Effects of dopamine on the immunity of white shrimp

* Litopenaeus vannamei *

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Abstract

The total haemocyte count (THC), phenoloxidase activity, respiratory burst, superoxide dismutase (SOD) activity, phagocytic activity and clearance efficiency in response to pathogen *Vibrio alginolyticus* were measured when the white shrimp *Litopenaeus vannamei* (20.0 ± 1.5 g) were injected individually with dopamine at 10^{-8}, 10^{-7} and 10^{-6} mol shrimp^{-1}, respectively. For the shrimp that received dopamine at 10^{-7} and 10^{-6} mol shrimp^{-1}, the THC decreased by 25% and 39%, phenoloxidase activity decreased by 15% and 32%, respiratory burst decreased by 21% and 36%, and SOD activity decreased by 50% and 63%, respectively, after 4 h. The phagocytic activity and clearance efficiency of shrimp that received dopamine at either dose decreased significantly after 2 h. The THC, phenoloxidase activity, respiratory burst, SOD activity, phagocytic activity and clearance efficiency returned to normal values after 16, 8, 8, 24, 16 and 4 h, respectively, for the shrimp that received dopamine at either dose. In another experiment, *L. vannamei* which had received dopamine at 10^{-8}, 10^{-7} and 10^{-6} mol shrimp^{-1} were challenged after 1 h by injection with *V. alginolyticus* at 1.0 × 10^5 colony-forming units (cfu) shrimp^{-1} and then placed in seawater of 20%. The cumulative mortality of shrimp that received dopamine at either dose was significantly higher than that of shrimp that received saline after 8 h, and of shrimp that received saline at the termination of the experiment (48 h after the challenge). It is therefore concluded that dopamine administration at 10^{-6} mol shrimp^{-1} or less causes immune modulation of *L. vannamei*.

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Keywords: *Litopenaeus vannamei*; Dopamine; Total haemocyte count; Phenoloxidase activity; Respiratory burst; Superoxide dismutase activity; Phagocytic activity; Clearance efficiency

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1. Introduction

White shrimp *Litopenaeus vannamei*, which is native to the Pacific coast of central and south America, was introduced to the Eastern hemisphere in 1985, and has since become the primary species cultured in Thailand, Taiwan, and China [1]. Since 2001, shrimp farmers have experienced disease problems causing production declines in farmed *L. vannamei*. The symptoms differ from those of TSV (Taura Syndrome Virus) in Taiwan [2]. A bacterium *Vibrio alginolyticus* isolated from the diseased *L. vannamei* with whitish musculature and inactivity is considered to be a secondary and opportunistic pathogen, and cause mortality of shrimp under ammonia stress [3,4].

In teleosts, the primary response to physiological stress involves the release of corticosteroids and catecholamines. These then induce hyperglycaemia as a secondary response [5]. Several biogenic amines which function mainly as neuroregulators (neurotransmitter and neuromodulators), including serotonin, dopamine, octopamine, histamine, noradrenaline (norepinephrine), adrenaline (epinephrine), tryptamine and tyramine have been identified and quantitatively measured in crayfish *Pacifastacus leniusculus* and other decapod crustaceans [6–8]. Among the biogenic amines, the presence of dopamine and serotonin in the crustacean nervous system is well established [9].

It is known that when Pacific oyster *Crassostrea gigas* are subjected to mechanical disturbance, noradrenaline and dopamine are released into the circulatory system, subsequently decreasing immune functions [10], and increasing susceptibility to *Vibrio splendidus* infection [11]. It is also known that when the abalone *Haliotis tuberculata* are subjected to a 15 min mechanical disturbance, noradrenaline and dopamine are released, and immune parameters such as haemocyte counts decrease, as do migratory activity, phagocytic and respiratory burst capacity of the haemocytes [12]. However, there is no knowledge on the release of biogenic amines and their potential role in immunosuppression of penaeid shrimps under stress.

