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ORIGINAL ARTICLE

Dietary potassium partially compensates the requirement of aqueous potassium of *P. vannamei* reared in medium saline inland groundwater

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Abstract

A 15-day trial was conducted to determine the effect of dietary potassium on the requirement of aqueous potassium in *P. vannamei* under inland saline water (ISW). Two experimental diets viz. control (C) and control with 1% KCl (K) were fed to *P. vannamei* as six treatments viz. FW (reared in freshwater and fed on C), ASW (reared in artificial seawater and fed on C), ISW (reared in inland saline water and fed on C), ISW+eK (reared in potassium fortified ISW and fed on C), ISW+fK (reared in ISW and fed on K) and ISW+½eK+fK (reared in 50% K fortified ISW and fed on K). The samples were collected on selected intervals (0^{th} day, 1^{st} day, 7^{th} day and 15^{th} day), and mortality was recorded continuously. Total mortality (100%) was observed in FW and ISW. 100% survival was recorded in ASW and ISW+½eK+fK, while a lower survival was observed in ISW+fk. The haemolymph osmotic and ionic concentrations were lowest in FW and ISW. The principal ions (Na^+, K^+ and Cl^-) and osmolality of ISW+½ eK+fK were restored to the level of ASW within 15 days. Na^+K^+ATPase activity was increased in ISW while lowered by K supplementation. HSP70 expression was upregulated in ISW fortified with K partially or entirely. However, the groups reared in ISW and ISW supplemented with feed potassium alone could not enhance HSP protection up to the level of ISW+eK and ISW+½ eK+fK groups. Overall, 50% of aqueous potassium can be compensated with 1% dietary KCl, without affecting survival and ionic homeostasis.

KEYWORDS
HSP70, Inland saline groundwater, Ions, *P. vannamei*, Sodium-potassium ATPase

1 | INTRODUCTION

Salinization has affected agriculture, environment and water resources negatively in various countries such as United States, Australia and India (Fielder et al., 2001). Worldwide, approximately 380 million ha of land had become unsuitable for agriculture due to soil and groundwater salination (Lambers, 2003). Inland saline groundwater (ISW) differs in its specific ionic concentration from seawater (Forsberg & Neill, 1997). The ionic concentration of ISW varies by location and type of soil due to uneven salt precipitation (Gong et al., 2004). ISW usually contains more calcium (Ca^{2+}) and less potassium (K^+) in the Indian sub-continent (Jain et al., 2002). There is a scope for culturing aquatic animals using saline groundwater. Mariculture, rather than the usual agriculture, has been

recommended for improving the productivity of the salt-affected lands (Payne, 1983; Stickney, 1981) and the economic loss of farmers due to low agricultural productivity from salinized farms. ISW aquaculture is not subjected to tides, storms, pathogens and parasites (Partridge, 2008), which reduces or eliminates the disease outbreak and hence produces disease-free stock

P. vannamei (Pacific white shrimp) is indigenous to the Pacific coast from Northern Peru to Mexico and is species of choice for cultivation in ISW due to its euryhaline nature (Re et al., 2012). However, low potassium levels (5 – 15 mg L^{-1}) in ISW affect the osmoregulatory capacity, growth and survivability of shrimps (Boyd et al., 2007; Davis et al., 2005). Potassium is directly involved in the activation of sodium-potassium ATPase / Na^+/K^+ ATPase, (Mantel & Farmer, 1983) which is the essential ion transporter involved in osmoregulation. Na^+/K^+ ATPase is situated in the gill epithelium at the basolateral surface (Lucu & Towle, 2003). Inadequacy of K^+ concentration can potentially counter the ability to osmoregulate (Bursey & Lane, 1971). Na^+/K^+ ATPase helps in the movement of Na^+ out of the cells and K^+ into the cells with the help of ATP. Therefore, Na^+/K^+ ATPase influences fluid volume significantly. High mortalities in white pacific shrimp (*P. vannamei*), western king prawn (*Penaeus latisulcatus*) and black tiger prawn *(Penaeus monodon*) post-larvae were found due to the low K^+ concentration (McGraw & Scarpa, 2003; Prangnell & Fotedar, 2005). An imbalance in the haemolymph ionic concentration particularly K^+ and Na^+ affects ATPase activity and may lead to mortality (Prangnell & Fotedar, 2005). Osmotic stress due to ionic imbalance leads to the expression of the stress-induced proteins such as antioxidant enzymes and HSP70. HSP70 is a cellular protecting protein synthesized by erythrocytes constitutively and induced by environmental stress. HSP70 acts as molecular chaperons, and prevents misfolding and aggregation of proteins. The antioxidant enzymes also referred as stress enzymes mainly include superoxide dismutase (SOD), catalase and glutathione peroxidase. Among these, SOD is the enzyme which is first activated during stress.

Marine vertebrates and invertebrates can be successfully cultured in ISW only if the deficiency of minerals (K^+) is eliminated by fortifying in water or through dietary supplementation. K^+ is a monovalent ion that gets transported mainly through body surface and gills, making its fortification in the culture water more adequate. However, some authors suggested intestinal potassium transporters involved in symport with nutrients in shrimps (Castagna et al., 1998; Simmons et al., 2012). In this respect, the scope of supplementing potassium in the feed also becomes meaningful as it can reduce the pressure on the environment when receiving additional salts through the KCl addition. Commercially available muriate of potash (KCl) is commonly used to augment the K^+ levels in ISW. The supplementation of K^+ in the diet of shrimps reared in ISW could enhance the osmoregulatory capacity and can potentially become environment friendly and cost-effective strategy (Roy et al., 2010). Although there were attempts to supplement KCl in the diet of *P. vannamei*, Davis, Saoud, & Henry, 2007; Jahan et al., 2018) in inland saline waters, there are hardly any studies on the effect of dietary potassium on the requirement of environmental potassium in a medium saline (10 ppt) inland groundwater. Thus, the current research was planned to evaluate the effect of the addition of potassium in the diet of *P. vannamei* cultured in ISW by replacing the environmental potassium.

