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Why living marine Catfish *Plotosus lineatus* in Persian Gulf is unique amongst teleosts?

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Abstract

Plotosidae marine catfish *Plotosus lineatus* reportedly have an extra-branchial salt secreting dendritic organ (DO) unlike other marine teleosts. 10 days acclimation in three salinity ranging from [seawater (SW control) 34‰, brackishwater (BW) 3‰ and hypersaline water (HSW) 60‰] was used to investigate the osmoregulatory abilities of *P. lineatus* through measurements of physiological parameters including blood chemistry, Na^+/K^+-ATPase (NKA) activity and ion transporter expression in DO and gills using immunohistochemistry. A significant osmoregulatory challenge results from elevated mortality (36%), plasma osmolality and ions, and hematocrit in HSW. At all salinities DO NKA activity and protein were significantly higher than gills. DO mass thus total DO NKA activity was higher, indicating higher overall capacity at HSW. NKA and NKCC1 co-localization was rare in gills ionocytes while it was observed strong in DO parenchymal cells. Using IB detected NKCC1 expression only in DO which was highest at HSW and DO NKA-IR cells showed apically CFTR localization. Taken together, the observed high NKA activity in DO, strong basolateral localized with NKCC1 and apical CFTR indicates the presence of the conserved secondary active Cl^- secretion mechanism found in other ion transporting epithelia.

Keywords: Plotosus lineatus, dendritic organ, gill, osmoregulation, Na^+/K^+-ATPase.

1. Introduction

1) In teleost fishes gills, kidney and digestive tract are involved in maintenance of body fluid balance as osmoregulatory organs (Marshall and Grosell, 2006). Gills are the organ to facilitate either excretion of monovalent ions (Na+, K+, and Cl-) or compensatory active uptake in SW and freshwater (FW), respectively to maintain plasma osmolality within a narrow range due to first direct sense of external osmotic changes and environmental salinity (Evans, 2008; Takei and Hwang, 2016). Hypoosmotic marine teleosts loss water to their environment by the osmosis while it can be compensated by drinking SW thus absorbing solute (Na^+ and Cl^-)-linked water transport from ingested water in the intestine (Grosell, 2010). However, in FW fishes which are hyperosmotic to the environment the osmoregulatory role of the intestine is minor (Takei and Hwang, 2016). However, substantial ion uptake by the intestine is occurring in feeding fishes (Wood and Bucking, 2011).

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2) The active regulation of the osmolality and ion levels of body fluids in FW or SW fishes is occurring (Edwards and Marshall, 2012). In euryhaline teleost species the plasma osmolalities of FW and SW vary, which are maintained at less than 480 mOsm/kg H_2O in SW (Varsamos et al., 2005). In euryhaline teleosts the effects of changing salinity on electrolytes and plasma osmolality have been reported (e.g. Christensen et al., 2012; Kang et al., 2008; Sardella et al., 2008; Tait et al., 2017; Malakpour Kolbadinezhad et al., 2018a). The amphidromous Plotosidae catfish *Plotosus lineatus* have recently invaded the Persian Gulf (https://www.oceandocs.org/handle/1834/9339) and Mediterranean (Lanzing, 1967; Froese and Pauly, 2018) while is native to Indo-Pacific coastal waters from Japan to the Red Sea and East Africa.They are found in marine and BW, and also in FW (Froese and Pauly, 2018).
3) The dentritic organ (DO), from early life stages, is very close to the urogenital papilla and a small fleshy external organ situated on the ventral caudal surface in both sexes of Plotosidae catfishes (Hirota, 1895; Lanzing, 1967; Laurenson et al., 1993). The glandular acini parenchymal cells of the DO are covered by a stratified squamous epithelium (Van Lennep, 1968; Van Lennep and Lanzing, 1967). The cellular similarity of *P. lineatus* DO to fish gill ionocytes and elasmobranch rectal gland cells (Pucke and Umminger, 1979; Van Lennep and Lanzing, 1967; and Van Lennep, 1968) suggested based on descriptive morphological studies. The specialized salt glands from marine tetrapods (e.g., lachrymal gland of marine turtles, the nasal salt gland of marine birds, and lingual glands in sea snakes, SW crocodiles; Shuttleworth and Hildebrandt, 1999; Kirschner, 1980) and the rectal glands from elasmobranchs have similar characteristics of parenchymal cell and similar mechanism of NaCl excretion to the teleost gill chloride cells (secondary activity Cl^- secretion) has been proposed (da Silva et al., 1977; Singer et al., 1998; Marshall and Grosell, 2006).
4) Since the molecular machinery of the (gill and DO) in *P. lineatus* as osmoregulatory organs are unknown, using a combination of enzymatic analysis, immunoblotting and immunohistochemistry the present study addressed molecular mechanisms and osmoregulatory indicators including (plasma ion levels and osmolality) in fish acclimated to different salinities covering the natural range of Plotosidae catfishes living and a hypersaline as more challenging condition that can be present in estuaries (Lanzing, 1967; Young and Potter, 2002). The possibly of a conservatory mechanisms for ion transport in salt secretory cell similar to other vertebrate salt glands also addressed.

