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Animal Health

Original

The Response of the Phenoloxidase Enzyme Following a Non-Lethal Heat Shock (NLHS) in *P. vannamei* Shrimp

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ABSTRACT

Background: Penaeus vannamei is affected by the emergence of diseases that cause high mortality rates and economic losses. **Aim.** To stimulate the immune system in Penaeus vannamei shrimp after non-lethal heat stress (NLHS). **Materials and methods:** Two NLHS conditions (acute and chronic) were developed. The acute challenge lasted 30 minutes, with sample collection at the following time points: 0, 30 minutes, 3, 6, and 12 hours. The chronic challenge lasted 5 minutes per day for seven days. Samples were collected at 0 and 7 days to evaluate immune response and resistance to osmotic stress. Shrimp acclimated to 28°C were considered the control group. **Results:** The ANOVA analysis of the acute challenge determined that the NLHS in *Penaeus vannamei* under our cultivation conditions is 37°C. An increase ($P<0.05$) in the enzyme phenoloxidase was detected through two-way ANOVA with interaction and HSD Tukey analysis at 6 and 12 hours post-NLHS (0.04593 and 0.05268×10^{-1} mg), when compared to 30 minutes and the control (0.0003037 and 0.001217×10^{-1} mg). The chronic NLHS showed a high stimulation of phenoloxidase ($P<0.05$) at 7 days (4.233 ± 10^{-1} mg). While in the control group it was only 1.392 ± 10^{-1} mg, survival under osmotic stress at 0 ppm, analyzed through a Student's t-test, demonstrated

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high survival of 85% in the NLHS group (P<0.05), compared to 20% in the control group. **Conclusions:** The results demonstrate that an NLHS of 37°C stimulates the immune system and increases survival when challenged with salinity at 0 ppm.
Keywords: NLHS, immune system, *Penaeus Vannamei* (Source*: AGROVOC*)

INTRODUCTION

Among the many challenges faced by aquaculture development is the prevention and control of diseases (Ramírez-Jiménez *et al.*, 2015). The production of penaeid shrimp, whether wild or farmed, is largely affected by the presence of pathogens. These pathogens exist naturally in the environment or opportunistically coexist with the shrimp culture, invading the crustaceans during periods of injury and stress (Rodríguez-Álvarez *et al*., 2020).

The immune system of invertebrates, unlike that of vertebrates, is characterized by the absence of immunoglobulin-type molecules and lymphoid cells. In crustaceans, the immune response is based on humoral and cellular effectors, which work together to eliminate potentially infectious microorganisms (Moreno, Salas & Gutiérrez, 2016). The innate immune response in shrimp is based on pathogen-associated molecular patterns (PAMPs), which can be recognized by pattern recognition receptors (PRRs) (Andino Lozano & Romero Ramírez, 2014). Humoral components are considered (Fonseca, 2016) to include anticoagulant proteins, agglutinins, phenoloxidase enzyme, antimicrobial peptides, and free radicals.

Recent studies show that understanding the immunological processes associated with physiological biochemistry allows for determining the health status of shrimp. The variation in osmotic capacity and plasma metabolite concentration has been used to determine the physiological state in relation to size, molting phase, dissolved oxygen, and broodstock quality (Peres-Jar *et al*., 2011). Temperature is the most important environmental factor in the cultivation of penaeid shrimp. The biochemical or physiological adjustments that occur in any adaptation depend on metabolic reactions involving enzymes that are entirely dependent on this factor for their development (Pérez & Balam, 2012).

The effect of temperature on enzymes causes an animal's metabolic rate to increase exponentially with body temperature (Díaz-Iglesias *et al*., 2004). Heat tolerance and resistance are adaptive strategies that species have developed under different thermal regimes. In penaeid shrimp, water temperature affects performance, including metabolic rates of growth and ingestion, fertility, behavior, and survival (Godínez *et al*., 2003).

Studies conducted by(Loc *et al*., 2013; Junprung, Supungul and Tassanakajon, 2019) show that exposing aquatic organisms to non-lethal temperatures stimulates the immune system and protects them against biotic and abiotic stressors.

The studies preceding the exposure of shrimp to non-lethal heat stress (NLHS) do not take into account the time required for the immune system to be stimulated with the activation of the

phenoloxidase enzyme. This aspect is of great interest for preventing routine challenges in aquaculture conditions, such as salinity, temperature, and the presence of pathogenic bacteria. Based on the aforementioned considerations, the objective of this study was to evaluate the stimulation of the phenoloxidase enzyme following a non-lethal heat shock (NLHS) in adult *P. vannamei* shrimp.

