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Research Article

Metabolic and osmoionic effects of the recombinant crustacean hyperglycemic hormone (rCHH-A) of the Pacific white shrimp *Penaeus vannamei* on specimens acutely exposed to extreme salinities

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ABSTRACT. The crustacean hyperglycemic hormone (CHH) is a multifunctional neuropeptide that plays a central role in crustacean metabolism and physiology. Experiments were conducted to examine the metabolic and osmoionic capabilities of the white shrimp *Penaeus vannamei* injected with its recombinant CHH-A (rCHH-A) peptide and acutely transferred from iso- (26 ppt) to hypo- (10 ppt) and hyper-osmotic (40 ppt) conditions. Hemolymph glucose, protein, osmoregulatory capacity (OC), and ionoregulatory capacity (IC) for sodium, chloride, potassium, and calcium were evaluated at four post-injection times (0.5, 1, 2, and 24 h). The rCHH-A peptide had hyperglycemic activity in all salinity conditions, obtaining maximum values at 1 h post-injection. However, in shrimp transferred to hyper-osmotic condition, rCHH-A caused the most significant reductions in OC (2 h), chloride IC (2 h), and total proteins (0.5-2 h) compared to the phosphate-buffered saline (PBS) control. Contrastingly, in shrimp transferred to hypo-osmotic conditions, rCHH-A decreased OC significantly from 2-24 h, strongly decreased chloride and potassium IC at 1 h post-injection, and increased total protein concentration in the hemolymph (1-2 h) when compared to PBS control. Concerning calcium, the rCHH-A injection decreased calcium IC at 10 ppt (1 h) and 26 ppt (2 h), providing insight into a potential role for CHH-A in calcium regulation. The results suggest that glucose and protein mobilization could enhance energy for osmo-ionic regulation under extreme osmotic conditions. This research study contributed to understanding crustacean endocrinology in *P. vannamei* and related euryhaline crustaceans. Further research should be performed to understand the osmo- and ionoregulatory mechanisms of the different CHH variants in crustaceans exposed to other stress conditions and the relationship with intermediary energy metabolism regulation.

Keywords: *Penaeus vannamei*; crustacean hyperglycemic hormone; glucose; osmoregulatory capacity; ion concentrations; recombinant peptide

INTRODUCTION

The Pacific white shrimp *Penaeus vannamei* is one of the most important euryhaline crustaceans and an economically important species with a complex migration pattern in its life cycle. The species inhabits estuaries as postlarvae and juvenile, while as an adult, it returns to the open sea for reproduction (Castille & Lawrence 1981a, Gong et al. 2004). *P. vannamei* behaves as a hyper-osmoregulator, actively uptaking salts from the surrounding water at salinities below the iso-osmotic point (~26 ppt) and as a hypo-osmoregulator excreting

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ions to the environment at high salinities (Castille & Lawrence 1981a, Díaz et al. 2001, Chong-Robles et al. 2014). Although this species can tolerate a wide range of environmental salinities (~1-40 ppt), at extreme salinities, the animals spend more energy to compensate for the cost of osmoregulation (Li et al. 2007). Despite the increasing importance of the shrimp aquaculture industry worldwide, the specific study of shrimp endocrinology has left behind the effort to study other crustacean species (Huberman 2000).

Osmoregulation entails the adjustment of the osmotic pressure (OP) of intra- and extracellular fluids (e.g. hemolymph) by regulating the concentrations of ions and other osmolytes with the external environment (Péqueux 1995). However, diverse factors, such as gender, body size, nutritional status, and developmental stage, can also directly or indirectly influence shrimp's ion and osmoregulatory capacity (Castille & Lawrence 1981b, Mena-Herrera et al. 2011, Li & Cheng 2012).

The two main mechanisms of performing osmoregulation in crustaceans are anisosmotic extracellular regulation (AER) by the gill and antennal glands to maintain body fluid osmolarity and intracellular isosmotic regulation (IIR) that controls osmolites in the tissues (Pequeux 1995, Shinji et al. 2012b). In decapod crustaceans, osmoregulatory mechanisms seem to be highly regulated by factors found in the eyestalks. Eyestalk ablation disrupts water content and ion concentrations in tissues and hemolymph, compromising the ability of crustaceans to compensate for salinity variations (Diwan & Laxminarayana 1989, Charmantier-Daures et al. 1994). Osmotic alterations caused by eyestalk removal can be reverted through tissue re-implantation or by injecting tissue extracts into affected crustaceans (Mantel 1985, Charmantier et al. 1988, Kamemoto 1991, Charmantier-Daures et al. 1994). The X-organ-sinus gland complex (XO-SG), located in eyestalks, is the main neuroendocrine organ in decapod crustaceans (Hopkins 2012). The CHH superfamily peptides -secreted from the XO-SG- include the molt-inhibiting hormone (MIH), gonad-inhibiting hormone (GIH), mandibular organ-inhibiting hormone (MOIH), and crustacean hyperglycemic hormone (CHH) (Chung et al. 2010, Chen et al. 2020). This CHH family includes two CHH orthologs, the insect ion transport peptide (ITP) and the ITP-like peptide (ITP-L), identified in insects. ITP peptide shares 40% sequence identity with CHHs. It is known to stimulate salt and water reabsorption by the hindgut of the ileal epithelium in locust *Schistocerca gregaria* (Audsley et al. 1992a,b, Gäde 2004). Precursors encoding peptides related to ITP and CHH have been identified in other insect orders (Webster et al. 2012, Zitnan & Adams, 2012). Despite the similarities between CHH and ITP, studies on ionoregulatory processes controlled by CHHs remain limited. CHH peptides are the most abundant neurohormones of the CHH family and have been identified from many crustacean species, but studies on their biological activities remain limited (Webster et al. 2012). These peptides are involved in different physiological processes, such as carbohydrate (Camacho-Jiménez et al. 2015) and lipid metabolism (Santos et al. 1997), molting (Chung & Webster 2005, Zarubin et al. 2009), reproduction (De Kleijn et al. 1998, Tsutsui et al. 2005), host immune response modulators (Wanlem et al. 2011, Wang et al. 2017). Recently, CHH has also been suggested to promote ammonia excretion by regulating ion channels, activating proteins, and exocytosis (Zhang et al. 2020).

Concerning osmoregulation, experiments with different crustaceans have revealed that purified or recombinant CHH can modify water and ion concentrations within organisms (Serrano et al. 2003, Prymaczok et al. 2016), probably by regulating ion transport in gills (Spanings-Pierrot et al. 2000). However, the relationship between CHH functioning and its effects on the main osmotic effectors in the hemolymph has not been entirely understood yet. Several CHH variants have been isolated from the eyestalks of *P. vannamei*. CHH-B1 and -B2 variants are formed by alternative splicing of the *chhB* gene. These variants have shown high *in vivo* hyperglycemic activity (Camacho-Jiménez et al. 2015, 2017a). The injection of these recombinant variants promoted changes in the osmo-ionic regulation in shrimp acutely exposed to different salinities (Camacho-Jiménez et al. 2018). The CHH-A variant instead is encoded by the *chhA* gene and consists of three exons and two introns, and a proteinase processing site (GK) in the 3'terminal region (Lago-Lestón et al. 2007, Sánchez-Castrejón et al. 2008). This variant has a high sequence identity (98.6%) to the mature LvCHH peptide (GenBank accession N°ADL27417) reported by Liu et al. (2014), which has been recombinantly expressed in *Escherichia coli* with a fusion tag at the N-terminus. The injection of rLvCHH into white shrimp increased the Na^+-K^+ ATPase (NKA) gene expression in shrimp gills, suggesting its participation in osmoregulation (Liu et al. 2014). Previously, our research group reported that CHH-A mRNA expression varies in shrimp acclimated to different temperature and salinity combinations within a range of 20-32°C and 10-40 ppt, respectively (Lago-Lestón et al. 2007). However, the

physiological and metabolic functions of the CHH-A variant have not been determined under salinity stress conditions yet. Therefore, this study reports the expression of an active recombinant *P. vannamei* CHH-A peptide with a free C-terminus (rCHH-A) in *Pichia pastoris*. The metabolic and osmoionic capabilities of euryhaline *P. vannamei* were also examined after rCHH-A injection and acute transfer of shrimp from iso-osmotic to hypo- and hyper-osmotic conditions.