2. Material and Methods

2.1. Animals

Purchased striped eel catfish *Plotosus lineatus* (~8–9 g) (Thunberg, 1787) specimen were acquired from TMC Portugal transported to the Laboratory of Molecular Physiology, Interdisciplinar Centre for Marine and Environmental Research (CIIMAR), Porto and were acclimated to a 50 L tank with seawater (SW) 34‰, natural photoperiod and biological and mechanical filtration with UV sterilization (HW-303B, Sun Sun, China) and temperature control (26–28°C) for two weeks to avoid any confounding effects of handling stress on osmoregulation (Biswas et al., 2006). The instant Ocean salt was used to make up saltwater. Diced fish fillets was used twice daily for Fish fed with during this period but not fed 2 days before samplings. Periodic 20% water changes were also made. Water salinity, pH (range pH 7.7–7.9), temperature (range at 26–28˚C) and dissolved oxygen (340i multimetric probe, WTW Measurement Systems Inc. Weilheim, Germany) as well as daily monitoring of fish behaviour were done. The animal protocols were approved by the CIIMAR-University of Porto

and Direction General Veterinarian (DGV). The used fish were according to (Decreto-Lei no.197/96), the Portuguese Animal Welfare Law.

2.2. Salinity acclimation

Groups 5-6 fish were transferred to 30 L tanks for 10 days acclimation in three salinity ranging from [seawater (SW control) 34‰, brackishwater (BW) 3‰ and hypersaline water (HSW) 60‰] which was changed in a stepwise fashion, 5‰ per day, from 34‰ (main tank) to 60‰ or 3‰. A stock solution of 100‰ was prepared by suing Instant Ocean® sea salts and diluted to the appropriate salinities. Aquaria water change 20% occurred weekly.

2.3. Sampling

Individual marine catfish were netted then euthanized in a separate smaller tank (1L) with an overdose of ethyl-m-amino benzoate-MS-222 (1:5000, pH 7.5 adjusted with $NaHCO_3$; Pharmaq UK), weighed (±0.01 g) and total length (mm) measured. Blood was collected using heparinized capillary tubes following caudal transection then centrifuged at 13000xg for 5min at room temperature (Heraeus Pico 17 Centrifuge, Thermo Scientific). Hematocrit (Hct) was measured. The isolated plasma was then frozen in liquid nitrogen and kept at −80°C. The following organs were collected: gill and dendritic organ (DO) were snap frozen in liquid nitrogen and were then immediately stored at -80°C. The gastrointestinal tract of *P. lineatus* is relatively short and without a stomach. The anterior intestine was heavily pigmented and easily distinguished from the unpigmented posterior intestine. Gill filaments samples from the second arch on the left side and DO were also excised, immersed in 100µl of ice-cold SEI (150 mM sucrose, 10mM EDTA, 50mM imidazole, pH 7.3) buffer and frozen at −80°C. In additional sets of six individuals, the body cavities were opened and then immersion fixed in 10% neutral buffered formalin (NBF 10%) for 24 h and then stored in 70% ethanol at 4°C.

2.4. Ion quantification

Plasma samples were also analyzed by flame photometry (PinAAcle 900T Atomic Absorption Spectrophotometer; Perkin Elmer Waltham MA). The colormetrically method used for measuring of chloride concentration in plasma samples (Küffer et al., 1975). Plasma osmolality was determined in fresh samples using freezing-point depression osmometry (Micro-Osmometer, Roebling Co. Berlin Germany) and reported as mOsm kg^{-1} (Malakpour Kolbadinezhad et al., 2012).