MATERIALS AND METHODS

P. vannamei shrimp collection

P. vannamei shrimp weighing between 18-20g were collected from the Yagüacam Larval Development Center, located in Cumanayagua, Cienfuegos, Cuba, at a density of 20 shrimp per tank. The temperature and salinity during the experiment were 28 ± 1 °C and 34 ± 1 g L^{-1}. The seawater used maintained a pH of 8.5 ± 0.5, was sterilized and filtered with 1 μm ultraviolet radiation, and was continuously illuminated and aerated throughout the experiment. Water exchanges were performed daily. Feeding was based on Artemia franciscana (Aquatic Eco-Systems Inc., Apopka, FL, USA) at a rate of 8-10 Artemia nauplii per shrimp every two hours, along with industrial feed.

Determination of non-lethal heat shock (NLHS) temperature in P. vannamei shrimp.

To determine the appropriate NLHS temperature for *P. vannamei* shrimp, five groups of 20 shrimp each were acclimated to 28ºC. Four of these groups were abruptly exposed to an acute NLHS of 34, 36, 37, and 38ºC for 30 minutes and then immediately transferred to ambient temperature for recovery. Survival was evaluated after 6 hours. The group of shrimp that were not heated and maintained at 28ºC was considered the control group. Samples were collected at various time points (0, 30 minutes, 3, 6, and 12 hours) to evaluate the total protein content and the specific enzymatic activity of phenoloxidase. The experiment was conducted with three replicates.

Kinetics of phenoloxidase enzymatic activity

Once the acute temperature stress was completed, samples were collected at various times (0, 30 minutes, 3, 6, and 12 hours). They were macerated and suspended in 1 mL of phosphate-buffered saline (PBS). Then, the protein content and the specific enzymatic activity of phenoloxidase were evaluated at each time point.

Chronic NLHS in P. vannamei shrimp

P. vannamei shrimp (18-20g body weight) acclimated to 28 ± 1ºC were abruptly subjected to chronic thermal stress at a temperature of 37 ± 0.2ºC for 5 minutes over 7 days. Aeration conditions were controlled by maintaining dissolved oxygen (DO) and a salinity of 10 ppm. After the heating process was completed, the shrimp were immediately transferred to their ambient temperature (28°C) for recovery. Samples were collected at two times (0 and 7 days) in both groups (control

and treated) to evaluate immunological parameters. The unheated shrimp (28ºC) were considered the control group.

Protein concentration

The total protein concentration in the sample supernatant was determined using the Bradford method (1976). The experiment was conducted in triplicate, and the protein concentration was calculated using a standard curve, for which a bovine serum albumin (BSA) solution (Sigma-Aldrich USA) in H_2Odd was used. The total protein concentration was divided by the number of shrimp present in each sample. The results were expressed in µg of protein per animal.

To determine the protein concentration in the precipitate of the samples, 100 µL of H_2Odd was added to the precipitate. Then, 10 µL from each sample was taken and solubilized in 190 µL of 1 mol L^{-1} NaOH at 80°C for 60 minutes. The protein concentration was determined using a standard curve, for which an ASB solution in 1M NaOH was used.

Specific enzymatic activity of Phenoloxidase (PO)

The enzymatic activity of phenoloxidase was determined through spectrophotometric detection of the formation of a chromogen from L-dihydroxyphenylalanine (L-DOPA) (Sigma-Aldrich, USA) (Hernández-López, Gollas-Galván, and Vargas-Albores, 1996). One unit of phenoloxidase (PO) activity was defined as the variation of 0.001 absorbance (Abs) units per minute.

Quality of Litopenaeus vannamei shrimp (osmotic stress)

After the experiment was completed, 50 out of the 60 individuals in each group were selected to evaluate their quality based on their resistance to osmotic stress through a change in salinity from 30 ppm to 0 ppm for one hour. After this period, the salinity was restored to 30 ppm, and survival was assessed by counting the live animals.

Statistical analysis

The statistical analysis of the experimental data was performed using GraphPad Prism version 8.0 (2016, GraphPad Software, USA). A normality analysis of the obtained data was performed using the Kolmogorov-Smirnov tests. Homogeneity of variances was also verified using Bartlett's test.

