










Transcriptional response of immune-related genes in *Litopenaeus vannamei* cultured in recirculating aquaculture systems with elevated CO₂

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ABSTRACT - This short-term study evaluated the effect of non-lethal high CO₂ concentration on the transcriptional response of immune-related genes of Pacific white shrimp (*Litopenaeus vannamei*) cultured in recirculating aquaculture systems (RAS). Two experimental groups were created: high CO₂ (47.67±2.04 mg L⁻¹) and low CO₂ (2.0±1.93 mg L⁻¹). Shrimp of 8.85±1.20 g were placed randomly at a density equivalent to 100 individuals m⁻³ and were monitored at 6, 12, 18, and 24 h. The transcriptional response of immune-related genes was analyzed by qPCR. Gene expression of hemocyanin, prophenoloxidase, and heat shock protein 60 was downregulated at 24 h, suggesting affectations on oxygen transportation, melanization, and protein functioning of *L. vannamei* under high CO₂ concentrations. Also, gene up-regulation of lipopolysaccharide- and β-glucan-binding protein and cytosolic manganese superoxide dismutase can impair the bacterial recognition and antioxidant defense of shrimp exposed to high CO₂ concentrations. These results suggest that concentration at about 47 mg L⁻¹ of CO₂ can significantly influence the transcriptional response modulation of immune-related genes.

Keywords: aquaculture, gene expression, intensive system

1. Introduction

Worldwide, crustacean farming reached a production of 9,386,500 tons in 2018; Pacific white shrimp (*Litopenaeus vannamei*) is the most representative and widely cultivated species, contributing 53% of total shrimp production (FAO, 2020). Due to aquaculture exponential growth and the adaptability of *L. vannamei* to intensive farming, recirculating aquaculture systems (RAS) has become an eco-sustainable alternative to traditional systems used for shrimp farming (Chen et al., 2019). Higher stocking densities in RAS require high feeding rates, thus increasing organic matter decomposition and CO₂ concentrations (Good et al., 2010). Therefore, high carbon dioxide (CO₂) levels are a characteristic of these culture systems (Khan et al., 2018).

High CO₂ concentrations contribute to the system acidification (Skov, 2019), which can negatively affect growth, physiology, energy metabolism, and immunity of fish (Dennis III et al., 2015; Good et al., 2018; Khan et al., 2018; Almroth et al., 2019; Hermann et al., 2019; Mota et al., 2019; Machado et al., 2020; Pan et al., 2020; Mota et al., 2020), crustaceans (Fehsenfeld et al., 2011; Rathburn et al., 2013; Johnson et al., 2015; Zheng et al., 2015; Chang et al., 2016; Meseck et al., 2016), and mollusks (Bibby et al., 2008; Wang et al., 2016; Clements et al., 2021). High non-lethal (23.8 mg L⁻¹), lethal (59.12 mg L⁻¹), and safe (5.9 mg L⁻¹) CO₂ levels for *L. vannamei* production in RAS systems were determined (Furtado et al., 2017), but concentration above 20 mg L⁻¹ reduces tissue oxygenation and increases the ventilation rate (Furtado et al., 2016). Consequently, high CO₂ concentrations in RAS cause blood acidosis during hypercapnia and could impair oxygen transport and general metabolic processes of *L. vannamei* (Johnson et al., 2015; Summerfelt et al., 2015; Chen et al., 2019). However, information on the effects of high non-lethal CO₂ concentration on the physiology, behavior, and production performance of shrimp farmed in RAS remains limited. Therefore, the objective of the present short-term study was to determine the effect of non-lethal high CO₂ concentration on the transcriptional response of immune-related genes of Pacific white shrimp cultivated in RAS.

2. Material and Methods

The research was conducted in Ciudad Obregon, Sonora, Mexico (27°29'03.6" N, 109°56'4.2" W), and animal use was conducted with ethical standards and approved by the institutional Ethics and Biosafety Committee (2020-04).

