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Expression studies on NA^+/K^+-ATPase in gills of *Penaeus monodon* (Fabricius) acclimated to different salinities

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The decapod crustacean *Penaeus monodon* survives large fluctuations in salinity through osmoregulation in which Na^+/K^+-ATPase (NKA) activity in the gills plays a central role. Adult *P. monodon* specimens were gradually acclimatized to 5, 25 and 35‰ salinities and maintained for 20 days to observe long term alterations in NKA expression. Specific NKA activity assayed in gill tissues was found to be 3 folds higher at 5‰ compared to 25‰ (isosmotic salinity) and 0.48 folds lower at 35‰. The enzyme was immunolocalized in gills using mouse α-5 monoclonal antibody that cross reacts with *P. monodon* NKA α-subunit. At 5‰ the immunopositive cells were distributed on lamellar tips and basal lamellar epithelium of the secondary gill filaments and their number was visibly higher. At both 25‰ and 35‰ NKA positive cells were observed in the inter-lamellar region but the expression was more pronounced at 25‰. Gill architecture was normal at all salinities. However, the 1.5 fold increase in NKA α-subunit mRNA at 5‰ measured by quantitative RT-PCR (qRT-PCR) using EF1α as reference gene was not statistically significant. The study confirms the osmoregulating ability of *P. monodon* like other crustaceans at lower salinities. It is likely that significant increase in NKA transcript level happens at an earlier time point. At higher salinities all three methods record only marginal or no change from isosmotic controls confirming the hypothesis that the animal largely osmoconforms in hyperosmotic environment.

Keywords: Asian tiger shrimp, Giant tiger prawn, Immunolocalization, Ion transport, Osmoregulation, Salinity

Penaeus monodon (Fabricus 1798), is a euryhaline decapod crustacean having the capacity to survive dramatic changes in environmental salinities and exhibits hypo- and hyper-osmotic regulation at salinities above and below the isosmotic concentrations (24–26‰), respectively[1]. The central role of Na^+/K^+-ATPase (NKA) in osmoregulatory ion transport in gills of decapods and other crustaceans is well established[2-6]. Although other tissues like integument, antennal glands[7] and intestine[8] might contribute to the osmoregulatory function to a minor extent, gills are known to be the primary site of osmoregulation, where the epithelial cells present a large membrane surface that is particularly elaborate in the basolateral region facing the body fluid. The abundant mitochondria in these cells fulfill the high ATP requirement for functioning of ion pumps like NKA and H^+-ATPase.

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The functional NKA is composed of two essential subunits (α and β) that are noncovalently paired to form αβ-heterodimer[9]. The α-subunit is the catalytic subunit and contains binding sites for ATP, Na^+, K^+ and the cardiac glycoside, ouabain that inhibits the enzyme activity. The β-subunit is a Type-II glycosylated polypeptide believed to assist in the folding and placement of the α-subunit into the cell membrane[10]. A third, nonessential γ-subunit has been identified in mammals[11], and is thought to play a role in modulating Na^+, K^+ and ATP-binding affinities to the enzyme complex[12,13]. Pongsomboon *et al*.[14] have reported the sequences of the complete transcripts of α and β subunits from *P. monodon* that code for 112 and 34 kD protein products (NCBI Accessions EF672699 and ABV65905).

The NKA activity is tightly regulated by salinity changes in the aquatic environment. The regulation may be categorized into short-term and long term. While short term control may be exercised by alteration in the enzyme's kinetic behaviour or translocation of the protein between the cell membrane and intracellular stores, long-term

regulation may involve *de novo* synthesis of new enzymes or the degradation of pre-existing enzyme molecules[15].

In this context, the present study was conducted to record the changes in gill NKA expression and activity in *P. monodon* samples gradually acclimatized to three salinities and maintained for 20 days. Understanding its role in salt homeostasis may enable transferring of salt tolerance trait to other organisms. In addition, determining the response of NKA gene promoters to varying salt concentrations can lead to their future use in *in vitro* expression systems. Here, we report changes in NKA enzyme activity, its transcript levels and immunolocalization in gills of adult *P. monodon* specimens after long term acclimation to hypo-, iso- and hyper-osmotic salinities.

