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"El saber de mis hijos hará mi grandeza"

Dose-dependent effect of recombinant CHH-B1 on osmoregulatory capacity and Na^+/K^+-ATPase expression in bilaterally eyestalk-ablated shrimp *Litopenaeus vannamei*

Efecto dosis-dependiente de la CHH-B1 recombinante sobre la capacidad osmorreguladora y la expresión de la Na^+/K^+-ATPasa en camarones *Litopenaeus vannamei* sometidos a ablacion bilateral del pedúnculo ocular

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ABSTRACT

Osmoregulation in crustaceans is under neuroendocrine control by the crustacean hyperglycemic hormones (CHHs), which modify water and ion concentrations in diverse species. Previous studies suggested that CHH variant B1 (CHH-B1) has effects on the osmoregulatory responses of the Pacific white shrimp *Litopenaeus vannamei*. For a better understanding of the molecular action mechanisms of CHH-B1 in osmoregulation, in this work, a biological assay was done by injecting different dosages (5, 10, 50, 100, 250, 500, and 1000 pmol) of recombinant CHH-B1 (rCHH-B1) into bilaterally eyestalk-ablated shrimp under hyper-osmotic conditions. The gene expression of Na^+/K^+-ATPase (NKA) was quantified by RT-qPCR in gills of shrimp injected with rCHH-B1. In addition, osmoregulatory capacity (OC) of shrimp was estimated as the difference between the osmotic pressure (OP) of the external medium and that of hemolymph. The results indicate that CHH-B1 regulates the OC of shrimp during hypo-regulation by modulating Na^+/K^+-ATPase at transcriptional level in a dose-dependent way. Our results suggest that CHH has direct participation in the control of osmo-ionic regulation mechanisms, not only in *L. vannamei* but in crustaceans in general.

Keywords: *Litopenaeus vannamei,* shrimp, crustacean hyperglycemic hormone, osmoregulatory capacity, Na^+/K^+-ATPase

RESUMEN

La osmorregulación en crustáceos está bajo control neuroendócrino por las hormonas hiperglucémicas de crustáceos (CHHs), las cuales han mostrado modificar las concentraciones de agua e iones en diversas especies. Estudios previos sugieren que la CHH variante B1 (CHH-B1) tiene efectos en las respuestas osmorreguladoras del camarón blanco *Litopenaeus vannamei*. Para una mejor compresión sobre los mecanismos de acción moleculares de CHH-B1 en osmoregulación, en este trabajo, se realizó un ensayo biológico inyectando diferentes dosis (5, 10, 50, 100, 250, 500 y 1000 pmol) de CHH-B1 recombinante (rCHH-B1) a camarones bilateralmente ablacionados del pedúnculo ocular bajo condiciones hiper-osmóticas. La expresión génica de la Na^+/K^+-ATPasa se cuantificó por RT-qPCR en branquias de camarones inyectados con rCHH-B1. Además, se estimó la capacidad osmorreguladora (CO) de los camarones como la diferencia entre la presión osmótica (PO) del medio externo y la de la hemolinfa. Los resultados indican que CHH-B1 regula la CO de camarones durante la hipo-regulación mediante la modulación de la NKA a nivel transcripcional de manera dosis-dependiente. Nuestros resultados sugieren que CHH tiene una participación directa en el control de los mecanismos de regulación osmo-iónica, no solo en *L. vannamei* sino en crustáceos en general.

Palabras claves: *Litopenaeus vannamei*, camarón, hormona hiperglucémica de crustáceos, capacidad osmoreguladora, Na^+/K^+-ATPasa

INTRODUCTION

The white shrimp (*Litopenaeus vannamei*) is a commercial penaeid species with a distribution range throughout the Pacific coast of Sonora, Mexico, and the north of Peru (Holthius, 1980), which tolerates a wide range of environmental salinities (~1-40 ppt). At salinities under iso-osmotic point (~26 ppt), penaeid shrimp hyper-regulate solute concentrations in hemolymph, whereas at high salinities they behave as hypo-regulators (Díaz *et al*., 2001; Chong-Robles *et al*., 2014).