MATERIALS AND METHODS

Protein expression and purification of *Penaeus vannamei* rCHH-A

The cDNA encoding the mature CHH-A peptide was cloned in the pPICZαA vector to express recombinant mature peptide (rCHH-A). Total RNA extracted from the eyestalks of juvenile *P. vannamei* shrimp was used to synthesize the first-strand cDNA as previously described (Camacho-Jimenez et al. 2015). The *chh-A* transcript (GenBank accession N°AY434016.1) was amplified by polymerase chain reaction (PCR) using the forward primer 5'-TCACCAAACGCTCGCTCTTC-3' and reverse primer 5'-TGCATGCCAGTGCTTTATTCG- 3' (Lago-Lestón et al. 2007). The reaction mixture (50 µL) included 1X Green GoTaq® Flexi Buffer (Promega, Madison, WI, USA), 3 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 M of each primer, 1.25 U of GoTaq® DNA polymerase, and 2.0 µL of cDNA. The cycling conditions were an initial denaturation step of 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 60°C for 45 s, and 72°C for 45 s, and a final extension step of 72°C for 5 min. The amplified PCR product (~600 bp) was purified with a PureLink™ PCR purification kit (Thermo Fisher Scientific, Carlsbad, CA, USA) and used as a template in a second PCR reaction. For cloning the mature *chh-A* transcript into the pPICZαA vector (Invitrogen, Life Technologies, Carlsbad, CA, USA), specific primers were designed based on the open reading frame sequence of Liv-CHH (Lago-Lestón et al. 2007) and the cloning sites of the expression vector. The forward primer 5'- CCGCTCGAGAAAAGATCGCTCTTCGACCCTTC- 3' includes a *Xho*I site (underlined) and deletes the *Ste*13 signal cleavage site (two E-A repeats) between the α-factor secretion signal sequence and the mature *chh-A* transcript (bold). The reverse primer 5'- GCTCTAGACTACCCGACCATCTGGACAGC- 3' includes a *Xba*I site (underlined) and a stop codon (bold). The codon for the last residue of the natural CHH-A peptide (lysine) was removed from the reverse primer so that the donor residue glycine (G) could be amidated *in vitro* if required in future experiments. The mature *chh-A* transcript was amplified by PCR (100 µL) using the previously purified cDNA PCR fragment (1.0 µL). PCR mix and settings were identical to those described above. The purified *chh-A* fragment and the pPICZαA vector were digested with *Xho*I and *Xba*I restriction enzymes (Promega, Madison, WI, USA) and ligated with T4 DNA ligase (New England BioLabs, Ipswich, MA, USA) according to the manufacturer's protocol. The ligation product was used to transform *Escherichia coli* DH5α cells by electroporation in a Bio-Rad (Richmond, CA, USA) Micropulser (2.5 kV, 1 pulse, ~5 ms). Transformant colonies were screened for zeocin (Thermo Fisher Scientific, Carlsbad, CA, USA) resistance and evaluated by PCR using 5'AOX1 and 3'AOX1 primers (Thermo Fisher Scientific, Carlsbad, CA, USA). The constructed vector was verified by sequencing (SeqxCel, San Diego, CA, USA). The pPICZαA-CHH-Ab vector was linearized with *Sac*I (Promega, Madison, WI, USA) and integrated into the *Pichia pastoris* X-33 genome by electroporation (2.0 kV, 1 pulse, ~5 ms) described in the manual (version G) of the EasySelect Pichia Expression Kit (Thermo Fisher Scientific, Carlsbad, CA, USA). The transformants were screened for zeocin resistance on YPDS plates (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar, and 100 µg mL^{-1} zeocin), and PCR and sequencing evaluated the integration of the construct into the yeast genome.

The recombinant mature peptide (rCHH-A) was expressed and purified according to the methods described by Camacho-Jiménez et al. (2015) with some modifications. A fresh colony of *P. pastoris* strain carrying pPICZαACHH-Ab vector inserted in its genome was grown in 3.0 mL of YPD medium with 100 µg mL^{-1} zeocin and incubated for 18 h at 30ºC and 200 rpm. The culture (2 mL) was transferred into 1000 mL of BMGY medium (1% yeast extract, 2% peptone, 0.67% yeast nitrogen base (YNB), 4 µg mL^{-1} D-biotin, 100 mM potassium buffer pH 6.0 with 1% glycerol), and incubated under the same conditions. When cell density reached an OD_{600} of 4.0, 10 aliquots (100 mL each) of the BMGY culture were collected and concentrated by centrifugation (2000 *g* for 5 min). The cell pellets were resuspended in 20 mL of BMMY medium with 1% methanol in 250 mL baffled flasks. Fresh methanol was added every 12 h to maintain the induction for 24 h. At the end of induction, cultures were centrifuged at 2000 *g* and 4ºC for 5 min. The supernatant was recovered and stored at -20ºC until further procedures.

The supernatant (200 mL) was precipitated with 50% ammonium sulfate (AS) and dialyzed against phosphate-buffered saline (PBS) 1X (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4, 1.8 mM KH_2PO_4, pH 7.4) using a 3 500 MWCO Slide-A-Lyzer dialysis cassette (Thermo Fisher Scientific, Carlsbad, CA, USA) following the manufacturer's instructions. Dialyzed protein was purified by reverse phase high-performance liquid chromatography (RP-HPLC) using an Agilent 1100 system (Agilent Technologies, Wilmington, DE, USA) equipped with a multiple wavelength detector. The separation was performed on a C18 TSK-Gel octadecyl-4 PW column (4.6×150 mm, 7 µm, Tosoh, Tokyo, JP). The column was appropriately equilibrated, and after sample injection, an isocratic step with 0.12% trifluoroacetic acid (TFA) in deionized water (solvent A) was applied for 5 min, and then eluted using a linear gradient 0-70% of solvent B (100% acetonitrile, 0.1% TFA) at a flow rate of 0.8 mL min^{-1} for 55 min. Ultraviolet (UV) absorbance was monitored at 280 nm, and 5 min fractions were collected from 30 to 50 min retention times. The fractions were lyophilized. Resuspended in PBS 1X and analyzed by tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) (14%) (Schägger 2006) with Coomassie brilliant blue (R-250) staining and also by Western Blot immunodetection (Sambrook & Russel 2001) to confirm the presence of rCHH-A.

For Westen blot, protein bands separated by Tricine-SDS-PAGE were transferred to a 0.45 µm Trans-Blot Transfer Medium nitrocellulose membrane (Bio-Rad, Richmond, CA, USA) using the semidry blotting technique (Sambrook & Russell 2001), in a Semidry Electroblotter System (Apollo, Continental Lab Products, San Diego, CA, USA). The membrane was blocked in PBS 1X with 0.05% (v/v) Tween-20 (PBST) and 5% nonfat milk (w/v) at room temperature for 2 h. The membrane was incubated with a primary rabbit anti-CHH-A polyclonal affinity antibody (GenScript, Piscataway, NJ, USA) diluted in PBST containing 1% nonfat milk (diluted 1:50) for 4 h at room temperature. The anti-CHH-A polyclonal antibody was produced using an antigen, a KLH-conjugated synthetic peptide designed from 14 residues of the N-terminal region of the *P. vannamei* CHH-A neurohormone. Finally, the membrane was incubated in a secondary antibody solution containing a goat anti-rabbit IgG (whole molecule) peroxidase monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA) diluted in PBST with 1% nonfat milk (1:2000) for 4 h. The membrane was washed three times with PBST between incubations for 15 min. The signal was detected using 3,3',5,5' -tetramethylbenzidine (TMB) peroxidase reagent (Thermo Fisher Scientific, Carlsbad, CA, USA).

To determine the N-terminal sequence of rCHH-A, the purified protein was separated by Tricine-SDS-PAGE and blotted onto a 0.2 µm Sequi-Blot PVDF membrane (Bio-Rad, Richmond, CA, USA). The bands (detected in the previous Western blot) were excised and sequenced by Edman degradation using a Procise 491 Protein Sequencing System (Applied Biosystems, Foster City, CA, USA), employing the Pulsed liquid PVDF method.

The concentration of the purified protein was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer's protocol. The purified rCHH-A was stored at -80°C until biological assays.

Salinity stress assay with rCHH-A

The *P. vannamei* postlarvae (PL_{18}) used in this experiment were obtained from the shrimp farm Acuacultura Mahr located in La Paz, Baja California Sur, Mexico. The PL was placed in 2000 L reservoirs with aerated seawater (35 ppt and 28 ± 1°C) and fed daily with commercial shrimp diet Aquaprofile 35 (Agribrands Purina, México, S.A. de C.V., MX) at a rate of 5% of their wet weight divided into three rations. Residual food and feces were siphoned daily from the tanks, and seawater was fully exchanged. Shrimp were grown under these conditions until they reached the sub-adult stage. This study was approved by the Centro de Investigación Científica y de Educación Superior de Ensenada (CICESE) ethical committee.

Sub-adult shrimp (9-14 g) were placed in individual 3.5-L containers in 200 L reservoirs with aerated seawater at 26 ± 1°C, which is the preferred temperature for *P. vannamei* within this weight range (González et al. 2010) and salinity of 26 ppt the iso-osmotic point for this species (Díaz et al. 2001). The iso-osmotic salinity was adjusted by mixing seawater with chlorine-free fresh water through a valve system and continuously monitored with a portable refractometer (ATAGO). The tank water was exchanged at a 50% daily rate, and salinity was adjusted when necessary by regulating the flux of mixed water. The tanks were connected to a recirculation system in which seawater and freshwater were purified through 5 and 10 mM bag filters, activated carbon filters, and UV-light treatment. The animals were acclimated for 10 days under these conditions before the experiment; then, the intermolt stage for each shrimp was estimated, calculated as half of the time between two successive

molts indicated by exoskeleton deposition (Camacho et al. 2015). Shrimp in intermolt fasted for 24 h before the salinity trial. Hypo- and hyper-osmotic salinities were achieved by diluting sea water with chlorine-free fresh water or adding natural sea salt.