Environmental stressors like hypoxia, ammonia, nitrite and Cu^{2+} have been reported to cause reduction in immune ability of blue shrimp *Litopenaeus stylirostris* [13], as well as white shrimp *L. vannamei* [3,14,15]. Dopamine has been found to mimic the action of CHH (crustacean hyperglycaemic hormone) in increasing the level of glucose in tiger shrimp *Penaeus monodon* [8]. It is assumed that penaeid shrimp under such stress may increase their levels of biogenic amines including dopamine, which subsequently leads to immunosuppressive effects and increases susceptibility to pathogen infection. Accordingly, the purpose of the present study is to examine: (1) the effect of dopamine on the susceptibility of *L. vannamei* to *V. alginolyticus*, and (2) the immune response of *L. vannamei* injected with dopamine. For the latter purpose, we examined THC (total haemocyte count), phenoloxidase activity, respiratory burst, SOD (superoxide dismutase) activity, phagocytic activity and clearance efficiency of shrimp to *V. alginolyticus*.

2. Materials and methods

2.1. *L. vannamei*

*L. vannamei* juveniles (18–22 g) were obtained from a commercial farm in Pingtung, Taiwan, and acclimated in the laboratory for 2 weeks before experimentation. Only shrimps in the intermoult stage were used for the study. The molt stage was determined by the examination of uropods in which partial retraction of the epidermis could be distinguished [16]. For the susceptibility experiment, test and control groups were comprised of 10 shrimps each in triplicate. For the determination of immune parameters, tests were carried out in eight replicate test groups consisting of one shrimp each in 20 l PVC tanks containing 10 l aerated test solution. In all tests, the shrimps were fed twice daily with a formulated shrimp diet (Shinta
Feed Company, Pingtung, Taiwan). No significant difference in weight was observed among the treatments. During the experiments, water temperature was maintained at 27 ± 1 °C, pH 7.8–8.2 while salinity was maintained at 20%o.

2.2. V. alginolyticus

A known pathogenic strain of *V. alginolyticus* (CH003), which had been isolated from diseased *L. vannamei* in Pingtung, Taiwan, was used for the study [4]. Stocks were cultured on tryptic soy agar (TSA supplemented with 2% NaCl, Difco) for 24 h at 28 °C and transferred to 10 ml tryptic soy broth (TSB supplemented with 2% NaCl, Difco) for 24 h at 28 °C as the culture for the test. The broth culture was centrifuged at 7155 × g for 20 min at 4 °C. The supernatant fluid was removed and the bacterial pellet was re-suspended in saline solution (0.85% NaCl) at 5 × 10⁶ and 1 × 10⁸ colony-forming units (cfu) ml⁻¹ for the susceptibility test, as well as for the tests of phagocytic activity and clearance efficiency, respectively.

2.3. Test solution

Dopamine (Sigma product No. H-8502) was dissolved in sterile saline (0.85% NaCl) to concentrations of 5 × 10⁻⁴, 5 × 10⁻³ and 5 × 10⁻² mol l⁻¹, respectively, before injection.

2.4. Effect of dopamine on the susceptibility of *L. vannamei* to *V. alginolyticus*

*L. vannamei* was injected individually into the ventral sinus of the cephalothorax with 5 × 10⁻⁴, 5 × 10⁻³ and 5 × 10⁻² mol l⁻¹ dopamine solution (around 20 µl) to reach doses of 10⁻⁸, 10⁻⁷ and 10⁻⁶ mol shrimp⁻¹, respectively. Challenge tests were conducted after 1 h with the injection of 20 µl of bacterial suspension (5 × 10⁶ cfu ml⁻¹) resulting in 1 × 10⁵ cfu shrimp⁻¹ into the ventral sinus of the cephalothorax. The shrimp that received no dopamine, and then received *V. alginolyticus* at 1 × 10⁵ cfu shrimp⁻¹ served as the challenged controls. The shrimp that received dopamine at 10⁻⁶ mol shrimp⁻¹, and then received saline (20 µl), however, served as the unchallenged controls (Table 1). Experimental and control shrimp (10 aquarium⁻¹) were kept in 60 l glass aquaria containing 40 l of seawater at 20%o. Therefore, there were a total of five treatments. Each treatment was conducted with 30 shrimp. The experiment lasted 48 h.

2.5. Effect of dopamine on the immune parameters of *L. vannamei*

*L. vannamei* was injected individually in the ventral sinus of the cephalothorax with 5 × 10⁻⁴, 5 × 10⁻³ and 5 × 10⁻² mol l⁻¹ dopamine solution to reach doses of 10⁻⁸, 10⁻⁷ and 10⁻⁶ mol shrimp⁻¹, respectively.