Osmoregulation in crustaceans involves the adjustment of the osmotic pressure (OP) of the intra- and extracellular fluids (e.g., hemolymph) (Péqueux 1995; Charmantier *et al*., 2009). In euryhaline species, such as *L. vannamei*, the extracellular regulation comprises changes in active ion transport, urine production, and permeability of the body surface to water and salts (Henry *et al*., 2012). The osmoregulation is subjected to a neuroendocrine control. The X-organ/sinus gland complex (XO/SG) located in eyestalks appears essential for the regulation of osmoregulatory mechanisms (Kamemoto, 1976; Charmantier *et al*., 1984; Mantel, 1985). The crustacean hyperglycemic hormones (CHHs) are the most abundant neuropeptides produced in the XO/SG complex (Webster *et al*., 2012). Classic gland ablation experiments in lobsters and crayfish have shown that eyestalk removal alters water and ion concentrations in crustaceans, an effect that can be restored by the injection of CHHs purified from SG tissue (Charmantier-Daures *et al*., 1994; Serrano *et al*., 2003).

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Moreover, chromatographic fractions containing CHH raised Na^+ influx in isolated gills from crabs (Spanings-Pierrot, 2000). This evidence suggests that CHHs may have a prime role in controlling the osmoregulatory processes in decapod crustaceans.

In *L. vannamei*, the cDNAs encoding different CHH variants isolated from the eyestalks were cloned and sequenced (Lago-Lestón *et al*., 2007; Ventura-López *et al*., 2016). The variants named CHH-B1 and CHH-B2 are originated from the same gene through alternative splicing events (Lago-Lestón *et al*., 2007). Notably, the expression of CHH variant B1 (CHH-B1) is strongly influenced by environmental salinity and temperature (Lago-Lestón *et al*., 2007). Moreover, the ability of CHH-B1 peptide to elicit hyperglycemia and hyperlipidemia in shrimp hemolymph has been proven by the injection of recombinant peptides (Sánchez-Castrejón *et al*., 2008; Camacho-Jiménez *et al*., 2015, Montiel-Arzate *et al*., 2020). Administration of recombinant CHH-B1 also has demonstrated to restore the osmoregulatory capacity (OC) of *L. vannamei* acclimated to hyper-osmotic salinity (Camacho-Jiménez *et al*., 2017a), suggesting that this variant participates in the response to salinity stress and the hydromineral balance in white shrimp.

The Na^+/K^+-ATPase (NKA) pump is a main driving force for ion transport in the cells of aquatic organisms, including crustaceans (Lucu and Towle, 2003). In *L. vannamei*, NKA α-subunit mRNA expression in posterior gills responds to changes in salinity conditions, suggesting its importance for shrimp survival to osmotic stress (Sun *et al*., 2011). Moreover, we have recently demonstrated that NKA mRNA expression in posterior gills of intact *L. vannamei* shrimp maintained at iso-osmotic salinity (26 ppt) was significantly up-regulated 3 h post-injection of rCHH-B1. However, the acute transference of shrimp from 26 ppt to hypo-osmotic salinity (8 ppt) showed a significant decrease of the NKA expression 1 h post-injection of rCHH-B1. In contrast, the NKA transcripts were significantly up-regulated 1 h post-injection of rCHH-B1 in animals acutely exposed to hyper-osmotic conditions (45 ppt). These results suggested that CHH-B1 could be directly involved in regulating ion transport mechanisms (Camacho-Jiménez *et al*., 2018).

Due to the importance of this molecular mechanism for the osmo-ionic regulation, we examined *in vivo* the dose-dependent effects of rCHH-B1 on the hemolymph OC and its relation with the mRNA expression of the NKA catalytic α-subunit in gills of bilaterally eyestalk-ablated shrimp acclimated to hyper-osmotic salinity.

MATERIAL AND METHODS

Animals

L. vannamei post-larvae (PL) were brought to CICESE's Marine Biotechnology wet laboratory from a shrimp farm located in La Paz, Mexico. The PL were grown to sub-adults in 2000 L reservoirs filled with seawater (35 ± 1 ppt, 28 ± 1°C) under constant aeration. Sub-adult shrimp were individually placed within 3.5 L containers inside 200 L reservoirs with recirculated seawater (35 ppt, 26°C) with aeration. Shrimp were fed once a day with commercial pelleted feed, and remanent food and feces were siphoned from the containers. Animals were maintained under these conditions for 10 days until the assays.