To examine the rCHH-A effect on metabolic and osmotic variables under salinity stress, 2 µg of the purified protein dissolved in 50 µL of sterile PBS 1X or 50 µL of sterile PBS 1X (control) were injected into shrimp through the arthrodial membrane of the fifth pair of pereiopods with a 1 mL sterile hypodermic syringe 31G (Plastipak, BD, México). rCHH-A and PBS 1X were injected into shrimp maintained under iso-osmotic conditions (26 ppt) (control salinity) or immediately transferred to either hypo- (10 ppt) or hyper-osmotic water (40 ppt). Un-injected shrimp were taken from the acclimation reservoirs at the iso-osmotic condition to monitor the stress of the injection and used to determine the basal levels at 0 h for the measured responses (un-injected control). After salinity change, different groups of shrimp (n = 4-8) injected with rCHH-A or PBS 1X were collected for hemolymph sampling at 0.5, 1, 2, and 24 h of exposure.

Hemolymph was collected (120 µL) from the thoracoabdominal membrane using a 1 mL sterile hypodermic syringe 27G (UltraFine; BD, Mexico) and separated into two parts. One part of the hemolymph (50 µL) was diluted (1:2) in shrimp salt solution (SSS) (450 mM NaCl, 10 mM KCl, 10 mM EDTA Na_2, 10 mM HEPES, pH 7.3, 850 mmol kg^{-1}) (Vargas-Albores et al. 1993). Another part was mixed with heparin ammonium salt solution (Sigma-Aldrich, Saint Louis, MO, USA) (0.8 mg mL^{-1}, pH 7.3) in a 1:3 dilution. The plasma fraction of the hemolymph was isolated by centrifugation at 4400 *g* and 4°C for 5 min and stored at -80°C.

Hemolymph metabolites

Glucose levels in hemolymph samples with SSS anticoagulants were analyzed in triplicate using a glucose oxidase diagnostic kit (Point Scientific, Cantor, MI, USA), according to the manufacturer protocol. Hemolymph protein concentration was determined by triplicate in 96-well plates using a Protein Assay kit (Bio-Rad, Hercules, CA, USA) with the SSS anticoagulant samples and bovine serum albumin (BSA) (Lafon Laboratories, Mexico) for the standard curve. The data were expressed in mg dL^{-1}.

Osmoregulatory and ionoregulatory capacity

The hemolymph and water osmotic pressure (OP) were analyzed from 10 µL fresh samples in a vapor osmometer (Wescor 5200, South Logan, UT, USA), and the data were expressed in mmol kg^{-1}. The osmoregulatory capacity (OC) was calculated as the difference between the OP of hemolymph minus the OP of the external medium (Lignot et al. 2000).

The concentrations of sodium, chloride, potassium, and calcium ions from water tanks and heparinized plasma samples were analyzed in 96-well plates in triplicates using the commercial Stambio/Licon kits (Stanbio, North Main, TX, USA). The ionoregulatory capacity (IC) for each ion was calculated as the difference in ion concentration between hemolymph and external medium (Camacho-Jiménez et al. 2018). The results were expressed in mmol L^{-1}.

Statistical analysis

Data from the salinity stress assay were tested for normality with Shapiro-Wilk's test and variance equality with the Brown-Forsythe test. Because data sets were not normally distributed or homoscedastic, they were analyzed by a non-parametric Kruskal-Wallis test followed by Dunn's test. When required, punctual comparisons between experimental groups were made with Mann-Whitney. The significance level was settled at $P \leq 0.05$, and all the analyses and graphics were done with SigmaPlot 14.0 software (Systat Software, San Jose, CA, USA). Data are shown as a median ± 95% confidence interval (CI) (n = 4-8).

Ethics approval consent to participate: This research followed the guidelines specified by the research permits from CICESE (permit number: 2S.3.1 ORGA_ACUA_2021_01).

RESULTS

Protein expression and purification of *Penaeus vannamei* rCHH-A

The mature CHH-A transcript was cloned into the pPICZαA vector producing the pPICZαA-CHH-Ab vector (Fig. 1). Sequencing of vector pPICZαA-CHH-Ab showed no mutations, and the reading frame with the α-factor protein secretion signal was corroborated. Five *Pichia pastoris* transformants were screened by PCR; all contained the expected 735 bp amplicon. The analysis of the transformants by sequencing confirmed the integration of the mature CHH-A sequence into the genome of *P. pastoris* X-33, producing the X-33 CHH-Ab strain. Since the codon for the lysine residue was removed from the construction, the rCHH-A peptide obtained has 73 aa and a predicted molecular average mass of 8691.82 Da in its reduced form with the free C-terminal G residue.

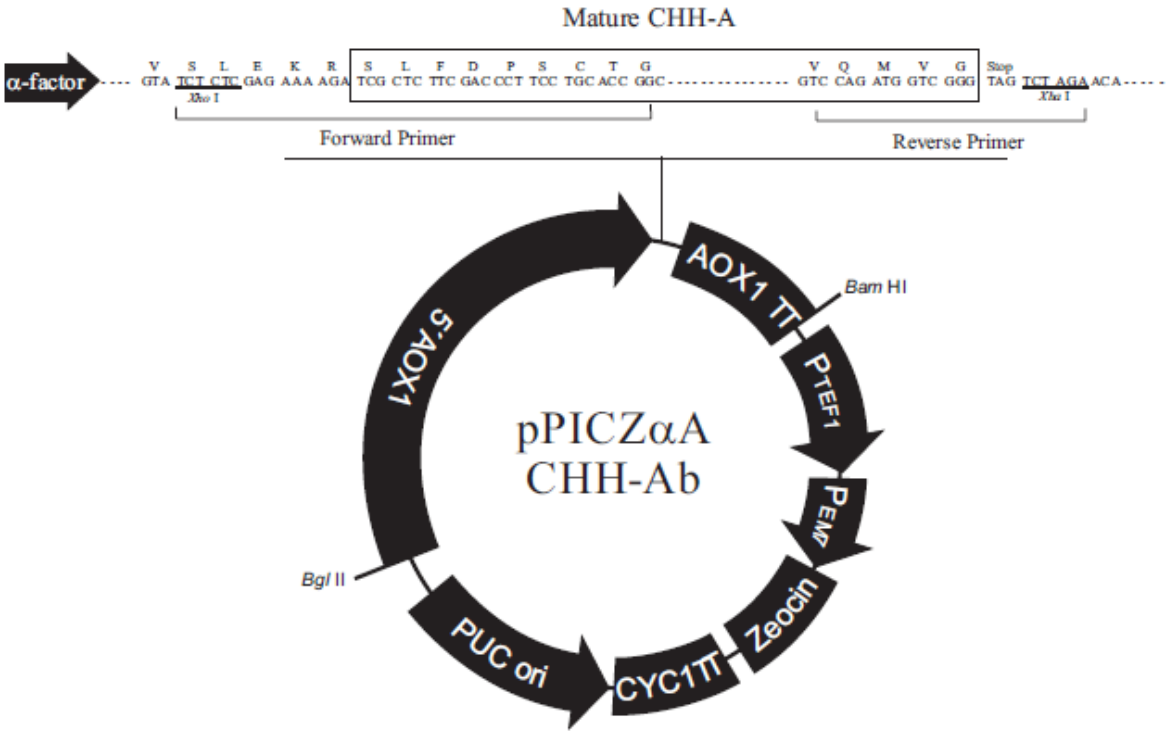

Figure 1. Map of the pPICZαA-CHH-Ab construction for expressing the mature CHH-A peptide in *Pichia pastoris*. The vector contains the α-factor signal peptide and the methanol-inducible P_{AOX1} promoter. The CHH-A is integrated into the genome upon transformation into *Pichia* through homologous recombination.

The rCHH-A protein was expressed in the *P. pastoris* X-33 CHH-Ab strain by adding 1% methanol into the induction media for 24 h and purified with AS precipitation followed by RP-HPLC. The chromatographic profile of the precipitated sample (Fig. 2) resulted in four principal fractions (F) that had retention times from 30 to 50 min (corresponding from F6 to F9). A major absorbance zone with a high peak was observed in F7 (35-40 min). The Tricine-SDS-PAGE analysis from F6-F9 (see "C" in Figure 2 insets) revealed the presence of two protein bands in F7, a ~10 kDa band that agreed with the expected size of the reduced form of rCHH-A and a second one less intense band of ~15 kDa. Both bands were positive in the Western blot analysis with anti-CHH-A antibody (see "WB" in Figure 2 insets). No other bands were visualized in this fraction, indicating the removal of the most contaminant proteins after the RP-HPLC step.

The N-terminal sequencing analysis of the ~10 kDa band from the purified rCHH-A sample revealed that the first 15 amino acids (SLFDPSXTGVFDRQL, where X is a black cycle corresponding to a cysteine (C) residue) matched 100% of the expected deduced sequence for mature rCHH-A. The N-terminal amino acid sequence obtained for the ~15 kDa band suggested the formation of the non-covalent aggregates of denatured rCHH-A molecules observed for other recombinant *P. vannamei* CHH peptides expressed in *P. pastoris* (Camacho-Jiménez et al. 2015, 2017a). Moreover, small proportions of the sequence GLOGTA (where O is 4-hydroxy-proline) were detected in the ~10 kDa band and the ~15 kDa band. This sequence corresponds to collagen fragments and peptone components in the culture media used to express CHH-A and has been observed in other recombinant peptides previously purified by this method (Camacho-Jimenez et al. 2015). The final yield of the purified rCHH-A was 12.19 mg L^{-1} of culture medium.