<table>
<thead>
<tr>
<th>Dopamine (mol shrimp⁻¹)</th>
<th>Bacterial dose (cfu shrimp⁻¹)</th>
<th>Cumulative mortality (%), time after challenge (h)</th>
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<td>4</td>
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<tr>
<td>10⁻⁶ Saline</td>
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<tr>
<td>10⁻⁸ 1 × 10⁵</td>
<td>0b</td>
<td>16.7 ± 3.3⁹</td>
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<td>10⁻⁷ 1 × 10⁵</td>
<td>0b</td>
<td>33.3 ± 8.8⁹</td>
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<tr>
<td>10⁻⁶ 1 × 10⁵</td>
<td>0b</td>
<td>33.3 ± 8.8⁹</td>
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</table>

Data in the challenge groups in the same column with different superscripts are significantly different. (p < 0.05) among treatments. Values are mean ± S.E. (n = 30 shrimp in each case).
Shrimp that received saline (20 μl) served as controls. There were four treatments (saline, \(10^{-8}, 10^{-7}\) and \(10^{-6}\) mol dopamine shrimp\(^{-1}\)) with five sampling times (2, 4, 8, 16, and 24 h). Each shrimp for each treatment and time were used for the studies. In addition, eight shrimp without any treatment were used as the initial control group.

At 0, 2, 4, 8, 16, and 24 h after injections, haemolymph (100 μl) was withdrawn from the ventral sinus of each shrimp into a 1 ml sterile syringe (25 gauge) containing 0.9 ml anticoagulant solution (trisodium citrate 30 mM, sodium chloride 0.34 M, EDTA 10 mM, pH 7.55, osmolality adjusted with 0.115 M glucose to 780 mOsm kg\(^{-1}\)). They were divided into two parts. A drop of the anticoagulant-haemolymph mixture (100 μl) was placed on a haemocytometer to measure THC (Leica DMIL, Leica Microsystems, Wetzlar GmbH, Germany). The remainder of the haemolymph mixture was used for subsequent tests.

Following the procedures of Hernández-López et al. [17], phenoloxidase activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA). The details of the measurements were described previously [3]. The optical density of the shrimp’s phenoloxidase activity was expressed as dopachrome formation per 50 μl haemolymph.

As described previously [3], the respiratory burst of haemocytes was quantified using the reduction of NBT (nitroblue tetrazolium) to formazan as a measure of superoxide anion (O\(_2^-\)). The optical density at 630 nm was measured in triplicates using a microplate reader (Model VERSAmax, Molecular Devices, Sunnyvale, CA, USA). Respiratory burst was expressed as NBT-reduction per 10 μl haemolymph.

Superoxide dismutase (SOD) activity was measured by its ability to inhibit superoxide radical dependent reactions using the Ransod Kit (Randox, Crumlin, UK). Briefly, the reaction mixture (1.7 ml) contained 0.05 mM xanthine and 0.025 mM 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) dissolved in 50 mM CAPS (pH 10.2) and 0.94 mM EDTA. In the presence of xanthine oxidase (80 U l\(^{-1}\), 250 μl), superoxide and uric acid were produced from the xanthine. The superoxide radical then reacted with INT to produce a red formazan dye. The optical density was measured at 505 nm, 37 °C, and the rate of reaction was estimated from the absorbance readings 30 s and 3 min after adding xanthine oxidase. A reference standard SOD was supplied with the Ransod Kit. One unit of SOD was defined as the amount required to inhibit the rate of xanthine reduction by 50%. Specific activity was expressed as SOD units ml\(^{-1}\) [18].