Expression and purification of rCHH-B1

Recombinant CHH-B1 (rCHH-B1) was expressed in the methylotrophic yeast *Pichia pastoris* and purified by reversed-phase high-performance liquid chromatography (RP-HPLC) as previously reported, with minor modifications (Camacho-Jiménez *et al*., 2015). Briefly, *P. pastoris* (strain X-33) with the pPicZαA-CHH-B1a plasmid for CHH-B1 expression integrated into its chromosomes was cultured in YPD medium for 18 h (30 °C and 200 rpm). The YPD culture (0.5 mL) was used to inoculate BMGY medium (500 mL), which was maintained until an OD_{600}= 4. The BMGY culture was centrifuged (2,500 x *g*, 5 min), and cells were in BMMY medium (100 mL) supplemented with 2% methanol as an inducer for recombinant protein expression. The induction was maintained for 24 h (30 °C, 200 rpm) with methanol supplementation every 12 h. After induction, culture supernatant was collected by centrifugation (2,500 x *g*, 5 min). Proteins from the supernatant were precipitated with 50% ammonium sulfate, and the concentrate was dialyzed with phosphate-buffered saline (PBS) 1X. The recombinant protein was separated from other dialyzed proteins by RP-HPLC using a C18 column (TSKgel® Octadecyl-4PW 4.6 mm × 150 mm, Tosoh, Tokyo, Japan) and a gradient of acetonitrile (0–55%) with 0.1% TFA. The recovery of recombinant protein was confirmed through Westen blot immunodetection using an anti-CHH-B1/B2 antibody. Proteins recovered after RP-HPLC were separated by Tricine-SDS-PAGE (12.5%), transferred to a nitrocellulose membrane (0.45 µm) (Bio-Rad, San Diego, CA, USA), and then incubated with the rabbit anti-CHH-B1/B2 polyclonal antibody (1:500) (GenScript, Piscataway, NJ, USA) followed by incubation with a peroxidase-conjugated goat anti-rabbit IgG antibody (Sigma-Aldrich, Saint Louis, MO, USA) (1:5000). Visualization of positive bands was done with the 1-Step TMB Blotting reagent (Pierce, Rockford, IL, USA). The BCA Protein Assay Kit (Pierce) was used to determine the concentration of rCHH-B1. The recombinant protein was stored at -80°C until dose-response experiment.

Biological assay and sample collection

A dose-response *in vivo* assay was done as described by Camacho-Jiménez *et al*. (2015) using bilaterally eyestalk-ablated shrimp . This experiment had the aprovation from CICESE's ethical committee. Fifty-four sub-adult shrimp (12.89 ± 2.41 g) were transferred to individual containers (3.5 L) inside tanks (200 L) filled with seawater (35 ± 1 ppt, 26 ± 1 °C) under constant aeration. Daily, animals were fed with shrimp pelleted diet (4% of their wet weight), debris and feces were siphoned from containers, and seawater was totally exchanged. The intermolt stage was calculated as half of the time between two consecutive molting events indicated by

exoskeleton deposition in the containers. During the first counted intermolt, one eyestalk was extirpated from each shrimp by cutting and cauterization, whereas the remaining eyestalk was removed at the second registered intermolt stage. Some of the removed eyestalks were collected for sinus gland (SG) extract preparation. SG were dissected from eyestalks, frozen (-80 °C) and homogenized in cold PBS 1X with a pestle. The homogenized sample was centrifuged (10,500 x *g*, 4 °C, 15 min), and the collected supernatant was lyophilized and resuspended in 50 µL of PBS 1X every 2 glands. The SG extract was stored at -80 °C before activity assay.

Bilaterally eyestalk-ablated shrimp in intermolt were fasted for 24 h before the experiment. To establish the dose-dependent effect of rCHH-B1 on osmoregulation, various doses of rCHH-B1 peptide (5, 10, 50, 100, 250, 500, and 1000 pmol) were diluted in 50 µL of PBS 1X and injected into shrimp through the arthrodial membrane with a 1 mL sterile syringe (31 G). As a negative control, a group of animals was injected with 50 µL of PBS 1X. The positive control group consisted of shrimp injected with a pair of SG extracted in PBS (50 µL). One hour after injection, six shrimp from each dosage treatment and control (n= 6) were sampled. Hemolymph was collected from shrimp with a sterile syringe (1 mL, 27 G) and immediately placed on ice before analysis. After that, posterior gills (~50 mg) were dissected from each animal and immersed in RNA stabilizing solution (25 mM sodium citrate, 10 mM EDTA, 70% $(NH_4)_2SO4$ (w/v), pH 5.2). Tissue samples were stored at -80°C before RNA isolation.

Osmoregulatory capacity (OC)

The osmotic pressure (OP) was measured from hemolymph samples with a vapor pressure osmometer (VAPRO 5520, Wescor, South Logan, UT, USA). The OP of the external medium was also determined from seawater in the experimental reservoirs. The osmoregulatory capacity (OC) was calculated by subtracting OP of the external medium from that of hemolymph. Because penaeid shrimp are hyper-regulators of hemolymph OP under hyper-osmotic conditions of salinity, the OC data was expressed as hypo-OC (Lignot *et al*., 1997).