Effect of rCHH-A on hemolymph metabolites

Glucose levels (Fig. 3) were analyzed over time in the hemolymph of shrimp acclimated to iso-osmotic conditions (26 ppt), injected with rCHH-A and PBS, and then acutely transferred to hypo- or hyper-osmotic conditions (10 and 40 ppt, respectively). The basal hemolymph glucose level (0 h) in un-injected shrimp acclimated to the iso-osmotic condition (26 ppt) was 12.31 ± 0.99 mg dL^{-1}. Regardless of salinity, rCHH-A and PBS caused an increase in hemolymph glucose levels in comparison with the un-injected control. The

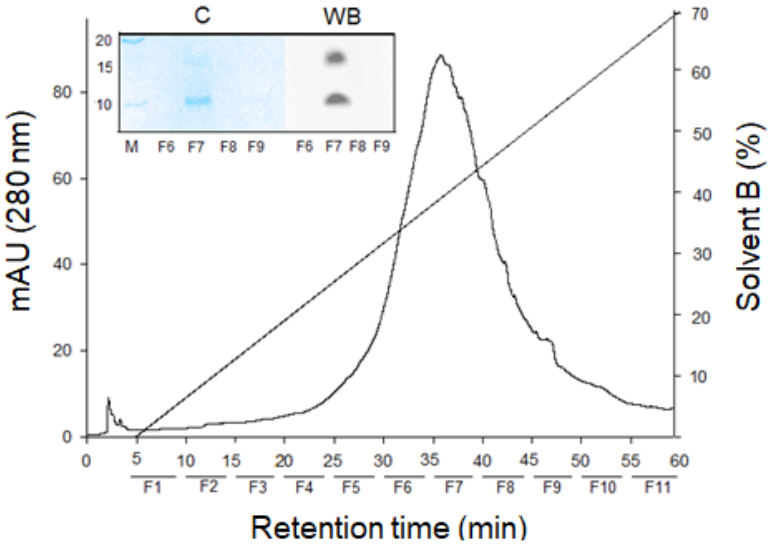

Figure 2. The reversed-phase high-performance liquid chromatography (RP-HPLC) profile of precipitated and dialyzed recombinant CHH-A. The chromatogram is divided into 5-min fractions (F1-11). The transverse line indicates the gradient of solvent B. C: Coomassie blue stained Tricine-SDS-PAGE (14%), WB: Western blot analysis of purified protein with anti-CHH-A antibody.

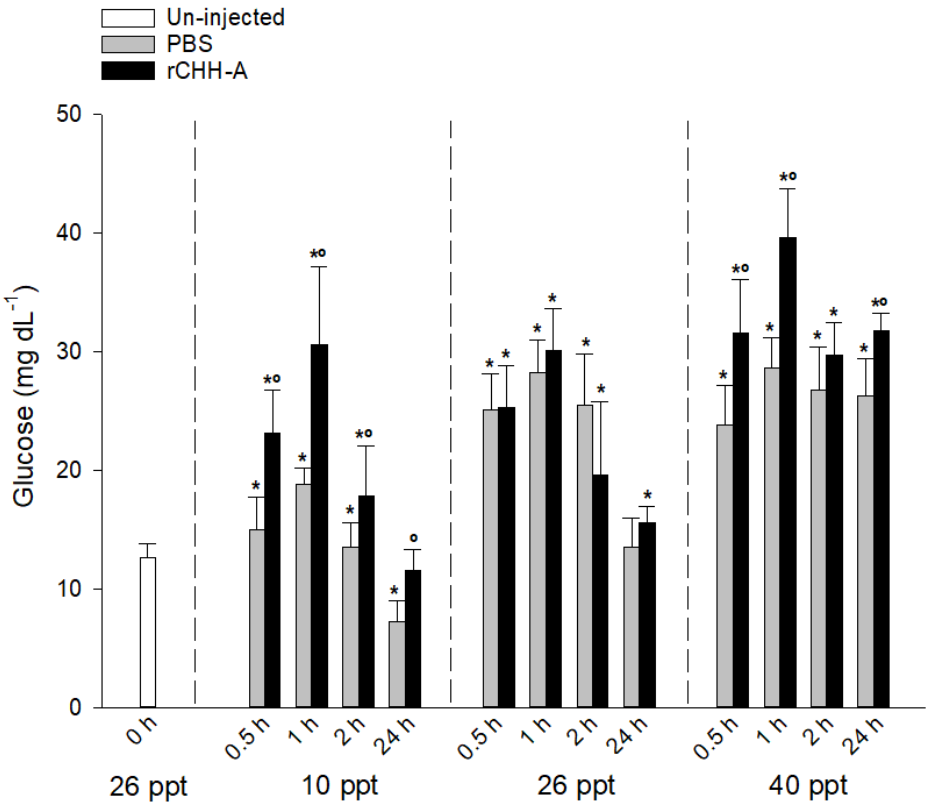

Figure 3. Effect of recombinant CHH-A on shrimp *Penaeus vannamei* glucose levels in hypo- (10 ppt), iso- (26 ppt), and hyper-osmotic (40 ppt) conditions. Data are expressed as median ± 95% confidence interval (n = 4-8). °Indicate differences in rCHH-A treatment from phosphate-buffered saline (PBS) control in each sampling time ($P < 0.05$). *Indicate significant differences from un-injected shrimp at 0 h (basal levels) ($P < 0.05$).

hyperglycemic activity in hormone treatments reached the highest values at 1 h post-injection in the three salinities (29.77 ± 4.55 mg dL^{-1} at 26 ppt, 33.90 ± 5.83 mg dL^{-1} at 10 ppt, and 38.43 ± 4.64 mg dL^{-1} at 40 ppt) ($P < 0.05$). However, at 26 ppt, which is the iso-osmotic salinity for *P. vannamei*, the increase in glucose concentration by rCHH-A was not significantly different from the values in the PBS group at any sampled time ($P > 0.05$). However, in shrimp exposed to acute hypo-osmotic salinity, rCHH-A promoted significant hyperglycemia from 0.5 to 24 h post-injection compared to the corresponding PBS control ($P < 0.05$). The challenge with rCHH-A at high salinity raised glucose levels principally at 0.5 and 1 h post-injection, in contrast to PBS ($P < 0.05$).

The total protein concentrations (Table 1) obtained from the hemolymph samples varied among salinities. In general, the highest total protein concentrations were found in shrimp transferred to hypo-osmotic salinity ($P < 0.05$), while the lowest ones were found at hyper-osmotic conditions ($P < 0.05$). In animals at 10 ppt, the rCHH-A and PBS injection promoted a rise in hemolymph proteins in comparison to un-injected shrimp (188.74 ± 9.15 mg mL^{-1}), which lasted 24 h ($P < 0.05$). However, hemolymph protein in hormone treatment at 10 ppt was only significantly different from the values in PBS control at 1 and 2 h post-injection, respectively ($P < 0.05$). At iso-osmotic conditions, shrimp injected with rCHH-A showed a significant difference after 0.5 h post-injection (206.95 ± 7.39 mg mL^{-1}) and 24 h concerning PBS (256.84 ± 22.41 mg dL^{-1}) and 0 h controls ($P < 0.05$). Nonetheless, no significant changes were found due to rCHH-A treatment in later sampling times ($P > 0.05$). Conversely, in animals exposed to high salinity, rCHH-A showed significant differences from the uninjected and PBS groups from 0.5 to 2 h post-injection ($P > 0.05$). After 24 h, the protein concentration in the rCHH-A treatment was not different from the PBS group ($P = 0.745$).

Effect of rCHH-A on osmoregulatory capacity

The shrimp exposed to hypo-osmotic salinity (10 ppt) had the highest osmoregulatory capacity (OC). They were hyper-osmotic concerning the external medium (hyper-OC) (Fig. 4). At this salinity, the OC values were significantly higher than those in un-injected shrimp kept at 26 ppt (0 h) (41.22 ± 7.3 mmol kg^{-1}) ($P < 0.05$). The injection of rCHH-A significantly reduced the OC of shrimp at 2 h (334.54 ± 19.75 mmol kg^{-1}) and 24 h (321.94 ± 10.33 mmol kg^{-1}) post-injection in comparison to the PBS at their respective sampling times ($P < 0.05$).

In animals maintained at 26 ppt, the OC was closer to zero than in the other two salinities since their OP values were similar to those in the environment. Under these conditions, OC values were higher after 24 h from rCHH-A injection (28.73 ± 7.91 mmol kg^{-1}) than those shown by PBS control (15.49 ± 5.05 mmol kg^{-1}) ($P = 0.008$). However, the hormone did not cause a significant effect on the un-injected control ($P = 0.056$).