Material and Methods

Acclimatization of shrimp—Shrimp weighing approximately 20±2 gm were procured from Pancham Aqua Farm, Saffale, Maharashtra, where they were being maintained at 18‰ salinity. At CIFE, Mumbai they were maintained in 1000 l FRP tanks (30 shrimp/ tank) with aeration and shelters and stabilized at 18‰. The animals were fed *ad lib* with artificial pellet feed (Charoen Pokphand, Thailand) thrice a day. From day 2, salinity was either increased or decreased by 2‰ per day, by adding treated seawater of 50‰ or freshwater until it reached 5, 25 or 35‰ in different tanks. There were two tanks for each treatment and shrimp were maintained for 20 days after acclimation.

Tissue collection—Five shrimp specimens were collected from each treatment group and stunned in ice. Gill tissue was dissected out aseptically and stored appropriately for RNA isolation, enzyme assay and immunocytochemistry. For RNA isolation, the tissue was rinsed in DEPC treated water and transferred to tubes containing RNAlater™ (Qiagen, Hilden, Germany) following manufacturer's instructions and stored at −80 °C till further use. For enzyme assay, the tissue was stored in chilled 0.33 M sucrose and stored at −80 °C. For immunocyto-chemical study the tissue was fixed in Davidson's fixative and left overnight at 4 °C. No distinction was made between anterior and posterior gill during collection and the entire tissue from one side constituted one sample.

Quantification of NKA α-subunit transcript by Real Time PCR—Gill tissue was collected from five individuals of each group. Gill tissue was preserved in RNAlater and total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, USA) following manufacturer's protocol and quantified using a biophotometer (Eppendorf, Hamburg, Germany). Total RNA integrity was further confirmed by agarose gel electrophoresis. RNA was treated with DNase I (Fermentas, Waltham, USA) following manufacturer's instructions to remove any DNA contamination. Single stranded cDNA was synthesized from 2 µg of DNase–treated total RNA by 200 units of RevertAid Mu-LV Reverse Transcriptase enzyme (Fermentas, Waltham, USA) in a final reaction volume of 20 µl using 100 pmol of oligo dT primer (Fermentas, Waltham, USA) as per manufacturer's protocol.

The cDNA served as template for relative quantification of NKA expression by Real Time PCR. Specific primer pair NKA-qRT-F (5′CGCCGTAA CTCCATTGTCCAC) and NKA-qRT-R (5′GAAGA CCCTTGTCCATGCCTG) designed against the reported *P. monodon* NKA alpha sequence (NCBI Acc. No. EF672699) was used to amplify a fragment of 120 bp in the ABI 7500 Real Time PCR machine (Applied Biosystem, Carlsbad, USA). EF1α transcript was used as the internal control, and specific primers EF1α-qRT-F (5′AAGAAGAACAGAGGCCACCGA3′) and EF1α-qRT-R (5′GCGACTCATCCCTCAGCCGT3′) designed against the reported sequence (NCBI Acc. No. DQ021452) amplified a 150 bp sequence. The 12.5 µl reaction mix contained 6.25 µl of Maxima SYBR Green qPCR Master Mix (Fermentas, Waltham, USA), 0.25 µl (10 pmol) each of forward and reverse primers, 4.75 µl of nuclease free water and 1 µl (25 ng) of cDNA. The default thermal profile was used which consisted of initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min. Each sample was run in duplicate. Melt curve analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. Comparative C_T method was used to estimate relative expression of NKA. Fold change in expression at 5‰ and 35‰ was calculated using the formula 2^-delta delta C_T where 25‰ was taken as the isosmotic condition. Statistical significance of the data was tested using ANOVA in Microsoft Excel software.

