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Glycine Betaine Levels and BADH Activity of Juvenile Shrimp *Litopenaeus vannamei* in Response to *Vibrio* Bacterial Infection and Sudden Hyperosmotic Stress

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Abstract: High evaporation rates due to solar intensity and low precipitation could represent a challenging culture environment in northwestern Mexico, generating osmotic stress in shrimp due to high salinity. Bacterial infections by pathogenic *Vibrio* strains are highly virulent in shrimp culture. This study evaluated betaine aldehyde dehydrogenase (BADH) activity and glycine betaine (GB) levels in *Litopenaeus vannamei* under high salinity levels plus experimental infection with virulent *Vibrio parahaemolyticus*. At 35 ppt (control group) and 40 ppt after infection, GB levels increased two-fold in the gills except at 45 ppt and were significantly higher at 50 ppt. The highest GB levels were in the hepatopancreas of the uninfected group at 45 ppt. In the gills, BADH activity decreased after 2 h of exposure at 40 and 45 ppt; at 50 ppt, there was a significant increase in the uninfected groups. However, upon infection, activity increased at all salinities except 50 ppt. In the hepatopancreas of the uninfected groups, the highest activity was at 40 ppt and this was lowest at 50 ppt after 8 h. In the muscles, BADH was detectable at all salinities; infection caused an increase in its activity at 45 and 50 ppt. Despite sudden exposure to high salinity plus experimental infection, our results show that Litopenaeus vannamei does not inhibit BADH activity, allowing GB synthesis, which may play a role in shrimp survival under these conditions.

Keywords: glycine betaine; betaine aldehyde dehydrogenase; *Litopenaeus vannamei*; *Vibrio parahaemolyticus*; salinity

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1. Introduction

Shrimp aquaculture has become an important industry worldwide, providing direct and indirect job sources leading to national and regional socio-economic benefits. However, this industry confronts several challenges, such as improving its practices to meet environmental regulations and controlling disease outbreaks [1,2]. Additionally, some of these farming premises are subject to extreme weather conditions, like the shrimp farms located in Northwest Mexico, a region with very high environmental temperatures and low rain precipitation, generating high salinities in their ponds. Salinity is an important environmental variable in aquatic ecosystems that plays a key role in the growth, immunity, osmolality, and survival of crustaceans [3,4]. Recently, several published studies have shown that high salinity stress has a significant effect on antioxidant activity as the expression levels of antioxidant-related genes in gill tissues were significantly increased [4,5]. Furthermore,

acute salinity stress increases the concentration of reactive oxygen species (ROS), resulting in oxidative stress in aquatic animals [6,7].

The shrimp species *Litopenaeus vannamei* is distributed in tropical marine environments of the Eastern Pacific coast of North, Central, and South America [8], and they are being farmed worldwide given their reasonable growth rate, some genetic lines of disease resistance, and osmoregulatory abilities, amongst other physiological performances. *Litopenaeus vannamei* undergo a biphasic life cycle, meaning pelagic larval stages known as nauplius, zoea, and mysis are followed by benthonic decapodid, juvenile, and adult stages. The osmoregulatory capabilities of this species have allowed their farming in a vast water salinity range from freshwater to water at 50 ppt salinity [9,10].

