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Effect of dietary vitamin E on reproductive performance and vitellogenin gene expression in broodstock of *Litopenaeus vannamei*

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Abstract

Vitamin E (VE) is one of the most important micronutrients that influence the performance of shrimp reproduction. The effects of different dietary VE levels (0, 100, 300 and 500 mg kg^{-1}) were investigated on the reproductive performance and vitellogenin (Vg) gene expression in *Litopenaeus vannamei* females which were co-fed with fresh food (two times daily) or experimental diets (two times daily) for 30 days. Sampling was carried out at the first day of the trial, prior to eyestalk ablation (ESA) (day 21) and after ESA (day 30). The reproductive parameters including hepatopancreatic index, absolute fecundity, egg diameter, latency period, and Vg gene expression were higher in the shrimp fed with VE supplemented diets than the control group (p<0.05). After ESA, the Vg gene expression significantly increased in all groups (p<0.05). As a measure of reproductive status of Pacific white shrimp, the present study evaluated correlations of Vg gene expression in hepatopancreas with the number and diameter of oocytes in gonads. Accordingly, Vg is a precursor of vitellin, which is the major yolk protein accumulated in ooplasm. These results provide evidence that dietary VE with 300 mg kg^{-1} may present a novel strategy and play a positive impact on reproductive performance and Vg gene expression of female shrimp.

Keywords: Vitellogenin gene expression, *Litopenaeus vannamei*, Vitamin E, Reproductive performance

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Introduction

The Pacific white shrimp, (*Litopenaeus vannamei*, Boone 1931) is one of the most significant shrimp species in aquaculture all over the world. It is because of its high survival, high tolerance to environmental changes and rapid growth in intensive culture systems and greater resistance to viral diseases in comparison with other shrimp species (Briggs *et al*., 2004). Although *L. vannamei* is commercially the most remarkable shrimp species, the development of its aquaculture has been limited by the artificial ripening of female gonads (Lem, 2006). Currently, its propagation is based on domesticated populations under biosecure condition (Moss and Crocos, 2001), which result in a continuous increase in the proportional share of this species in global shrimp production (Valderrama and Anderson, 2012). In this context, using domesticated broodstocks as an alternative for the wild ones as well as supplying artificial dry diets rather than fresh food organisms (*i.e.* squid, *Artemia* biomass, bloodworms) increased the biosecurity in hatcheries through reducing the risk of transmission of pathogenic bacteria and viruses (Wouters *et al*., 2002). However, pond-reared brooders are generally unpredictable in reproductive performance which could be attributed to their dietary deficiencies and/or general rearing conditions (Palacios *et al*., 2000). Moreover, artificial dry diets do not perform as well as fresh food for shrimp broodstock (Wouters *et al*., 2002). Nutrition plays a crucial role in the growth, reproduction, immunity, and overall health status of any organism. Maternal nutrition, before and at the time of conception, is very critical for ensuring the birth of a full-term viable and healthy offspring (Williams, 1994). Dietary requirements of shrimp are generally higher in sexually matured adults than in non-reproductive adults and juveniles, but knowledge in this field is still limited. During maturation, which can take from 3 days to 1 month, the weight of the ovaries increases four to eight folds (Ravid *et al*., 1999). The nutrition of broodstock shrimps often affects the gonadal development, fecundity, egg and sperm quality, embryonic development, and post larval quality (Lavens and Sorgeloos, 2000; Garcıa-Guerrero *et al*., 2003; Du *et al*., 2006). During ovarian development, dietary and maternal reserves are mobilized and transported into the oocytes to fulfill the nutritional requirements for growth and development of the embryo and the yolk sac larvae or fry till the start of exogenous feeding (Fernandez-Palacios *et al*., 2011). The manipulation of maternal immunity transfer can be used to enhance the survival rate of larvae (Swain and Nayak, 2009).