Estimation of NKA enzyme activity—NKA activity was assayed according to the method of[16]. The method is based on ouabain sensitive hydrolysis of

adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and is enzymatically coupled to oxidation of nicotinamide adenine dinucleotide (NADH, reduced form) which was directly measured in a UV-Visible spectrophotometer at 340 nm. Briefly, a preparation of imidazole buffer (50 mM, pH 7.5) was used to prepare a basic reaction mixture and salt solution. Sucrose EDTA imidazole (SEI) buffer consisted of 150 mM sucrose, 10 mM EDTA and 50 mM imidazole (pH 7.3), and this was used to prepare 0.1% sodium deoxycholate (SEID) stock solution. SEID was diluted 5-fold with SEI to obtain working SEID solution prior to sample homogenization. An assay mixture (solution A) containing 4 U lactate dehydrogenase (LDH) mL^{-1}, 5U pyruvate kinase (PK) mL^{-1}, 2.8 mM phosphoenolpyruvate (PEP), 0.7 mM ATP, 0.22 mM NADH, and 50 mM imidazole (pH 7.5) was made just prior to the assay. The solution is stable for 2-3 days at 4 °C. Assay solution B was as above but also contained 0.5 mM ouabain. A salt solution containing 189 mM NaCl, 10.5 mM $MgCl_2$, 42 mM KCl and 50mM imidazole (pH 7.5) was prepared in advance and is stable for several weeks at 4 °C. Standards for the assay were prepared as 0, 5, 10, 15 and 20 nmol in working SEID solution. All the steps in enzyme assay were carried out at 4 ºC.

Five samples were taken from each group and each sample was run in duplicate. Pieces of gill tissue stored at −80 °C (30-40 mg) were quickly added to tubes containing 300 µl of working SEID solution and homogenized for 30 s on ice using a hand-held Tissue Tearor (USA). Homogenates were then centrifuged at 5000 ×*g* for 5 min and the supernatant was used as the enzyme source. The assay was conducted by adding and mixing 25 µl supernatant to solution A and salt mixture (3:1 ratio) in cuvette. Then the linear rate of NADH disappearance was measured at 340 nm for 10 min. The same protocol was repeated for solution B plus salt mixture (3:1 ratio) per sample in the cuvette. Protein was estimated by the method of Lowry *et al*. (1951)[17] using bovine serum albumin as standard. Specific activity was expressed as millimoles of ADP formed/min/mg protein. The entire estimation was done twice on two separate occasions with different sets of animals for confirmation. Statistical comparisons were done between different exposure groups by one-way ANOVA followed by Tukey's test using SPSS 13.0 software package. $P \leq 0.05$ was accepted as the level of statistical significance.

Immunohistochemical localization of NKA—After overnight fixation in Davidson's fixative the gill tissue was processed according to Lightner (1996)[18]. Paraffin sections of 3 µm were cut in a microtome (Leica, RM 2125RT) and mounted on poly-L-lysine coated slides. Sections were then pre-incubated for 10 min in 0.01 mM Tween 20, 150 mM NaCl in 10 mM phosphate buffer, pH 7.3. Thereafter, the sections were first treated for 5 min with 50 mM NH_4Cl in phosphate-buffered saline (PBS), pH 7.3. Following washes (3×5 min) in PBS, the slides were incubated for 10 min with a blocking solution (BS) containing 5% bovine albumin and 0.1% gelatin in PBS. The slides were incubated at 4 °C overnight in wet chamber with mouse monoclonal primary antibody (α5) against the α-subunit of the avian NKA (Developmental Studies Hybridoma Bank, USA)[19] diluted (1:2000) in 10% normal goat serum in PBS (PBSS). After being washed (4×5 min) in PBSS to remove unbound antibodies, the sections were incubated for 2 h in droplets of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma) at 1:500 dilution in PBSS. Following extensive washes in PBS (4×5 min), sections were mounted in Flour preserve (Calbiochem) anti-fading mounting agent. The slides were stored at 4 °C and observed under Digital Fluorescence Microscope (Zeiss Axiophot A1) at 100× magnification.

Results

Effect of salinity on NKA transcript level—Fold changes in NKA transcript level in *P. monodon* gill tissue at hyposmotic (5‰) and hyperosmotic (35‰) salinities compared to that at isosmotic salinity (25‰) were estimated using comparative C_T method of real time PCR and are presented in Fig. 1a. The 1.5 fold increase in the transcript at 5‰ was not statistically significant at 5% level when the data was analyzed after removing outliers. At 35‰ the transcript level was same as that of isosmotic controls ($P \leq 0.05$).