2 | MATERIAL AND METHODS

2.1 | Ethics statement

The study conducted was in full compliance with the existing legislation on animal welfare in India, following the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Ministry of Environment & Forests (Animal Welfare Division), Govt. of India, on care and use of animals in scientific research.

2.2 | Experimental site and design

The experiment was conducted on *P. vannamei* juveniles at the central wet laboratory at ICAR-Central Institute of Fisheries Education, Rohtak, Haryana, to study the effects of K^+ supplementation to practical diets of *P. vannamei* reared in low saline (10 ppt). The experimental shrimps were obtained from Jajar farm, Haryana, and were kept in a 2000 L capacity FRP tank for two weeks. During acclimatization, shrimps were fed with a commercial diet (crude protein 35%) without supplemental potassium. The medium used for the rearing of shrimps was of three types – inland saline groundwater original (ISW) and fortified (ISW+eK, ISW+½eK), artificial seawater (ASW) and freshwater (FW). The inland saline water of 10 ppt was taken from borewell and stored in 12000 L storage tanks. ASW preparation was prepared according to the composition given by Kester et al., 1967 (sodium chloride −6.8 gL^{-1}, sodium sulphate −1.14gL^{-1}, potassium chloride −0.19gL^{-1}, sodium bicarbonate - 0.056gL^{-1}, potassium bromide −0.028gL^{-1}, boric acid- 0.007gL^{-1}, calcium chloride −0.42gL^{-1} and magnesium chloride - 3gL^{-1}).

The experiment was conducted for 15 days. The experimental set up consisted of triplicate groups for each treatment. The treatments were FW (group reared in freshwater and fed with control diet), ASW (group reared in artificial saline water with control diet), ISW (group reared in inland saline water (10 ppt) and fed with control diet), ISW+eK (group reared in Inland saline water (10 ppt) fortified with 100% of K^+ as that of ASW and fed with control diet), ISW+fK (group reared in Inland saline water (10 ppt) and fed with a diet supplemented with 1%KCl) and ISW+½eK+fK (group reared in Inland saline water (10 ppt) fortified with 50% of K+ compared to ASW and fed with a diet supplemented with 1% KCl). The inland saline water of 10 ppt was selected as this is the most common salinity encountered in the inland saline region of India. Other treatments such as freshwater (FW) and artificial sea water (ASW) were selected because they are the standard rearing environments for commercial aquatic species. ASW was

considered to have a balanced ionic composition, which taken as a positive control. The 100% potassium fortification of ISW is a common practice followed by farmers in the region.

2.3 | Analysis of physico-chemical parameters from water

Water quality parameters, such as dissolved oxygen, temperature, pH, free carbon dioxide, carbonate hardness, ammonia-nitrogen and nitrite-nitrogen, were recorded every fortnight during the experimental period and was analysed following the standard methods (APHA, 2005). A Flame Emission Photometer (Electronics, India) was employed to determine potassium content in water. The photometer was operated according to the manufacturer's instructions. A blank solution (containing no potassium) and potassium calibration standards of 2, 4, 8 and 10 mg potassium per litre were made. The instrument was fixed at zero with the blank solution. The emission was determined at 766.5 nm, and the calibration curve was prepared.

2.4 | Diet preparation

All of the ingredients mentioned in Table 1 were accurately measured and kept in plastic trays. After the addition of water, the preweighed ingredients were combined to form a dough. The dough was put in an autoclave bag and kept in a pressure cooker for cooking/ steaming for about twenty minutes. The steamed dough was taken out and cooled. The required amount of the oils, BHT, vitamins and minerals mixture were added to the dough. In addition to these ingredients, KCl was added to the dough. All the components were correctly mixed, and the dough was pressed through a pelletizer whose diameter of the dye was 1 mm to produce standardized sized pellets. The pellets were air-dried and packed in airtight containers. The containers were marked as per the treatments.

2.5 | Proximate analysis of diets

The crude protein (CP), ether extract (EE), ash content and crude fibre (CF) of the experimental diets were determined by using standard methods (AOAC, 1995). The nitrogen-free extract (NFE) of the diets was estimated using the following equation:

NFE (%) =100 – [CP (%) + EE (%) + Ash (%) + CF (%)].

Digestible energy (DE) was estimated by using the following equation (Halver, 1976):

DE (kcal/100 g) = {CP (%) X4 + EE (%) X9 +NFE (%) X4}.

2.6 | Survival (%)

Based on the number of shrimps, survival was determined at the end of the experiment by using the following equation:

TABLE 1 Formulation and proximate composition of different experimental diets (isonitrogenous) fed to *P. vannamei*

Ingredient composition (%)	Diets	
	D_1	D_2
Fish meal	15	15
Shrimp waste meal	5	5
Defatted soybean	30	30
Gelatin	5	5
Wheat flour	14	14
Starch	16	16
Fish oil	3	3
Vegetable oil	3	3
BHT[a]	0.02	0.02
Vit-Min[b]	1.5	1.5
Stay C	0.1	0.1
Choline chloride	0.2	0.2
Lecithin	0.5	0.5
Cholesterol	0.2	0.2
CMC[c]	2	2
Cellulose	4.48	3.48
KCl[d]	0	1
Total	100	100
Proximate composition (on dry matter basis)		
Dry matter (%)	90.4 ± 0.03	89.8 ± 0.05
Crude protein (%)	36.16 ± 0.05	36.21 ± 0.01
Ether extract (%)	6.71 ± 0.2	6.91 ± 0.4
Nitrogen-free extract (%)	40.91 ± 0.31	40.57 ± 0.45
Crude fibre (%)	6.48 ± 0.1	6.52 ± 0.4
Total ash (%)	9.75 ± 0.5	9.80 ± 0.0
Digestible energy (Kcal/100 g)	394.55 ± 1.0	395.35 ± 2.8

D1 – Control diet, D2 – Control diet +KCl.