For the present study, two RAS with six circular tanks (0.9 × 1.10 m) each and with a capacity of 700 L were used. One RAS with six tanks was used to receive the additional CO₂ through a diffuser from a pressurized CO₂-gas bottle until achieving dissolved concentrations of 47.67±2.04 mg L⁻¹ for the high treatment. The remaining six tanks did not receive CO₂ (control treatment), so the levels were 2.0±1.93 mg L⁻¹ CO₂. One hundred and eighty shrimp (8.85±1.20 g) were randomly distributed in the 12 tanks at a density of 15 individuals per tank with a working volume of 150 L, equivalent to 100 shrimp m⁻³ density. The RAS remained with aeration, without water changes, and feeding was suspended during the test time (24 h). Afterwards, one shrimp per replicate (six per treatment) was collected at different times (6, 12, 18, and 24 h) to obtain 400 µL of shrimp hemolymph according to that described by Martínez-Porchas et al. (2020). The hemolymph was centrifuged at 3,500 rpm for 10 min at 4 °C, the plasma was discarded, and the cell pellet was resuspended in TRIzol for RNA extraction and frozen at -70 °C until analysis.

The physicochemical parameters were measured during the sampling points. Dissolved oxygen (DO) and temperature were measured with an oximeter (YSI 55, Yellow Springs), salinity with a refractometer (Hanna RB80, Hanna Instruments), and pH with a portable submersible potentiometer (Hanna HI 98127, Hanna Instruments).

Total RNA was extracted with the TRIzol reagent. Concentration and purity of RNA were analyzed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific), and an A260: A280 ratio between 1.8 and 2.2 was ensured. Total RNA was treated with RNA-free DNase (Promega®). The cDNA was synthesized using the ImProm-II™ Reverse Transcription System (Promega®) with oligo d(T)20 (T4OLIGO), using 500 ng of total RNA. The cDNA was diluted with 80 µL of ultrapure water, and 5 µL were used as template for the quantitative real time PCR (qPCR) reaction.

Transcriptional response was analyzed based on five immune-related genes and β-actin as reference gene (Table 1) (Zhang et al., 2013). The qPCR amplifications were performed in final reaction volumes of 15 µL following the instructions of MyTaq DNA polymerase (Bioline™) with 0.2 µM of each primer (T4OLIGO), 0.0125 µM of EvaGreen® 20X (Biotium), and 5 µL of cDNA. The qPCR was performed on a StepOne Real Time PCR System (Thermo Fisher Scientific). Conditions for qPCR were initial denaturation for at 95 °C for 10 min, followed by 40 denaturation cycles at 95 °C for 15 s, and annealing/extension at 60 °C for 1 min. An analysis of the dissociation curve (60–95 °C at a temperature transition rate of 0.5 °C s⁻¹) was performed for each pair of primers. The levels of gene-relative expressions

Table 1 - Specific primers used for qPCR amplifications of immune-related genes of Pacific white shrimp, *Litopenaeus vannamei*

Gene	Primer name	Forward/reverse sequence	Amplicon length	GenBank accession number
β-Actin	β-ActinF	5'-CCACGAGACCACCTACAAC-3'	125	AF300705
	β-ActinR	5'-AGCGAGGGCAGTGATTTC-3'		
Hemocyanin (Hc)	HcF	5'-GTCTTAGTGGTTCTTGGGCTTGTC-3'	123	X82502
	HcR	5'-GGTCTCCGCTCTGAATGTCTCC-3'		
Prophenoloxidase (proPO)	pPOF	5'-CGGTGACAAAGTTCCTCTTC-3'	121	AY723296
	pPOR	5'-GCAGGTCGCCGTAGTAAG-3'		
Lipopolysaccharide- and β-glucan-binding protein (LGBP)	LGBPF	5'-CCATGTCCGCGGTGGAA-3'	120	EU102286
	LGBPR	5'-GTCATCGCCCTTCCAGTTG-3'		
Cytosolic manganese superoxide dismutase (cytMnSOD)	MnSODF	5'-TGTTGACACAAGCCATTGACGA-3'	97	DQ005531
	MnSODR	5'-CCAGCCAGAGCCTTCACTCC-3'		
Heat shock protein 60 (HSP60)	HSP60F	5'-ATTGTCGCGAAGGCTATC-3'	101	FJ710169
	HSP60R	5'-ATCTCCAGACGCTCCAT-3'		

were calculated according to the $2^{-\Delta\Delta CT}$ equation (Livak and Schmittgen, 2001). Data from relative gene expression were transformed with $\log_{10} + 1$ to achieve normal distribution according to that described by Rodríguez-Anaya et al. (2020).