Effect of salinity on NKA specific enzyme activity—The specific activity of NKA in *P. monodon* gill was almost 3 folds higher at 5‰ (0.074±0.03 millimoles of ADP formed/min/mg protein) compared to the controls at 25‰, while it was 0.48 folds lower at 35‰ (0.025±0.004 and 0.012±0.003 millimoles of ADP formed/min/mg protein at 25 and 35‰, respectively; Fig. 1b).

Effect of salinity on NKA localization and recruitment in gill—Immunofluorescence studies on

gill sections showed highest reactivity in gills of shrimp acclimatized to 5‰ salinity (Fig. 2a-d), while immunostained regions were least at 35‰. The negative control that was not exposed to primary antibody showed no immunofluorescence. At 5‰ salinity, the immunopositive cells were found distributed on the lamellar tips and basal lamellar epithelium of the secondary gill filaments and their number was visibly higher than that observed at higher salinities. At both 25‰ and 35‰ NKA positive cells were observed in the interlamellar region but the expression was significantly more pronounced at 25‰ compared to 35‰. As can be seen in the phase contrast images (Fig. 2e-h), gill architecture was normal at all salinities.

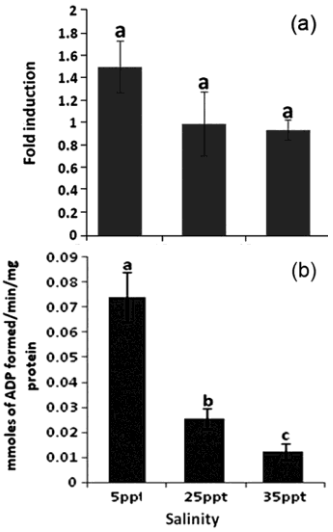

Fig. 1—Effect of different salinities on (a) Na^+/K^+-ATPase mRNA transcript; and (b) Na^+/K^+-ATPase activity in *Penaeus monodon* gill. Each bar diagram represents mean ± S.D. Different lower case letters show significant variation (P <0.05).

Discussion

The importance of NKA activity in gills of osmoregulating euryhaline crustaceans has been widely documented[20]. Towle *et al*. (1976)[21] reported that the NKA activity in gills of blue crab, *Callinectes sapidus*, increased when the animals were transferred from 34 to 5‰ salinity. However, there have been ambiguities regarding whether these changes in activity result from enzyme activation or from increased transcription. Using Western blotting, Lucu and Flik[22] showed that when the shore crab *C. maenas* is acclimated to low salinity for 3 wk, the amount of NKA alpha subunit doubles in plasma membranes purified from gills compared to animals that have been exposed to high or low salinities for only 4 h. The present study shows that in *P. monodon* gills too, the NKA activity increases almost 3 folds when the animals are acclimated to 5‰ salinity for 20 days. Immunodetectable NKA levels also increased markedly at 5‰, however, transcript levels as measured by qRT-PCR do not show a statistically significant increase at the lowest salinity.

Earlier studies on NKA alpha subunit mRNA in *C. sapidus* gills could not detect salinity related changes using semi-quantitative RT-PCR[23,24]. NKA expression in the antennal gland, which is also known to be an osmoregulatory organ in crustaceans, was studied by Buranajitpirom *et al*.[25]. *P. monodon* specimens were reared in 7, 15 and 30 ppt seawater and NKA expression was reported to be enhanced at