Vitellogenesis is the process during which synthesis of Vg and Vn, occurs as the main yolk proteins associated with lipids and carbohydrates. Vg is an important nutritive source for maturation and development of oocytes (Tseng *et al*., 2001; Zapata *et al*., 2003)

causing a significant increase in their diameter (Meusy and Charniax-Cotton, 1984). In oviparous animals, female maturation is characterized by the synthesis of a major yolk protein called Vn, a glycolipophosphoprotein (200 to 500 kDa), which is important for providing nutrition to developing embryos. Then, Vg of hemolymph which is associated with the secondary vitellogenesis takes place in the oocyte and Vg undergoes further modifications to become Vn. Vn which is also the common form of yolk, accumulates within the ovary during the process of vitellogenesis in crustaceans and forms the nutritive material necessary for the successful development of an embryo that remains independent of its mother (Meusy and Payen, 1988; Yehezkel *et al*., 2000; Abdu *et al*., 2002).

VE is a lipid soluble vitamin that comprises four tocopherols and four tocotrienols in nature. Among them, α-tocopherol has the highest VE activity (NRC, 1993). Rather than its VE activity, α-tocopherol is a potent biological antioxidant able to protect biological membranes and lipid components containing unsaturated fatty acids against attack from oxygen free radicals. VE was originally considered as a dietary factor of animal nutrition, which is an important factor in reproduction functions. In aquaculture, VE is used for the fortification of feed to improve the growth, protection of eggs during early development, resistance to stress and diseases as well as survival of fish and shrimp (Vismara *et al*., 2003; Maulana *et al*., 2017). Like the higher vertebrates, VE deficiency affects reproductive performance, causing immature gonads and lower hatching rate and threatened survival of offspring (Izquierdo *et al*., 2001). VE is digested in the small intestine and stored in some tissues such as adipose tissue and liver (Pour *et al*., 2011). In this way, the objective of the present study was to determine the effect of dietary VE (four levels: 0, 100, 300 and 500 mg kg^{-1}) in broodstock diet on reproductive performance (egg diameters, hepatopancreatic index, absolute fecundity, and latency period) and mRNA level of Vg gene in the hepatopancreas of female *L. vannamei*.

Materials and methods

Experimental diets

A practical basal diet formulated to contain approximately 55% crude protein, 12% crude lipid, 13% ash, 4.4% fiber, 10% moisture and 18.7 MJ kg^{-1} of digestible energy was used to meet all known nutritional requirements for *L. vannamei* brooders (Goimier *et al*., 2006) via Lindo software (Copy right 1999, release 6.1, USA). The experimental diets were supplemented with VE at the expense of cellulose (Merck, Germany) at the combined concentrations of 0, 100, 300 and 500 mg kg^{-1}. In this regard, all dry ingredients were mixed for 30 min, then oil and sufficient distilled water were added to form a soft dough that was mechanically extruded by using an

electric grinder to obtain pellets of 3 mm in size. The pellets were dried in a convection oven at 50°C for 24 h and stored in re-sealable plastic bags at -20°C until their use.

Experimental design

The third-generation families of *L. vannamei* taken from an imported strain (SPF_3, domesticated in Hawaii) were transferred from pond (0.1 acre) in a private aquaculture farm in Bandar Rig (Boshehr, Iran) to a private hatchery in Genaveh (Seydan Jonoub, Genaveh, Boshehr, Iran). Upon arrival, the animals were treated against filamentous bacteria, fungal and protozoan infections with formalin (100 ppm, for 30 sec) according to Alday-Sanz (2010), then were acclimated to the experimental conditions for a week. A total of 120 female and male *L. vannamei* (33.2±2.4 g; Mean±Standard Error) at a ratio of 1.5:1 were randomly allocated to 12 black circular tanks (0.8 m diameter×0.6 m high) at a density of 10 shrimp $tank^{-1}$ in an open-flow system. The tanks were supplied with filtered running seawater with 100% daily water exchange. Average values of water temperature, salinity, dissolved oxygen and pH were 28.6±1.7°C, 31.8±4.1 ‰, 6.2±0.9 mg L^{-1} and 7.5±0.4, respectively and the photoperiod was 10L:14D (light: darkness). The shrimps were co-fed with fresh food [squid (40%), polychaetes (15%) and chicken liver (40%)]; twice a day (0600 and 1800 h) accounting for a total daily supply of 20% of wet weight biomass and experimental diets two times daily (1200 and 2300 h) accounting for a total daily supply of 5% of wet weight biomass 4 times a day.