To evaluate the effect of temperature on survival and the kinetics of phenoloxidase enzymatic activity, mean comparison techniques were used, including one-way and two-way analysis of variance with interaction. Additionally, HSD Tukey was employed for multiple comparisons, while the Student's t-test was used for independent samples in assessing survival under osmotic stress. In the case of protein concentration determination, a simple linear regression analysis was performed.

RESULTS AND DISCUSSION

Non-lethal temperature in juvenile Litopenaeus vannamei

The results of the single ANOVA with interaction in determining an appropriate NLHS (Fig. 1) show high survival rates among the control groups (28), 35, 36, and 37°C, with no significant differences between them ($P > 0.05$), where 100% of the shrimp can survive at a maximum non-lethal temperature of 37°C. However, significant differences ($P < 0.05$) were observed in the group heated to 38°C when compared to the other NLHS groups and the control, reducing the survival rate to 50%. The maximum non-lethal temperature of 37°C was used in the following experiments.

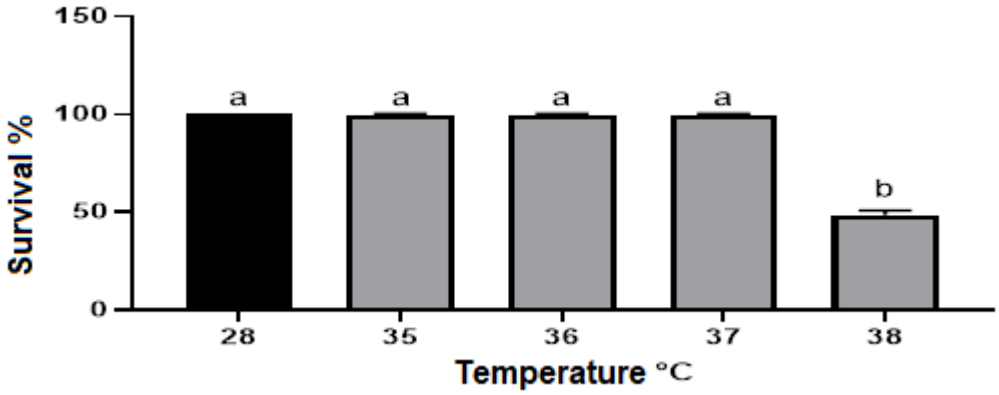

Figure 1. Determination of a non-lethal heat shock (NLHS) temperature in *Litopenaeus vannamei* shrimp The T bar corresponds to the standard error (SE) value; different letters indicate significant differences ($p < 0.05$) between groups according to the HSD Tukey test.

One of the main threats in marine shrimp farming is the emergence of infectious diseases, primarily those caused by viruses and bacteria (Lightner & Redman, 1998). The fundamental factor that causes an infection to develop into disease at the host level is stress (Overstreet, Cooper, & Katz, 1978). This is considered the set of physiological responses of an organism reacting to an environmental or metabolic disturbance to maintain homeostasis (Schreck, 2000) and can be temporally classified according to its duration into: Acute stress occurs in response to a sudden event (minutes, hours, or few days), while chronic stress arises from prolonged or recurrent environmental events (weeks) (Zacarías-Soto, 1997).

Animal responses to temperature help infer the heat tolerance and resistance limits (Fried, 1991). Within these limits are the preferred temperature or temperature referendum, avoidance temperatures, incipient lethal temperatures, and critical temperatures (Brett, 1946). In aquatic organisms, the preferred temperature can be differentiated between the acute temperature preferendum and the final preferendum. While acute temperature preferendum is influenced by acclimation temperature, the final preferendum remains unaffected (Díaz *et al.*, 2002). The more a cell is subjected to heat stress, the greater its tolerance to it, in a phenomenon known as heat

tolerance. The stress response is triggered when the temperature rises 10–15°C above the organism's optimal temperature, with a margin of only 5°C (Marchena López, 2020).

The results obtained from NLHS at 37°C in our experiment for adult Litopenaeus vannamei following acute stress are considered a good indicator of the species' environmental heat requirements. During this period, the physiological parameters of the organisms remain stable. (Nichelmann, 1983) stated that, during these intervals, the organism is exposed to minimal stress, and its physiological functions are optimized, which is reflected in adequate reproductive conditions. A physiological disturbance may be present at 38°C, where only 50% of the shrimp survived. Heat shocks are essential for survival. The temperature value obtained is consistent with those reported by Paschke *et al*. (2013), Junprung, Supungul & Tassanakajon (2017), and Ulaje *et al.* (2020).

