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Effects of Salinity and Ca^{2+}: Mg^{2+} Ratio on Nutrient Profile, Physiological Responses and Clinical Signs of *Penaeus vannamei* (Boone, 1931) Reared in Inland Saline Ground Water

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ABSTRACT

Background: A 60-day experiment was conducted to evaluate the combined effect of salinity and Ca^{2+}: Mg^{2+} ratio on the nutrient profile, physiological responses, and clinical signs of *Penaeus vannamei* reared in inland saline ground water.

Methods: The study used three different salinities *viz*. T_1 (5 ppt), T_2 (10 ppt) and T_3 (15 ppt) with four different levels of Ca^{2+}: Mg^{2+} ratios such as 1:1, 1:2, 1:3, and 1:4. A total of 720 healthy post larvae of *P. vannamei* (mean body weight of 3.70±0.02 g) were stocked (20 shrimp/tank) in 36 circular plastic tanks (100 L capacity each). Each treatment was triplicated following the 3 × 4 factorial design.

Result: A significant increase of protein and ash contents and decrease of moisture content were noticed with increasing salinity. The study found no significant difference in the carbohydrate and crude lipid content of *P. vannamei.* A linear relationship between salinity and serum osmolality was observed which indicated significant increase of osmoregulatory capacity with increasing salinity. The treatment groups fortified with deficient level of Ca^{2+}: Mg^{2+} ratio showed prominent changes such as abdominal white muscle on dorsal side and muscle cramps. Based on the results, it can be concluded that the better physiological adoption of *P. vannamei* was observed in 10 and 15 ppt salinities of ISGW fortified with Ca^{2+}: Mg^{2+} ratio 1:3.

Key words: Clinical sign, ISGW, Nutrient profile, Osmoregulatory capacity, *P. vannamei*.

INTRODUCTION

Inland salinization has become a major risk to the natural resources, especially in the freshwater regions affecting an area of approximately one billion hectares worldwide (Srivastava and Kumar, 2015). In India, approximately 6.727-million-hectare land is affected by salinization (Arora and Sharma, 2017). Almost 75% salt- affected soils located in Maharashtra (0.61 million ha), Uttar Pradesh (1.37 million ha), Gujarat (2.23 million ha), West Bengal (0.44 million ha) and Rajasthan (0.38 million ha) region of India (Mandal *et al.,* 2018). Salinization is an important concern which builds up the water-soluble salts in the soil of inland areas and leads to the production of inland saline ground water (ISGW) which makes the inland ground water unfit for human consumption, crop production or any freshwater aquaculture production (Li *et al.,* 2020a). In the recent past, salinity in the inland areas was raised due to various anthropogenic activities which is significantly threatening the livelihood of various rural communities and causing major economic, social and environmental consequences (Chand *et al.,* 2012). As per the 2012-14 assessment data, owing to soil salinization in India, yearly 16.84 million tons of agriculture production is being wasted which valued at Rs. 230.20 billion (Mandal *et al.,* 2018). In this context, the agriculture farmers, in response to salinity issue are forced to shift their livelihood approaches (Ziaul and Zaber, 2013).

Generally, this unproductive ISGW resources can be well utilized by adopting various inland saline water aquaculture activities which involves the rearing of aquatic organisms having euryhaline nature (Jamil *et al.,* 2011). Moreover, aquaculture in ISGW provides nutritional security, employment generation and livelihood enrichment (Dorothy *et al.,* 2021). However, in contrast to brackish and sea water, IGSW has highly imbalanced ionic concentrations which makes the IGSW unsuitable for commercial aquaculture (Aklakur, 2017). In general, ISGW is deficient in potassium (K^+), high in calcium (Ca^{2+}) and contains variable level of magnesium (Mg^{2+}). On the other side, concentration of ionic rations (Calcium to magnesium ratio Ca^{2+}: Mg^{2+} ratio; sodium to potassium ratio Na^+: K^+ ratio) is crucial for aquaculture as it affects normal growth, survival and osmoregulation in crustaceans (Antony *et al.,* 2015). Additionally, salinity, a chemical characteristic of water, influences the physiological

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homeostasis of aquatic animals and it affects the growth performance and nutrient requirements of fish (Jiang *et al.,* 2019). The changes in salinity influenced the growth, survival, feed utilization and physiological activities of various fish species such as *Labeo rohita* (Murmu *et al.,* 2019) and *Pangasionodon hypophthalmus* (Lingam *et al.,* 2024). Therefore, to maintain a better physiological conditions and ideal growth of the cultured animals, water salinity and ISGW ionic concentrations need to be manipulated and maintained as per the physiological requirements of cultural animal (Garg *et al.,* 2022).