Eyestalk ablation and sampling

Before each sampling, the shrimps were placed in chilled ($4^{\circ}C$) and aerated seawater for 10 min to reduce stress, metabolic activity, and manipulation effect. After 21 days, female shrimps were ablated unilaterally (left eyestalk only) by cutting the eyestalk under water at the base of the peduncle and applying burn to the wound to minimize fluid loss and help coagulation. Gonad development was assessed daily with a lamp to observe the size and color of the gonad through the exoskeleton (Alday-Sanz, 2010). Female shrimps were sampled at day 21 (before ESA) and then again at day 30 (after ESA) post-initiation on the experimental diets. The hepatopancreas (before and after ESA) and ovaries (only after ESA) from the cephalothoracic region were dissected and weighed (n=3 samples per treatments), then transferred into liquid nitrogen for further analysis.

Reproductive performance assessments

The weights of shrimp and their hepatopancreas were measured to the nearest 0.1 g and the total body length was measured to the nearest 1 mm. Reproductive performance was evaluated in terms of hepatopancreatic index (HSI), gonadosomatic index (GSI), absolute fecundity, number of

eggs per female body weight, egg diameter and latency period (interval between ESA and first spawn). Standard formula was used to determine HSI and GSI as below (Fatima *et al*., 2013):

hepatopancreatic index (%): HSI= (hepatopancreas weight / body weight) × 100

gonadosomatic index (%): GSI = (gonad weight / body weight) × 100.

For determining absolute fecundity, three samples from three parts of the ovaries (head, mid and hind parts) were collected and weighed (0.1 g), then transferred in Gilson's fluid (5 mL; for two months; Simpson, 1951), a process which releases the oocytes by macerating the connective tissues of ovaries. The eggs' diameter was determined by using a light microscope (Nikon, Japan).

RNA Extraction and cDNA Synthesis

Total RNA was extracted from about 100 mg of hepatopancreas tissue by RNA extraction kit companies CinnaGen RNXTM (Iran) according to the manufacturer manual. Hepatopancreas RNA from *L. vannamei* females at 30^{th} day sampling (after ESA) post-initiation on the experimental diets were used to measure Vg gene expression. The specific Oligonucleotide primer for the Vg gene of *L. vannamei* based on the sequence reported recently by Raviv *et al*. (2006) (GenBank database Vg accession number: AY321153) and a housekeeping gene called the 18sRNA as a control for the expression of the Vg gene was used in this study. The sequence of primers is shown in Table 1.

The second step after extracting RNA was to reverse the DNA synthesis (Random hexamer method) for further steps, so the cDNA constructed in standard PCR reactions was used. To test the primers of Vg and 18sRNA (Designed by Rubin Teb, Iran), a conventional PCR was first performed using a cDNA library constructed from hepatopancreas tissue as a template and standard PCR reaction using the PCR machine (Corbbet Research, Australia). The real-time PCR mixture was prepared by mixing 0.5 µl cDNA (25 ng), 200 nM primer, 0.25 µL Tag DNA polymerase and DEPC-water to a final volume of 25 µL. Amplification was set at 95°C for 2 min for hot start denaturation followed by 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s and then incubated at 72°C for 10 min for final extension using a DNA Engine® Thermal Cycler (Corbbet Research, Australia). The PCR products were analyzed on a 1.2% agarose gel. After extracting the whole RNA from hepatopancreas tissue of *L. vannamei*, its quality was evaluated using a bio texture and electrophoresis on 1% agarose gel. The concentration of RNA samples was measured by using an absorbance assay of 260 nm wavelength. The absorption ratio was determined at 260 and 280 nm wavelengths using Eppendorf

Spectrophotometer (Germany) and the DNase enzyme was used to remove DNA from the RNA extraction product.