All data are presented as mean±SE. Data collected at 6, 12, 18, and 24 h were evaluated by one-way analysis of variance. If any significance was observed, Tukey's test was performed for a comparison of the means. Statistical analysis was performed with Statgraphics Centurion XVI. Significance was set at 95% probability levels.

Variables were analyzed according to the following mathematical model:

$$Y_{ij} = \mu + \beta_i + \varepsilon_{ij}$$

in which Y_{ij} = observed variable, μ = overall mean, β_i = effect of CO_2 level, and ε_{ij} = random error associated to each observation.

3. Results

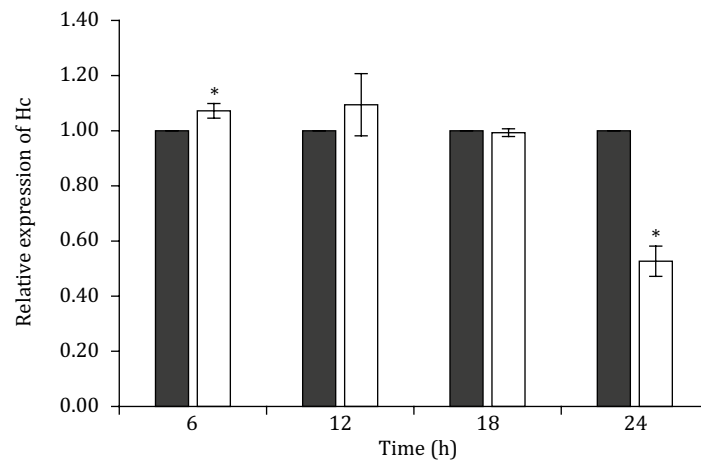
Carbon dioxide treatments did not affect ($P > 0.05$) salinity, temperature, and dissolved oxygen, while pH was affected by high CO_2 level (Table 2).

The transcriptional response of immune-related genes of white shrimp exposed to high CO_2 was determined in comparison with the shrimp response subjected to low CO_2 . Hemocyanin (Hc) gene expression was up-regulated ($P < 0.05$) at 6 h but down-regulated ($P < 0.05$) at 24 h (Figure 1). Prophenoloxidase (proPO) gene expression was upregulated ($P < 0.05$) at 6 and 12 h but downregulated ($P < 0.05$) at 24 h (Figure 2). Lipopolysaccharide- and β-glucan-binding protein (LGBP) gene expression was upregulated ($P < 0.05$) at 6, 12, 18, and 24 h (Figure 3). Cytosolic manganese superoxide dismutase (cytMnSOD) gene expression was downregulated ($P < 0.05$) at 12 and 18 h but upregulated ($P < 0.05$) at 24 h (Figure 4). Heat shock protein 60 (HSP60) gene expression was downregulated ($P < 0.05$) at 12, 18, and 24 h (Figure 5).

Table 2 - Water quality parameters measured during a 24 h test with different concentrations of carbon dioxide (CO_2)

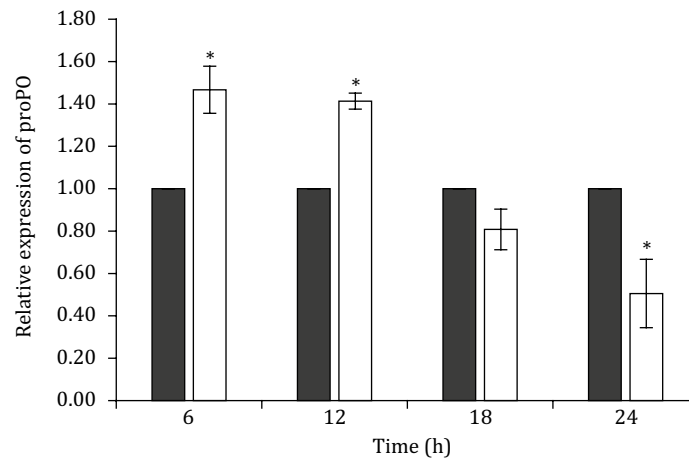
CO_2 treatment	Salinity (g L ⁻¹)	Temperature (°C)	Dissolved oxygen (mg L ⁻¹)	pH
Low	43.29±2.54a	30.43±1.24a	4.68±0.25a	7.71±0.04a
High	42.98±2.29a	30.45±1.23a	4.81±0.20a	6.63±0.64b