The organisms exposed to hyper-osmotic salinity (40 ppt) behaved as hypo-regulators since they had a lower OC than shrimp transferred to iso- and hypo-osmotic salinities. The OC values obtained after rCHH-A treatment were very similar in all the sampled times except for the 2 h in which the OC decreased significantly around 20% (-348.50 ± 4.66 mmol kg^{-1}) in comparison with the other values for the same treatment at 0.5 h (-449.80 ± 11.30 mmol kg^{-1}) ($P < 0.001$), 1 h (-442 ± 15.52 mmol kg^{-1}) ($P = 0.006$), and 24 h (-412.67 ± 10.60 mmol kg^{-1}) ($P = 0.004$). The OC value in rCHH-A group at 2 h was also significantly different from the one obtained for the PBS treatment (-384.33 ± 14.01 mmol kg^{-1}). After 24 h post-injection, the hemolymph OC values in shrimp injected with rCHH-A treatment (-412.67 ± 10.60 mmol kg^{-1}) and PBS control (391.00 ± 29.70 mmol kg^{-1}) did not show significant differences between them ($P = 0.304$).

Effect of rCHH-A on hemolymph ion concentrations

The most abundant ions in the hemolymph of un-injected shrimp were sodium and chloride, which account for more than 90% of the total ions in all salinities. The sodium and chloride ions concentrations in the un-injected control were 311.76 and 185.57 mmol L^{-1}, respectively. Calcium and potassium were the least abundant ions with concentrations in the un-injected control of 3.05 and 1.27 mmol L^{-1}, respectively.

When shrimp were acutely transferred from 26 to 10 ppt salinity, all ions were maintained at higher concentrations in the hemolymph than in the external medium (hyper-IC) (Figs. 5a-b, 6a-b). The exception was the sodium IC obtained with PBS at 0.5 h showing a hypo IC response that was significantly different from rCHH-A injection at 0.5 h (55.85 ± 8.34 mmol L^{-1}) ($P = 0.009$) (Fig. 5a). However, sodium IC values for rCHH-A at 10 ppt were not significantly different from PBS at other sampling times (1-24 h).

Table 1. Total protein concentration in the hemolymph of *Penaeus vanamei* after rCHH-A injection at different salinities. Data are expressed as median ± 95% CI (n = 4-8). *Differences for un-injected shrimp acclimated at 0 h ($P < 0.05$). °Differences for PBS control at the given salinity and sampling time ($P < 0.05$). PBS: phosphate-buffered saline.

	Salinity (ppt)		
	10	26	40
Total protein (mg mL^{-1})			
Un-injected		192.65 ± 7.95	
PBS (0.5 h)	227.99 ± 11.85*	250.63 ± 15.99*	102.15 ± 13.54*
PBS (1 h)	241.67 ± 8.95*	195.99 ± 13.90	130.86 ± 7.46
PBS (2 h)	222.92 ± 11.11*	198.18 ± 10.07*	114.06 ± 5.56*
PBS (24 h)	224.10 ± 6.56*	263.47 ± 13.41*	119.31 ± 7.25*
rCHH-A (0.5 h)	230.89 ± 7.89*	206.13 ± 6.69*°	122.56 ± 2.04*°
rCHH-A (1 h)	218.98 ± 4.41*°	192.32 ± 9.44	103.54 ± 10.54*°
rCHH-A (2 h)	234.58 ± 2.25*°	195.24 ± 10.85	99.28 ± 13.02*°
rCHH-A (24 h)	226.37 ± 3.43*	203.19 ± 10.49°	106.22 ± 23.56*

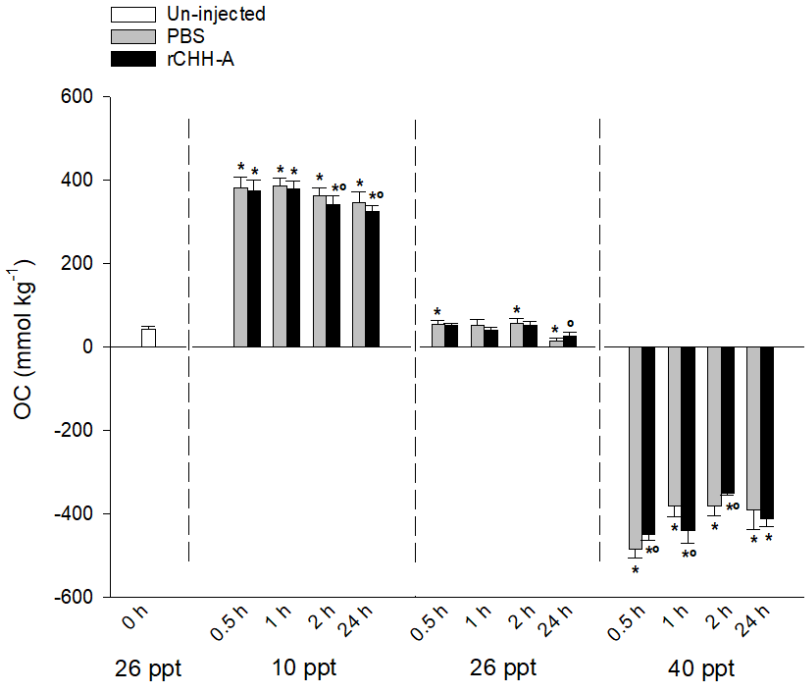

Figure 4. Effect of recombinant CHH-A on shrimp *Penaeus vannamei* osmoregulatory capacity (OC) in hypo- (10 ppt), iso- (26 ppt), and hyper-osmotic (40 ppt) conditions. Data are expressed as median ± 95% confidence interval (n = 4-8). °Indicate differences in rCHH-A treatment from phosphate-buffered saline (PBS) control in each sampling time ($P < 0.05$). *Indicate significant differences from un-injected shrimp at 0 h (basal levels) ($P < 0.05$).

For chloride IC (Fig. 5b) at low salinity, the values were significantly higher than the un-injected control ($P < 0.001$), except for the hormone treatment at 1 h ($P = 0.773$). At this time, the rCHH-A treatment showed a significant reduction of ~40% from the levels of the PBS treatment (177.98 ± 7.21 mmol L^{-1}) ($P = 0.001$). No difference between rCHH-A and PBS treatments was observed in all the other sampled times at 10 ppt. The potassium IC (Fig. 6a) was significantly higher in all the shrimp exposed to low salinity than in shrimp exposed to 26 and 40 ppt ($P < 0.001$). Under these conditions, rCHH-A promoted a significant decrease at 0.5 h post-injection (9.57 ± 0.41 mmol L^{-1}) and 1 h (7.92 ± 0.35 mmol L^{-1}) when compared to the PBS treatment (10.90 ± 0.04 and 9.24 ± 0.39 mmol L^{-1}, respectively) at the same salinity ($P < 0.05$). After 2 h, the potassium IC in hormone treatment significantly increased over PBS control (10.17 ± 0.41 mmol L^{-1})

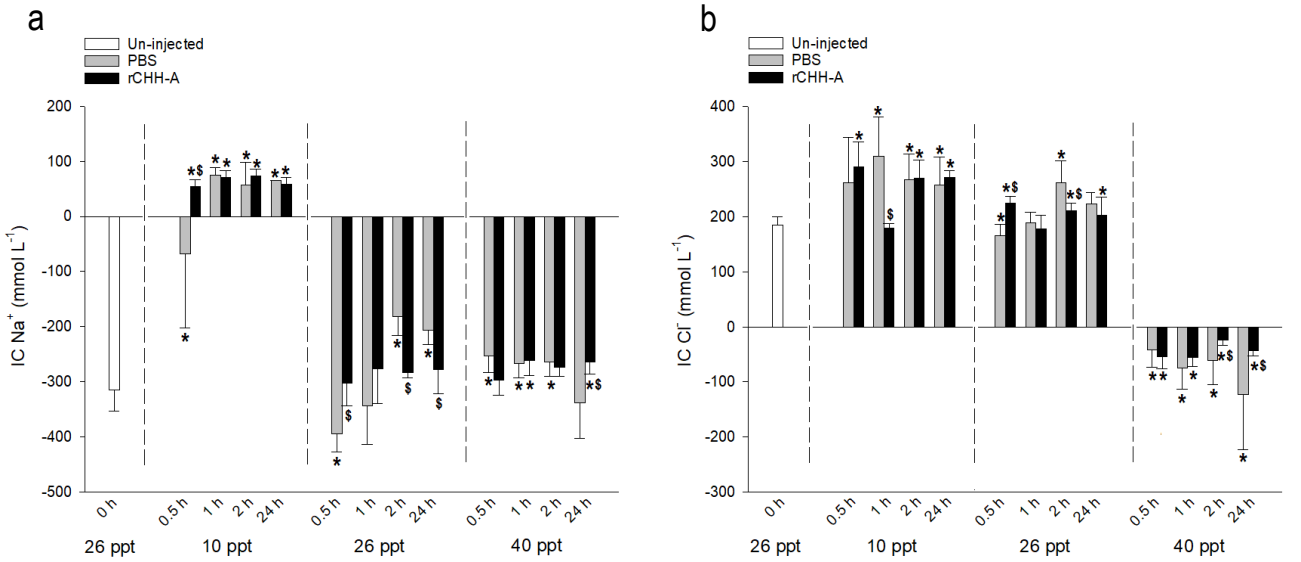

Figure 5. Effect of recombinant CHH-A on the ionoregulatory capacity (IC) of major ions in shrimp *Penaeus vannamei* hemolymph under hypo- (10 ppt), iso- (26 ppt) and hyper-osmotic (40 ppt) conditions. a) Sodium, b) chloride. Data are expressed as median ± 95% confidence interval (n = 4). *Indicate significant differences from un-injected shrimp at 0 h (basal levels) ($P \leq 0.05$). $^{\$}$Indicate differences in CHH-A treatment from phosphate-buffered saline (PBS) control in each sampling time ($P \leq 0.05$).