Quantification of Vg gene expression by real-time RT-PCR

To evaluate the mRNA levels of Vg gene in hepatopancreas tissue in real-time PCR test, comparative method ΔΔCt (device Rotor Gene corbett-3000, Australia) was used. The test method was based on Cyber green color application. In this study, 18sRNA gene was used as an internal control gene and due to the expression of the constant gene of 18sRNA, Vg gene expression in hepatopancreas tissue of female shrimp was evaluated. Negative control samples (containing distilled water) without any cDNA were considered in each reaction. For each replication, 4 samples (2 samples VgRTF and VgRTR and 2 samples 18s F and 18s R) were prepared for Real Time PCR reaction, for 12 repetitions, 48 micro tubes 0.2 ml samples were prepared for 12 replicates. 24 micro tubes containing sample cDNA and Vg primer and 24 micro tubes containing the cDNA of the sample and 18sRNA primer were also prepared. Before transferring micro tubes to the Real Time-PCR, the solution in the bottom of the micro tubes was centrifuged for a few seconds. The primers for real-time PCR method were used to assess the expression of Vg gene. The sequence of these primers is listed in Table 1. Data were analyzed using the comparative C_T method ($2^{-\Delta\Delta CT}$) described by Livak and Schmittgen (2001), so that, in quantitative analysis, Real-time PCR based on threshold counts (Ct) of the tested specimens (treatments fed diets containing different amounts of VE, treated with dietary foods without VE) with control samples (negative control) and using the formula ΔΔCt, the ratio of target gene to reference gene was calculated using the following formula: $R=2^{-\Delta\Delta Ct}$.

Statistical analysis

The relative expression levels of Vg gene were calculated by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Data were analyzed using the SPSS ver. 19.0 (Chicago, Illinois, USA). All data are presented as means ± standard error of the mean. Arcsine transformations were conducted on all percentage data to achieve homogeneity of variance before statistical analysis. A One-way ANOVA was performed to check differences among treatments for different analyzed variables, whereas differences between different sampling times (days) were tested by One-way ANOVA and the post-hoc Duncan test was performed when significant differences were found among them. The level of significant difference was set at $p<0.05$ for all statistical tests.

Table 1: The sequence of primers used in this study.

Gene	Forward primer	Reverse primer
Vg	5'-AATACAAGAACGTGAGGGATAGGAA-3'	5'-AGGCAATCACACACTTGTATATTTGTATTTC-3'
18S RNA	5'-TCGGAACCCGAGGTAATGATT-3'	5'-CTCTAGCGTCGCAGTACGAATG-3'

Results

Growth and reproductive performances

The total body length and weight, weight gain, survival, specific growth rate, condition factor, HSI, GSI, absolute fecundity, number of eggs per female body weight, egg diameter and latency period of the experimental groups are summarized in Table 2.

The results of the present study showed that the morphometric parameters (growth performance), survival and growth performance of females broodstock shrimp were not affected by dietary VE levels. According to the result, total length of female shrimps was not different between treatments and control (p>0.05), although the highest and lowest values of SGR and weight gain rate were observed in female shrimps fed with 300 mg kg^{-1} VE supplemented diet and control group, respectively (Table 2). In regard to reproductive parameters, except for absolute fecundity, eggs diameter and number of eggs per female body weight, other parameters were significantly affected by dietary VE supplementation. It was also demonstrated that elevated levels of VE significantly increased reproductive performance (p<0.05).

Table 2: Morphometric and reproductive parameters of female shrimp (*L. vannamei*) fed diets supplemented with different levels of VE (mean ± *SE*, *n* = 3).