[a]Butylated hydroxy toluene.; [b]Composition of vitamin mineral mix (PRE-EMIX PLUS) (quantity/kg) –Vitamin A, 55,00,000 IU; Vitamin D3, 11,00,000 IU; Vitamin B2, 2,000 mg; Vitamin E, 750 mg; Vitamin K, 1,000 mg; Vitamin B6, 1,000 mg; Vitamin B12, 6 mcg; Calcium pantothenate, 2,500 mg; Nicotinamide, 10 g; Choline Chloride, 150 g; Mn, 27,000 mg; I, 1,000 mg; Fe, 7,500 mg; Zn, 5,000 mg; Cu, 2,000 mg; Co, 450L- lysine, 10 g; DL- Methionine, 10 g; Selenium, 125 mg.; [c]Carboxy methyl cellulose.; [d]Supplemental Potassium chloride.

Survival(%) = Total no. of animal harvested / Total no. of animal stocked X 100.

2.7 | Sample collection

Sampling was done on the 0, 1, 7 and 15th day of the experiment. As the mineral absorption, metabolism and resultant physiological effects are acute changes, a time point study of 2 weeks is deemed sufficient for understanding the physiological consequences. Three

TABLE 2 Primer sequences used for quantitative RT-PCR analysis of HSP 70 mRNA expression

Primer name	Sequence	Size(Base pairs)	GenBank Reference
HSP 70-F*	CCTCCTACGTCGCCTTCACAGACA	233	AY645906
HSP-R**	GGGGTAGAAGGTCTTCTTGTCTCCC		
Beta actin-F*	TGGACTTCGAGCAGGAGATG	138	JF288784.1
Beta actin-R**	GGAATGAGGGCTGGAACAGG		

*Forward Primer, **Reverse Primer, Size- no of base pairs, GenBank Reference-NCBI

shrimps from each group were selected randomly, and haemolymph was drawn to analyse haemolymph osmolality and ionic concentration. Haemolymph was collected from the pericardial cavity by injecting a 25-gauge needle and 1-cc syringe under the carapace at the cephalothorax–abdominal junction. The gills and hepatopancreas were collected into a 1.5 mL Eppendorf centrifuge tube (RNase-free), stored in Qiagen's RNA later™ at −80°C until analysis. The gills and hepatopancreas collected for enzyme analysis were homogenized with chilled phosphate buffer saline (pH 7.5) using a tissue homogenizer (REMI Equipments, Mumbai, India). The homogenate was centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was stored at 4°C until use.

2.8 | Analysis of haemolymph osmolality and ionic concentration

A vapour pressure osmometer (Wescor) and Eschweiler blood analyser (Combiline) were employed as per the manufacturer's instructions to assess haemolymph osmolality (reported as mmol kg^{-1}) and ionic concentration respectively.

2.9 | Superoxide dismutase (SOD) activity

SOD was measured using the method defined by Misra and Fridovich (1972). The method is based on the oxidation of epinephrine–adrenochrome transition by the enzyme. An assay mixture consisting of carbonate–bicarbonate buffer, tissue sample and freshly prepared epinephrine was made. Immediately, with the help of Shimadzu–UV spectrophotometer, change in absorbance at 480 nm was read for 3 min. SOD was expressed as unit activity (amount of protein required to give 50% inhibition of epinephrine auto-oxidation).

2.10 | Na^+ K^+ ATPase activity

Measurement of Na^+/ K^+ – ATPase was performed by the method described by McCormick (1993). In brief, the reaction mixture consisted of lactate dehydrogenase (LDH), pyruvate kinase (PK), phosphoenolpyruvate (PEP), ATP, NADH, ouabain and imidazole (pH 7.5) buffer. A standard of 0–20 nMol ADP was used parallely. Accurately 1 ml of the reaction mixture and 50 µl of the sample was added to the cuvette. The OD was read at 240 nm for 2–10 minutes. Na^+ K^+ ATPase activity is measured as a difference in ATP hydrolysis in the absence and presence of ouabain expressed as micromoles of ADP per milligram of protein per hour.

2.11 | Analysis of HSP 70 mRNA expression

Total RNA was isolated from gill tissues of experimental shrimps using TRIzol reagent (Invitrogen, USA) as per manufacturer's guidelines. RNA concentration and purity at 260 and 280 nm were determined using Nanodrop spectrophotometer (Thermoscientific, USA). To remove the genomic DNA contamination, extracted RNA was treated with RNase-free DNase I (Thermo scientific, USA) before cDNA synthesis according to manufacturer's instructions. The mRNA pool was converted into its complementary DNA using the first-strand cDNA synthesis Kit (Thermo Scientific, USA) following the protocol provided by the manufacturer and stored at −80°C until for quantitative real-time PCR (RT-qPCR).

RT-qPCR was performed with an Aria Mx Real-Time PCR system (Model no. G8830A, Agilent Technologies, USA) using maxima R SYBR green PCR master mix (2x) (Fermentas, USA) as per manufacturer's protocol. The reaction mix was prepared by adding 12.5µL of PCR master mix, 1µL of each forward and reverse primer, 1µL of the cDNA and nuclease-free water to make up volume to 25µL. The β-actin was chosen as a housekeeping gene and primers for RT-qPCR of HSP70 and β-actin genes of *P. vannamei* (Table 2) was synthesized from Eurofins Genomics, Bangalore. The annealing temperature for the primer combinations used was optimized by using gradient PCR (Thermo Fisher). The annealing temperature for HSP70 was fixed at 57°C. The mRNA quantified was measured in terms of threshold cycle (CT) value and at the end of each PCR reaction, melting curve analysis of the amplified products was conducted. 2-ΔΔCT method (Livak & Schmittgen, 2001) was used to evaluate the relative mRNA expression of HSP70.