2.5. Na+/K+-ATPase activity assay

The Na^+/K^+-ATPase (NKA) activity was measured according to McCormick (1993) with modifications by Wilson et al. (2007). After thawing samples in 300 µl SEI (250 mM sucrose, 10 mM EDTA, 50 mM imidazole pH 7.3) buffer, sodium deoxycholate was added to a final concentration of 1%. Tissue was homogenized using a Precellys 24 bead homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) at 5800 RPM for 2x15s and then immediately centrifuged at 15,000 x g for 5 min at 4°C. Ten µl of supernatant were run in two duplicate sets for the ATPase assay at 340nm with a temperature controlled microplate reader (Powerwave 340; Biotek, Winooski, VT) and Gen5™ reader control and data analysis software for 10-20 min at 25°C. The assay mixture was in one set of wells while the other was contain set assay mixture plus ouabain (1 mM, Sigma– Aldrich Chemical Co.; St. Louis MO) to specifically inhibit NKA activity. Total protein was determined in the remaining

supernatant using the Bradford (1976) at 600nm and the results are expressed as μmol ADP mg^{-1} protein h^{-1}. Since DO mass increased under HSW conditions, the DO NKA activity is also expressed per DO correcting for body mass.

2.6. Immunohistochemistry (IHC)

Immunofluorescence localization was performed according to Reis-Santos et al. (2008). Briefly, a series of xylene baths and a descending ethanol series to water (rehydrating) done on the cut and dewaxed paraffin serial sections. Antigen retrieval was performed on some sections (Shi et al., 2011) by pretreatment with 0.05% citraconic anhydride (pH 7.3) for 30min at 98°C (Namimatsu et al., 2005) and then with 1% sodium dodecyl sulfate (SDS) in PBS for 5 min (Brown et al., 1996). All sections were then blocked with 5% normal goat serum (NGS) and then incubated with primary rabbit or mouse antibodies to the α-subunit of NKA (αR1), NKCC/NCC (T4) overnight at 4°C, rinsed in TPBS (0.05% tween-20 phosphate buffered saline, pH 7.4), followed by incubation with secondary goat anti-mouse Alexa Fluor 568 and/or goat anti-rabbit Alexa Fluor 488-conjugated antibodies for 1h at 37°C. Sections were stained with DAPI (4',6-diamidino-2phenylindole) and viewed on a Leica DM6000B wide field epifluorescence microscope and micrographs taken with a digital camera (DFC340FX, Leica Microsystems, Wetzlar, Germany) using Leica LAS AF acquisition software. Figures were assembled in Photoshop CS3.

2.7. Statistics

Data are presented as means ± standard error of the mean (SEM). Statistical differences of protein, mRNA expression between groups were determined using one-way analysis of variance (ANOVA) followed by the post hoc Student-Newman-Keuls (SNK) test (SigmaPlot 11.0 Systat Software, Inc.) in juveniles exposed to different salinities. Data were square root or log transformed in the case of a failed normality test. The fiducial limit was set at 0.05.

3. Results

3.1. Osmoregulatory indicators

Plasma osmoregulatory indicators are presented in Table 1. Plasma Na^+ concentrations correlated positively across the range of acclimation salinity while plasma $Cl^-$and Ca^{2+} concentrations and osmolality were significantly higher in HSW compared with SW and BW acclimated animals. Plasma osmolality was more than 50% higher in HSW acclimated fish. Hematocrit showed a positive correlation with salinity where BW values were half of HSW. Acclimation salinity had no effect on plasma K^+ concentration. There was mortality (36%) only with HSW acclimation but not in other salinity groups.

3.2. NKA activity

In SW *P. lineatus*, the specific NKA activity is lowest in the gill and twenty times higher in the DO (Fig. 1A). In response to salinity acclimation, similar patterns of NKA specific activity were detected in the gill and DO with significantly higher activity in SW acclimated fish compare to both BW and HSW salinities (Fig. 1B, F).

Table 1: Fish morphometrics: condition factor (K) and DO mass (mg DO per g body mass); plasma Na^+, Cl^-, K^+, and Ca^{2+} concentrations and osmolality and hematocrit of *P. lineatus* acclimated to [brackish water (BW) 3‰, seawater (SW control) 34‰, and hypersaline water (HSW) 60‰]. Data are means ± SEM. (n=7-9). Salinity difference within a given parameter that do not share the same letter(s) are significantly different from one another.