parameter	Dietary VE levels (mg/kg)			
	control	100	300	500
Morphometric parameters				
BW_i (g)[1]	33.1 ± 2.1	33.3 ± 1.8	33.4 ± 3.1	32.7 ± 2.6
BW_f(g)[2]	39.3 ± 2.5	41.0 ± 1.7	41.4 ± 2.8	41.1 ± 2.9
TL_i (cm)[3]	15.3 ± 0.4	15.4 ± 0.7	15.5 ± 0.8	15.1 ± 0.5
TL_f (cm)[4]	17.2 ± 0.7	17.9 ± 0.4	18.1 ± 0.6	17.9 ± 0.9
Survival rate (%)	79 ± 2.1	85 ± 2.8	81.3 ± 2.6	86.7 ± 1.5
Weight gain rate (%)	18.73 ± 2.7	23.12 ± 2.1	23.95 ± 3.6	25.69 ± 2.8
Specific growth rate (%/day)	0.6 ± 0.2	0.7 ± 0.4	0.7 ± 0.3	0.8 ± 0.2
Condition factor (%)	0.8 ± 0.1	0.6 ± 0.0	0.7 ± 0.1	0.7 ± 0.1
Reproductive parameters				
Hepatosomatic index (%) before eye stalk ablation	$^{B}3.1 \pm 0.5^{a}$	$^{B}3.4 \pm 0.6^{ab}$	$^{B}3.9 \pm 0.3^{b}$	$^{B}3.6 \pm 0.2^{ab}$
Hepatosomatic index (%) after eye stalk ablation	$^{A}2.8 \pm 0.3^{b}$	$^{A}2.7 \pm 0.5^{ab}$	$^{A}2.4 \pm 0.2^{a}$	$^{A}2.6 \pm 0.4^{ab}$
Gonadosomatic index (%)	2.5 ± 1.3^{a}	3.6 ± 1.2^{ab}	4.5 ± 0.8^{b}	4.0 ± 0.5^{ab}
Absolute fecundity ($\times 10^3$)	167.1±15.7	174.3±18.4	176.2±19.6	174.7±12.1
Number of egg ($\times 10^3$) per g of female's body weight	4.42±0.3	4.61±0.5	4.66±0.7	4.63±0.2
Eggs diameter (µm)	117.28±29.6	122.35±37.4	123.55±41.5	122.65±31.9
Latency period (days after ESA to spawning)	8.0 ± 0.6^{a}	7.0 ± 0.6^{a}	6.0 ± 0.2^{b}	7.0 ± 0.6^{a}

Different lower-case letters correspond to statistical differences among experimental groups within the same sampling (days) tested by One-way ANOVA, whereas different upper-case letters indicate differences between different sampling times tested by One-way ANOVA.

[1]BW_i: initial body weight.

[2]BW_f: final body weight.

[3]TL_i: initial total length.

[4]TL_f: final total length.

Hepatopancreatic Vg mRNA expression

The results of this study indicated that exogenous VE supplementation contributes to the increased expression of Vg mRNA in the hepatopancreas and improved reproductive performance in shrimp females fed VE diets. After 30 days of feeding trials, results from this study showed significant differences in expression of Vg mRNA among the shrimps that were fed on different VE diets. The expression of Vg mRNA in the hepatopancreas peaked in *L. vannamei* fed with the 300 mg VE kg^{-1} diet, with a lower expression observed at lower and higher VE percentages and control groups (Fig. 1). The expression of the Vg mRNA was increased more in response to VE compared to the control, with a mean expression ratio of 5.2±0.3 in the control group and 55.4±4.3 in the VE supplementation groups. There was a significant difference between two groups treated with different levels of dietary VE at 100 and 500 mg kg^{-1} with a mean expression ratio of 46.4±3.8 and 51.9±4.3, respectively, and 300 mg VE kg^{-1} diet with a mean expression ratio of 67.9±4.7.