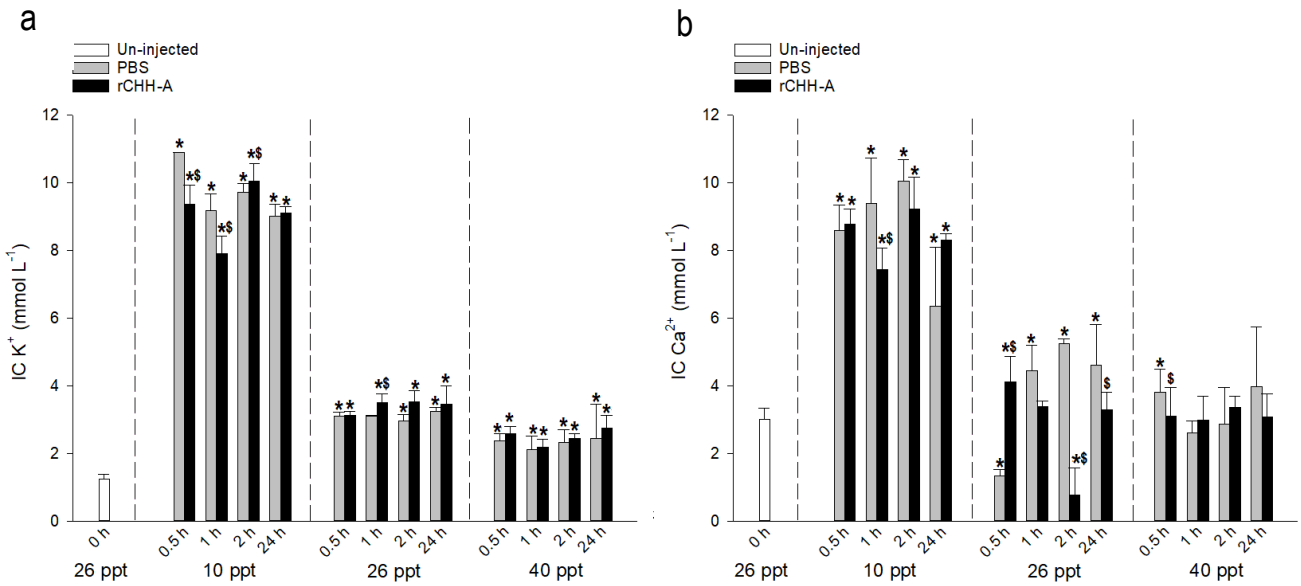

Figure 6. Effect of recombinant CHH-A on the ionoregulatory capacity (IC) of minor ions in shrimp *Penaeus vannamei* hemolymph under hypo- (10 ppt), iso- (26 ppt) and hyper-osmotic (40 ppt) conditions. a) Potassium, b) calcium. Data are expressed as median ± 95% confidence interval (n = 4-8). *Indicate significant differences from un-injected shrimp at 0 h (basal levels) ($P \leq 0.05$). $^{\$}$Indicate differences in CHH-A treatment from phosphate-buffered saline (PBS) control in each sampling time ($P \leq 0.05$).

(P = 0.014) and decreased again to PBS levels at 24 h post-injection (9.09 ± 0.24 mmol L^{-1}) (P = 1.000). Similarly to potassium IC, calcium IC values were significantly greater in shrimp at 10 ppt compared to the un-injected control shrimp (P = 0.001). The calcium IC at 10 ppt (Fig. 6b) showed a significant reduction in values at 1 h after rCHH-A injection (7.47 ± 0.49 mmol L^{-1}) compared to PBS control (9.27 ± 0.89

mmol L^{-1}) (P = 0.003). No significant changes were detected in the other sampling times compared to the PBS control (P > 0.05).

At 26 ppt salinity, sodium IC was hypo-ionic to ambient values, contrasting with the hyper-ionic behavior of the other ions IC (Figs. 5-6). At this salinity, sodium IC values in the organisms injected with rCHH-A were very similar over the sampling time (-281.65 to -302.1 mmol L^{-1}) and also compared with the un-injected control (-311.76 mmol L^{-1}) (P > 0.05). However, PBS samples showed variations over time for 0 h control and rCHH-A treatment. The highest difference between the sodium IC showed by rCHH-A and PBS groups at this salinity were at 2 h post-injection, in which the values were -281.65 ± 8.11 and -177.50 ± 23.57 mmol L^{-1}, respectively (P < 0.001). Chloride IC (Fig. 5b) showed a significant increment at iso-osmotic salinity after 0.5 h post-injection of rCHH-A (228.17 ± 9.76 mmol L^{-1}) in comparison to PBS (157.57 ± 22.69 mmol L^{-1}) (P < 0.001). After 2 h post-injection rCHH-A, the chloride IC values (211.93 ± 14.91 mmol L^{-1}) were very similar to hormone IC sodium values at 0. 5 h post-injection but significantly lower than PBS treatment values (264.54 ± 35.67 mmol L^{-1}) (P = 0.002). No significant differences were found after 24 h post-injection of rCHH-A and PBS (P > 0.05).

Concerning potassium IC (Fig. 6a), the values shown by shrimp at 26 ppt were significantly higher than the un-injected control, which had a lower IC value (1.27 mmol L^{-1}) (P < 0.05). Under iso-osmotic conditions, rCHH-A caused a significant increase in potassium IC after 1 h (3.49 ± 0.31 mmol L^{-1}) in contrast to PBS control (3.1 ± 0.05 mmol L^{-1}) (P = 0.004). No differences with PBS were found at later sampling times (P > 0.05). Interestingly, at 26 ppt salinity, a strong increase (~65%) in calcium IC was observed at 0.5 h post-injection of rCHH-A (3.89 ± 0.56 mmol L^{-1}) (Fig. 6b), in contrast to PBS control (1.34 ± 0.23 mmol L^{-1}) (P < 0.001). However, calcium IC values in rCHH-A treatment were reduced over time, having a lower IC value at 2 h (1.06 ± 0.64 mmol L^{-1}) in comparison with PBS (5.30 ± 0.19 mmol L^{-1}) (P = 0.001). Calcium IC was also lower than measured at 10 and 40 ppt (P < 0.05). At 24 h post-injection, the IC values in rCHH-A group were restored to un-injected levels (3.05 ± 0.47 mmol L^{-1}) (P = 0.244), but they were still significantly lower than shrimp injected with PBS (4.83 ± 1.13 mmol L^{-1}) (P = 0.006).

At 40 ppt salinity, sodium IC showed a hypo-ionic response (Fig. 5a). Under this salinity condition, rCHH-A injection did not cause any significant change in sodium IC from 0.5 to 2 h post-injection compared with PBS values (P > 0.05). The sodium IC values of rCHH-A at 24 h remained very similar to the other sampling times (-266.95 ± 14.84 mmol L^{-1}) (P = 0.023). However, it differed from the PBS control, which showed an increase in the hypo IC values for sodium (-339.79 ± 45.93 mmol L^{-1}) compared to the rCHH-A treatment. However, this value was not significantly different from the un-injected control (-311.76 ± 29.44 mmol L^{-1}) (P = 0.413). Chloride IC at 40 ppt was hypo-ionic (Fig. 5b) and hence lower than under iso- and hypo-osmotic salinities, including the un-injected control (185.57 ± 28.40 mmol L^{-1}) (P < 0.05). A significant decrease (rise in concentration) was observed at 2 h (-28.18 ± 11.42 mmol L^{-1}) and 24 h (-38.04 ± 8.09 mmol L^{-1}) post-injection of rCHH-A treatment compared with the PBS control at the same time (-61.82 ± 37.59 and -122.86 ± 69.10 mmol L^{-1}, respectively) (P < 0.05).

Potassium and calcium IC of organisms transferred to 40 ppt showed a hyper-ionic response (Figs. 6a,b, respectively). The potassium IC value in the un-injected control (1.27 ± 0.15 mmol L^{-1}) was significantly lower than in rCHH-A and PBS values obtained over time (P < 0.05). However, no differences were observed between rCHH-A and PBS treatments at 40 ppt (P > 0.05). By comparison, calcium IC values in un-injected animals (3.05 ± 0.47 mmol L^{-1}) were not different from those in shrimp injected with PBS and rCHH-A treatments and transferred to hyper-osmotic conditions (P < 0.05). The recombinant hormone only showed a significant difference in the IC levels for calcium after 0.5 h injection compared with the PBS treatment (3.00 ± 0.56 and 3.99 ± 0.55 mmol L^{-1}, respectively) (P = 0.033).