One of the strategies used by aquatic animals to cope with acute high salinity exposure, is the accumulation of organic solutes (polyhydric alcohols, free amino acids, and quaternary ammonium compounds). One of these types of molecules is glycine betaine (GB), that is used as a non-disturbing osmolyte by plants, bacteria, invertebrates, and vertebrates to compensate for hypertonic stress [11]. Furthermore, GB is one of the main osmolytes accumulated in the whiteleg shrimp in response to osmotic stress [12]. GB is a quaternary ammonium compound that accumulates to protect cells or tissues during osmotic stress [13]. GB has different functions in living organisms; it can act as a methyl donor for methionine synthesis [14,15] or as an osmoprotectant, protecting proteins against salt denaturalization [16,17]. In fish and marine invertebrates such as horseshoe crabs and oyster, GB synthesis takes place mostly in osmoregulatory tissues regulated by ionic concentration in the external milieu [18–21]. In several organisms, GB is synthesized by the irreversible oxidation of betaine aldehyde by the enzyme betaine aldehyde dehydrogenase (BADH EC 1.2.1.8) [22–24]. In *Litopenaeus vannamei*, under osmotic stress, the hepatopancreas and gills showed BADH activity [25,26]. However, its expression is modulated in a tissue-specific manner when salinity varies [27]. These results suggest a critical participation of BADH in the osmotic-stress response of *Litopenaeus vannamei*. However, despite these physiological adaptations to high salinities, the crustacean's physiological ability to cope with high salinities within a commercial shrimp farming pond may be impaired by bacterial infections, which are relatively common within the farming ponds. Shrimp acute hepatopancreatic necrosis disease (AHPND) is considered to be a relatively new farmed-penaeid-shrimp bacterial disease [28] caused by virulent strains of *Vibrio parahaemolyticus* and related *Vibrio* species. AHPND-associated mortalities occur early in the production cycle, usually within 30 to 35 days of stocking, and because of this, AHPND was initially referred to as early mortality syndrome [29]. Since 2009, AHPND has progressively spread as an epidemic, devastating shrimp production across much of the shrimp farming region in Asia. Eventually, the disease reached the Western Hemisphere [30,31]. In the Northern states of Mexico, including Nayarit, Sinaloa, and Sonora, a *Vibrio parahaemolyticus* (*Vp*) strain caused acute hepatopancreatic necrosis disease (AHPND) and dropped the shrimp farming production by 65% when it was first detected in 2013 [30].

Therefore, the objective of this work was to evaluate some key osmoregulatory responses of the juvenile *Litopenaeus vannamei* at sudden high salinities under an AHPND *Vibrio* bacterial infection.

2. Materials and Methods

2.1. Animal Maintenance

Litopenaeus vannamei postlarvae (0.0065 ± 0.009 g) were obtained from a local larval production laboratory and were transported by car inside plastic bags half-filled with seawater inside rigid insulated polystyrene boxes. The total transport time was 2 h, and all postlarvae arrived at the CIAD experimental facilities in good shape with 100% survival.

At CIAD, PL were transferred to eight 400 L fiberglass containers connected to a closed circulation system and were grown up until they reached the average target size of 1.3 g. The animals were fed Api Camarón® Raceway five times daily during this time. Water quality was monitored daily and kept at 28 °C, 35 ppt salinity, and 5.6 mg O_2. In addition, during this time, constant monitoring of the primary pathogens that affect shrimp (white spot syndrome virus; *Hepatobacter penaei*; Infectious hypodermal and hematopoietic necrosis virus; *Enterocytozoon Hepatopenaei*; Taura syndrome virus and Acute hepatopancreatic necrosis disease) was carried out to ensure that the postlarvae population remained free of pathogens before being used.

2.2. *Vibrio parahaemolyticus* Strain

Vibrio parahaemolyticus (*Vp*) is a Gram-negative halophilic bacterium, slightly curved with a polar flagellum, that does not ferment sucrose, so on TCBS agar (citrate thiosulfate bile salts sucrose), it grows in circular, colorless colonies with a green center of 2 to 3 mm [32]. The *Vibrio* bacterial strain used (HP19-21) to carry out the bacterial infection was isolated from shrimp hepatopancreas during mortality events in the 2021 farming season on TCBS agar and subsequently identified by molecular testing using the primers shown in Table 1.

Table 1. Primer sequence for amplification of *Vibrio* and virulent genes.

Primer Name	Target Species	Primer Sequence (5′-3′)	Amplicon Size	Reference
*Vp*tl-450-F *Vp*ttl-450-R	*V. parahaemolyticus*	AAAGCGGATTATGCAGAAGCACTG GCTACTTTCTAGCATTTTCTCTGC	450 bp	[33]
*Pir*A-F *Pir*A-R	AHPND toxin	TGACTATTCTCACGATTGGACTG CACGACTAGCGCCATTGTTA	284 bp	[34]
*Pir*B-F *Pir*B-R		TGATGAAGTGATGGGTGCTC TGTAAGCGCCGTTTAACTCA	392 bp	

2.3. *Vibrio* Bacterial Infection

Additionally, the selected *Vp* strain (HP19-21) was used to perform a preliminary bioassay only at the control salinity (36 ppt) to evaluate the mortality rates in juvenile shrimps following an immersion infection protocol [35] to ensure that the selected *Vp* strain was a pathogenic *Vibrio* strain.