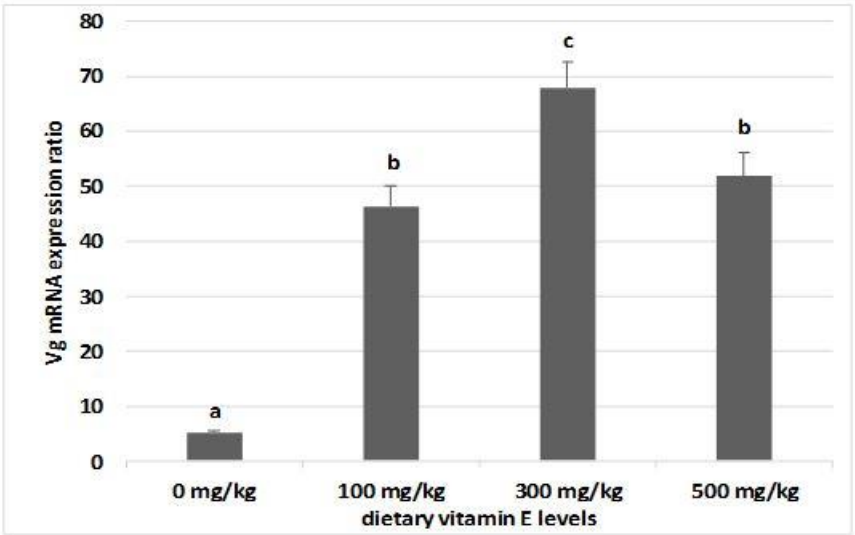

Figure 1: Vg mRNA expression ratio in the hepatopancreas of female *L. vannamei* fed with four experimental diets. The mean expression ratio±standard error of mean (SEM) (n=3) in the different groups are shown, with treatment at the x axis and expression ratio at the y axis. Statistical significance is indicated with different letters (p<0.05).

Discussion

Growth and reproductive performance

VE supplementation was positively correlated with weight gain and feed efficiency for hybrid striped bass after the 12-week feeding trial (Kocabas and Gatlin, 1999), but it seems that the character of the trade-off between reproduction and somatic growth apparently leads to similar growth performance in different groups, since during oogenesis vast, nutrient resources are spent on ovary activity and vitellogenesis (Sainz-Hernández *et al*., 2008). VE and particularly its most active form, a-tocopherol, is one of the

most important lipid-soluble vitamins and antioxidant molecules present in cell membranes and biological systems. It is a membrane associated vitamin that functions as a radical scavenger inhibiting the peroxidation of lipids (HUFA) in cellular membranes. It is a potent antioxidant that prolongs the life of erythrocytes and plays an essential role in cellular respiration (Hung *et al*., 1981). These results demonstrated that VE could offer a protective role against the adverse influence of reactive oxygen and other free radicals (NRC, 2011), and VE supplementation was necessary to protect against lipid oxidation for normal growth of shrimp. Free radicals, such as superoxide, hydroxyl ions and nitric oxide all contain an unpaired electron. These radicals can have a negative effect on cells causing oxidative damage that leads to cell death. Antioxidants, such as VE and vitamin C, prevent cell damage by binding to the free radical and neutralizing its unpaired electron. It is assumed VE used in the diet of fish and other aquatic animals, including shrimp can promote growth rate and reproductive performance (NRC, 2011). Also, the values of survival rate were not statistically different between experimental treatments and control (p>0.05). Similarly, Darvishpour *et al*. (2012) reported that VE supplementation had no significant effects on the growth and survival rate of *L. vannamei* PLs 15 days after feeding trial. According to the results, total length of post larvae was not different between treatments and control, although the highest and lowest values of SGR and total weight of PLs were observed in T_3 (VE) (SGR: 58%/day) and T_1 (VA), control (SGR: 30%/day), respectively. Linn *et al.* (2014) reported that weight gain and SGR were the lowest values in red sea bream (*Pagrus major*) fed without supplementation of VE compare to those fish fed with supplementation of 100, 200 and 400 mg VE kg^{-1} diet. Survival rate, condition factor and feed intake had not significantly different among the treatments. Wilson *et al*. (1984) also reported that dietary VE supplementation had no effect on growth rates and feed efficiency of channel catfish. One of the most possible reasons, which might have caused these different results among the above-mentioned studies, was the feeding trial duration and also diet biochemical compositions. Moreover, other studies also reported that inclusion of the dietary VE led to an increase in growth performance of other decapod species in juvenile stage such as *L. vannamei* (He *et al*., 1992; He *et al*., 1993), *Macrobrachium rosenbergii* broodstock (Cavalli *et al*., 2001), also improved survival of larval *Penaeus japonicas* (Kanazawa, 1985).