Different letters in the same column indicate statistical difference ($P < 0.05$) according to Tukey's HSD test.



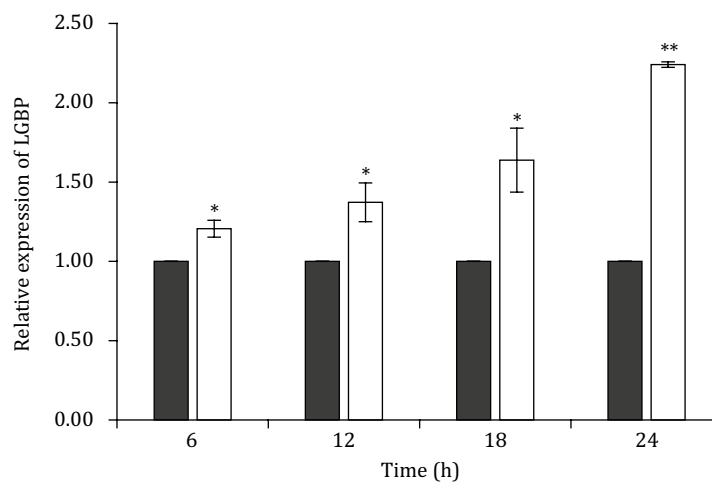
Data are presented as mean±SE, n = 6 each group.
Low CO₂ treatment is represented by black bar and high CO₂ is represented by white bar.
Significant differences compared with low CO₂ treatment: *P<0.05.

Figure 1 - Transcriptional response of hemocyanin (Hc) in *L. vannamei* under high CO₂ level.



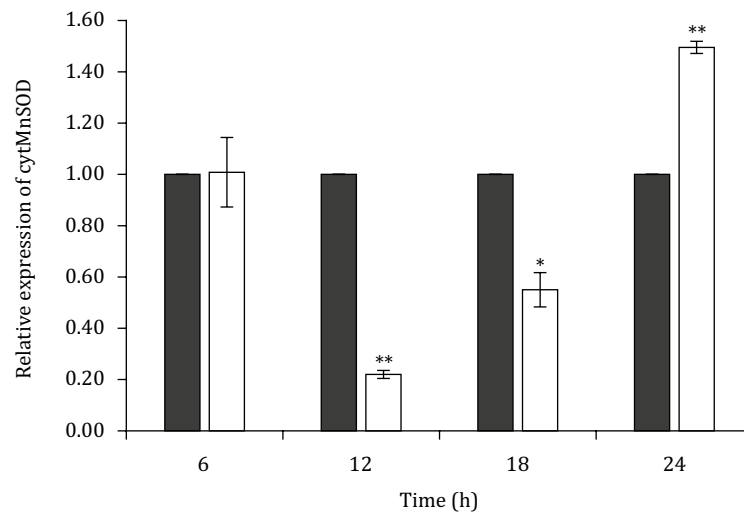
Data are presented as mean±SE, n = 6 each group.
Low CO₂ treatment is represented by black bar and high CO₂ is represented by white bar.
Significant differences compared with low CO₂ treatment: *P<0.05.

Figure 2 - Transcriptional response of prophenoloxidase (proPO) in *L. vannamei* under high CO₂ level.