2.12 | Statistical analysis

Statistical analysis was assessed by applying one-way analysis of variance (ANOVA) using Statistical Package for the Social Sciences, SPSS software (version 22.0). Duncan's multiple range test was used for post hoc to analyse differences among the mean ($p < 0.05$) of various treatments at 5% probability level. All data are expressed as means ±standard error.

3 | RESULTS

3.1 | Physico-chemical parameters of water

The temperature, pH, dissolved oxygen, total hardness and total alkalinity of experimental water were found to be in the range of 27°C - 28.4°C, 7.49 to 8.09, 7.6 to 8.0 mgL^{-1},1800–3500 mgL^{-1} and 290–314 mgL^{-1} respectively. The free CO2 was not perceptible. The ammonia and nitrite concentration were within acceptable limit. K^+ concentration was found to be in the range of 104 – 107.5 mgL^{-1}in ASW, 9.4 – 9.7 in ISW and in fortified water (ISW+eK) it was maintained at 107– 107.5 mgL^{-1} while for the water used for the treatment ISW+½eK+fK, the potassium content was 51.3–53.5 mgL^{-1}.

3.2 | Proximate composition of experimental diets

Data pertaining to the proximate composition of different experimental diets are given in Table 1. The dry matter (%) ranged from 90.4 to 89.8%. CP (%), EE(%), CF(%), NFE (%) and ash content(%) of the diets were in the range of 36.16 to 36.2, 6.71 to 6.91, 6.48 to 6.52, 40.9 to 40.57 and 9.75 to 9.8 respectively. Digestible energy (Kcal/ 100 g) was within the range of 394.55 to 395.35.

3.3 | Effect of K^+ supplementation on survival

The survival rate of the different treatment groups is given in Figure 1. The highest survival percentage was recorded in ISW+eK+fK (100 ± 0%) and ASW (100.0 ± 0%). In ISW+eK, 71.4 ± 6% survival was recorded, whereas the lowest survival percentage was observed in FW and ISW, that is the shrimps in freshwater and inland saline groundwater without any fortification could not survive after 6 h and 12 days respectively.

3.4 | Effect of K^+ supplementation on haemolymph osmolality

Haemolymph osmolality was recorded for 15 days at selected intervals and is depicted in Figure 2. In FW, the osmolality has been reduced rapidly, and the shrimps were not able to maintain homeostasis, and they died within a day. In ASW, almost constant osmolality has been observed till the end of the experiment. In ISW and ISW+fK, osmolality decreased continuously, and after 12 days of the experiment, mortality occurred in ISW. The ISW+eK group maintained similar osmolality levels to that of ASW till the first day. However, the osmolality levels started to decrease after the first day, and the final osmolality on 15th day was significantly ($p < 0.05$) lower than the ASW treatment group. In ISW + ½ eK+fK group, osmolality increased after one day, but it was also lower than that of ASW while similar to that of the ISW+eK group.

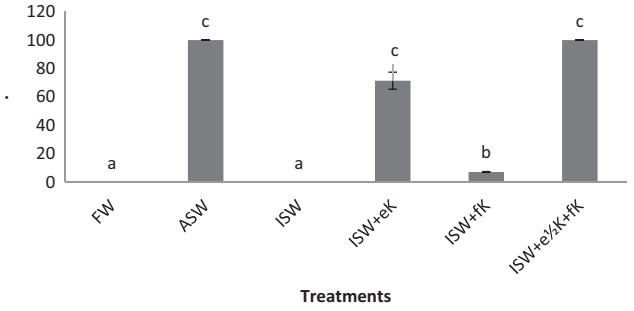

FIGURE 1 Survival % of *P. vannamei* in different treatment groups as on the 15th day of experiment. The bars bearing different superscripts differ significantly ($p < 0.05$). Data expressed as Mean ±S.E

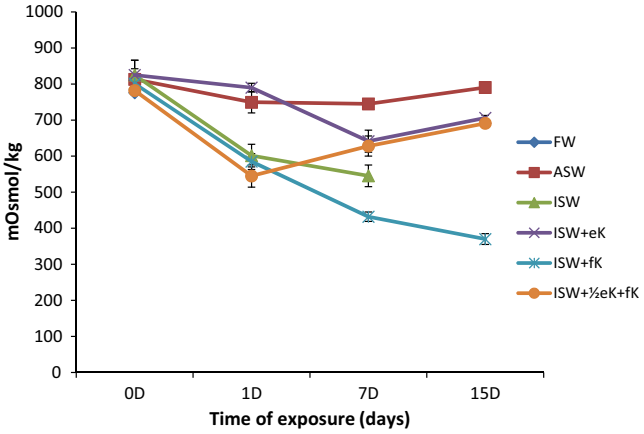

FIGURE 2 Haemolymph osmolality (mOsm/kg) of *P. vannamei* of different treatment groups at different time intervals. The bars bearing different superscripts differ significantly ($p < 0.05$). Data expressed as Mean ±S.E (n = 6)

There was no significant difference at 0 days among different treatment groups. High osmolality, similar to ASW, was observed in ISW+eK treatment on the first day. There was no significant difference between these treatments on the first day. However, the osmolality of dietary K-supplemented groups was lower than ASW and ISW+eK groups on the first day. Haemolymph osmolality of the ASW group was significantly higher ($p < 0.05$) than all other groups on 7th day. At the final sampling (15th day), there were no significant difference between ISW+eK and ISW + ½eK+fK groups; however, the level was significantly lower ($p < 0.05$) than that of ASW.