***NKA* mRNA quantification by RT-qPCR**

The effect of rCHH-B1 on *NKA* mRNA expression in *L. vannamei* gills was measured by real-time quantitative PCR (RT-qPCR) according to Camacho-Jiménez *et al*. (2018). Gill samples of shrimp from each treatment and control (n= 6) were pooled in groups of two organisms (n= 3) to add ~50 mg of tissue (same amount per shrimp). Total RNA was isolated from gills tissue samples and treated with DNAse I with the Direct-zol™ RNA MiniPrep kit (Zymo Research, Irvine, CA, USA). Total RNA (~1 µg) was reverse-transcribed for cDNA synthesis with SuperScript® III Reverse Transcriptase and oligo $(dT)_{20}$ primer (Invitrogen, Life Technologies, Carlsbad, CA, USA). A specific fragment for *NKA* catalytic α-subunit (122 bp) was amplified by RT-qPCR in a StepOnePlusTM Real-Time PCR System (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). The reaction mix (10 µL) included 5 µL of Power SYBR® Green PCR Mix (Applied Biosystems, Life Technologies), cDNA (25 ng), 0.1 µM of LvαATP_F forward primer (5'-AGCAAGGCCATCAACGATCT-3') and 0.1 µM of LvαATP_R reverse primer (5'-GCCCACTGCACAATCACAAT-3') (Li *et al*., 2009). Cycling conditions were as follows: an initial denaturation cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 20 s and 60 °C for 1 min. Each cDNA sample was analyzed in triplicates. In each run, a non-template control (negative control) was included in triplicates. After the last extension step, a melting curve was run from 60 to 95 °C with an increase of 0.3 °C each 15 s to validate the amplification specificity. The absolute copy number of *NKA* transcripts in samples was assessed by interpolating the quantification cycle (Cq) values of each sample to a standard curve ($R^2 \geq 0.99$) constructed with serial dilutions (10^7-10^2 copies) of a pCR™ 2.1-TOPO® plasmid (Life Technologies) carrying the 122 bp *NKA* fragment (Camacho-Jiménez *et al*., 2018). The copy number of *NKA* transcripts in each sample was reported per ng of cDNA. The efficiency of primers was calculated using raw fluorescence data from the standard curve with LinRegPCR Software 2016.0 (Ruijter *et al*., 2009), which was 91.6%.

Statistical analyses

Data were analyzed for normality and homoscedasticity by Shapiro-Wilk and Brown-Forsythe tests, respectively. To determine statistical differences in OC between PBS control and neuropeptide doses, one-way ANOVA with Fisher's LSD test was performed. Because *NKA* expression data did not satisfy normality and homoscedasticity criteria, a Kruskal-Wallis test with Dunn's *post hoc* test was performed to find differences between hormone treatments and negative control. All the statistical analyzes were done with SigmaPlot 14.0 (Systat Software) with a significance level settled at $p < 0.05$. The data were plotted as mean ± standard deviation.

RESULTS

Expression and purification of rCHH-B1

The expression and purification of the native recombinant protein were corroborated by Western blot analysis (Fig. 1). The results showed rCHH-B1 as a ~10 kDa band, which is close to the predicted mass of 8.8 kDa. This band was identified as rCHH-B1 by N-terminal sequencing in a previous study (Camacho-Jiménez *et al*., 2015). No additional bands were detected during the analyses, confirming the elimination of contaminating proteins.

Effect of rCHH-B1 dosage on the hypo-OC of eyestalk ablated shrimp

The effect of the purified rCHH-B1 on the osmoregulatory capacity was evaluated in bilaterally-eyestalk ablated shrimp acclimated to 35 ppt salinity by a dose-response *in vivo* assay. Compared to PBS control (-287.27 ± 13.35 mmol kg^{-1}), rCHH-B1 increased the hypo-OC of the shrimp (Fig. 2) due to a decrease in the osmotic pressure of the hemo-

