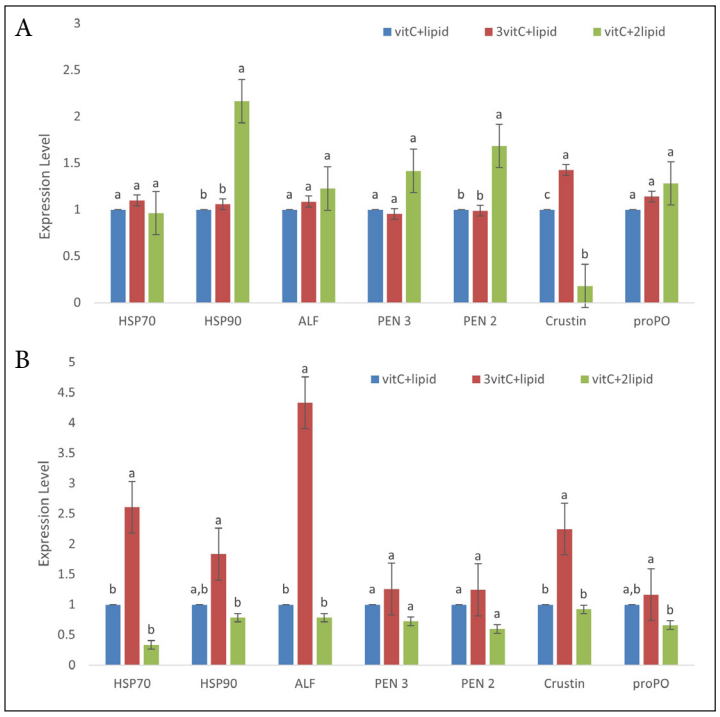

Figure 2. Mean fold gene expressions of heat shock protein70 (HSP70) gene, heat shock protein90 (HSP90) gene, anti-lipopolysaccharide factor (ALF) gene, penaeidin 3 (PEN 3) gene, penaeidin 2 (PEN 2) gene, crustin, and prophenoloxidase (proPO) system gene measured at (**A**) Day 7 and (**B**) Day 15 of culture from the hemolymph of *L. vannamei* subjected to thermal stress, fed vitC and lipid-supplemented diets (vitC+lipid, *3vitC*+lipid, and vitC+*2lipid*). Gene expressions are presented in geometric mean. Data are mean ± SE. Different superscripts indicate significant differences ($P < 0.05$).

The THC of *L. vannamei* subjected to thermal stress and fed vitC+lipid, *3vitC*+lipid, and vitC+*2lipid* is shown in Figure 1. At Day 7, vitC+*2lipid* resulted in a significantly higher ($P < 0.05$) THC of 7.88 ± 3.23 × 10^5 cells ml^{-1} than vitC+lipid, which resulted in a THC of 4.71 ± 2.89 × 10^5 cells ml $^{-1}$. Meanwhile, *3vitC*+lipid resulted in THC of 7.07 ± 5.55 × 10^5 cells ml $^{-1,}$ which is not significantly different ($P > 0.05$) from that of vitC+lipid and vitC+*2lipid*. At Day 15, *3vitC*+lipid resulted in a significantly lower ($P < 0.05$) THC of 5.44 ± 1.84 × 10^5 cells ml $^{-1}$ than vitC+lipid and vitC+*2lipid* with THC of 9.63 ± 3.28 × 10^5 cells ml $^{-1}$ and 9.98 ± 5.29 × 10^5 cells ml $^{-1}$ respectively.

3.3 Expression of immune-related genes in *L. vannamei*

The mean fold expressions of the immune-related genes at Days 7 and 15 from the hemolymph samples of *L. vannamei* subjected to thermal stress are illustrated in Figure 2. Compared with the vitC+lipid diet, the vitC+*2lipid* diet at Day 7 of culture resulted in a significantly upregulated HSP90 and PEN 2 ($P < 0.05$) at 2.16-fold and 1.69-fold, respectively. Notably, the vitC+2lipid diet resulted in a significantly downregulated Crustin ($P < 0.05$); however, it is not downregulated in shrimp fed with vitC+lipid or *3vitC*+lipid diets. The experimental diets resulted in HSP70, ALF, PEN 3, and proPO levels being not significantly expressed.