Many authors reported that ionic manipulation in ISGW had significant impact on the physiology, growth and survival of various species such as Black tiger prawn (Antony *et al.,* 2015), *Macrobrachium rosenbergii* (Raizada *et al.,* 2015), *Cyprinus carpio* (Jahan *et al.,* 2020), *Anabas testudineus* (Talukdar *et al.,* 2021), *Penaeus vannamei* (Jana *et al.,* 2021; Paswan *et al.,* 2024) and GIFT (Paul *et al*., 2023). Recently, shrimp farming with *Penaeus vannamei* is blooming in inland salt affected areas of India. At global level, there are about 20 different species of shrimps cultured among them Pacific white leg shrimp *P. vannamei* is the most cultured one, because of fast growth, high economic value, higher stocking density and tolerance of wide range of salinity and temperature (Lakra *et al.,* 2014). With impetuous efforts from Regional Centre of ICAR- Central Institute of Fisheries Education, Mumbai, India, had successfully developed cultural practices for profitable aquaculture of *P. vannamei* in Inland Saline Ground Waters areas of Haryana (CIFE, 2015). However, many farmers are facing problems while rearing of *P. vannamei* in salt affected inland areas because of the lack of knowledge on the ionic composition if ISGW. Besides that, based on the ISGW salinity the ionic concentration will vary and variability is again region specific. Keeping all this in mind, the present study was performed to assess the effect of salinity and Ca^{2+}: Mg^{2+} ratio on physiology, nutrient profile and clinical changes of *P. vannamei* juveniles reared in ISGW.

MATERIALS AND METHODS

A 60 days experiment was carried out at ICAR-Central Institute of Fisheries Education, Mumbai, Rohtak centre, Haryana, India (28°53′43″N, 76°36′23″E). *Penaeus vannamei* juveniles were acquired from a shrimp culture farm near by centre and carefully transported to the wet laboratory using oxygenated polythene bags filled with same salinity of inland saline ground water (ISGW) in the centre. The juveniles were acclimatized for 15 days and then stocked in circular plastic tanks (100 L capacity). The tanks were filled with 4/5th of its capacity at different salinity ISGW waters such as 5 ppt, 10 ppt and 15 ppt and fortified with four different levels of Ca^{2+}: Mg^{2+} rations such as 1:1, 1:2, 1:3 and 1:4. Following standard protocol by (Nunes and Lopez, 2001). ISGW obtained by tube well from the rearing site had 15 ppt which was further diluted with freshwater for maintaining 10 ppt and 5 ppt. Commercial grade of potassium chloride (KCl) was used for fortification to maintain potassium ion (K^+) levels as of sea water. Similarly, $CaCO_3$ and $MgCl_2$ were used for maintaining the ratio of Ca^{2+}: Mg^{2+} ratio, respectively (Goldberg, 1963). The experimental mediums of different salinities and various levels of Ca^{2+}: Mg^{2+} ratio waters were prepared and stored in 1000 L capacity of fibre reinforced plastic tanks (total 12 tanks). Prior to water filling in tanks, Sodium hypochlorite (10 ppm) was applied to disinfect the tanks and water. Digital refractometer (Hanna Instruments, Model HI 96822, Carrollton, TX, USA) was used to test the experimental salinity and it was further cross checked with manual titration method (Argentometric method).

The initial body weight of shrimp was calculated by measuring the individual weight of the randomly selected shrimp (30 nos). A total of 20 animals in each tank were randomly distributed and each treatment was maintained in triplicate following the factorial design (3×4). Totally 36 experimental tanks and 720 animals were used in the trial. Continuous aeration was provided and 30% water was exchanged at every day to maintain the water quality. Initially, shrimp juveniles were fed @7% of body weight and as the experiment progressed it was gradually reduced up to 3%. Commercially available shrimp feed procured from Avanti feed company. The composition of feed was protein (35%), fat (5%), fibre (2%) and moisture (12%) fed twice in a day (09.00 hrs and 21.00 hrs). The physico-chemical parameters such as water salinity and temperature were observed on daily basis and dissolved oxygen, pH, hardness, total alkalinity were estimated on weekly basis (APHA, 2005).