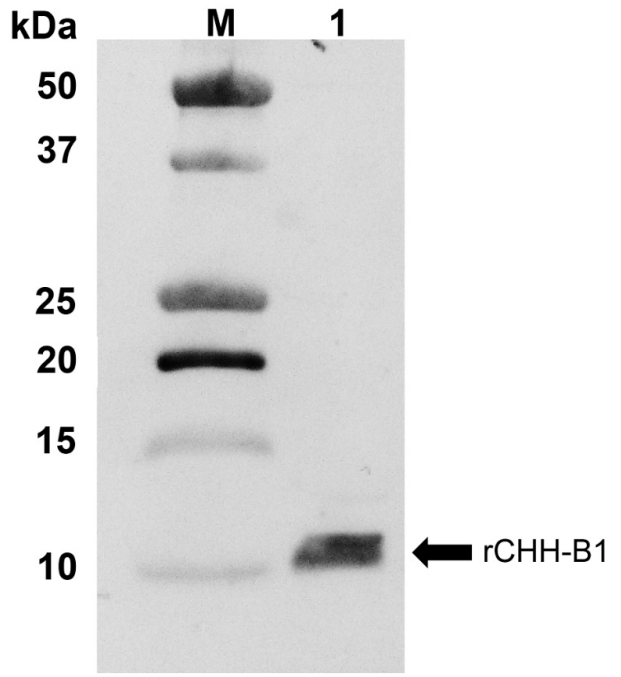

Figure 1. Immunodetection of rCHH-B1 by Western blot. M, molecular weight marker Precision Plus Protein All Blue (Bio-Rad); 1, recombinant protein purified by RP-HPLC. The arrow indicates the band corresponding to rCHH-B1.
Figura 1. Análisis por Western blot de rCHH-B1. M, marcador de pesos moleculares Precision Plus Protein All Blue (Bio-Rad); 1, proteína recombinante purificada por RP-HPLC. La flecha indica la banda correspondiente a rCHH-B1.

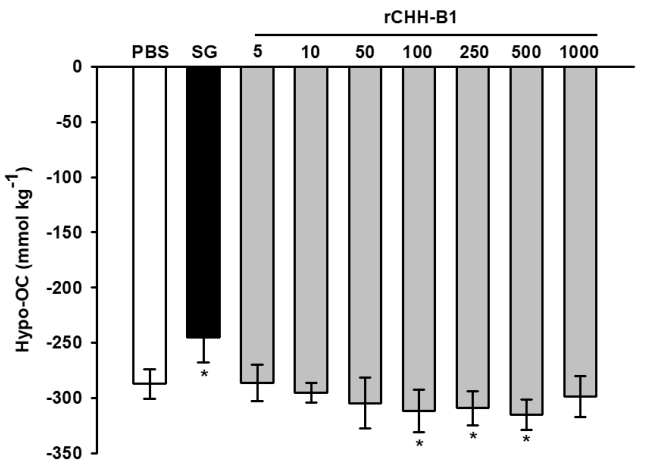

Figure 2. Dose-response effect of rCHH-B1 on the hypo-OC of *L. vannamei*. PBS, negative control; SG, sinus gland extract; rCHH-B1, doses of rCHH-B1 (5-1000 pmol). Data are expressed as mean ± standard deviation (n= 6, per dosage or control). Asterisks (*) indicate significant differences with respect to PBS control ($p < 0.05$).
Figura 2. Efecto dosis-respuesta de rCHH-B1 sobre la hipo-CO de *L. vannamei*. PBS, control negativo; SG, extracto de glándula del seno; rCHH-B1, dosis de rCHH-B1 (5-1000 pmol). Los datos se expresan como media ± desviación estandar (n = 6, por dosis o control). Los asteriscos (*) indican las diferencias con respecto al control PBS ($p < 0.05$).

lymph. The effect was significant ($p < 0.05$) starting from the 100 pmol dose (-311.78 ± 19.15 mmol kg^{-1}) and showed no differences ($p > 0.05$) with the effect of higher doses of hormone (250-500 pmol). The shrimp injected with SG showed a significant decrease ($p < 0.05$) in the hypo-OC (-245.00 ± 22.63 mmol kg^{-1}) with respect to PBS due to an increase of the hemolymph OP.

Effect of rCHH-B1 dosage on *NKA* expression

The expression of *NKA* in the posterior gills decreased in response to rCHH-B1 injection in a dose-dependent manner (Fig. 3). The reduction in *NKA* mRNAs was significant ($p < 0.05$) with respect to the PBS control (15.76 ± 4.39 $x10^3$ copies of *NKA* transcript per ng of cDNA) starting from the 50 pmol dose (9.51 ± 2.88 $x10^3$ copies of *NKA* transcript per ng of cDNA), which was lower than the effective dose found for the OC. The expression reached minimum values ($p < 0.05$) by injecting 1000 pmol of rCHH-B1 (3.68 ± 0.81 $x10^3$ copies of *NKA* transcript per ng of cDNA). Conversely, the SG extract increased the *NKA* transcripts (41.95 ± 0.92 $x10^3$ copies of *NKA* transcript per ng of cDNA) in comparison to the PBS control ($p < 0.05$).