3.5 | Effect of K^+ supplementation on haemolymph ionic concentrations

Haemolymph potassium concentration of *P. vannamei* is depicted in Figure 3. The K^+ concentration in FW was analysed at 0th day. However, at rest of the intervals the analysis could not be achieved

due to mortality of shrimps. The K^+ concentration decreased in ISW and ISW+fK after every interval. In ASW, ISW+eK and ISW+½eK+fK, K+ decrease in the level of K^+ was observed at first day. However, the concentration increased on 7th day and was found to be almost similar at 15th day. There was no significant difference in the K^+ concentration in different treatment groups at 0th and 1st day of the experiment ($p < 0.05$). The high concentration of haemolymph potassium was found in ISW+½eK+fK on the 7th day and also at the end of the experiment.

Haemolymph sodium concentration has been depicted in Figure 4. The concentration of Na^+ in FW was determined at 0th day. However, owing to the mortality of shrimps, the study could not be done at remaining intervals. In ISW and ISW+fK, decrease in sodium concentration was observed throughout the experimental period. On first day, high and low sodium concentration was observed in ASW and ISW respectively. However, ISW+eK did not differ significantly with ASW ($p < 0.05$). There was no significant difference among the treatments ISW+eK, ISW+fK and ISW+½eK+fK, ($p > 0.05$). On the 7th day, high concentration was observed in ISW+½eK+fK; there was no significant difference with ASW ($p > 0.05$). At the end of the experiment, a significantly ($p < 0.05$) higher concentration of sodium was observed in ISW+½eK+fK ($p > 0.05$).

Calcium levels did not show any particular trend within the treatments at different intervals and are depicted in Figure 5. However, it varied when compared between the treatments at the same intervals. On the 0th day, the calcium concentrations were similar ($p < 0.05$) in all the groups. Calcium concentration in FW could not be measured at other intervals as shrimps could not survive beyond a day. Calcium concentration was observed to be high in ISW on the first day. However, there was no significant difference among ISW+eK, ISW+fK and ISW+½eK+fK ($p > 0.05$). On the 7th day and 15th day of the experiment, a significantly ($p < 0.05$) higher concentration of calcium was observed in ISW+½ eK+fK ($p < 0.05$).

Haemolymph chloride concentration is depicted in Figure 6. There was no significant difference in the level of Cl^- among the

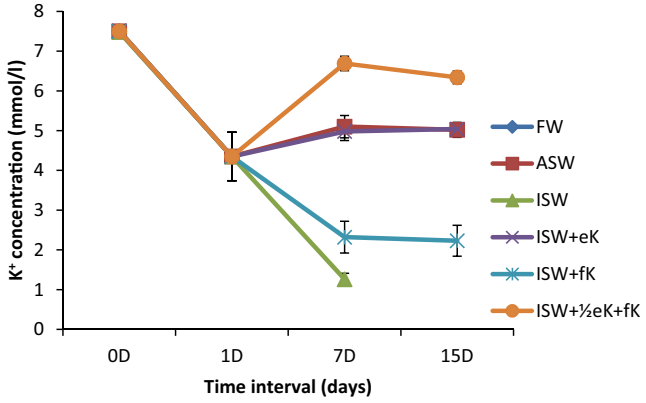

FIGURE 3 Haemolymph potassium (K^+) concentration of *P. vannamei* in different groups at different time intervals. The bars bearing different superscripts differ significantly ($p < 0.05$). Data expressed as Mean ±S.E (n = 3)

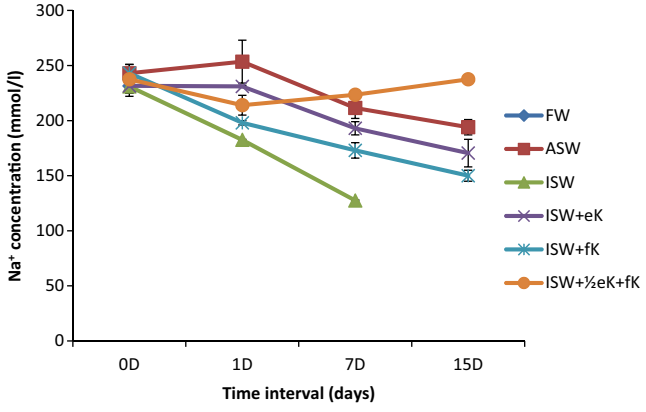

FIGURE 4 Haemolymph sodium (Na^+) concentration of *P. vannamei* in different groups at different time intervals. The bars bearing different superscripts differ significantly ($p < 0.05$). Data expressed as Mean ±S.E (n = 3)

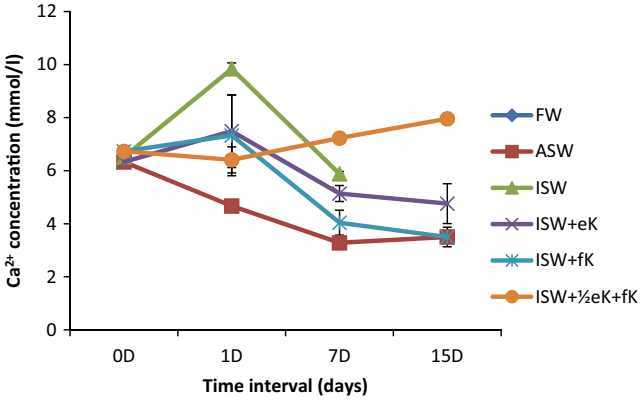

FIGURE 5 Haemolymph calcium (Ca^{2+}) concentration of *P. vannamei* in different groups at different time intervals. The bars bearing different superscripts differ significantly ($p < 0.05$). Data expressed as Mean ±S.E (n = 6)