DISCUSSION

Expression of rCHH-A

To contribute to understanding the physiological and metabolic function of the this variant in *P. vannamei*, a recombinant version of the CHH-A variant was expressed in *Pichia pastoris*. The identity of the hormone was confirmed by immunodetection with an anti-CHH-A antibody and N-terminal sequencing. The yield obtained in this study (12.9 mg L^{-1}) was higher than those reported for other recombinant CHH peptides expressed in *P. pastoris* and purified by RP-HPLC (Ohira et al. 2003, Treerattrakool et al. 2003, Camacho-Jiménez et al. 2015, 2017a). Therefore, sufficient amounts of rCHH-A were recovered to study the effects on some metabolic and osmoregulatory

variables in *P. vannamei* shrimp acutely transferred from iso- (26 ppt) to hypo- (10 ppt) and hyper- (40 ppt) osmotic conditions.

The mature rCHH-A peptide has 74 amino acids. Even though the native CHH-A peptide sequence seems to be amidated at the C-terminal (Lago-Lestón et al. 2007), the rCHH-A peptide with a free C-terminal (G) caused a significant hyperglycemic response in shrimp injected with a dose of 2 µg. Nevertheless, a dose-response *in vivo* assay could be useful in future studies to determine whether this variant is minimal and maximal biological activities. Although the C-terminal amidation can be essential for the hyperglycemic activity of some CHH neuropeptides (Mosco et al. 2008, Chang et al. 2010), it is not a requisite in some peptides with a free C-terminus (like CHH-B1) (Camacho-Jiménez et al. 2015). This case also appears to be the same for some naturally amidated hormones expressed with a free C-terminus, such as CHH-B2 (Camacho-Jiménez et al. 2017b) and LvCHH (Wanlem et al. 2011) from *P. vannamei*, as well as Pem-CHH2 and PemCHH3 from *Penaeus monodon* (Udomkit et al. 2004).

The CHH-A variant from *P. vannamei* has a high or total sequence identity to other CHH peptides that have been given different names: LvCHH (Liu et al. 2014), rLV-CHH (Wanlem et al. 2011), and rLvCHH I (Wang et al. 2017). The injection of rLV-CHH was observed to cause *P. vannamei* shrimp to have a high immune response against the pathogenic bacteria *Vibrio harveyi* (Wanlem et al. 2011). Additionally, the incubation of hemocytes with the rLvCHH I peptide, which has 100% identity to the CHH-A variant, increased the expression of antimicrobial peptides in *P. vannamei* (Wang et al. 2017). CHH-A also shows a high sequence identity (98.6%) to the mature rLv-CHH, which has been suggested to be involved in intracellular signaling pathways of phagocytic activity in white shrimp hemocytes (Xu et al. 2019). Interestingly, Liu et al. (2014) reported an increase in the Na^+-K^+ ATPase (NKA) gene expression in shrimp gills injected with the rLv-CHH peptide, suggesting its participation in osmoregulation. However, this peptide's physiological functions have yet to be determined under salinity stress.

Effect of rCHH-A on glucose metabolism

The results showed a substantial rise in the hemolymph glucose levels at 0.5-1 h after rCHH-A injection into shrimp acutely exposed to extreme salinities (10 and 40 ppt). These results agree with the hyperglycemic activity reported at 1 h post-injection of the non-amidated rLV-CHH peptide into shrimp acclimated to hypo-osmotic conditions (15 ppt) (Wanlem et al. 2011) and with the rCHH-B1 and rCHH-B2 variants principally at 45 ppt salinity (Camacho-Jiménez et al. 2018). The strong capacity of rCHH-A to rapidly raise glucose concentration in hemolymph agrees with the main role attributed to CHH peptides in regulating glucose dynamics between hemolymph and tissues. In response to environmental and endogenous stimuli, CHH is secreted from OX-SG and stimulated glycogenolysis in glycogen storage organs (i.e. hepatopancreas and muscle). It leads to a rise in glucose in the hemolymph, which can be subsequently uptaken by tissues to satisfy energy demands, especially during stressful situations (Fanjul-Moles 2006). Thus, it is not rare that rCHH-A elicited the strongest hyperglycemic response in high salinity since energy expenditure for physiological compensation is typically increased at salinities that are not iso-osmotic (Valdez et al. 2008).

Effect of rCHH-A on osmoregulation

Salinity is one of the most important abiotic factors that influence the distribution and abundance of crustaceans and their general physiology (Romano & Zeng 2011). To assess the osmotic effects of rCHH-A in *P. vannamei* acutely exposed to different salinities, OC and IC were used as physiological performance indicators. These indicators have been applied in crustaceans to evaluate salinity tolerance and the effect of endocrine factors on osmoregulation (Charmantier et al. 1988, 1989, Charmantier-Daures et al. 1994, Camacho-Jiménez et al. 2018). The hemolymph OP is known to vary with sodium and chloride concentration gradients, which are the predominant ionic constituents of shrimp hemolymph (Chen & Chen 1996). According to the results of this study, the most abundant ions in the hemolymph of un-injected shrimp in all salinities were sodium and chloride, representing more than 90% of the total ions. This concentration agrees with the osmotic concentration reported by Castille & Lawrence (1981a) by these active solutes in the hemolymph. At the same time, potassium and calcium were minor osmolytes. The hemolymph was iso-osmotic at 26 ppt (the acclimation salinity) with an OC close to zero, which agrees with the expected pattern for *P. vannamei* at an iso-osmotic point (25-27 ppt) since less active processes are needed to regulate the inner solute content (Díaz et al. 2001). Shrimp acutely transferred to hypo-osmotic conditions showed higher OP, sodium, and chloride concentrations than the external medium (shown as hyper-OC and hyper-IC). The opposite (shown as hypo-OC and hypo-sodium and chloride IC regulation) was found in shrimp transferred to hyper-

osmotic conditions. The data obtained herein are according to the expected regulatory patterns for *P. vannamei* at salinities above and below iso-osmotic points (Castille & Lawrence 1981a, Díaz et al. 2001, Mena-Herrera et al. 2011). Despite sodium concentrations not changing significantly at 10 and 40 ppt in response to rCHH-A, the inner chloride concentration was modified considerably in these extreme salinities.

Interestingly, at 40 ppt, a disruptive effect of rCHH-A was observed by a strong reduction of the OC at 2 h post-injection and chloride IC at 2 and 24 h post-injection, suggesting that shrimp were hypo-regulating hemolymph chloride. These results suggest that -besides the hyperglycemic response- salt exchange mechanisms with the environment were active. These response patterns differ from those previously reported for *P. vannamei* after the injection with rCHH-B1 and rCHH-B2 peptides, followed by acute transference to different salinities (Camacho-Jiménez et al. 2018). The rCHH-B1 peptide increased the hypo-OC of shrimp 1 h post-injection at 45 ppt, whereas rCHH-B2 increased the hypo-OC 6 h post-injection at 26 ppt. However, both peptides reduced the IC levels for sodium and chloride ions at 45 ppt suggesting that potential CHH variants of *P. vannamei* have differential roles in osmoregulation, depending on salinity conditions. These differential responses of the CHH variants can contribute to *P. vannamei*'s capacity to adapt rapidly to new salinities by increasing and decreasing the hemolymph osmotic concentration (Pannikar 1968, Díaz et al. 2001). A previous study also supports this hypothesis demonstrating that gene expression patterns at eyestalks for CHH-B1 and CHH-B2 peptides respond differently to changes in acclimation salinities, regardless of temperature conditions (Lago-Lestón et al. 2007). The injection of purified CHH into *Pachygrapsus marmoratus* crabs maintained in hyper-osmotic conditions (36.7 ppt) also produced a strong and specific hyperglycemic response and a direct effect on branchial osmoregulatory processes (Spanings-Pierrot et al. 2000).

Interestingly, the biological activity of the ion transport peptide, which belongs to the subfamily of the CHHs, has been described to elevate the intracellular cyclic AMP stimulating chloride, potassium, and sodium ion reabsorption across the ileon of *Schistocerca gregaria* (Audsley et al. 1992a, 2013). However, another putative messenger is anticipated to inhibit acid secretion in tissues (Gäde 2004). Kings et al. (1999) reported that the chemically synthesized ITP had biological activities identical to the ITP purified from the corpora cardiaca of *S. gregaria*. At the transcriptional level, the *P. vannamei* neuropeptide named Lv-ITP, which is 95.8% identical to the CHH-B1 sequence, decreased the expression levels in the posterior gill when shrimp were exposed to extreme salinity conditions, suggesting its participation in osmoregulation or/and ionic transport (Tiu et al. 2007).

Gills are the main osmoregulatory organs in crustaceans and have epithelial cells rich in proteins involved in ion transport, such as the NKA, the primary driving force for the active exchange of NaCl between tissues and the environment (Henry et al. 2012). The NKA expression and activity are sensitive to high and low salinity in penaeid shrimp (Huong et al. 2010, Sun et al. 2011, Shekhar et al. 2014). The involvement of CHH in NKA regulation has been investigated in the euryhaline crab *Portunus trituberculatus*, in which CHH gene knockdown by RNA interference strongly reduced NKA activity in gills (Sun et al. 2019). Liu et al. (2014) demonstrated that rLv-CHH protein injection to *P. vannamei* shrimp raised NKA activity in gills when acclimated to 31 ppt salinity, mainly at 1 h post-injection. These results suggested that rLv-CHH is involved in the osmosis responses of shrimps.