2.6. Phagocytic activity and clearance efficiency of *L. vannamei* to *V. alginolyticus*

*L. vannamei* that received saline or dopamine were the same as those described above. Tests were carried out on eight shrimp. Twenty microlitre of bacterial suspension (1 × 10\(^8\) cfu ml\(^{-1}\)) resulting in 2 × 10\(^8\) cfu shrimp\(^{-1}\) was injected into the ventral sinus of each shrimp. After injection, the shrimp were kept for 1 h in a separate tank containing 40 l of water at 25.0 ± 1.0 °C. Then, 200 μl of haemolymph was collected from the ventral sinus and mixed with 200 μl of sterile anticoagulant. This mixture was divided into two equal sub-samples, one to measure phagocytic activity and the other to measure clearance efficiency. The methods for the measurements of phagocytic activity and clearance efficiency were described previously [3]. Two hundred haemocytes were counted. Phagocytic activity, defined as percentage phagocytosis (PR) was expressed as:

\[
PR = \frac{\text{phagocytic haemocytes}}{\text{total haemocytes}} \times 100
\]

Clearance efficiency was measured following the method of Adams [19]. The 200 μl volume of diluted haemolymph was further diluted to 20 ml with saline solution. Three 50 μl portions of this diluted haemolymph sample were spread on separate TSA plates and incubated at 27 °C for 24 h before colonies
were counted using a colony counter. The numbers of colonies from shrimp that received saline were the control values, and the numbers of colonies from shrimp that received dopamine at $10^{-8}$, $10^{-7}$ and $10^{-6}$ mol shrimp$^{-1}$ after 2, 4, 8, 16 and 24 h were the test values. Clearance efficiency to *V. alginolyticus*, defined as percentage inhibition (PI), was calculated as:

$$PI = 100 - \frac{\text{cfu in test group}}{\text{cfu in control group}} 	imes 100$$

2.7. Statistical analysis

A multiple comparison (Tukey) test was conducted to compare the significant differences among treatments using the SAS computer software (SAS Institute Inc., Cary, NC, USA). Percent data (susceptibility study) were normalised using an arcsin transformation before analysis. For statistically significant differences, it was required that $p < 0.05$.

3. Results

3.1. Effect of dopamine on the susceptibility of *L. vannamei* to *V. alginolyticus*

All the unchallenged control shrimp that received dopamine at $10^{-6}$ and were then injected with saline survived. By contrast, death occurred after 4 h for the challenged shrimp that received dopamine at $10^{-6}$ mol shrimp$^{-1}$. After 8–48 h, the mortality of shrimp that received dopamine at $10^{-8}$, $10^{-7}$ and $10^{-6}$ mol shrimp$^{-1}$ was significantly higher than that of shrimp that received saline (Table 1).

3.2. Effect of dopamine on the immune parameters of *L. vannamei*

No significant difference in THC was observed among the four treatments after 2 h. The mean (±S.E.) THC was 98.0 ± 11.0 × 10$^5$ cells ml$^{-1}$ in the control shrimp. The THC of shrimp decreased by 25% and 39% after 4 h, and decreased by 20% and 20% after 8 h for the shrimp that received dopamine at $10^{-7}$ and $10^{-6}$ mol shrimp$^{-1}$, respectively. No significant difference in THC was observed among the four treatments after 16 and 24 h (Fig. 1A).

Phenoloxidase activity of shrimp that received dopamine at $10^{-7}$ and $10^{-6}$ mol shrimp$^{-1}$ decreased by 15% and 38% after 2 h, and decreased significantly by 19% and 32% after 4 h. No significant difference in phenoloxidase activity, however, was observed among four treatments after 8, 16 and 24 h (Fig. 1B).

Respiratory burst of shrimp that received dopamine at $10^{-8}$, $10^{-7}$ and $10^{-6}$ mol shrimp$^{-1}$ decreased significantly by 29%, 30% and 42%, respectively after 2 h. Respiratory burst of shrimp that received dopamine at $10^{-6}$ mol shrimp$^{-1}$ decreased significantly by 36% after 4 h. No significant difference in respiratory burst, however, was observed among the four treatments after 8, 16 and 24 h (Fig. 2A).