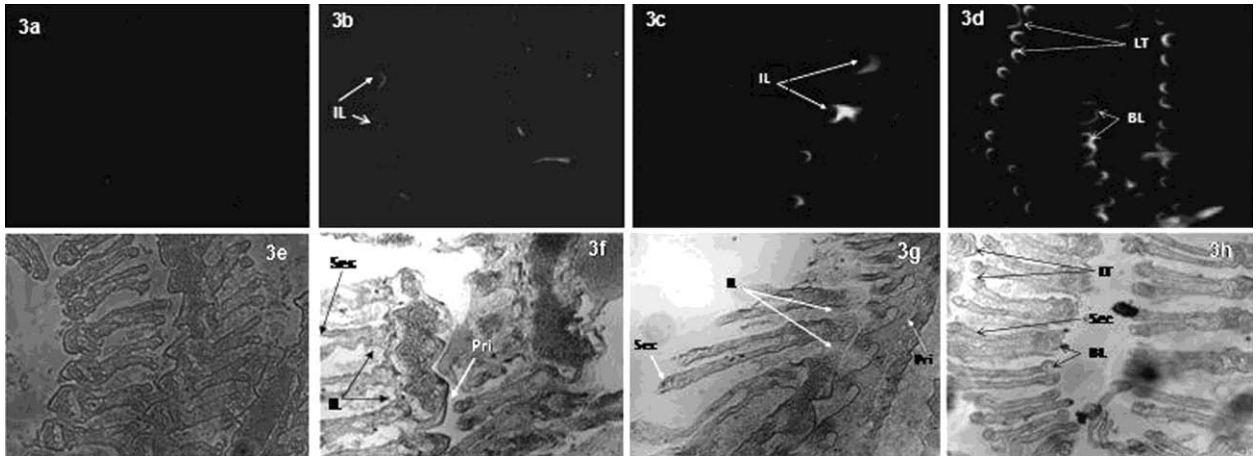

Fig. 2—Immunolocalization of Na^+/K^+-ATPase in *Penaeus monodon* gills acclimatized to 5, 25 and 35‰. a-d: Fluorescence images. [a, Negative control; b, 35‰ salinity; c, 25‰ salinity; d, 5‰]; and e-h: Phase contrast images. [e, Negative control; f, 35‰ salinity; g, 25‰ salinity; h, 5‰ (LT, Lamellar Tips; BL, Basal Lamellar epithelium; IL, Inter Lamellar epithelium; Pri, Primary lamellae; and Sec, Secondary Gill Filament)].

lower salinity when estimated by enzyme assay and Western blotting. Havird *et al*.[26] performed a meta-analysis on 59 published studies that used qRT-PCR to examine expression of a number of osmoregulatory genes including NKA in teleosts and crustaceans in response to salinity transfer. They concluded that at hyperosmotic salinities crustaceans tend to osmoconform but when transferred to lower salinity they switch to osmoregulation accompanied by upregulation of NKA. Although statistically significant changes could not be recorded in qRT-PCR in the present study, the results indicate the need for a larger sample size or estimation at earlier time points to confirm the increase at 5‰. Further in their study, for most genes upregulation was recorded between 1-3 days although it persisted longer for NKA. At 35‰ no fold change from control was recorded supporting osmoconformation at higher salinities. However, most of the studies on crustaceans have not used internal control genes that are essential for qRT-PCR reliability. Shekar *et al*.[27] identified differentially expressed genes in *P. monodon* exposed to low salinity stress (3‰) through suppression subtractive hybridization and validated 13 selected genes including NKA by qRT-PCR. The highest gene expression levels were observed for NKA (34.28-folds) in gill tissues in response to low salinity stress (3‰) at 2 wk post salinity stress using β actin as internal control. We failed to record such a phenomenal fold change at 5‰ after 20 days of transfer. The internal control has a great significance in qRT-PCR estimations and in our experience β actin is not a stable reference gene for estimating salinity induced changes in mRNA expression in *P. monodon* with its expression increasing at higher salinity (unpublished results). Chung and Lin[28] also used β actin as internal control while estimating salinity related changes in euryhaline marine crab *Scylla paramamosain*. They detected enhanced specific enzyme activity of NKA in gill 2, of crabs acclimated to 5‰ salinity for 12 h compared to those at 25 or 45‰ salinity. Nevertheless, it recorded only 1.8 fold increase in NKA mRNA at 5‰ salinity, while 15.8 fold increase was registered in animals at 45‰. The authors could not explain the unexpected increase in mRNA levels observed at 45‰ salinity where the crab is not osmoregulating and almost osmoconforming. We used EF1α as internal control in the present study and its expression was observed to be stable across salinities. This was ascertained by one way ANOVA of C_T values obtained for EF1α across salinities (Table 1).