Moreover, Camacho-Jiménez et al. (2018) reported that rCHH-B1 and rCHH-B2 up-regulated NKA mRNA expression at 26 ppt (3 h) and 45 ppt (1 h). Later experiments showed that the effect of rCHH-B1 on NKA expression occurs in a dose-dependent way (Camacho-Jiménez et al. 2021). Then, rCHH-A could regulate NKA-dependant ion transport through its metabolic effect. This response could explain OC changes provoked by rCHH-A beyond 2 h at iso- and hypo-osmotic conditions when the hyperglycemic effect had disappeared. Furthermore, since the recombinant PO-CHH from *Callinectes sapidus* increased the cGMP production in different tissues, including gills (Katayama & Chung 2009), the fact that CHH peptides can activate directly regulatory signal transduction pathways of ion transport in this tissue cannot be discarded.

Even though potassium and calcium are minor ions in *P. vannamei* hemolymph, they can limit shrimp survival since they are considered the essential ions affecting growth, molting, and feeding (Davis et al. 2004). During the experiment, both ions were hyper-regulated in basal shrimp exposed to the three salinities. The potassium and calcium ions concentration increased with salinity, causing a significant reduction of IC values at 26 and 40 ppt salinities. The rCHH-A injection of shrimps submitted to hypo-osmotic conditions reduced the potassium IC at 0.5 h and more strongly at 1 h post-injection due to increased ion

concentration. This response can be associated with the possible changes in ion transport promoted by the hormone since potassium concentrations can be regulated to some extent by NKA activity (Mena-Herrera et al. 2011).

The results obtained in this study corroborate that fluctuation in salinity is an important environmental factor affecting hemolymph calcium levels in crustaceans (Hagerman 1983). The significant reduction observed in IC calcium (1 h at 10 ppt) and the initial increase (0.5 h at 26 ppt) followed by a significant reduction at 2 h suggests that rCHH-A might participate in maintaining physiological homeostasis of *P. vannamei* principally at low salinities. In this sense, Li & Cheng (2012) reported in *P. vannamei* that calcium tends to vary more during the molt cycle at salinities below 34 ppt. Calcium transport occurs in gills, hepatopancreas, and antennal glands through mechanisms that require energy (Ahearn et al. 2004). Then, considering the primary role of CHH peptides in energy reserve mobilization, they could be candidates as endocrine calcium transport regulators.

The role of CHH in osmoregulation has been studied in ablated crayfish *Astacus leptodactylus* and lobsters *Homarus americanus* exposed to different osmotic environments. The eyestalk removal affected the ability to regulate hemolymph OP and ions; meanwhile, the injection of purified CHH peptides improved the osmoregulatory performance of ablated animals (Charmantier-Daures et al. 1994, Serrano et al. 2003). Concordantly, the results obtained in this research study suggest that CHH peptides are essential for white shrimp tolerance when encountering changes in salinity conditions, for example, during the migration or at estuarine habitats (Castille & Lawrence 1981a).

Relationship between metabolism and osmoregulation

Hemolymph osmolarity and glycemia have been proposed as physiological stress biomarkers in invertebrates (Galgani 1987). CHH-mediated hyperglycemia is a typical response of crustaceans to stressful situations (Webster 2015). Glucose is a known energy source that can provide energy for active osmoregulation (Spanings-Pierrot et al. 2000). In this study, in both hypo- and hyper-osmotic conditions, the most significant osmo-ionic changes coincided with the hyperglycemic response observed from 1-2 h post-injection of rCHH-A. This result suggests its possible participation in mobilizing energy reserves to support the active mechanisms for the movement of ions between hemolymph and other tissues. Concordantly, CHH secretion from eyestalks in response to osmotic stress has been shown to occur in the stenohaline lobster *Homarus americanus* exposed to hyper-saline or diluted seawater (Chang 2005) and in *P. vannamei* exposed to low salinity (Shinji et al. 2012a).

Salinity, age, and the physiological state of crustaceans influence -to a different extent- the utilization of dietary protein as an energy source and growth (Shiau et al. 1992). The atomic ratio of oxygen consumed to nitrogen excreted (O/N) in *P. vannamei* shrimp is <40, indicating that amino acids are the predominant energy substrates for crustaceans (Comoglio et al. 2004, Coelho et al. 2019).

Thus, in addition to changes in hemolymph glucose, rCHH-A significantly affected the total protein concentrations in shrimp exposed to all salinity conditions. Even though protein utilization in penaeid shrimp is usually higher at salinities below the iso-osmotic point, an increase in hemolymph total protein concentration was observed at 10 ppt salinity, suggesting its release to hemolymph to lower osmolality from other tissues. This suggestion agrees with Shinji et al. (2012b) studies that showed the release of some amino acids from the muscle tissue to the hemolymph of *P. vannamei* during the hypo-osmotic adaptation to regulate osmotic differences between hemolymph and muscle.

At 26 ppt salinity, total proteins increased during the first half-hour post-injection of rCHH-A and recovered rapidly after one hour. In environmental conditions near *P. vannamei* isosmotic point (25-27 ppt), less osmotic energy is required (Díaz et al. 2001), and carbohydrates are preferentially catabolized over lipids and proteins (Valdez et al. 2008).

The protein concentrations found in shrimp at 40 ppt were the lowest of the experiment. They decreased even more by the effect of rCHH-A injection (1-2 h), suggesting an accelerated utilization of circulating proteins compared to 10 and 26 ppt salinities. This response could allow shrimp to cope with the energy demand caused by acute osmotic stress since the standard metabolic rate of *P. vannamei* has been shown to increase at salinities above and below the iso-osmotic point (Valdez et al. 2008). Penaeid shrimp has been suggested to shift metabolic substrates (carbohydrates, lipids, and proteins) in response to salinity changes (Rosas et al. 1999). These results might also explain those in protein concentrations in the hemolymph of shrimp exposed to hypo-osmotic conditions in this study, in which increases were followed by decreases, suggesting mobilized proteins could have also catabolized at low salinity to enhance energy for osmoionic regulation.

However, under osmotic stress -especially at low salinity conditions- proteins not only can provide metabolic energy for active compensation of NaCl loss but also supply ammonium ions, which can be excreted by NKA instead of potassium cations (Rosas et al. 1999, Díaz et al. 2001, Henry et al. 2012). Ammonium is continually released through the gill epithelium of crustaceans, representing 40-90% of the total excreted nitrogen (Hartenstein 1970).

Maintaining osmolyte concentrations in hemolymph and tissues requires a considerable proportion of total metabolic energy generated by organisms (Hagerman & Uglow 1982). Protein breakdown may also produce organic osmolytes, such as peptides and free amino acids (FAA), as effectors to counteract external osmotic pressure and ion imbalances in tissues and fluids (McNamara et al. 2004). In *P. monodon*, the distribution of FAA in hemolymph and muscle varies with salinity, and it is not the same at high (45 ppt) and low (15 ppt) salinities (Fang et al. 1992). Freire et al. (1995) demonstrated that FAA pools in *Macrobrachium olfersii* in hemolymph respond to the injection of homogenates from eyestalks and other neuroendocrine tissues, suggesting its hormonal control. The mobilization of potential fuels in insects, like carbohydrates and lipids, are known to be hormonally controlled by small peptides like the adipokinetic hormone and the red pigment-concentrating hormone (Gäde 2004).

The transcriptome analysis also deepens knowledge of the molecular mechanisms triggered in *P. vannamei* cultivated under different salinities. Differentially expressed genes related to ion transport and amino metabolism, and energy has been observed when freshwater or low salinity conditions are compared with seawater-cultivated shrimp, suggesting an increased energy demand for osmoregulation and homeostasis (Zhang et al. 2016, Farhadi et al. 2022).

CONCLUSIONS

This study documented the metabolic and osmotic effects of the recombinant CHH-A peptide in *P. vannamei* shrimp acutely exposed to extreme salinities. The recombinant hormone treatment changed hemolymph glucose and total protein levels. In addition to its high hyperglycemic activity, the CHH-A variant proved to mobilize proteins and enhance energy for osmo-ionic regulation in *P. vannamei* to maintain hemolymph composition under extreme hypo- and hyper-osmotic conditions, which allowed the normal physiological and metabolic activities of the shrimp. The results obtained in this study can provide a useful resource for future research on *P. vannamei* osmoregulation and other euryhaline crustaceans. Furthermore, studies need to be performed to understand the osmo- and ionoregulatory mechanisms of different CHH variants in crustaceans exposed to other stress conditions and their relation with the regulation of intermediary energy metabolism. Particularly interesting would be to examine the possible influence of the C-terminal amidated version of the CHH-A variant on the hyperglycemic activity and osmoregulation as it has been observed with other naturally amidated variants (Katayama et al. 2002, Camacho-Jiménez et al. 2017b).

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