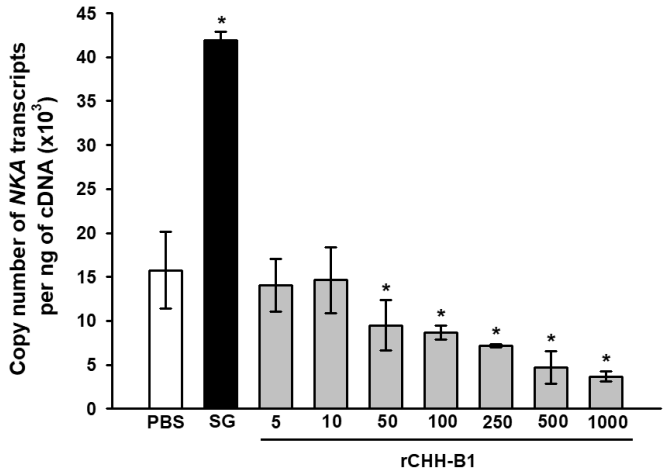

Figure 3. Dose-response effect of rCHH-B1 on *NKA* expression in posterior gills of *L. vannamei*. PBS, negative control; SG, sinus gland extract; rCHH-B1, doses of rCHH-B1 (5-1000 pmol). Data are expressed as mean ± standard deviation (n= 3, per dosage or control). Asterisks (*) indicate significant differences with respect to PBS control ($p < 0.05$).
Figura 3. Efecto dosis-respuesta de rCHH-B1 sobre la expresión de *NKA* en branquias posteriores de *L. vannamei*. PBS, control negativo; SG, extracto de glándula del seno; rCHH-B1, dosis de rCHH-B1 (5-1000 pmol). Los datos se expresan como media ± desviación estandar (n = 3, por dosis o control). Los asteriscos (*) indican las diferencias con respecto al control PBS ($p < 0.05$).

DISCUSSION

The CHHs are neuropeptides of crustaceans with a well-established role in carbohydrate metabolism (Fanjul-Moles, 2006). Experiments injecting rCHH-B1 into eyestalk-ablated *L. vannamei* have demonstrated its participation in controlling the hemolymph glucose levels (Camacho-Jiménez *et al*., 2015). The CHH-mediated hyperglycemia has been proposed as an adaptive response to cope with increases in the energy needs of tissues during stressful situations

(Chang, 2005). Nonetheless, the CHHs are recognized as pleiotropic hormones with multiple functions proposed in the physiology of crustaceans, including osmo-ionic regulation (Chung *et al*., 2010; Webster *et al*., 2012). Interestingly, CHH-B1 mRNA levels were up-regulated in *L. vannamei* eyestalks during exposure to extreme salinities, pointing to a role in osmoregulation under osmotic stress (Lago-Lestón *et al*., 2007). Additionally, the relative expression of the *chh* transcripts showed to be sensitive to salinity, being higher at salinities far from iso-osmotic point (26 ppt). Recent studies have shown that rCHH-B1 injection into *L. vannamei* shrimp acutely exposed to iso-osmotic (26 ppt) and hyper-osmotic salinity (45 ppt) increased *NKA* α-subunit mRNA expression in posterior gills (Camacho-Jiménez *et al*., 2018). However, since this study was done with non-ablated shrimp, the effect of other endocrine molecules present in the eyestalks could not be ruled out. Diverse biogenic amines that are produced in the eyestalks may affect metabolism and osmoregulation in a way that can be or not dependant on CHH action (Liu *et al*., 2008; 2009; Lorenzon *et al*., 2005). Moreover, according to experiments in *Penaeus monodon*, other eyestalk peptides, like the red concentrating hormone, can elicit changes in NKA activity in gills, suggesting that it may exert an overlapping endocrine function to CHHs in osmoregulation (Sathapondecha *et al*. 2014). Eyestalk ablation is used to eliminate the primary source of neuropeptides in crustaceans to test their individual effects. Although this surgical procedure causes impairments in metabolism and osmoregulation by itself (Charmantier-Daures *et al*., 1994; Sainz-Hernández *et al*., 2008), it is still a classic approach for the functional characterization of CHHs, as it completely eliminates XO-SG, which is the main tissue secreting CHH (Chang *et al*., 2010; Liu *et al*., 2014; Mosco *et al*., 2015).