The present study demonstrated that within a 1-month experimental period, females fed the basal diet with supplementations of VE could successfully perform reproductive process. This study is the first report on the interactive effects of VE for

reproductive process and Vg gene expression of *L. vannamei*. Interestingly, dietary vitamins such as VE for spawners directly affected the egg and larval quality of shrimp. Penaeid shrimp broodstock fed VE deficient diets were reported to have low post-ablation survival, retarded ovarian development and low hatchability (Alava *et al*., 1993; Cahu *et al*., 1995; Du *et al*., 2006). Reproductive parameters were significantly affected by dietary VE supplementation. Before ESA female shrimps fed 300 mg VE kg^{-1} had the highest and the control diet had the lowest HSI values (p<0.05). Moreover, the fact that ESA led to a significant decrease in HSI in all groups may be due to mobilization of lipids from the hepatopancreas to the ovaries. It also might be attributed to the concomitant reduction in the levels of gonad inhibiting hormone produced by the sinus gland (Sainz-Hernández *et al*., 2008). This result was supported by higher plasma HDL concentration in all groups after ESA which indicates an increased lipid mobilization from hepatopancreas to ovaries (Arshadi *et al*., 2018). The effects of dietary VE supplementation on increased HSI were reported in *M. japonicas* (Alava *et al*., 1993). Moreover, the dietary VE levels increased GSI and HSI values of female *L. vannamei* (Du *et al*., 2006). In this experiment, increasing dietary VE levels resulted in increased weight of hepatopancreas and hepatosomatic index (%) before eye stalk ablation of *L. vannamei*; thus, it may be partly related to accumulations of lipids largely stored in hepatopancreas for maturation process. Even though the mechanism for this relationship may not be fully clarified in the present study, it is reasonable to suggest that VE as lipophilic antioxidant against lipid peroxidation and damage to cell membrane (Halver, 2002) had a positive effect on lipid absorption and utilization in the hepatopancreas. Those hypotheses were also supported by the fact that dietary supplementation of VE enhances the synthesis of egg yolk precursors in hepatopancreas of crustaceans (Cavalli *et al*., 2003; Katre, 1977). Deficiency of VE has been shown to retard the ovarian development of *Marsupenaeus japonicus* (Alava *et al*., 1993). In this experiment, the highest and lowest values of average GSI were observed in female shrimps fed with 300 mg kg^{-1} VE diet and control, respectively (p<0.05), while in treatments 100, 300 and 500 mg kg^{-1} were not significantly different (p>0.05). Female shrimps fed with VE diets had a higher fecundity and egg diameter than control group, but dietary VE did not have any significant influence on fecundity and eggs diameter of females *L. vannamei*. This result was in agreement with previous reports for crustacean species (Cahu *et al*., 1995; Cavalli *et al*., 2003; Du *et al*., 2006; Maulana *et al*., 2017). Furthermore, shrimps fed with 300 mg

kg^{-1} diet had a shorter latency period than the other groups (p<0.05). The results of this study indicated that exogenous VE supplementation contributes to the VE pools and improves reproductive performance in shrimp females fed VE diets. Watanabe *et al*. (1985) indicated that the incorporation of dietary VE into the eggs occurred together with lipids. Cavalli *et al*. (2001) found a high correlation between deposition of lipids and VE in the ovary of *M. rosenbergii* concluding that this was in line with the antioxidative function of VE. Effects of dietary VE on reproductive performance have been documented for several penaeid shrimp species. Dietary VE which has positive effects on ovarian maturation in *M. japonicus* (Alava *et al*., 1993) plays an important role in maturation process, enhances larval development of *L. vannamei* (Wouters *et al*., 2001b; Maulana *et al*., 2017), and improves hatchability in *P. indicus* and *L. vannamei* (Cahu *et al*., 1995; Du *et al*., 2006). The VE is absorbed by the intestine and metabolized in the liver and is further stored in the skin as well as gonads during maturation and in muscle tissue during growth (Tizkar *et al*., 2016). VE and vitellogenesis are closely related to the development of oocytes in fish through prostaglandins, which is enzymatically synthesized using essential fatty acids. The addition of VE maintains the fatty acids synthesis due to its function as an antioxidant (Asaikkutti *et al*., 2016). In shrimps, VE contributes to ovarian development and optimal breeding performance (Ai and Chen, 2001). Also, research has shown that crayfish (*Procambarus clarkii*) fed 200 mg kg^{-1} VE have improved spawning rates and higher number of spawning females (Li, 2007). However, excessive VE in the feed may contribute to very high levels of the vitamin in shrimp and inhibit their growth and reproduction (Naessens *et al*., 1997). Similar results were observed in this experiment showing that reproductive performance could increase with the elevation of dietary VE supplemented levels.