Water osmolality and haemolymph osmolality (mmol/kg) were recorded using a cryoscopy osmometer (Osmomat 030). The osmoregulatory capacity was calculated by using the given formula; Osmoregulatory capacity = Mean osmolality of the shrimp - Mean osmolality of the rearing medium. The whole-body nutrient composition of *P. vannamei* was estimated following scientific methods of AOAC (1995). Moisture content was estimated by keeping the pre-weighed fish sample from each replicate in the hot air oven at 110°C for 24 hrs and re-weighed. After moisture estimation, the dried samples were grinded and stored in muffle furnace for further analyses. Protein, lipid and ash contents were estimated by Kjeldal, Soxhlet and furnace, respectively. The collected data of proximate parameters and osmolality were subjected to two-way analysis of variance (ANOVA) using SAS Institute 2010, SAS/ STAT user guide, VERSION 9.3. SAS INSTITUTE, CARY, NC, USA (1-940) for windows. Post hoc comparison of different treatment groups mean values was carried out using Duncan's multiple range tests. All the data were expressed as mean ± standard error with a statistical significance value of $p<0.05$.

RESULTS AND DISCUSSION

Nutrient profile analysis

The proximate composition of *P. vannamei* reared in different salinities of ISGW fortified with various level of

Table 1: Proximate composition of different experimental group of *P. vannamei* reared in ISGW fortified with different levels of Ca^{2+}: Mg^{2+} ratio.

Treatments (Salinity)	Ca^{2}: Mg^{2+}	Moisture	Carbohydrate	Crude protein	Ash	Crude lipid
5 ppt	1:1	79.41^{a}±0.52	1.70^{a}±0.33	16.03^{c}±0.43	1.24^{b}±0.32	1.09^{a}±0.21
	1:2	79.75^{a}±0.21	1.11^{a}±0.17	16.22^{c}±0.52	1.37^{b}±0.18	1.55^{a}±0.11
	1:3	79.28^{a}±0.41	1.12^{a}±0.50	16.94^{bc}±0.51	1.46^{b} ±0.23	1.18^{a}±0.17
	1:4	78.45^{abc}±0.11	1.55^{a}±0.23	17.06^{bc}±0.45	1.66^{ab}±0.50	1.27^{a}±0.29
10 ppt	1:1	78.51^{ab}±0.52	1.08^{a}±0.20	17.68^{abc}±0.51	1.20^{b}±0.28	1.45^{a}±0.25
	1:2	77.63^{abcd}±0.31	1.07^{a}±0.16	17.94^{abc}±0.29	1.18^{b}±0.19	2.18^{a}±0.35
	1:3	77.22^{abcd}±0.21	1.42^{a}±0.35	17.94^{abc}±0.31	1.19^{b}±0.28	2.22^{a}±0.23
	1:4	75.27^{d}±0.34	1.16^{a}±0.43	19.96^{a}±0.11	1.73^{ab}±0.31	1.86^{a}±0.38
15 ppt	1:1	76.02^{bcd}±0.43	1.13^{a}±0.46	19.39^{ab}±0.43	1.18^{b}±0.44	2.28^{a}±0.26
	1:2	76.22^{bcd}±0.19	1.35^{a}±0.35	19.24^{ab}±0.46	1.53^{b}±0.24	1.64^{a}±0.14
	1:3	75.58^{dc}±0.38	1.80^{a}±0.29	19.15^{ab}±0.34	1.35^{b}±0.13	2.05^{a}±0.53
	1:4	75.83^{bcd}±0.51	1.60^{a}±0.16	18.68^{abc}±0.21	2.23^{a}±0.26	1.65^{a}±0.43

Abbreviation: K^{+} =potassium; Mg^{2+} = magnesium; Ca^{2+} =calcium; Ca^{2+}: Mg^{2+} = calcium: magnesium ratio

T_1- 5 ppt; Ca^{2+}: Mg^{2+} (1:1, 1:2, 1:3 & 1:4), T_2- 10 ppt; Ca^{2+}: Mg^{2+} (1:1, 1:2, 1:3 & 1:4) and T_3- 15 ppt; Ca^{2+}: Mg^{2+} (1:1, 1:2, 1:3 & 1:4). Values in the same column with different superscripts differ significantly ($P<0.05$) for each parameter. Data expressed as means ± S.E.M (n=3). Two-way ANOVA was used following Duncan multiple range test in SAS - 9.3.