For the infection exposure to *Vp*AHPND at all salinities tested, the HP19-21 pathogenic strain was cultured for 24 h in TSB at 37 °C, and the bacterial growth was followed by the OD600nm using a spectrophotometer. An adaptation of an immersion infection protocol was followed [35]. Briefly, juvenile shrimps were immersed with the *Vp* strain at a final cellular density of 5×10^6 CFU mL^{-1} for two hours at each salinity tested.

2.4. Hyperosmotic Stress (Infection Experimental Groups and Control)

According to salinity levels measured over some years at commercial shrimp farms in Northwest Mexico, we selected salinities of 40, 45, and 50 ppt as the conditions for sudden hypersalinity stress [36]. The hyperosmotic stress was added by using instant ocean salts to control seawater (35 ppt) to reach the targeted high salinities. Four experimental infection groups, including 15 healthy shrimps (1.3 ± 0.2 g) per group, were placed in 20 L polyethylene containers for each salinity treatment (35, 40, 45, and 50 ppt) in triplicate and *Vp* was added for 2 h immersion at the bacterial *Vp* density (5×10^6 CFU mL^{-1}); these groups were named as 2 h post-infection (2H *pi*). After 2 h of *Vp* exposure, animals were transferred to salinity containers (35, 40, 45, and 50) without *Vp* and left for 6 h and named as 8 h post-infection (8H pi). The four uninfected experimental groups were also exposed

for two hours to seawater without *Vp* at each salinity level tested, named as 2 h control (2H C), and then transferred into 20 L polyethylene salinity containers for 6 h and named as 8 h control (8H C).

2.5. Animal Sampling

Two sampling times were used: first, after 2 h of exposure to both *Vp*/high salinity (2H *pi*) and 2 h of exposure to high salinity (2H C); and second, after 6 h exposure to high salinities with 2 h exposure to *Vp* (8H *pi*) and after 6 h exposure to high salinities with 2 h exposure to high salinities (8H C). At each sampling time, the gills, hepatopancreas, and muscle (abdominal segment 1) tissues were collected, frozen immediately on dry ice, and stored at −80 °C for further analysis.

2.6. Crude Extract Preparation

Crude extracts of hepatopancreas, gills, and muscle abdominal segment 1 tissues were prepared to quantify glycine betaine and BADH activity using the protocol outlined in [26]. A total of 0.1 g of tissue was homogenized with 500 µL of extraction buffer (0.1 M Tris-HCl, pH 8.5, 1 mM EDTA, 10% (*v*/*v*) glycerol, 14 mM β-mercaptoethanol, 10 µL of protease inhibitor, and 100 µL of PMFS), then centrifuged at 13,000 rpm for 20 min at 4 °C and the supernatant was separated. Each value (n) represents the median of 3 animals individually analyzed per container, and each experimental group was composed of 3 containers. Therefore, the total number of experimental units analyzed per treatment was 9.

2.7. Glycine Betaine Quantification

A water-soluble quaternary ammonium compound (QAC) assay measured the glycine betaine concentration in crude extracts (hepatopancreas, gills, and muscle abdominal segment 1) [37]. Raw extracts (0.5 mL) were diluted with 0.5 mL of 2 N H_2SO4 to evaluate the total QAC levels. After adding 0.2 mL of 1 M KI-I2 to the diluted extracts, samples were incubated for 16 h at 4 °C. This was followed by centrifugation at 10,000 rpm for 20 min, and the supernatant was separated and discarded. The periodide crystals were dissolved in 1,2-dichloroethane and incubated for 2 h at 4 °C. Additionally, the choline concentration in samples was quantified. A total of 0.5 mL of KPi buffer (0.2 M, pH 6.8) was used to dilute the sample extracts (0.5 mL). As previously described, choline periodide was precipitated and analyzed for total QAC. Total QAC and choline concentrations were measured at 365 nm. Calculations for the glycine betaine concentration used the following formula: [Glycine betaine] = [QAC] − [Choline].