Hepatopancreatic Vg mRNA expression

The hepatopancreas plays a major role in vitellogenesis of penaeid shrimp. In *L. vannamei*, exogenous Vg synthesis in the hepatopancreas have been reported to be the major sites of synthesis of Vg (Tsutsui *et al*., 2004; Mak *et al*., 2005; Fatima *et al*., 2013). Studies on *L. vannamei* have identified Vg gene, *LvVg* (Raviv *et al*., 2006), in the hepatopancreas. Vg is a precursor protein of Vn (a major egg yolk protein that plays a major role in vitellogenesis). In other words, it is an important reproductive process in oviparous animals. VE, as lipid-soluble extracellular and intracellular antioxidants, protects the highly unsaturated fatty acids, and plays an important role in cellular respiration, gene expression, biosynthesis of DNA

and coenzyme Q[1] within the cells (Leeson and Summers, 2001; Casagrande *et al*., 2018). Dietary VE has important long-term effects on liver gene expression with potential downstream effects on extrahepatic tissues (Rimbach *et al*., 2010). Furthermore, in rat brains, a significant number of genes were found to be regulated by VE. These VE sensitive genes encode for proteins associated with hormones and hormone metabolism, nerve growth, apoptosis, dopaminergic neurotransmission, and clearance of amyloid-β and advanced glycated end products (Rota *et al*., 2005). In fact, it is speculated that high requirement for RNA and DNA during oogenesis invests this vitamin for oogenesis at the expense of growth performance. VE regulates sex hormone secretion and promotes yolk protein synthesis, which is beneficial for gonad development (Cavalli *et al*., 2000). Increasing dietary VE levels increased the expression of Vg and Vg receptor genes, which revealed that VE had a positive effect on ovarian development and promoted yolk protein synthesis. This result was similar to the previous study in *L. vannamei* (Arcos *et al*., 2011). Also, with adequate VE levels, the expression of Vg mRNA significantly increased as reported in *Macrobrachium nipponense* female shrimp (Li *et al*., 2018). Under stress, crustaceans reduce activity frequency and store nutrients for normal physiological metabolism (Yu, 2007). Overall, inadequate or excessive VE levels negatively affect ovarian maturity (Naessens *et al*., 1997). This phenomenon was observed with the high VE supplemented group. As a nutrient regulatory site, the hepatopancreas play important roles in crustaceans. With the highest VE level, the reproductive performances were decreased, followed by a decrease in the expression of Vg mRNA. Accordingly, excessive VE levels in the feed affect egg quality and reproductive performance (Wouters *et al.*, 2001a). Taken together, these results might imply that suitable VE supplementation could protect tissues structure and reproductive-related gene expression, while excessive VE level would cause damage to the organization and suppress gene expression of female shrimp.

In conclusion, for optimal reproductive performance and expression of Vg mRNA, *L. vannamei* require 300 mg kg^{-1} dietary VE. The reproductive performance and expression of Vg mRNA significantly increased with 100–500 mg kg^{-1} VE. VE at the level of 300 mg kg^{-1} had positive effects on the hepatopancreas, expression of Vg mRNA and ovaries in female shrimps. Meanwhile, inadequate or excessive dietary levels of VE had inhibitory effects on expression of Vg

[1] Coenzyme Q_{10} (CoQ_{10}) is an essential component of the mitochondrial electron transport chain responsible for different functions, among them its action as an antioxidant compound.

mRNA in female shrimp. In addition, shrimps fed with 300 mg kg^{-1} VE had higher mRNA expressions of Vg. The results of this study can be useful for future study, to provide reference data for effective aquatic feeds of female shrimp. Therefore, further study should be conducted on the effect of dietary VE on ovary-related another genes regulation of female shrimp.

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