Ca^{2+}: Mg^{2+} ratio was evaluated at the end of the experiment and presented in the (Table 1). There was a significant difference observed in moisture, crude protein and total ash contents of *P. vannamei* among the various treatment groups. However, the study found no significant difference in the carbohydrate and crude lipid contents of *P. vannamei,* which indicates that Ca^{2+}: Mg^{2+} ratios do not seem to have any impact on the carbohydrate and lipid contents of *P. vannamei*. In the case of moisture an inverse relationship was observed with salinity where increasing salinity decreased the moisture content. Similarly, previous studies also recorded that the moisture content of experimental animals was significantly decreases as the salinity of rearing medium increases (Liang *et al.,* 2008). The present study found increasing of salinity and Ca^{2+}: Mg^{2+} ratio levels had significantly increased the protein content of the shrimp which indicated that change in Ca^{2+}: Mg^{2+} ratio and salinity levels significantly affects the protein deposition in shrimp. The study found protein in the range of 19.39 to 16.03 % which was like the previous report of (Sriket *et al.,* 2007) where they reported 17 and 21% of crude protein content in shrimps, respectively. Lipid, a major energy resource, varies based on the environmental fluctuations (Pillay and Nair, 1973). On the other side, increasing salinity has also raised the ash content (1.18 to 2.23). However, similar ranges of ash content were reported in black tiger and white shrimps at 0.95 and 1.47%, respectively, by (Gokoglu *et al.,* 2008).

Physiological responses

The details of osmolality parameters are represented in (Table 2). At the end of experiment, haemolymph osmolality was recorded and it ranged between 568 to 632 mOsmol/Kg. There was no significant difference in haemolymph osmolality of all three salinity levels fortified with Ca^{2+}: Mg^{2+}. In contrast, significant difference was observed in the water osmolality among the treatments fortified with different levels of Ca^{2+}: Mg^{2+} ratio. However, no interaction effect was observed among the treatment groups in water osmolality due to Ca^{2+}: Mg^{2+} and salinity. The variation in water osmolarity, among the treatment groups, was due to difference in salinity of waters. The higher (445.67 mOsmol/Kg) and lower (210.66 mOsmol/Kg) osmoregulatory capacity were found in T_1 fortified with Ca^{2+}: Mg^{2+} ratio of (1:2) and T_3 with of (1:4), respectively. There was a decreasing trend in osmoregulatory capacity with increasing salinity. Haemolymph osmolality did not vary significantly among the different treatment groups fortified with various levels of Ca^{2+}: Mg^{2+} ratios. There is a linear relationship existing between salinity and serum osmolality where increasing medium salinity elevates the serum osmolality (Tantulo and Fotedar, 2006). A similar study conducted in ISGW by (Singh *et al.,* 2020) found serum osmolality of Amur carp increased with increasing salinity from 5 to 15 ppt. Similarly, a linear relationship was recorded between salinity and serum osmolality of present study. Mellor and Fotedar (2011) reported that increase of blood osmolality in juveniles of Murray cod (*Maccullochella peelii peelii*) when the rearing medium salinity was increased which is similar to the results of present study. A decreasing trend was observed in osmoregulatory capacity where increasing medium salinity lowered the osmoregulatory capacity which agreed with the earlier studies of (Tantulo and Fotedar, 2007). However, there is no significant difference in osmoregulatory capacity among the various salinity treatment groups which may be due to no interactive effect of Ca^{2+}: Mg^{2+} and salinity.