2.8. BADH Activity Assay

BADH activity was measured spectrophotometrically by monitoring the reduction of NAD+ by the increase in extinction at 340 nm, following the methodology in [38]. The measurement was performed using 0.1 M Hepes-KOH, pH 8.0, 0.1 mM EDTA, 0.5 mM betaine aldehyde, and 1.0 mM NAD in a final volume of 0.4 mL. The reaction started by adding 16 µL of the extract. The enzymatic activity of BADH was expressed as mU/mg of protein. The protein concentration was quantified using Bradford's method, using BSA as a standard [39].

2.9. Statistics

An analysis of variance (ANOVA) was performed on all data using the NCSS (2020) software. A completely randomized design was used to evaluate the effect of salinity and experimental infection on the glycine betaine concentration and BADH activity in each tissue using Tukey–Kramer with a significance level of 5%.

3. Results

Figure 1 shows PCR amplicons from the *Vibrio parahaemolyticus* AHPND strain isolated from shrimp hepatopancreas (HP19-21): 1A: Lane M; molecular weight; Line 1 and (+) positive amplicons (450 bp) for *Vibrio parahaemolyticus*; and 1B: Lane M; molecular weight; Line 1 and (+) positive amplicons (284 and 392 bp) for AHPND.

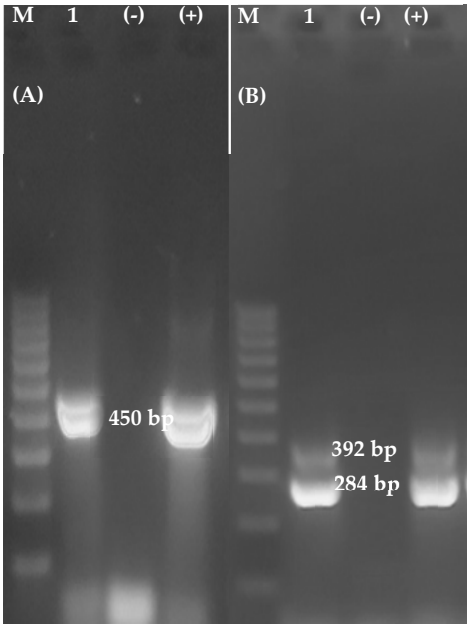


Figure 1. Electrophoresis. (**A**) *Vibrio parahaemolyticus*; (**B**) AHPND M: 100 bp DNA marker, 1: HP19-21; (-): negative control; and (+): positive control.

Analysis of the mortality values (Figure 2) revealed that after 42 h of exposure to *Vp*AHPND HP19-21, juvenile shrimp reached the highest 60% cumulative mortality, maintaining a final survival rate of 40%. When exposed to all salinities tested in this study, the juvenile-shrimp cumulative mortalities were less than 15% after 2 h of exposure to *Vp*AHPND HP19-21 and 0% during the 6 h of exposure to all salinity values tested. In the control group, the sudden salinity changes up to 2 h show that the GB content increases to about twice its levels in the gills at all salinities tested vs. 35 ppt (Figure 3A). However, when coupled with the *Vp* infection time (up to 2 h), GB increased to more than twice its level in the same tissue only at 50 ppt.