Table 1—Suitability of EF1a as internal control

[Duncan's Test : Formation of one subset indicates statistical similarity between all treatments]

Treatment	N	Subset for alpha = 0.05
		1
25 ppt	4	14.5391
35 ppt	5	14.6628
5 ppt	4	15.2638
Sig.		0.487

Means for groups in homogeneous subsets are displayed.

Observations in this study show that the abundance and distribution of NKA fluorescent cells in *P. monodon* gills varies at different salinities. It has been reported that 25‰ salinity is isosmotic for *P. monodon*[29] and in animals maintained at this salinity NKA activity was restricted to inter-lamellar region only. At 5‰, when the animal is hyper-osmoregulating, the NKA positive cells could be detected on basal lamellar epithelium and also on the lamellar tips of the secondary gill filaments with a large increase in abundance. There are concurring reports on the occurrence and increased abundance of lamellar NKA positive cells in osmoregulating euryhaline fish adapted to freshwater, such as *Oncorhynchus keta*[30], Japanese eel *Anguilla japonica*[31], Japanese sea bass *Lateolabrax japonicus*[32], *Dicentrarchus labrax*[33], *Chanos chanos*[34] and *Acipenser persicus*[35]. Dang *et al.*[36] made an interesting observation that NKA positive cells that were confined to lamellar tips in tilapia maintained in freshwater covered the entire surface of the lamellae when the fish were exposed to distilled water. This might be due to the migration of the epithelial cells from the germinal center (filamental epithelium) which is found to be positive for proliferating cell nuclear antigen (PCNA) or due to *de novo* synthesis of NKA during salinity acclimation. Earlier cytoimmunochemical studies have revealed that in gills of euryhaline crabs NKA is located entirely in the basolateral membrane of gill epithelial cells[24]. There are reports of ultrastructural changes in lobster when transferred to low salinity such as increase in thickness of basolateral infoldings, appearance of numerous vesicles and an increase in height of the apical microvilli in the ion transporting epithelial cells[37].

In the present study, at 35‰ the NKA immunopositive cells were located only on the interlamellar region and the fluorescence was considerably reduced compared to 25 **‰**, an observation

that is supported by reduced enzyme activity and lower transcript level at this salinity. In *Eriocheir sinensis* too, the immunopositive cells were located only at the bases of the lamellae and inter-lamellar regions when the crab was acclimated to 25‰, while at 5‰ they covered the entire lamellae[38]. According to reports, lobsters maintained in seawater had low amount of immunopositive cells[39,40]. However, Shikano and Fujio[41] working on freshwater and seawater adaptations of newborn guppy *Poecilia reticulate*, found that NKA positive cells to be located only in inter-lamellar regions regardless of the salinity.

Only one isoform of α-subunit has been reported from *P. monodon* that has been used here for expression studies. More than one isoform of α-subunit has been reported from mammals[9] and several fish species like rainbow trout[42], the notothenid *Trematomus bernacchii*[43], *Fundulus heteroclitus*[44] and zebrafish *Danio rerio*[45], where the isoforms show different tissue distribution patterns most likely reflecting the physiological function. Although two α-subunit isoforms have been found in brine shrimp *Artemia franciscana*, α1 and α2, the function of α1 may be osmoregulatory as suggested by its presence primarily in osmoregulatory organs, including the salt gland, antennal gland and midgut[46]. Towle *et al.* (2001)[24] did not report any isoform in the gills of *Callinectus sapidus.*

The present study confirms that like other crustaceans, *P. monodon* osmoregulates at lower salinities as evidenced by the increased NKA enzyme recruitment and activity in gills 20 days post acclimation. Though it could not be statistically proven in this study, it is likely that this is accompanied by an increase in NKA transcript level at an earlier time point. At higher salinities NKA levels as estimated by all three methods vary only marginally from isosmotic controls confirming the hypothesis that the animal largely osmoconforms in hyperosmotic environment. The NKA β subunit promoter has been sequenced by us (NCBI Accession No. JX397998) and its response to various salt concentrations is being tested *in vitro* since procuring live *P. monodon* samples is difficult as it is not the preferred culture species at present.

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