Two hours after injections, no significant difference in SOD activity was observed for shrimp that received dopamine at $10^{-8}$, $10^{-7}$ and $10^{-6}$ mol shrimp$^{-1}$, or those that received saline. The SOD activity of shrimp that received dopamine at $10^{-8}$, $10^{-7}$ and $10^{-6}$ mol shrimp$^{-1}$ decreased significantly by 21%, 50% and 62% after 4 h, and decreased significantly by 62%, 92% and 96% after 8 h, respectively, when compared to the shrimp that received saline. The SOD activity of shrimp that received dopamine at $10^{-7}$ and $10^{-6}$ mol shrimp$^{-1}$ decreased significantly by 44% and 42% after 16 h, respectively, when compared to the shrimp that received saline. No significant difference in SOD, however, was observed among the four treatments after 24 h (Fig. 2B).
3.3. Phagocytic activity and clearance efficiency of *L. vannamei* to *V. alginolyticus*

At time 0 h, phagocytic activity was 5.5%. After 2 h, phagocytic activity decreased significantly to 4.1%, 4.2%, and 2.5% for the shrimp that received dopamine at $10^{-8}$, $10^{-7}$, and $10^{-6}$ mol shrimp$^{-1}$, respectively, as compared to the activity of shrimp that received saline. After 4 h, phagocytic activity decreased significantly to 3.9%, and 2.4% for the shrimp received dopamine at $10^{-7}$ and $10^{-6}$ mol shrimp$^{-1}$, respectively, as compared to the activity of shrimp that received saline. After 8 h, phagocytic activity decreased significantly to 3.8% for the shrimp that received dopamine at $10^{-6}$ mol shrimp$^{-1}$, as compared to the activity of shrimp that received saline. No significant difference in phagocytic activity, however, was observed among the four treatments after 24 h (Fig. 3A).
A similar trend was observed for clearance efficiency against V. alginolyticus. After 2 h, clearance efficiency decreased by 75%, 196%, and 344% for the shrimp that received dopamine at 10⁻⁸, 10⁻⁷ and 10⁻⁶ mol shrimp⁻¹, respectively, as compared to the shrimp received saline. However, no significant difference in clearance efficiency was observed among the four treatments after 4, 8, 16 and 24 h (Fig. 3B).

4. Discussion

In the present study, it was shown that white shrimp L. vannamei that received dopamine had an increased susceptibility to V. alginolyticus infection. Previously it was shown that shrimp had an increased susceptibility to V. alginolyticus infection by decrease in salinity, as well as the presence of ammonia, nitrite and copper sulphate in the rearing water [3,14,15,20]. For the Pacific oyster C. gigas that had been
challenged with pathogen *V. splendidus* and subjected to a mechanical stress, the mortality increased [11]. Injection of noradrenaline, a key component of the neuroendocrine stress response system also caused higher mortality in *C. gigas* [21]. Therefore, it is suggested that the physiological changes imposed by the stressors like salinity change, increased concentrations of ammonia and nitrite caused increases in the susceptibility of *L. vannamei* to *V. alginolyticus*.

Circulating haemocytes of *L. vannamei* displayed higher THC and phenoloxidase activities at the C stage and the lowest at the A stage [22]. In the present study, the *L. vannamei* used were at the C stage and were therefore considered to be similar with regard to defence. Circulating haemocyte count is also affected by extrinsic factors like temperature and salinity variations, as well as nitrite and Cu²⁺ in *L. vannamei* and *Litopenaeus stylirostris* [13–15,23].

Both THC and phenoloxidase activity of freshwater prawn *Macrobrachium rosenbergii* were significantly higher at pH 7.5–7.7 and 30–31 °C than they were at pH 4.6–5.0 or pH 9.0–9.5 and 33–34 °C [24].

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**Fig. 3.** Mean (±S.E.) phagocytic activity (A) and clearance efficiency (B) of *Litopenaeus_vannemei* that received dopamine at 10⁻⁸, 10⁻⁷ and 10⁻⁶ mol shrimp⁻¹, and that received saline. See Fig. 1 for statistical information.
Exposure of common shrimp *Crangon crangon* to PCB 15 (polychlorinated biphenyl 15) resulted in significantly decreased THC and phenoloxidase activity [25]. The phenoloxidase activity was significantly decreased in both *L. vannamei* and *M. rosenbergii* by exposure to ammonia-N at 0.55 mg l\(^{-1}\) or more [3,26]. Decreases in both THC and phenoloxidase activity occurred in *L. vannamei* following the exposure to 9.87 mg l\(^{-1}\) nitrite-N, as well as the exposure to 10 mg l\(^{-1}\) Cu\(^{2+}\) [14,15]. In the present study, *L. vannamei* that received dopamine at 10\(^{-6}\) mol shrimp\(^{-1}\) or less experienced decreased THC and phenoloxidase activity in 2h, indicating that dopamine down-regulated the immune ability of shrimp.