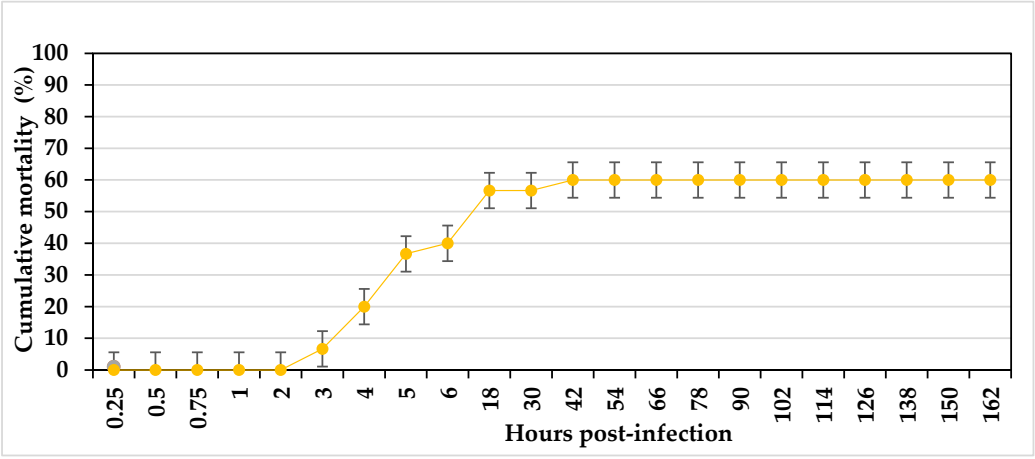


Figure 2. Cumulative mortality for juvenile *Litopenaeus vannamei* exposed to VpAHPND HP19-21 (5×10^6 CFU mL^{-1}).