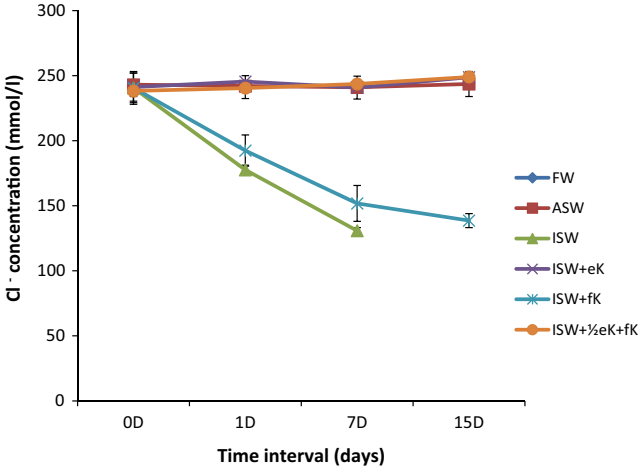

FIGURE 6 Haemolymph chloride (Cl^-) concentration of *P. vannamei* in different groups at different time intervals. The bars bearing different superscripts differ significantly ($p < 0.05$). Data expressed as Mean ±S.E (n = 6)

treatments at 0 days ($p < 0.05$). The Cl^- concentration in FW could not be assessed beyond the 0^{th} day of the interval as the shrimps could not survive at 0 ppt. There was no particular trend observed. In the ASW, ISW+eK and ISW+½eK+fK groups, the chloride concentration was maintained almost at the same level in all the time intervals. In ISW and ISW+fK, chloride concentration was observed to be reduced at every interval. High chloride concentration was observed in ASW on the first day; however, it did not differ significantly with ISW+eK and ISW+½eK+fK ($p > 0.05$). On day seven, a high concentration of chloride was observed in ISW+½eK+fK. Still, it did not differ significantly with ASW ($p > 0.05$), and at the end of the experiment, a significantly high chloride concentration was found in ISW+½eK+fK (P < 0. 05).

3.6 | Effect of K^+ supplementation on SOD activity

The SOD activity in the hepatopancreas of *P. vannamei* in different treatment groups at selected intervals (0D, 1D, 7D and 15D) is shown in Table 3. SOD activity on 0^{th} day in FW was found to be 38.48 ± 0.62. At other intervals, SOD was not measured as the shrimps died after 6 h of the experiment. In ASW, it remained at similar levels till the end of the experiment. SOD activity increased in ISW after every interval. In ISW+eK and ISW+½eK+fK, the activity decreased after 1^{st} day. In the ISW+fK group, the SOD activity has been observed to increase after the 7th day of the experiment. There was no significant difference in the treatment groups on the 0^{th} day ($p > 0.05$). There was a significantly higher activity ($p < 0.05$) observed in ISW on the first and 7th day of the experiment, and on the 15th day, significantly ($p < 0.05$) higher activity was observed in ISW+fK.

3.7 | Effect of K^+ supplementation on branchial NKA activity

Na^+/K^+- ATPase activity in gills of different treatment groups has been analysed at selected intervals (0^{th} day, 1^{st} day, 7^{th} day, 15^{th} day), and results are given in Table 4. The Na^+/K^+- ATPase activity in the gills of *P. vannamei* at selected intervals remained at similar levels in ASW. The Na^+/K^+- ATPase activity increased in all the other treatments on the 7th day and remained unchanged at the end of

TABLE 3 Superoxide dismutase (SOD) activity in the hepatopancreas of *P. vannamei*

Treatments	0D	1D	7D	15D
FW	38.48 ± 0.62	-	-	-
ASW	34.84 ± 3.09	31.44^{a}±1.47	31.48^{a}±0.62	38.06^{b}±0.13
ISW	38.68 ± 0.79	50.97^{c}±2.53	57.9^{c}±1.56	-
ISW+eK	36.72 ± 1.59	45.06^{b}±0.3	38.65^{b}±0.48	38.72^{b}±0.21
ISW+fK	34.89 ± 1.47	40.82^{b}±2.15	$34.1.45^{ab}$±1.07	55.56^{c}±2.5
ISW+½eK+fK	37.07 ± 0.73	41.07^{b}±0.97	34.85^{ab}±1.55	32.6^{a}±0.88
P-value	0.4300	0.001	0.001	0.001

SOD: specific activity expressed in 50% inhibition of epinephrine auto-oxidation/mg protein/min.
Mean values in the same column with different superscript differ significantly ($p < 0.05$).
Data expressed as Mean ±SE, (n = 3).

TABLE 4 Sodium-potassium adenosine triphosphate activity (Na^+/K^+-ATPase) in gills of *P. vannamei* exposed different environmental and dietary ionic concentrations

	Na+/K+ - ATPase (gills)			
Treatments	0D	1D	7D	15D
FW	1.80 ± 0.38	2.81 ± 0.59	-	-
ASW	2.50 ± 0.06	2.61 ± 0.44	2.49^{a}±0.09	2.58^{a}±0.20
ISW	1.88 ± 0.25	4.82 ± 0.35	18.34^{b}±4.00	-
ISW+eK	2.17 ± 0.37	3.61 ± 0.18	7.90^{a}±0.89	6.36^{b}±0.55
ISW+fK	2.67 ± 0.58	5.21 ± 0.16	16.17^{b}±4.05	12.53^{c}±0.83
ISW+eK+fk	1.71 ± 0.42	3.76 ± 0.21	7.15^{a}±0.74	8.51^{b}±0.97
P-value	0.421	0.281	0.007	0.001

Na+/K+ - ATPase: specific activity expressed in micromoles of ADP released per milligram protein per hour.
Mean values in the same column with different superscript differ significantly ($p < 0.05$). Data expressed as Mean ±SE, (n = 3).
The notation '-' indicates missing values, because of the complete mortality in the respective treatments.