Using Pacific oyster *C. gigas*, Lacoste et al. [27] reported that noradrenaline had a dose-dependent inhibitory effect on chemiluminescence (CL)-response at the physiological concentrations of 0.1 \(\mu\)M and above, and indicated that noradrenaline decreases the release of reactive oxygen species. They also documented that \(\beta\)-adrenergic receptors are present at the surface of oyster haemocytes which allow noradrenaline to down-regulate the CL-response. The releases of O\(_2^-\) (superoxide anion) and H\(_2\)O\(_2\) (hydrogen peroxide) were considered to play a more important role in shrimp microbial activity than OCl\(^-\) (hypochlorites) and MPO (myeloperoxidase) [28]. The injection of fungicide propiconazole into the white shrimp *L. vannamei* induced an increase of superoxide anion at day 6, but caused a dose-dependent decrease in superoxide anion at day 13 [23]. It was proposed that the decreased production of superoxide anion in hypoxic *L. stylirostris* was due to the decrease of THC, and that the activity of NADPH oxidase responsible for the production of superoxide anion was not affected under hypoxia [13]. Exposure of *L. vannamei* to 11.10 mg l\(^{-1}\) ammonia-N for 48 h, to 9.87 mg l\(^{-1}\) nitrite for 96 h, or exposure to 20 mg l\(^{-1}\) Cu\(^{2+}\) decreased its release of superoxide anion [3,14]. Herein, we found that treatment with dopamine at 10\(^{-6}\) mol shrimp\(^{-1}\) or lower for 4 h decreased both the release of superoxide anion and the activity of SOD. The present study suggests that the activity of NADPH oxidase responsible for the release of superoxide anion, decreased with the decrease in the activity of SOD responsible for scavenging superoxide anion for shrimp that received dopamine at 10\(^{-6}\) mol shrimp\(^{-1}\) in a short amount of time.

Phagocytosis is an important cellular defence mechanism, whereas clearance efficiency is an important humoral defence mechanism in molluscs and crustaceans [29,30]. A significant reduction of phagocytic activity and clearance efficiency against *V. alginolyticus* was observed in the white shrimp *L. vannamei* following exposure to 1 mg l\(^{-1}\) Cu\(^{2+}\) [15], to 11.21 mg l\(^{-1}\) ammonia-N [3], and when transferred to 5 and 15% from 25% seawater [20]. A significant reduction in phagocytosis of *Bacillus cereus* was also observed in the shore crab *Carcinus maenas* following 14 days exposure to 500 \(\mu\)g l\(^{-1}\) Cd\(^{2+}\) and 10 days exposure to 100 \(\mu\)g l\(^{-1}\) Cu\(^{2+}\) [31]. The phagocytosis and clearance efficiency for *Vibrio harveyi* decreased in *P. monodon* following exposure to 1.8–2.0 mg l\(^{-1}\) O\(_2\) for 6 h, when compared to control shrimp [32]. Noradrenaline had a dose-dependent inhibitory effect on phagocytosis at physiological concentration of 0.1 \(\mu\)M and above in the Pacific oyster *C. gigas* [21]. Similarly, we found that phagocytic activity and clearance efficiency to *V. alginolyticus* decreased in *L. vannamei* that received dopamine at 10\(^{-8}\), 10\(^{-7}\) and 10\(^{-6}\) mol shrimp\(^{-1}\). This correlated with the increased susceptibility of *L. vannamei* to *V. alginolyticus* when the shrimp were injected with dopamine. Therefore, dopamine is considered to serve as an immune modulator in *L. vannamei*.

In conclusion, the present study documented that the white shrimp *L. vannamei* experienced an increase in susceptibility to *V. alginolyticus* after injections of dopamine at 10\(^{-6}\) mol shrimp\(^{-1}\) or less. Dopamine seems to play a role in immune modulation by decreasing THC, phenoloxidase activity, respiratory burst, SOD activity, phagocytic activity and clearance efficiency.

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