Immune-related genes in shrimp fed with the *3vitC*+lipid diet that were not found to be significantly upregulated at Day 7, such as ALF and HSP70, were observed to be significantly upregulated ($P < 0.05$) at Day 15 of culture. Compared with the vitC+lipid diet, the *3vitC*+lipid diet significantly resulted in a 4.33-fold higher ALF expression, 2.61-fold higher HSP70 expression, 1.83-fold higher HSP90 expression, and 1.64-fold higher proPO expression ($P < 0.05$). However, the vitC+*2lipid* diet downregulated the

expressions of HSP70, HSP90, ALF, PEN3, PEN2, Crustin, and proPO.

4. DISCUSSION

Long-term hyperthermal stress above the critical thermal point disrupts normal physiological processes in shrimp. Extreme temperature conditions decrease food intake, impede molting and growth, and weaken the immune system of shrimps, leading to mortality (Roberts et al. 2010). Thermal stress also leads to increased incidence and prevalence of diseases in shrimp culture, such as susceptibility to WSSV and *Vibrio alginolyticus* (Cheng et al. 2005)

Vitamin C is a known immunostimulant, while lipids from fish oil reportedly have beneficial effects on the immunity of some aquatic organisms (Kumar et al. 2022). However, studies examining the effects of increased vitamin C and lipid intake on *L. vannamei* exposed to thermal stress are scarce. Therefore, the impact of these supplements on shrimp needs to be investigated to possibly improve the animals' immune response to thermal stress.

4.1 On growth indices and survival rate

The effects of increased dietary vitC and lipid supplementation on the growth and survival indices of *L. vannamei* exposed to thermal stress were not significantly different between groups ($P > 0.05$). However, shrimp fed diets with increased vitC and lipid supplementation had numerically better growth (i.e., weight gain, specific growth rate), survival, and feed conversion ratio. For comparison, *L. vannamei* reared at 25 ± 4 °C and fed only the optimal amounts of required nutrients already had significantly lower growth rates than shrimp reared at 25 ± 3 °C, 25 ± 1 °C, and 25 ± 1 °C (Guo et al. 2010). The physiological parameters of the shrimp at 25 ± 4 °C indicated a stressed state. Long-term hyperthermal stress affects the physiological processes of shrimp, resulting in stunted growth (Roberts et al. 2010). Water temperatures above 34 °C negatively affect the metabolic rate of *L. vannamei*, resulting in growth discrepancies and even retardation (Allan et al. 2006). In the present study, the growth and survival indices of *L. vannamei* postlarvae exposed to thermal stress and fed diets with increased vitC and lipid supplementation still showed better growth performance. These results may indicate that the increased supplementations promoted shrimp growth and survival. This is consistent with the results of Chien et al. (1999) with spiny dogfish *Terapon jarbua*, as the test animal, reared at 36 °C. The best growth performance was observed in dogfish fed diets with increased vitC supplementation. Mortality also decreased with increasing vitC supplementation levels, even with increasing water temperature.

The proximate analysis showed a reduced crude protein content of the *3vitC*+lipid diet. Despite this, shrimps fed *3vitC*+lipid resulted in the highest WGR and SGR. The effect of vitamin C on crude protein content necessitates further studies, as it may be affected by different factors. For instance, the proximate composition of the muscle of *L. vannamei* fed different levels of dietary vitamin E, lipid, and vitamin C showed varying crude protein levels (Ebadi et al. 2021). When vitamin E supplementation is increased in diets with the same vitamin C levels, crude protein content decreases. Meanwhile, crude protein content increases when vitamin C levels are higher than vitamin E in the same diet formulation. The same study also noted that vitamin C and E interaction significantly affected the antioxidant activity and shrimp growth, thereby improving growth indices and reducing dietary lipid levels. Van Gaal and De Leeuw (1989) suggested that nutrient combustion rate changes, such as increased lipid combustion, can impact protein levels. Mrigal carp (*Cirrhinus mrigala*) fingerlings fed diets of varying vitamin C levels improved body protein content and reduced body fat, which indicates the more pronounced effect of vitamin C (Zehra and Khan 2012). Fracalossi et al. (1998) likewise reported the same differences in weight gain and protein content as a function of the vitamin C-free and enriched diets. In the present study, the increased vitamin C supplementation may not directly compromise protein content, but it can interact with other dietary components to influence protein content and metabolism.