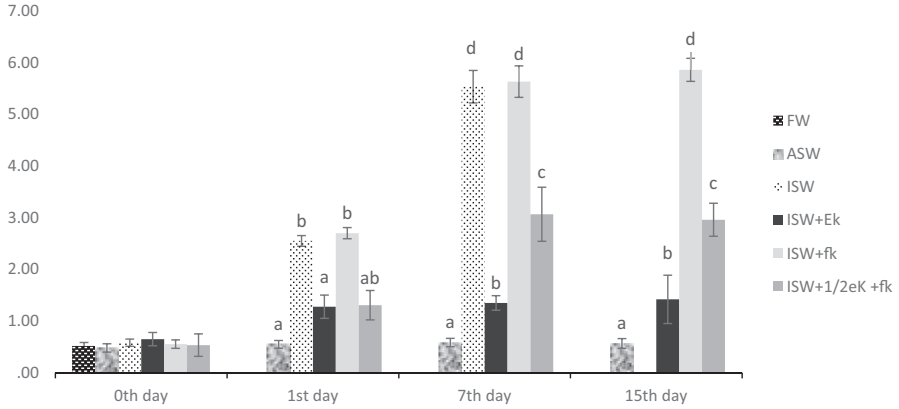

FIGURE 7 HSP 70 relative mRNA levels in *P. vannamei* expoesed to different osmotic conditions. The bars bearing different superscripts differ significantly ($p < 0.05$). Data expressed as mean ±S.E

the experiment. There was no significant difference observed in the activity of Na^+/ K^+- ATPase in the treatment groups on the 0^{th} day ($p > 0.05$). On day seven, significant differences were found among the treatments ($p < 0.05$) and high activity was observed in ISW but did not differ significantly with ISW+fK ($p > 0.05$). At the end of the experiment, significantly ($p < 0.05$), high activity was observed in the ISW+fK group.

3.8 | Effect of K^+ supplementation on branchial HSP70 expression

The branchial HSP70 expression is depicted in Figure 7. The mRNA expression of HSP70 was analysed in the ISW group up to 7 days as there was total mortality in that group before the next sampling interval (15^{th} day). Similarly, the FW group could not survive for 24 h, which resulted in the lack of expression data in the remaining intervals. There was an upregulation in Hsp 70 expression ($p < 0.05$) of *P. vannamei* when transferred to ISW (10 ppt) from ASW (10ppt) from the first day onwards. However, the expression was downregulated in ISW+ek and ISW+½eK+fK groups on the 1^{st}, 7^{th} and 15^{th} day. However, there was a significant difference between ISW+ek and ISW+½eK+fK groups, with higher values in the ISW++½eK+fK group.

4 | DISCUSSION

There is a minimum requirement of 1ppt environmental potassium for *P. vannamei* (McGraw & Scarpa, 2003). However, when the salinity increases, that is in the presence of other ions, there is a chance that this requirement of potassium will increase. In the present study, total mortality was observed for *P. vannamei* reared in inland saline water without any fortification of potassium either through diet or through the water. These results are in good agreement with the earlier studies in shrimps (Antony et al., 2015; Jahan et al., 2018; Tantulo & Fotedar, 2007). It might be due to the inadequate availability of potassium in the water and the feed. Compared with the other essential ions, K^+ is a minor constituent in freshwater (Horne, 1969). K^+ is a critical intracellular cation, whose levels are essential for maintaining the concentration of other vital minerals such as sodium and chloride (Robertson, 1953) and also includes maintaining osmolality, participating in vital nutrient transport, nerve impulse transduction and muscle contraction. (Robertson, 1953). The survival percentage of *P. vannamei* has been improved upon fortification of potassium in the environment, similar to the level of artificial seawater.

Further, it has been observed that the fortification of potassium in the feed alone has shown minimal improvement in survival. Thus, we attempted simultaneous supplementation of potassium in the diet as well as in the water. The diet potassium levels were chosen as 1% following the reports of earlier workers. When P. japonicus is supplemented with 0.9% potassium, survival and growth were optimal compared with a diet containing 1.8% potassium (Kanazawa et al., 1984). An extra-fortification of 1% potassium was administered to shrimp for optimal growth (Deshimaru & Yone, 1978). However, an additional dose of infeed potassium may not be utilized because the absorption is dependent on the presence of transporters in the intestine, which is not yet elucidated in the shrimps. The supplementation of potassium in the inland saline water was reduced to 50% of the total potassium present in ASW, and the potassium level in the diet was maintained at 1%. This treatment showed a survival rate to the tune of 100%, similar to that of ASW. Our study presents a novel solution to the farming of *P. vannamei* in inland saline water with only 50% fortification of potassium in the water.

At ten ppt saline water and FW as in the present study, the shrimps have to hyperosmoregulate as the average osmolality of shrimp haemolymph is well above that of the freshwater and ten ppt saline water. Gong et al.,(2004) reported that the osmoregulatory capacity and more stable osmolality had been found in shrimps fed with a diet containing potassium chloride, magnesium oxide, sodium chloride, phospholipids and cholesterol as compared to the shrimps fed with a diet containing no supplements. Shrimp osmolality in freshwater reduced to approximately 360 mOsm/Kg, after which all of them died within 6 h after transfer. Shrimps reared in inland saline water fortified with potassium in the feed and the environment, and the water alone could maintain the almost same osmolality. However, it was a little lower than those of the artificial seawater reared ones. Total mortality has been observed for the shrimps reared in unamended water. These results are similar to studies reported by Jain et al., 2005. Thus, from the present study, it is evident that potassium concentration in the external medium affects haemolymph osmolality

though the contribution of K to the haemolymph osmolality is less than 1% (Shimizu et al., 2001). The principal influence of K on osmolality may be due to the role of K^+ in osmoregulation by activation of Na^+/ K^+ ATPase pump, which regulate sodium concentration directly and chloride concentration indirectly (Saoud et al., 2007). The present study indicates that K^+ supplementation only in the diet could not be as effective as its supplementation both in the diet as well as in the external rearing medium for improving the osmoregulation and hence growth and survival of *P. vannamei*. Supplementation of potassium only in the diet reduces osmotic stress for only short periods (Saoud et al., 2007).