Kinetics of the phenoloxidase enzyme under acute temperature stress

The results of a two-way ANOVA with interaction in our experiment show an increase in the specific enzymatic activity of phenoloxidase (Fig. 2). High values were observed at 3, 6, and 12 hours post-challenge (0.04215; 0.04593; 0.05268 × 10^{-1} mg, respectively) in the NLHS group. Meanwhile, in the control group, the results remained consistent, with values close to zero at all time points.

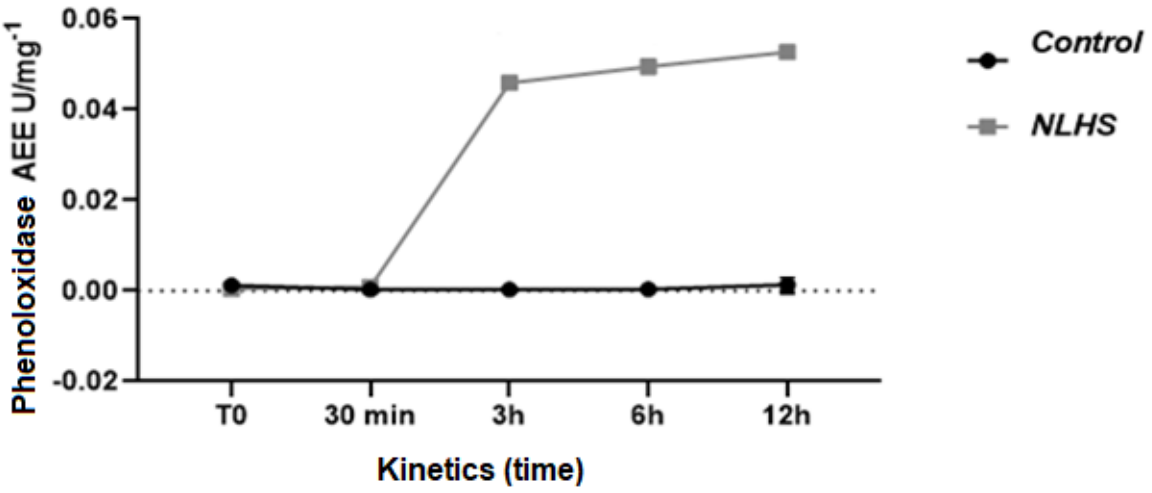

Figure 2. Kinetics of phenoloxidase enzymatic activity in adult *Litopenaeus vannamei* shrimp following acute NLHS at 37°C.

Among the defense mechanisms of shrimp, the prophenoloxidase (proPO) system, when activated, triggers a series of enzymatic reactions. It is located within the granules of granular and semi-granular hemocytes and can be released through antigen stimulation (Martínez & Saa, 2007). Once the granular content is released through degranulation, prophenoloxidase (proPO) is activated into phenoloxidase (PO). The activating enzyme is a trypsin-like serine protease, known as Prophenoloxidase Activating Proteins (ppA). Phenoloxidase (PO) is the enzyme responsible for

melanization observed in crustaceans and insects, as it catalyzes the oxidation of phenols into quinones, which then polymerize into melanin (López-Elías *et al*., 2016).

Phenoloxidase activity in our experiment was detected three hours after non-lethal heat stress, demonstrating that the defense system in penaeids is activated within a few hours post-challenge. This response alerts the immune system to the presence of a foreign element, which could compromise homeostasis. PO is activated, controlled, and regulated to minimize the damage caused by stress.

Our results align with those obtained in a study on the activation of prophenoloxidase (proPO) expression in *Macrobrachium rosenbergii*, where the highest enzyme activity was observed between 6 and 12 hours post-stimulation (Pascual, Rodríguez & Rosas, 2006). The same researchers noted that the increase in phenoloxidase activity occurred three hours post-infection with *Vibrio harveyi*. Other studies related to non-lethal temperature exposure, in this case in Artemia, demonstrated an enhanced immune response of phenoloxidase when compared to the control group (Pestana *et al*., 2016).