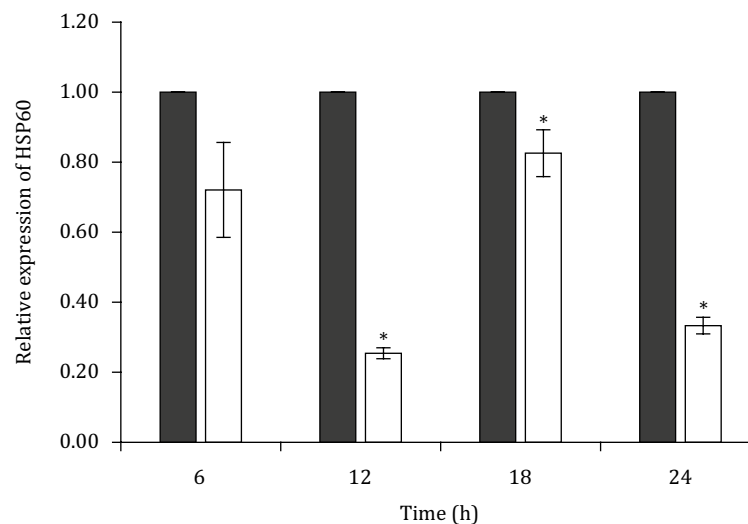
Data are presented as mean±SE, n = 6 each group.
Low CO₂ treatment is represented by black bar and high CO₂ is represented by white bar.
Significant differences compared with low CO₂ treatment: *P<0.05, **P<0.01.

Figure 3 - Transcriptional response of lipopolysaccharide- and β-glucan-binding protein (LGBP) in *L. vannamei* under high CO₂ level.



Data are presented as mean±SE, n = 6 each group.
Low CO₂ treatment is represented by black bar and high CO₂ is represented by white bar.
Significant differences compared with low CO₂ treatment: *P<0.05, **P<0.01.

Figure 4 - Transcriptional response of cytosolic manganese superoxide dismutase (cytMnSOD) in *L. vannamei* under high CO₂ level.



Data are presented as mean±SE, n = 6 each group.
Low CO₂ treatment is represented by black bar and high CO₂ is represented by white bar.
Significant differences compared with low CO₂ treatment: *P<0.05.

Figure 5 - Transcriptional response of heat shock protein 60 (HSP60) in *L. vannamei* under high CO₂ level.

4. Discussion

In RAS, the optimal CO₂ range is 5 to 10 mg L⁻¹, but high densities can produce concentrations above 20 mg L⁻¹ (Furtado et al., 2017). Although CO₂ concentrations between 20 and 60 mg L⁻¹ are not lethal, the pH hemolymph decreases, causing negative effects on shrimp metabolism (Furtado et al., 2016; Furtado et al., 2017), including transcriptional response of genes related to shrimp immunity (Zhou et al., 2010; Johnson et al., 2015). During this study, a significant decrease in water pH was observed in the high CO₂ treatment compared with the control treatment. Therefore, we hypothesized that a high CO₂ concentration between non-lethal levels could influence expression of genes related to oxygen transportation and hemolytic activity, melanization, pathogen recognition, antioxidant defense, and stress response of *L. vannamei* cultured in RAS.

Shrimp, as all organisms, regulate their physiological activity by modulating the transcriptional response of their genes for homeostasis maintenance (Fierro-Coronado et al., 2019). The hemocyanin function is related to oxygen transportation and non-specific innate immune defense (Zhang et al., 2009; Li et al., 2017). A 24-h short-term study with shrimp under hypercapnic hypoxia reported a decrease in hemocyanin gene expression due to a global reduction in shrimp protein synthesis (Johnson et al., 2015). Our results showed an upregulation of Hc gene expression at 6 h, but the gene expression significantly decreased over time suggesting an effect on oxygen transportation. Prophenoloxidase participates in melanization, and its activation promotes phagocytosis, encapsulation, and nodule formation for the protection against invading pathogenic microorganisms (Vazquez et al., 2009). Two long-term studies (1 to 14 days) with brine shrimp (*Artemia sinica*) evidenced an increase in proPO gene expression during the seventh day post water acidification (Zheng et al., 2015; Chang et al., 2016). Although ours was a short-time study, in which proPO gene expression increased at 6 and 12 h but decreased at 24 h, we agree that non-lethal high CO₂ can affect pathogen recognition via proPO-activating system.