Morphometrics			
mg DO /g body mass	1.25 ± 0.06 [a]	0.90 ± 0.04 [b]	2.65 ± 0.10 [c]
Plasma	**BW (3‰)**	**SW Control (34‰)**	**HSW (60‰)**
Na^+(mmol l^{-1})	119.8 ± 5.4[a]	152.6 ± 5.6 [b]	186.2 ± 16.7 [c]
Cl^- (mmol l-1)	125.5 ±4.2 [a]	127.8 ± 3.9 [a]	148.7 ± 8.0 [b]
K^+(mmol l^{-1})	4.6 ± 0.5	5.1 ± 0.4	5.1 ± 0.3
Ca^{2+} (mmol l^{-1})	2.6 ± 0.2 [a]	3.0 ± 0.2 [a]	3.8 ± 0.5 [b]
Osmolality(mOsm)	391.2 ± 25.1 [a]	374.4 ± 26.0 [a]	588.0 ± 48.7 [b]
Hematocrit (%)	15.3 ± 1.2 [a]	23.3 ± 2.8 [b]	29.2 ± 1.7 [c]

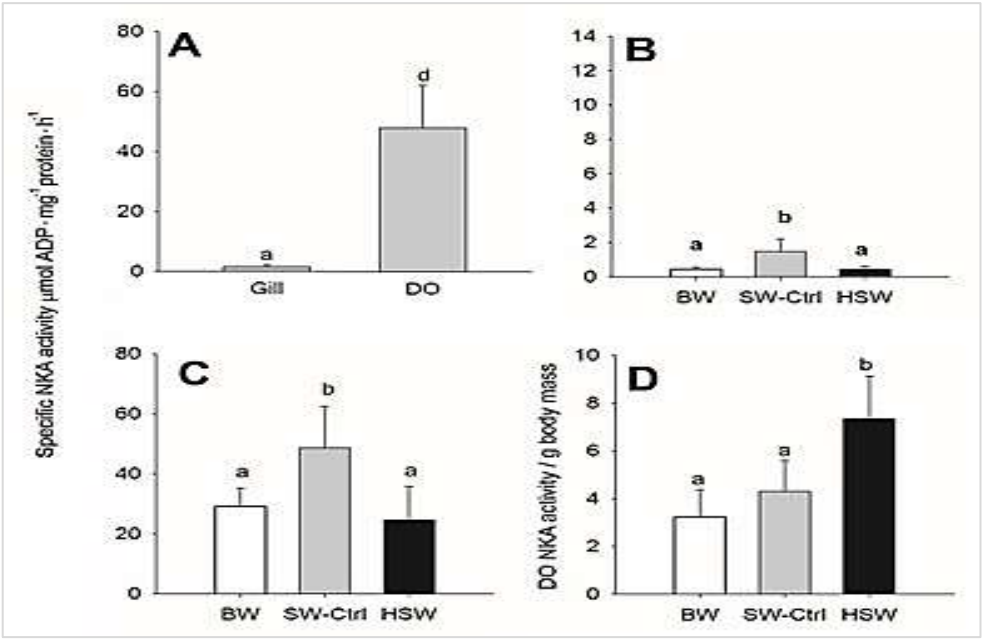

Fig. 1: Na^+/K^+-ATP (NKA) activity in gilla and dendritic organ of SW acclimated *P.lineatus* (A). Organ specific NKA activity [gill (B) and dendritic organ (C) in *P. lineatus* acclimated to brackishwater (BW) 3‰, seawater (SW control) 34‰, and hypersaline water (HSW) 60‰. Total DO NKA activity per g body mass (D). Values are means ± SEM. (n=5-6). Different lower case letters indicate a significant difference between organs (A) and with salinity within each tissue (A-D) ($P < 0.05$).

The mass of the DO in SW-control salinity acclimated fish expressed as a percentage of fish body mass was significantly lower compared to BW and HSW salinity groups (Table 1). However, in HSW salinity acclimated fish DO mass was greatest at 213% and 243 % of BW and SW fish, respectively. Since DO mass changed with salinity, we also expressed NKA activity on a whole organ basis corrected for fish mass. The expression of the total DO

NKA activity relative to fish body mass shows that in HSW fish DO NKA activity was 1.6 and 2.1 fold higher than in SW and BW fishes, respectively (Fig 1G).