Protein concentration

The protein concentration shows a notable increase in protein content in animals exposed to non-lethal heat stress. The highest values were observed at 6 and 12 hours (0.83 and 0.89 µg/animal^{-1}) when compared to T0 and 30 minutes (0.028 and 0.036 µg/animal) in the NLHS group. Meanwhile, in the control group, even lower values were observed.

This result is associated with the fact that protein serves as the primary energy resource, with an essential role in maintaining vital functions and responding to stress. The increase in protein concentration exhibited a linear trend, with an R^2 value of 0.9944.

In *Litopenaeus vannamei*, protein concentration serves as an indicator of development, good health condition, and proper nutrition—the latter being essential for evaluating immune function (Martín *et al*., 2012). Some authors (Bindels *et al*., 2015; Kumar and Pansari, 2016; Pestana *et al*., 2016; Srisapoome and Areechon, 2017; Di *et al*., 2019), found an increase in protein concentration in challenged *P. vannamei* to different *Bacillus* strains.

Chronic non-lethal heat stress (NLHS) in adult Litopenaeus vannamei

One of the main defense mechanisms in the immune system of crustaceans is the prophenoloxidase (proPO) system. Hemocytes, stimulated by the presence of foreign elements, release the enzyme in the form of an inactive zymogen (proPO). Once activated through a proteolytic cascade, it is converted into active phenoloxidase (PO) (Laria *et al*., 2005).

In this experiment with the chronic NLHS group in adult Litopenaeus vannamei shrimp (Fig. 3), individuals were acclimated to 28°C and then abruptly exposed to 37°C for 5 minutes per day over 7 days. After each heat exposure, the shrimp were returned to their ambient temperature. The results

indicate that following chronic temperature stress, phenoloxidase enzymatic activity can be stimulated, reaching values of 4.233 ± 10^{-1} mg, with significant differences ($P < 0.05$) when compared to the control group (1.392 ± 10^{-1} mg).

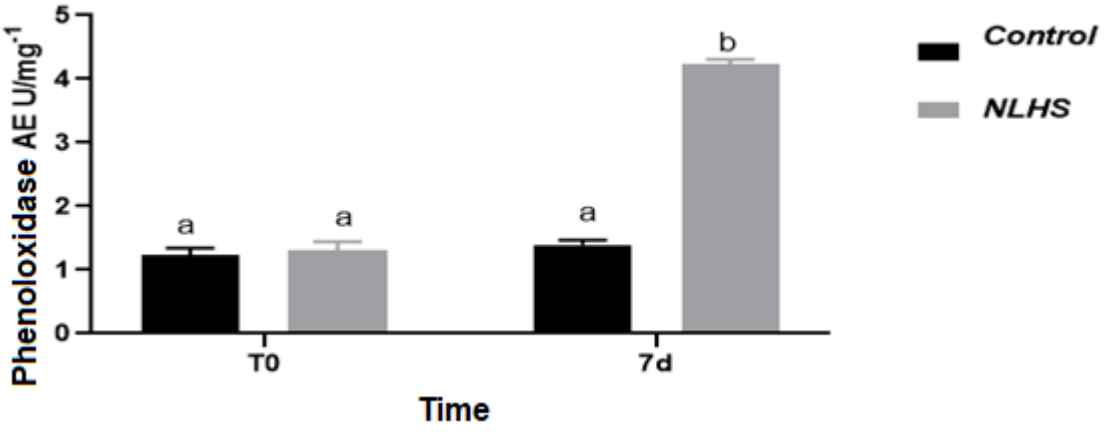

Figure 3. Specific phenoloxidase enzymatic activity in *Litopenaeus vannamei* shrimp exposed to chronic NLHS The data represent means and standard error (T-bars); different scripts indicate significant differences between groups (P < 0.05) according to Student's t-test.

Previous reports indicate that NLHS treatment can increase tolerance to pathogenic infections in aquatic organisms such as Artemia and the Asian green mussel (Baruah *et al.*, 2017). This demonstrates an increase in immunological parameters, which help protect these organisms against both biotic and abiotic stressors. The results of chronic NLHS treatment at 37°C in our experiment demonstrate that the immune system is stimulated with the activation of the phenoloxidase enzyme following non-lethal heat stress. This occurs due to an increase in circulating hemocytes and the proteolytic action of certain heat shock proteins, such as HSP70 and HSP90, which have been described following NLHS (Loc *et al.*, 2013; Junprung *et al.*, 2017).