In this study, the experiments were performed with bilaterally eyestalk-ablated sub-adult shrimp acclimated to hyper-osmotic conditions (35 ppt), in which organisms are hypo-osmotic with respect to the external medium. Interestingly, rCHH-B1 treatment increased the hypo-OC of eyestalk-ablated shrimp after 1 h within a dose range of 100-500 pmol due to a reduction in the hemolymph OP. These results agree with a previous study in which the injection of 226 pmol (2 µg) of rCHH-B1 increased hypo-OC of non-ablated shrimp after 1 h (Camacho *et al*., 2018). Thus, the evidence herein confirms that CHH-B1 acts on the osmoregulatory performance of shrimp during hypo-osmoregulation at high salinities besides its metabolic effects. Considering that in *L. vannamei,* the Na^+ and Cl^- ions comprise ~80% of the hemolymph OP at 35 ppt (Castille and Lawrence, 1981), the effect of rCHH-B1 on the hypo-OC could be related to changes in their concentrations, in part by modifying branchial ion transport. The rCHH-B1 peptide suppressed *NKA* expression in gills of eyestalk-ablated shrimp in a dose-dependent way, starting at a dosage that is a half lower than the effective dosage for the OC (100 pmol). These results indicate that CHH-B1 might regulate the OC of shrimp during hypo-regulation by modulating NKA activity. NKA modulation could occur through a signal transduction pathway that triggers changes at a transcriptional level.

In euryhaline hyper-osmoregulator crustaceans, the catalytic α-subunit of NKA located in the basolateral membrane of epithelial cells from gills provides the primary driving force for the uptake of Na^+ from diluted media by exchanging K^+ (Lucu and Towle, 2003). The NKA mRNA expression was stimulated in *L. vannamei* gills in response to salinity stress (Sun *et al*., 2011; Wang et al., 2012). A high NKA gene expression level has also been observed in the gill tissue of black tiger shrimp (*P. monodon*) exposed to high salinity conditions (55 ppt) (Shekhar *et al*., 2014). Additionally, a transient induction of the mRNA levels for the α-subunit of NKA has been reported during acclimation of the euryhaline blue crabs *Callinectes sapidus* from 35 ppt to 10 ppt salinity (Lovett *et al*., 2006). *NKA* expression in the euryhaline crab *Eriocheir sinensis* gills showed adaptive up-regulated expression response to the salinity changes (Zhang *et al*., 2018). In contrast, *NKA* expression in the *E. sinensis* gills was significantly downregulated in organisms acclimated to seawater (25 ppt) compared with the freshwater group (Yang *et al*., 2019). Nonetheless, in animals capable of hypo-osmoregulation, like *L. vannamei*, branchial NKA could also drive the movement of ions through the $Na^+/K^+/2Cl^-$ cotransporter and other transport proteins in cell membranes, as has been suggested in some marine teleosts (Evans *et al*., 2005). These results suggest the importance of tight regulation of NKA activity for the survival of crustacea to osmotic stress caused by both high and low salinities. The effect of rCHH-B1 on *NKA* expression found in this work, in which organisms were acclimated at 35 ppt, contrast with those reported in a previous study in which a transitory up-regulation in transcript copy number occurred in shrimp treated with rCHH-B1 (226 pmol) and acutely transferred to hyper-osmotic (45 ppt) conditions (Camacho-Jiménez *et al*., 2018). Since the optimum salinity range for *L. vannamei* growth is 33-40 ppt and survival is compromised at salinities above 40 ppt (Ponce-Palafox *et al*., 1997), the level of stress experienced by animals at 35 ppt probably was not the same as at 45 ppt. In agreement, shrimp acclimated to iso-osmotic salinity (26 ppt) and injected with rCHH-B1 or rCHH-B2, displayed a weaker effect on *NKA* induction than shrimp acutely transferred to 45 ppt. Moreover, the transference of animals injected with the recombinant peptides to low salinity (8 ppt) for 1 h did not induced *NKA* expression. However, the authors also reported a less-magnitude increase in shrimp under the same salinity conditions that not received hormone injection, which suggests that other endocrine factors involved in osmoregulation could be acting under salinity stress (Camacho-Jiménez *et al*., 2018).

Results presented in this study showed that eyestalk-ablated shrimp injected with the SG extract had an strong and opposite response to that of rCHH-B1 treatments. SG extract caused a reduction in hypo-OC (increase in OP) and an increase in *NKA* transcripts, suggesting an increase in ion uptake dependant on NKA activity. The SG extract contains a mixture of endocrine molecules (i.e., biogenic amines and

neuropeptides) that potentially have different effects on the osmo-ionic regulation of *L. vannamei* (Liu *et al*., 2008; 2009). Thus, differences among intact and eyestalk-ablated animals can be expected, as well as between the effects of SG extract and single CHH neuropeptides. In this sense, recent studies revealed that the rCHH-B2 variant decrease the hypo-OC in the hemolymph of bilaterally eyestalk-ablated *L. vannamei* exposed to 35 ppt (Camacho-Jiménez *et al*., 2017b). CHH-B2 has also been shown to increase the hemolymph ion concentrations and *NKA* expression in gills of non-ablated shrimp transferred to high salinity (45 ppt) (Camacho-Jiménez *et al*., 2018). Liu *et al*. (2014) reported that recombinant CHH peptide of *L. vannamei* (rLvCHH) increased the NKA activity in intact shrimp acclimated to 31 ppt.