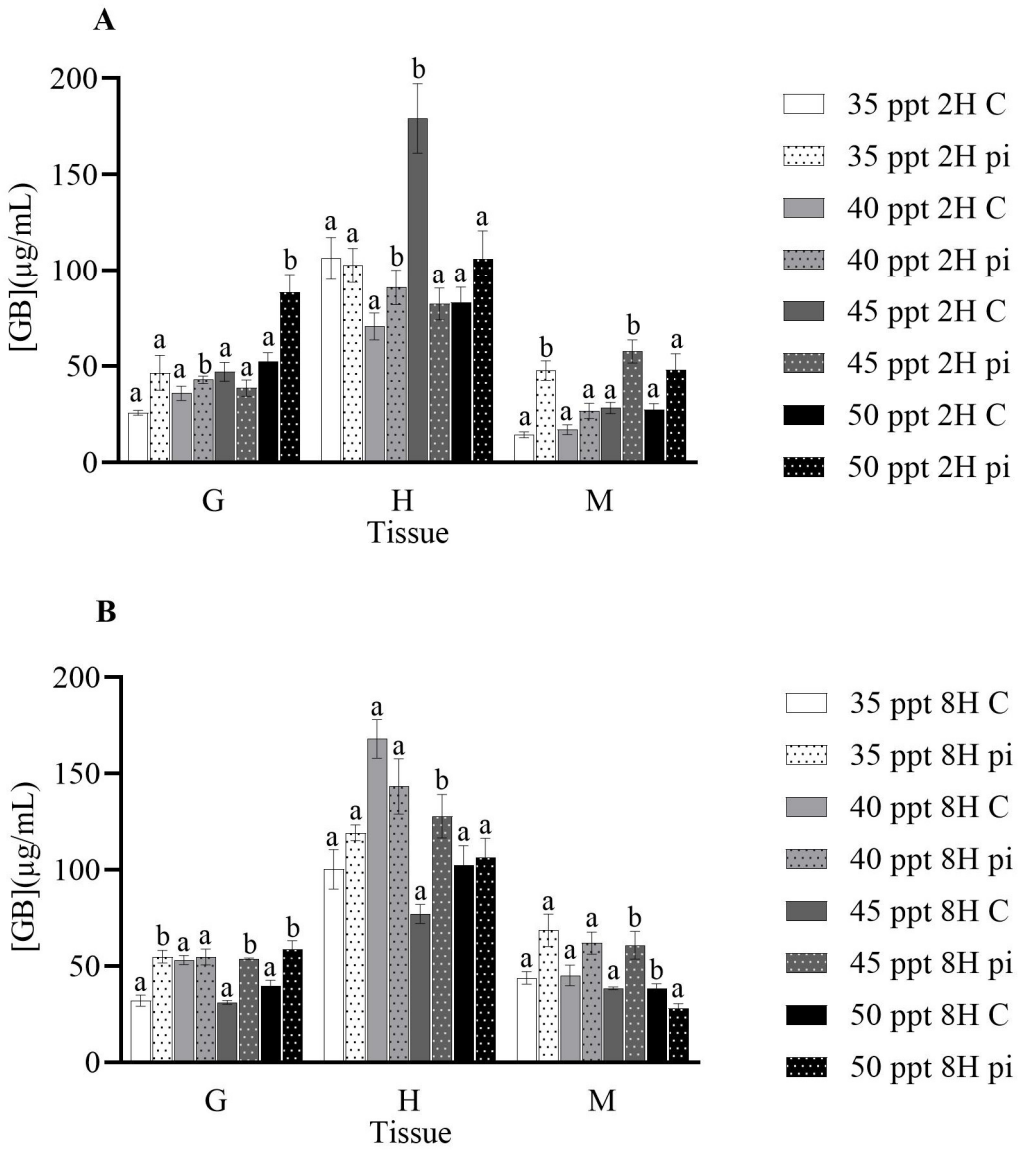


Figure 3. Glycine betaine levels in *Litopenaeus vannamei* gills (G), hepatopancreas (H), and muscles (M), exposed to 2 h sudden high salinities without *Vp* infection (2H C); sudden high salinities and 2 h *Vp* exposure (2H *pi*) (**A**), and 6 h exposure to high salinities: 8H C and 8 h exposure to high salinities plus *Vp* infection 8H pi (**B**). Data represent the mean ± SE. Different letters indicate significant differences between the control and experimental infection groups per salinity $n = 9$, $p < 0.05$.

The highest GB level (179.16 µg mL^{-1}) was recorded in the hepatopancreas of the uninfected group at 45 ppt and this is significantly higher than the GB levels in all of the treatments in those tissues. Regarding the muscle GB in the uninfected groups, the lowest level was found at 35 ppt and the highest at 45 ppt. In the muscles, the combination of salinity and infection illustrated that the GB content increased under all of the salinity conditions (Figure 3A).

In the uninfected groups, where shrimp were exposed to sudden high salinities and kept for 8 h total exposure (Figure 3B), there were slightly higher GB concentrations compared to those found at 2 h (Figure 3A), in the gills, hepatopancreas and muscles at 35, 40; 40, 50; and 35, 40, 45, and 50 ppt, respectively (Figure 3B). Muscle analysis showed that the GB content did not change in all of the salinities tested (Figure 3B). On the other hand, the sudden high salinity exposure plus 8 h *Vp* post-infection increased the GB content in the gills at 35, 45, and 50; in the hepatopancreas, GB increased under all salinity conditions except at 40 ppt; and in the muscle tissue, GB increased at all salinities except 50 ppt

(Figure 3B). BADH activity data in the uninfected groups showed that 2 h exposure to sudden high salinity caused the enzyme activity in the gills to decrease at 40 and 45 ppt, while at 50 ppt there was a significant increase in the BADH activity (Figure 4A). *Vibrio parahaemolyticus* infection caused an increase in enzyme activity at all salinities except at 50 ppt in the gills. Hepatopancreas BADH activity was higher than BADH activity in the gills and muscle; in this tissue, the activity decreased at 45 and 50 ppt vs. the activity at 35 ppt; under *Vp* infection conditions, BADH activity was lower at all salinities tested and was significantly lower at 35, 40, and 50 ppt (Figure 4A). In the muscle tissue, BADH activity was very low under all conditions tested (Figure 4A). After 8 h exposure to high salinities, an increase in the BADH activity was detected in the gills in the uninfected groups at 35 and 40 ppt, whereas combined with the *Vp* infection, it decreased at 35, 40, and 50 ppt (Figure 4B). In the hepatopancreas, the enzyme activity significantly increased only at 40 ppt but decreased at 45 and 50 ppt. The combination of salinity and infection caused higher enzyme activity, except at 40 ppt (Figure 4B). In the muscle tissue, the activity was detectable at all salinities; however, the infection increased its activity at 45 and 50 ppt (Figure 4B).