4.2 On nonspecific immune response, THC

When the effect of vitC and lipids on the THC of *L. vannamei* postlarvae exposed to thermal stress was determined, shrimp supplemented with vitC and lipids had significantly higher THC. In contrast to these findings, Díaz et al. (2013) reported a significant THC decrease when *L. vannamei* were exposed to their critical thermal maximum (36–41.6 °C), while de la Vega et al. (2008) found no significant differences in THC when *Penaeus monodon* were exposed to 35 °C after 24 h. Thermal stress also significantly reduced the THC of *L. vannamei* after 6–12 h of rearing in freshwater or seawater (Jia et al. 2014). However, in these reports, the test shrimps were only fed the required basal diet. The increased vitamin

C and lipid supplementation in the present study may have contributed to the increased THC, even when the shrimps were subjected to thermal stress. In other aquatic species, increased supplementation of vitamin C at 250.12 to 321.38 mg kg $^{-1}$ in red swamp crayfish (*Procambarus clarkii*) diet resulted in increased THC, improved growth, immunity, and antioxidant capacity (Kong et al. 2021).

Vitamin C plays a key role in hemocyte (blood cell) formation due to its direct role in collagen synthesis (Niu et al. 2009). Collagen, a structural protein, is critical for the stability of blood vessels and, thus, for the proper functioning of the bone marrow, where red blood cells are formed (Mercier et al. 2012). The antioxidant properties of vitamin C also protect red blood cells by scavenging free radicals and minimizing oxidative stress (Cheng et al. 2005). Although the role of fish oil in hemocyte formation is not well established, the mediating effect of fish oil on cell membrane integrity (Matesanz et al. 2010) is hypothesized to have beneficial effects on hemocytes. The structural stability and functionality of cell membranes are influenced by fatty acids esterified in phospholipids. Robust cell membranes contribute to efficient oxygen transport in the blood, and omega-3 fatty acids have been shown to increase the ability of hemoglobin to transfer oxygen by making red blood cells more flexible and deformable (Patel et al. 2013). Increasing hemocyte count is critical, especially when these cells are also affected by environmental stressors such as pH, salinity, and temperature (Muthaiyan 2020). They are among the most critical components of crustacean cellular immune response, as they help remove foreign material by influencing cell adhesion and cytotoxicity and induce hemocyte-mediated defense responses (Cheng et al. 2005).

Interestingly, the THC of shrimp fed the *3vitC*+lipid diet significantly decreased compared to those fed the vitC+lipid and the vitC+*2lipid* diets on day 15. This contrasts with the immediate reduction in THC observed by Cheng et al. (2005) and de la Vega et al. (2008). It could be that the beneficial effects of vitamin C peaked on day 7, and the shrimp had already acclimated to the increased vitamin C intake. THC could also be affected by treatment and timing of hemolymph collection. *L. vannamei,* infected with the infectious myonecrosis virus (IMNV), did not show an increase in THC until 17 days after the viral challenge ended (Sabry-Neto and Nunes 2015). This could help explain the (reduced) variability of THC in shrimp fed *3vitC*+lipid compared with vitC+lipid only and vitC+*2lipid*, as it might be influenced by treatment and sampling conditions. Pan et al. (2008) also noted that THC and the number of large granular cells could stabilize after six days without significant differences. In any case, studies on the long-term effects of vitamin C on the THC of shrimp exposed to thermal stress are recommended for a more conclusive impact of dietary supplementation.