Clinical signs

As the experiment progressed, the stocked shrimp displayed various morphological and clinical changes in the different salinity treatment groups fortified with Ca^{2+}:

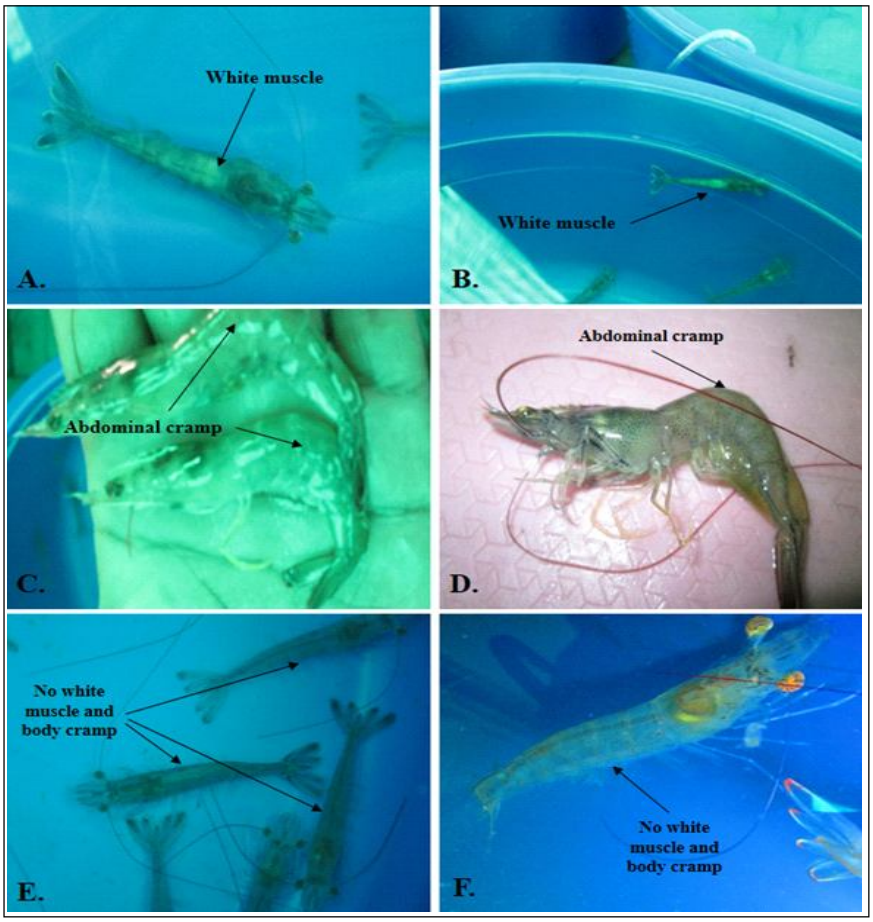

Fig 1: Photograph A and B showing the abdominal white muscle on dorsal side of body in the treatment T_1, T_2 and T_3 fortified with low Ca^{2+}: Mg^{2+} ratio such as 1:1, 1:2 and 1:4 except in 1:3. Photograph C and D indicate the whole body cramp in the treatment T_1, T_2 and T_3 fortified with low Ca^{2+}: Mg^{2+} such as 1:1, 1:2 and 1:4 except in 1:3. Photograph E and F indicates the healthy shrimp with no deformities or symptoms in Ca^{2+}: Mg^{2+} ratio 1:3 in the 10 ppt (T_2) and 15 ppt (T_3).

Table 2: Water and haemolymph osmolality of different experimental group of *P. vannamei* reared in ISGW fortified with different levels of Ca^{2+}: Mg^{2+} ratio.

Treatments (Salinity)	Ca^{2+}: Mg^{2+}	Haemolymph osmolality (mOsmol/Kg)	Water osmolality (mOsmol/Kg)	Osmoregulatory capacity (mOsmol/Kg)
	1:1	$568.00^{a}\pm14.21$	$134.00^{hi}\pm3.54$	$434.00^{a}\pm10.67$
5 ppt	1:2	$566.67^{a}\pm12.32$	$120.33^{i}\pm3.94$	$445.67^{a}\pm8.38$
	1:3	$577.33^{a}\pm09.02$	$140.33^{h}\pm4.54$	$437.00^{a}\pm4.48$
	1:4	$602.33^{a}\pm08.89$	$166.33^{g}\pm5.54$	$436.00^{a}\pm3.35$
	1:1	$611.00^{a}\pm08.32$	$228.00^{f}\pm3.54$	$383.00^{b}\pm4.78$
10 ppt	1:2	$630.00^{a}\pm09.63$	$255.33^{e}\pm6.51$	$374.67^{b}\pm3.12$
	1:3	$605.67^{a}\pm12.31$	$277.00^{d}\pm6.04$	$328.67^{b}\pm6.27$
	1:4	$631.33^{a}\pm10.23$	$288.00^{d}\pm4.04$	$343.33^{b}\pm6.19$
	1:1	$629.67^{a}\pm10.32$	$410.33^{ab}\pm8.14$	$219.34^{c}\pm2.18$
15 ppt	1:2	$631.00^{a}\pm11.21$	$336.00^{c}\pm5.11$	$295.00^{c}\pm6.10$
	1:3	$631.00^{a}\pm09.99$	$400.00^{b}\pm4.00$	$231.00^{c}\pm5.99$
	1:4	$632.33^{a}\pm09.01$	$421.67^{a}\pm4.34$	$210.66^{c}\pm4.67$