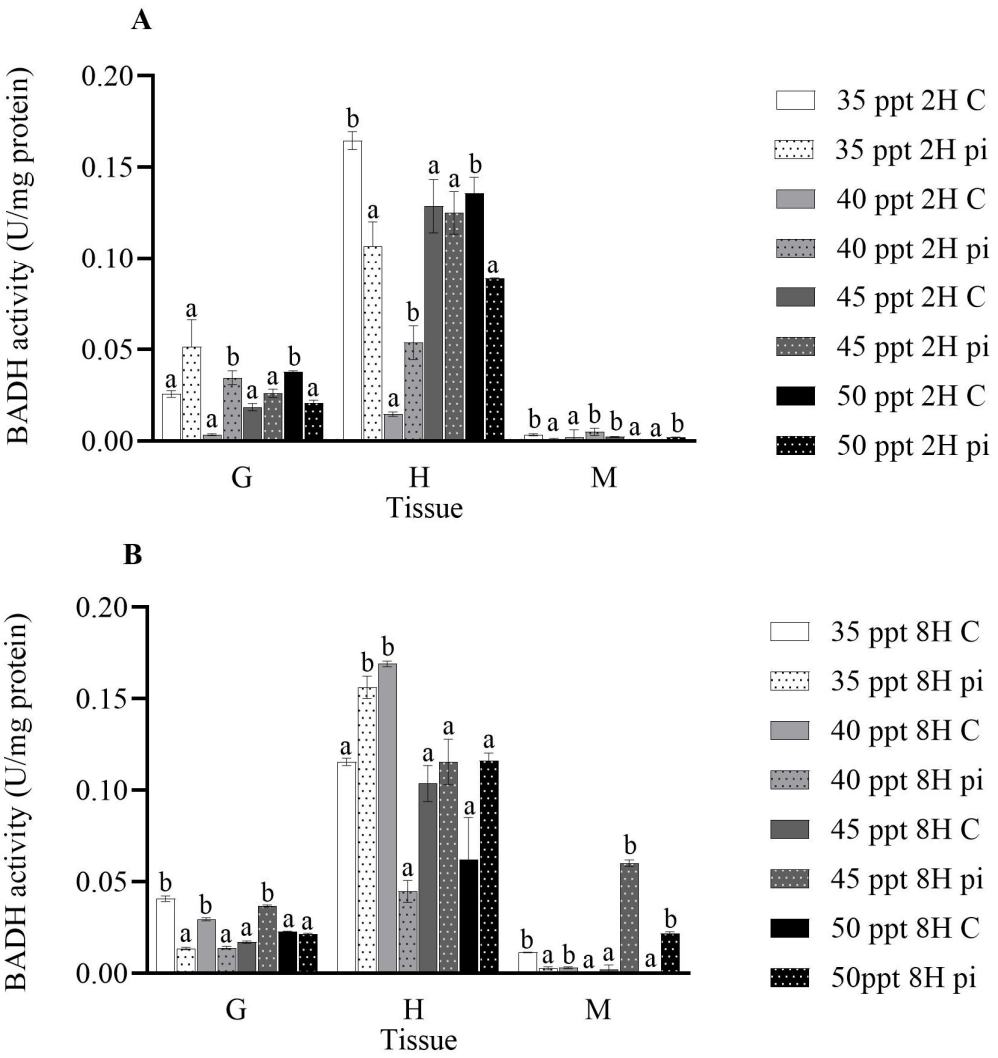


Figure 4. Effect of sudden high salinity exposure plus experimental *Vibrio parahaemolyticus* infection on betaine aldehyde dehydrogenase activity in *L. vannamei* gills, hepatopancreas, and muscle; (**A**) 2 h *pi*, (**B**) 8 h *pi*. Data represent the mean ± SE. Different letters indicate significant differences between the control and experimental infection groups per salinity $n = 9$, $p < 0.05$.

4. Discussion

The highest GB content was detected in the hepatopancreas tissue under all salinities tested and this pattern remained after 2 and 8 h *pi*. The gills and hepatopancreas are the primary osmoregulatory tissues in crustaceans [40–42]. Therefore, high GB levels agree with the hepatopancreas function; similar results were found by the authors in [26]. Furthermore, GB levels found following sudden exposure to higher salinities, in general, evoke an increased GB content in all tissues compared with the control salinity (35 ppt). This result might be associated with the osmoprotective role that GB plays in the cells of some crustaceans when they are exposed to hyperosmotic stress [43,44]. The accumulation of osmotically active compatible solutes, such as GB, is used by some organisms to assist growth and also to stabilize macromolecules against hyperosmotic stress [20].

Moreover, with the intensification of shrimp aquaculture practices, new bacterial pathogens have been detected in the farming environments, causing high mortalities, since some *Vp* isolates appear to be extremely virulent [30,35,45]. The virulence of *Vp* is variable depending on the virulence determinants [46,47]. As with some marine pathogens, *Vp* strains might become virulent by acquiring a plasmid that expresses toxins causing hepatopancreatic damage to the shrimp and resulting in high mortalities [30,48]. The virulence of the *Vp* strain used in this study is due to the pirA and pirB genes, causing acute hepatopancreatic necrosis disease (AHPND) and mortality rates in shrimp.