3.3. IHC

3.3.1. Gill

Strong NKA immunoreactivity (IR) was detected αR1 antibodies in large isolated ovoid cells throughout the cytoplasm with the exception of the apical region in the branchial epithelium of *P. lineatus* (Fig. 2A-B). This NKA cellular staining pattern is typical of teleost fish chloride cell or ionocyte tubular system, which is continuous with the basolateral membrane. A heterogeneous distribution of few branchial NKA-IR cells were limited to a few interlamellar regions over the leading edge of the filament and were absent from the lamella. There were no alteration on the NKA-IR cell distribution pattern in experimental salinities. The rarely detected colocalization of secretory Na^+:K^+:$2Cl^-$ cotransporter (NKCC1) expression with NKA-IR cells was in gill. (Fig. 2A). In some NKA-IR cells the apical localization of CFTR was detected with no apparent salinity dependent differences (Fig. 2B). Since there was no detected difference between salinity only seawater (SW control) 34‰ showed in Fig 2.

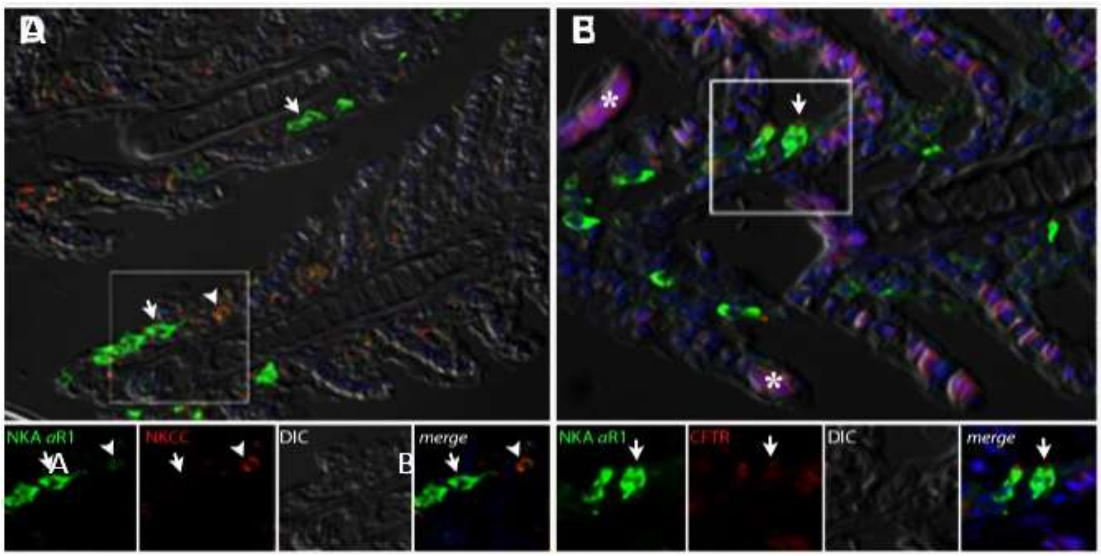

Fig. 2: Immunofluorescence localization of Na^+/K^+-ATPase (αR1, green A, B) with NKCC1 (T4, red A) and CFTR (red, B) in the gills of *P. lineatus*. Acclimation was performed in seawater (SW control) 34‰ (A-B). Since there was no detected difference between salinity only seawater (SW control) 34‰ showed in Fig 2. Sections were counter stained with DAPI nuclear staining (blue) and overlaid with the differential interference contrast (DIC) images. Insets show the separate channels of the framed area in the respective panels (A-B). In A arrowheads indicate NKCC1 IR cells and arrows NKA-IR cells. In B arrows indicate CFTR+NKA IR cells. Scale bar 100 µm.

3.3.2. Dendritic Organ

The external DO of *P. lineatus* are well vascularized and have branching irregular lobes. The strong NKA and NKCC1 immunoreactivity in parenchymal cells of large ovoid to pear-shaped in DO generally showed throughout the cell indicative of basolateral tubular system staining (Fig. 3A-B) while there is a subpopulation of more angular shape parenchymal cells with show noticeably stronger NKA-IR and lack NKCC-IR. The apical chloride channel CFTR was only observed in a SW control fish and was generally not detectable despite the use of antigen retrieval

techniques and positive immunoreactivity in gill indicating that species specific immunoreactivity problems were not an issue (Fig. 3). Since there was no detected difference between salinity only seawater (SW control) 34‰ represented in Fig 3.