4.3 On the expression of immune-related genes in *L. vannamei*

The effects of increased dietary vitC and lipid supplementation on the expression of the immune-related genes HSP70, HSP90, ALF, PEN 3, PEN 2, Crustin, and proPO of *L. vannamei* exposed to thermal stress were also investigated. Significant upregulation of target genes was found in shrimp with increased vitC and fish oil supplementation. Shrimp supplemented with vitC exhibited significant upregulations of HSP70, HSP90, ALF, Crustin, and proPO. Vitamin C is considered an immunostimulant due to its ability to enhance the nonspecific immune response of most crustaceans, which may explain the upregulations of the target genes observed in the present study. It is a cofactor and mediates enzymatic reactions and cytokine synthesis, regulating the immune responses in shrimp (Harrison and May 2009). In loaches, *Misgurnus anguillicaudatus*, reared at 34 °C, a diet supplemented with 1000 mg kg $^{-1}$ vitC, which is five (5) times higher than their optimal vitamin C requirement showed significantly higher HSP70, HSP90α, HSP90β expressions (Yan et al. 2017). It is hypothesized that the increased vitamin C supplementation may have reduced the damage caused by thermal stress. Wuchang bream, *Megalobrama* amblycephala, under thermal stress (34 °C) fed with increased vitamin C supplementation resulted in better antioxidant activity, nonspecific immunity, and HSP70s mRNA expression, increasing thermal stress resistance (Ming et al. 2012). Ebadi et al. (2021) reported that increased vitamin C supplementation in the diet significantly improved survival, growth performance, and antioxidant activity of *L. vannamei* when the temperature was controlled and in the optimal range. The same conclusion was observed by Lee and Shiau (2002), where high ascorbate supplementation, regardless of source, significantly improved the immunological indices of *P. monodon* shrimp. These studies show that the beneficial effects of vitamin C are still observed when experimental animals are under thermal stress, as in the present study.

Fish oil is known for its beneficial effects on nutrition, growth, and development because it

is the primary source of dietary lipids rich in highly unsaturated fatty acids or HUFAs (Zhou et al. 2007). In the present study, shrimp fed vitC+*2lipid* showed significantly upregulated HSP90 and PEN2. Other studies have also reported that fish oil positively affects immune-related gene expressions in animals under thermal stress. Chim et al. (2001) showed that increased levels of HUFAs in the diet resulted in better adaptation and resistance of *Penaeus stylirostris* to environmental stress (heat and salt stress) by altering cellular membrane composition. A diet enriched with HUFAs also increases the lipid fraction of circulating lipoproteins (Vargas-Albores 1995). In agreement with these studies, it only shows that a fish oil-enriched diet of *L. vannamei* subjected to thermal stress positively affects its immune-related genes and, therefore, immune response.

5. CONCLUSION

Increased supplementation of dietary vitamin C and lipid on *L. vannamei* postlarvae subjected to thermal stress significantly upregulated the expression of immune-related genes HSP70, HSP90, proPO, and AMPs, compared to shrimp fed only the minimum basal diet and nutrient levels. The increased supplementation also resulted in numerically better growth and survival indices and feed conversion ratio. The findings suggest that farmers involved in *L. vannamei* culture may increase dietary vitC and lipid supplementation up to 3 g kg^{-1} and 34 g kg^{-1}, respectively, to boost the immunity of the shrimp when exposed to thermal stress. The growth and survival of the shrimp are also unaffected by the increased supplementation levels. Nonetheless, an additional round of feeding experiments is recommended for short-term feeding trials to check and verify the growth and survival indices. Studies determining the long-term impact of further elevated levels of dietary vitamin C and lipid supplementations on shrimps subjected to thermal stress are recommended. The optimal profit generated when increased dietary vitamin C and lipid supplementations are added to the feed costs also calls for further studies.

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AUTHOR CONTRIBUTIONS

Dequito A: Conceptualization, Experimentation, Analysis, Writing – Final Review and Editing, Funding Acquisition, Project Administration and Supervision. **Sindo E:** Conceptualization, Experimentation, Analysis, Writing – Original Draft. **Traifalgar R:** Conceptualization, Writing – Review and Editing, Project Supervision. Dequito A. and Sindo E. contributed equally to this work.

CONFLICTS OF INTEREST

The authors affirmed that the work provided in this report does not present any conflicts of interest.

ETHICS STATEMENT

The study complied with the University of the Philippines Visayas Research Ethics Boards through the approved issuance of the Student Research Ethics Clearance (UPVREB #C-6) on March 7, 2022.

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