The shrimp farms in Northwest Mexico are located in regions where the intense solar incidence of the dessert areas give rise to high evaporation rates of the shrimp ponds, thereby increasing the salinity and generating a challenging farming environment. In this context of high salinities plus *Vp* infection, it is interesting to note that despite the *Vp* infection in the animals, GB levels accumulated in all tissues, particularly in the gills and muscle tissue, after 2 and 8 h *pi* at all salinities; however, this effect was not observed in the hepatopancreas.

BADH activity was higher in the hepatopancreas than in the gills, while in general, it was very low in muscle tissue under all conditions tested. Similar results were found by the authors of [26] in shrimp subjected to different salinities. *Vp* infection increased BADH activity only in the gills after 2 h of exposure. However, in the hepatopancreas, the BADH activity detected allowed the synthesis and accumulation of GB at higher levels than in the gills. A longer post-infection time decreased the BADH activity in the gills but not GB accumulation. These results demonstrating an increase in muscle and gill GB levels without an increase in BADH activity may be due to the transport of GB from the hepatopancreas into the muscle and gills since the authors of [26] found GB in the hemolymph and increases in GB concentration in all tissues analyzed in response to salinities higher than 35 ppt (seawater).

Interestingly, in the gills and muscle tissue at 35 ppt (control salinity) only the *Vp* infection induced increases in GB concentration, especially after 8 h of exposure to the *Vp* pathogen.

It has been reported that BADH might be involved in the response to the effects provoked by the white spot syndrome virus (WSSV) infection [49]. In this context, our results indicate that BADH might also play a role during a bacterial (*Vp*) infection. BADH activity increased under the control salinity (35 ppt) after 2 h *Vp* infection, probably causing the high GB levels detected in the gills and muscle tissue of the infected group at the control salinity. Osmotic stress induces adverse effects on cellular ion regulation, which may disrupt protein synthesis and damage [50,51]. Furthermore, the sudden high salinity stress with a pathogenic *Vp* exposure that might occur in the shrimp farms in Northwest Mexico exerted an osmoregulation mechanism through the BDAH activity and GB synthesis. Thus, our results have shown that despite the sudden exposure to higher salinities and

Vp infection, juvenile *Litopenaeus vannamei* shrimp keep their BADH activity, allowing GB synthesis. Hence, using the host–pathogen high salinity studies, further enzymatic antioxidant system and osmoregulatory studies are still needed for adequate response characterization to support the sustainable development of shrimp aquaculture.

5. Conclusions

We have described a bioassay challenge that identified BADH activity and GB synthesis in juvenile *Litopenaeus vannamei* shrimp regulated by osmotic stress and *Vp* exposure. In combination with different high salinity levels, *Vp* infection does not inhibit BADH activity, allowing GB synthesis, which may protect shrimp cell integrity and thus enable shrimp survival.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/aquacj5010004/s1, Figure S1: Electrophoresis pirAB. M: 100 bp DNA marker, 1: HP19-21, 2: P04-22, 3: P09-22, 4: P15-22, 5: P17-22, 6: P19-22, 7: P22-22, 8: P26-22, 9: P28-22, 10: P32-22, 11: P44B-22. (-): Negative control and (+): Positive control.; Figure S2: Cumulative mortality of strains of Vibrio parahaemolyticus and Vibrio alginolyticus.

Author Contributions: S.G.-J. and L.A.G.-A. designed the experiments; S.G.-J. and E.M.V.-S. discussed the results; S.G.-J. wrote the paper original draft; E.M.V.-S. executed data curation; J.C.Z.-A. and L.A.G.-A. conducted the experiments; C.M.-B. and J.C.Z.-A. performed all laboratory analysis and L.A.G.-A. and J.C.Z.-A. assisted with proofreading. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The white shrimp, *Litopenaeus vannamei*, is not considered to be an endangered or protected species and is widely farmed alongside coastal zones. Therefore, no specific authorization was required to work on the white shrimp used in this study.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflict of interests.

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