The specific defense mechanisms against bacteria, fungi, and viruses are activated by pattern recognition proteins such as lipopolysaccharide- and β -glucan-binding protein, which help in bacterial agglutination and removal by phagocytosis (Aguirre-Guzman et al., 2009). Gene expression of Gram-negative bacteria-binding protein, a pattern recognition protein similar to LGBP, was enhanced when brine shrimp was exposed to high CO₂ concentration (Zheng et al., 2015). In this study, the transcriptional response of LGBP gene significantly increased during the trial.

These results suggest that genes encoding for pattern recognition proteins are biologically responsive to water acidification; therefore, the bacterial recognition and removal could be affected by acidification stress.

Superoxide dismutase (SOD) is the main antioxidant defense pathway in response to oxidative stress caused by reactive oxygen species (ROS) (Campa-Córdova et al., 2002). Pacific oyster (*Crassostrea gigas*) under high CO₂ showed varied SOD gene expression, down- and up-regulation (Wang et al., 2016). Our data indicated that cytMnSOD gene expression decreased at 12 and 18 h but increased at 24 h, suggesting that water acidification can impair ROS metabolism, causing damage to proteins, lipids, and DNA by oxidative stress.

Heat shock proteins (HSP) play an important role in protecting organisms from almost any sudden change in the cellular environment that induces protein damage (Li, 2017), and their expression can take more time (>4 h) to reach the high expression levels under stress factors (Dennis III et al., 2015). Green crab (*Carcinus maenas*) under high CO₂ concentrations during more than seven days altered its HSP gene expression (Fehsenfeld et al., 2011). The transcriptional response data of the HSP60 gene indicated a downregulation during this study, but the bioassay duration could be a factor for not reaching the maximum expression levels. However, the protein function can be detrimental under high CO₂ concentration.

Our study demonstrated how non-lethal high CO₂ level influenced the transcriptional response of immune-related genes of *L. vannamei*. The gene expression modulation by water acidification promotes metabolic suppression, reduced protein synthesis and respiratory stress, and reduced metabolic scope, causing pathogen invasion, disease transmission, and host susceptibility (Chang et al., 2016; Wang et al., 2016). On the other hand, the energy destined to transcriptional response modulation can reduce shrimp muscular growth and productive performance (Silveira et al., 2018). Therefore, further long-term studies are necessary to determine how non-lethal high CO₂ concentrations influence growth, tissue histology, nutrient absorption, and physiologic response of shrimp cultured in RAS.

5. Conclusions

The transcriptional response of immune-related genes of *L. vannamei* cultured in recirculating aquaculture systems was affected by high CO₂.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: R. Casillas-Hernández, K.J. Arévalo-Sainz, M.B. Flores-Pérez and J.R. Gonzalez-Galaviz. Data curation: R. Casillas-Hernández, K.J. Arévalo-Sainz, M.B. Flores-Pérez and J.R. Gonzalez-Galaviz. Formal analysis: R. Casillas-Hernández, K.J. Arévalo-Sainz, M.B. Flores-Pérez and J.R. Gonzalez-Galaviz. Funding acquisition: J.R. Gonzalez-Galaviz. Investigation: R. Casillas-Hernández, F. Lares-Villa, R.A. Bórquez-López and J.C. Gil-Núñez. Methodology: K.J. Arévalo-Sainz, M.B. Flores-Pérez and J.G. Garcia-Clark. Project administration: J.R. Gonzalez-Galaviz. Resources: R. Casillas-Hernández and J.R. Gonzalez-Galaviz. Supervision: J.R. Gonzalez-Galaviz. Validation: L.Z. Rodriguez-Anaya and J.R. Gonzalez-Galaviz. Visualization: R. Casillas-Hernández, K.J. Arévalo-Sainz and M.B. Flores-Pérez. Writing-original draft: R. Casillas-Hernández, K.J. Arévalo-Sainz, M.B. Flores-Pérez and J.R. Gonzalez-Galaviz. Writing-review & editing: J.R. Gonzalez-Galaviz.

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