The significant ions contributing to osmolality and vital functions such as moulting are Na^+, K^+, Ca^{2+} and Cl^-. In the present study, the shrimp potassium concentration in haemolymph has been affected by its quantity in water and feed. The present study further reinstates that haemolymph potassium increased through supplementation of this ion through water and diet, whereas decreased in non-fortified waters. This is a regulation pattern, which has been demonstrated with several other penaeid species (Cheng & Liao, 1986; Dall & Smith, 1981; Lin et al., 2000).

Sodium and chloride have been identified as the significant ions contributing (88.4%) to haemolymph osmolality in marine shrimp (Chen & Chen, 1996). Sodium is the most predominant cation in haemolymph (Sowers et al., 2006), and Cl^- is the major anion in the extracellular medium. *P. vannamei* operates hyperosmotically at low salinity, and sodium can be quickly discharged through the sodium leak pathway in the gill ionocytes. Therefore, high Na^+/ K^+ ATPase activity is necessary to absorb enough sodium from the environment to compensate for the lost sodium (Liu et al., 2014). Sodium concentration in the haemolymph of *P. vannamei* was found lower than that of ASW in all the other groups, including freshwater. However, within 7 days, 100% potassium fortified the ISW group, and 50% K fortified, and 1% KCl fed group regained similar sodium levels to ASW. The chloride levels also showed the same trend as sodium, revealing potassium's effect on sodium and chloride transport. Our study concurs with Jahan et al., (2018) reports, who reported an increase in haemolymph osmolality with respect to infeed supplementation of potassium. The current study further describes that haemolymph calcium of *P. vannamei* does not vary much in amended and unamended waters, which corroborates with the study which reported that calcium ion concentrations remained constant under all low salinities (Huong et al., 2010). Our study also confirms that potassium supplementation does not influence calcium concentration. Moreover, calcium levels have to be maintained for moulting irrespective of the environmental variation. It could also be speculated that short-term exposure of 15 days to calcium deficient freshwater may not be sufficient to alter its serum concentration.

In the present study, the lowest SOD activities were observed in the ASW group. Further, SOD activities increased significantly ($p < 0.05$) in the groups reared in imbalanced waters and remained unchanged in the group fed with potassium and reared in 50% potassium fortified water. It indicates that potassium supplementation in feed and water was effective in ameliorating the stress, eliminating the need of SOD activity. Superoxide dismutase (SOD) activities in shrimp reared at low salinity were high (Li et al., 2008). Higher SOD activities in shrimp at lower salinity may enable shrimp to maintain health by scavenging the radicals produced (Li et al., 2008).

The primary site of active transport of Na^+ and Cl^- from the water into the fish's extracellular fluid is gills. Na^+ and chloride uptake is directly related to the activity of Na^+ K^+ ATPase, which is an essential enzyme for osmotic and ionic regulation in crustaceans. Previous studies also suggested the importance of Na^+-K^+-ATPase in the gills of crustaceans and teleosts under acute salinity stress (Gao et al., 2016). NKA activity increases when there is a reduction in osmolality, which explains the elevated activity levels in inland saline water, which was reviewed by Roy et al., (2010). High activity was found in shrimps reared in non-fortified water. Moreover, at the end of the experiment, among the survived groups, higher activity was found in shrimps fed with the potassium supplemented diet. It indicates that potassium supplemented in water is more effective than supplementation in the diet.

The expression of HSP70 is an indication of osmotic stress in euryhaline animals (Wu et al., 2008). In the present study, HSP 70 expression increased as a result of rearing in ISW. Another study observed that Hsp 70 expression levels in sea cucumber upregulated (Wang et al., 2014) at low salinity and high temperature levels. However, the potassium supplementation in any form (as aqueous or dietary) has reduced the induced levels of HSP 70. The group with partially supplemented aqueous potassium combined with dietary potassium have lower levels of HSP 70, indicating that the strategy has been effective at reducing HSP 70 than that of rearing in raw ISW and ISW with feed potassium alone.

5 | CONCLUSION

Shrimp farming (*P. vannamei*) in inland saline water (10 ppt) is prone to the challenge of ionic imbalance of these waters, mainly low potassium (K^+). From the present study, we concluded that potassium supplementation in the water is essential for rearing *P. vannamei* in ISW. Thus, we suggest that farmers growing shrimp in inland saline water with lower levels of K^+ should continue to supplement K^+. Further, the aqueous supplementation of KCl can be lowered to 50% by incorporating 1% KCl in the diet. An elevation of Na^+/K^+ ATPase activity in ISW indicates the ability of *P. vannamei* to enhance ion absorption with imbalanced potassium. However, the elevated Na^+/K^+ ATPase levels were reduced by potassium supplementation in feed and water. Further studies are necessary to evaluate the effects of different ions, which can modulate ion transporters to achieve an osmolality level similar to ASW.

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CONFLICT OF INTEREST

The authors declare that they have no competing that could influence the work documented in this paper.

AUTHOR CONTRIBUTION

Ifrah Zaffar contributed to experimentation, benchwork and manuscript drafting. Tincy Varghese contributed to overall guidance, designing of the experiment and manuscript correction. Subrata Dasgupta contributed to guidance in research and manuscript editing. Narottam Prasad Sahu contributed as member, Advisory Committee. Prem Prakash Srivastava contributed as member, Advisory Committee. Vungarala Harikrishna contributed to manuscript correction. Zahoor Mushtaq contributed to assay of gene expression. Showkat Ahmad Dar contributed to assistance sample collection and analysis. Satya Prakash contributed to water quality analysis. Gopal Krishna contributed to overall guidance for the work and as Principal investigator of Project.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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