Interestingly, CHH variants of *L. vannamei* are differentially distributed among tissues. LvCHH peptide is highly expressed in eyestalks, heart, nervous systems, muscle, and hepatopancreas (Liu *et al*., 2014), while CHH-B2 has been only detected in eyestalks (Lago-Lestón *et al*., 2007). Moreover, *L. vannamei* ion transport peptide (LvITP), a peptide with high sequence identity with CHH-B1, is expressed in gills, suggesting an osmoregulatory role in this tissue (Tiu *et al*., 2007). These dissimilarities between tissue distribution patterns of CHHs have been related to their structural variability, as well as to their functional diversity (Liu *et al*., 2015).

The involvement of CHHs in osmotic adaptation has been mostly studied in crustaceans upon transfer to hypo-osmotic environments. In lobster and crayfish species, the injection of purified CHH reverted the reduction in the ability to regulate the internal OP and/or Na^+ in eyestalk ablated animals kept in diluted media (Charmantier-Daures *et al*., 1994; Serrano *et al*., 2003), probably by promoting changes in the transepithelial potential and Na^+ influx across the posterior gills, as has been shown in *Pachygrapsus marmoratus* (Spanings-Pierrot *et al*., 2000). In agreement with our findings, Spanings-Pierrot *et al*. (2000) suggested that CHH could indirectly modulate the NKA activity in gills by controlling the metabolic energy available for this mechanism. rCHH-B1 has been demonstrated to cause hyperglycemia in eyestalk-ablated shrimp at 35 ppt (Camacho-Jiménez *et al*., 2015). Moreover, rCHH-B1 has proven to elicit triglycerides and phospholipids mobilization into hemolymph, making lipids available for uptake by tissues (Montiel-Arzate *et al*., 2020). In this sense, unsaturated phospholipid content in posterior gills tissue has been positively related to the level of NKA activity (Chapelle and Zwingelstein, 1984). Thus, the metabolic effects of CHH-B1 in hemolymph and gill tissue can aid shrimp to osmoregulate in changing environmental conditions. Alternatively, CHH may be directly involved in the activation of signal transduction pathways that control NKA activity. Specific binding sites for CHH have been detected in the gills tissue from *Carcinus maenas* and *C. sapidus*, where the hormone rises the cGMP concentration (Chung and Webster 2006; Katayama and Chung, 2009). Moreover, it has been predicted that the NKA α-subunit of the euryhaline crab *P. marmoratus* has a cAMP- and cGMP-dependent protein kinase phosphorylation site, while in the promoter region of the α-subunit gene potential recognition sites have been located for activating transcription factor/cAMP response element-binding protein (ATF/CREB) (Jayasundara *et al*., 2007). This family of transcription factors is involved in the cellular stress response in mammalian cell lines (Fawcett *et al*., 1999). In this sense, the existence of a signal transduction pathway dependant on CHH and cyclic nucleotides (as second messengers) that controls NKA expression must be elucidated.

CONCLUSIONS

Based on our results, CHH-B1 is involved in the osmotic regulation of *L. vannamei*, which is correlated with the transcriptional regulation of NKA activity in gills. The rCHH-B1 peptide had a dose-dependent suppressive effect on *NKA* expression in gills of eyestalk-ablated shrimp acclimated to 35 ppt. Even though these results support a regulatory role for CHH in osmo-ionic regulation in crustaceans, they contrast with previous evidence on CHH-dependant stimulation of Na^+ uptake and NKA activity in gills (Spanings-Pierrot *et al*., 2000; Liu *et al*., 2014; Camacho-Jiménez *et al*., 2018). However, salinity conditions seem to have a significant influence on gene expression and activities of CHH variants of *L. vannamei* (Lago-Lestón *et al*., 2007; Camacho-Jiménez *et al*., 2018). Moreover, osmoregulation appears to be a highly complex physiological process involving diverse endocrine molecules with unknown interactions among each other (Charmantier *et al*., 2009). Further research is needed to clarify the signal transduction pathways and the effects of the different CHH peptides of *L. vannamei* under diverse environmental and physiological conditions. The study of their effects in response to stressors, such as salinity fluctuations, could be interesting for aquaculture production since they impact the growth, health, and even survival of shrimp.

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