Some studies report a decrease in phenoloxidase activity related to changes in salinity, temperature fluctuations, and other external factors (Cabrera Machado, Jaime Ceballos & Galindo López, 2010). Therefore, it is necessary to optimize these parameters in shrimp farming.

Post-Chronic NLHS osmotic stress in adult P. vannamei shrimp

The determination of the quality of adult P. vannamei shrimp subjected to a temperature NLHS for 5 minutes at 37°C for 7 days and then transferred to ambient temperature (28°C) was evaluated based on their resistance to osmotic stress at 0 ppm (Fig. 4). In our experiment, the heated shrimp exhibited a high survival rate exceeding 80%, with significant differences ($P<0.05$) when compared to the control group (20%).

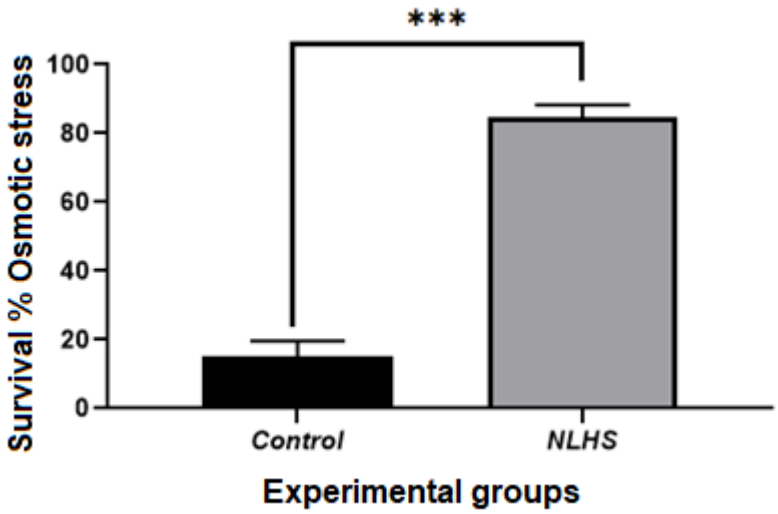

Figure 4. Osmotic stress at a salinity of 0 ppm in adult *P. vannamei* shrimp following chronic NLHS. The T bar corresponds to the SE value. Different scripts represent significant differences between the groups (P < 0.05) according to Student's t-test.

Shrimp of the genus Penaeidae tolerate a wide range of salinities, from freshwater to seawater at 150% (≈ 52 g/L1). They are excellent hypo- and hyper-regulators, and in an osmoregulation graph, they generally exhibit a pattern in the form of a broad plateau in the central part of their entire range, where the hemolymph concentration remains very constant. Slight variations in regulation levels and the position of this plateau correlate with their normal habitat distribution in nature (Cabrera Machado *et al*., 2010). However, this is quite contradictory, as noted by Le Moullac and Haffner (2000), since the energetic cost of regulation is lower when there is a low osmotic gradient between the hemolymph and the environment.

Similarly, it is minimal when the osmolarity of the environment is similar to the osmolarity of the hemolymph, or when the hemolymph is isosmotic with the external medium. Based on this hypothesis, the shrimp in the experimental group maintained a low regulatory cost, influenced by immune system stimulation following chronic temperature NLHS. They exhibited a high defensive capacity against stress and improved osmoregulatory ability compared to the unheated control group shrimp, which experienced a greater severity of osmotic regulation due to differences between the hemolymph and the environment, leading to high mortality in this group. Similar results to those obtained in this study were described in the exposure of *P. vannamei* to combinations of salinities and temperature. Where high survival rates were obtained (Piña-Valdez *et al*., 2015; Albines Nizama, 2019).

CONCLUSIONES

The exposure of adult *P. vannamei* to a 37°C NLHS stimulates the immune system with the activation of the phenoloxidase enzyme a few hours after the challenge. Shrimp exposed to NLHS achieve higher survival rates at low salinities compared to non-heated (Control) shrimp.

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AUTHOR CONTRIBUTION STATEMENT

Research conception and design: MGS, HCA, YCB, LCG, ESC, AAC, AAVP; data analysis and interpretation: MGS, AAC; redaction of the manuscript: MGS, HCA, YCB, LCG, ESC, AAC, AAVP.

CONFLICT OF INTEREST STATEMENT

The authors state there are no conflicts of interest whatsoever.