Abbreviation: K^{+} =potassium; Mg^{2+} = magnesium; Ca^{2+} =calcium; Ca^{2+}: Mg^{2+} = calcium: magnesium ratio T_1- 5 ppt; Ca^{2+}: Mg^{2+} (1:1, 1:2, 1:3 and 1:4), T_2- 10 ppt; Ca^{2+}: Mg^{2+} (1:1, 1:2, 1:3 and 1:4) and T_3- 15 ppt; Ca^{2+}: Mg^{2+} (1:1, 1:2, 1:3 and 1:4). Values in the same column with different superscripts differ significantly ($P<0.0001$) for each parameter. Data expressed as means ± S.E.M (n=3). Two-way ANOVA was used following Duncan multiple range test in SAS - 9.3.

Mg^{2+} ratio of 1:1, 1:2 and 1:4 (Fig 1). The study noticed an abdominal white muscle at dorsal side and whole-body cramp in all the shrimps reared in the Ca^{2+}: Mg^{2+} fortified groups, except 1:3 groups (A, B, C, D, E and F). In all the treatment groups, except 1:3 group, shrimps showed slower swimming behavior and steady mortality from 22nd day of the experiment. The deficiency of mineral in aquatic animals can be identified by their morphological features such as cramped body, white muscle and tail cut-off which ultimately leads to mortality due to ionic imbalances (Suguna, 2020). Similarly, the present study noticed behavioural, morphological changes and mortality in some treatment groups fortified with deficient level of Ca^{2+}: Mg^{2+} ratio. The more prominent changes such as abdominal white muscle and muscle cramps were recorded in all salinity groups fortified with Ca^{2+}: Mg^{2+} ratio of 1:1. These behavioural changes were started appearing on the 16th day and the shrimp were started showing mortality on 22nd day of stocking at 5 ppt with Ca^{2+}: Mg^{2+} of 1:1, followed by in other salinity groups fortified with a Ca^{2+}: Mg^{2+} of 1:1. Within a short span of time, white muscle was observed in 50% of the stocked shrimp.

CONCLUSION

The salinization of inland area is the major concern in Indian scenario. Nowadays, the salt affected land and water resources are used for aquaculture practices, as an adaptive strategy, but, due to its mineral variability the use of these resources is very limited. In the present study, *P. vannamei* was selected, as a euryhaline potential species, to evaluate physiological adaptation, nutrient profile and clinical signs under IGSW conditions of different salinities such as 5, 10 and 15 ppt fortified with various levels of Ca^{2+}: Mg^{2+} ratio (1:1, 1:2, 1:3 and 1:4). At 1:1, 1:2 of Ca^{2+}: Mg^{2+} ratios, the study observed that shrimps were not able to maintain the physiological homeostasis due to imbalance in ionic composition. However, optimal physiological conditions and healthy shrimps were observed at Ca^{2+}: Mg^{2} ratio 1:3 in T_2 and T_3 treatment groups and shrimp showed the physiological homeostasis at this ionic composition. Overall, it can be concluded that the fortification of 1:3 ratio of Ca^{2+}: Mg^{2+} (equivalent to seawater) in ISGW is sufficient to give better physiological homeostasis, nutrient profile and healthy shrimps. Therefore, farmer who are interested in shrimp farming with *Penaeus vannamei* at ISGW can fortify their water with 1:3 ratio of Ca^{2+}: Mg^{2+} to get optimal production. Further, research on optimization of stocking density and nutritional requirements of *Penaeus vannamei* in ISGW could help to develop a complete package of shrimp farming technology in the salt affected inland areas of India.

Conflict of interest

The authors declare that there is no conflict of interest.

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