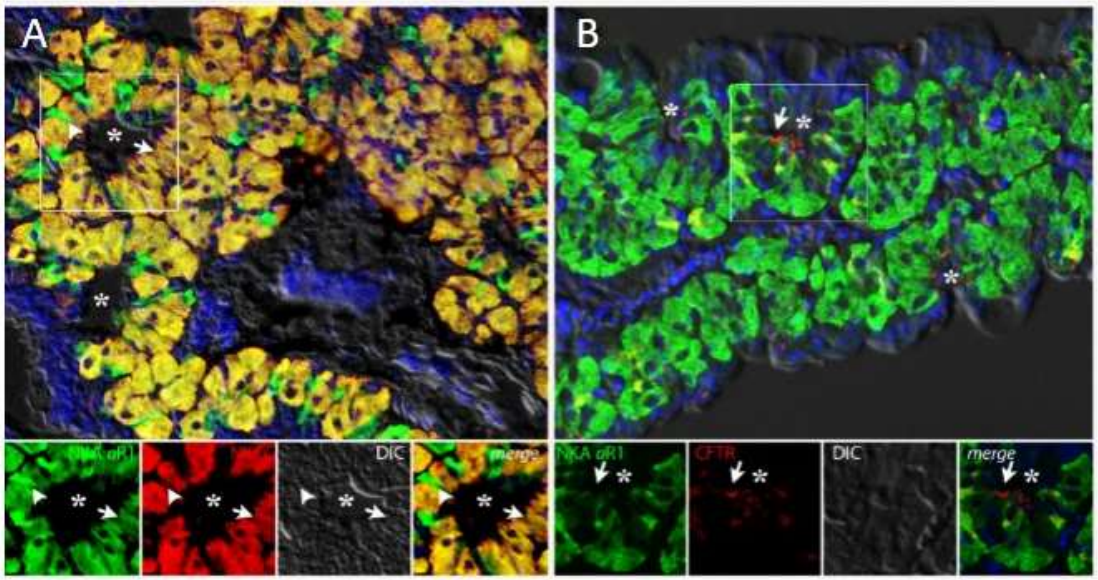

Fig. 3: Immunofluorescence localization of Na^+/K^+-ATPase (αR1, green A, B) with NKCC1 (T4, red A) and CFTR (red, B) in the DO of *P. lineatus*. The *P. lineatus* acclimated in seawater (SW control) 34‰. In A arrows indicate NKCC1+NKA IR cells and arrowheads NKA only IR cells. In B arrows indicate CFTR+NKA IR cells. Asterisks (*) indicate water. See Figure 2 caption for additional information. Scale bar 100 µm.

4. Discussion

Plotosus lineatus showed osmoregulatory capability (euryhaline) in a wide range of salinities from BW (3‰) to 34‰ (SW) as more natural salinity range of Plotosidae catfishes while HSW (60‰) conditions resulted to a challenge which only be encountered in closed or inverted estuaries (Lanzing, 1967; Young and Potter, 2002). Using HSW as challenging conditions allowed us to test the osmoregulatory abilities of *P. lineatus* (Gonzalez, 2012). The *P. lineatus* DO showed conserved mechanism of secondary activity Cl^- transport with NKA, NKCC1 possessing the molecular machinery for active NaCl secretion while the gills clearly represented a secondary role in ion regulation by detecting few ionocytes and low overall NKA expression.

4.1. Salinity acclimation and osmoregulatory responses

The measured plasma ion concentrations were in the range of other teleost species (see review by Freire and Prodocimo, 2007; Whittamore et al., 2012). However, in comparison to other studies in Plotosidae, the plasma Na^+, Cl^- and K^+ concentrations of *P. lineatus* in SW control in the present study were less than those of *P. lineatus* studied by Pucke and Umminger (1979), while Na^+ was not very different from *Cnidoglanis macrocephalus* (Kowarsky, 1973). In both of these studies osmolality was also lower. These detected differences might be due to a number of differences between the studies (sampling and analytical methods, acclimation temperatures 26-28°C versus 19-20°C, species differences). Salinity challenges typically alter plasma osmolality and electrolytes levels in euryhaline teleosts with an initial crisis stage followed by a regulatory stage (Madsen and Naamansen, 1989;

Jensen et al., 1998; Wang et al., 2009). *Plotosus lineatus* acclimated to HSW had higher plasma osmolality and ions (except K^+), and hematocrit. Together, these data indicate a systemic dehydration due to water loss by osmosis, and elevated plasma osmolality representing disturbances from internal fluid shift, which may be problematic resulting in a stress situation and mortality. Thus long-term survival in HSW is likely limited. As a corollary, the Plotosidae catfish *C. macrocephalus* in the closed hypersaline Wellstead Estuary (Australia) was not found in the most hypersaline areas of the estuary (55‰-112‰; Young and Potter, 2002). The increase in plasma osmolality at 60‰ cannot be accounted for by increases in measured inorganic osmolytes (fall 200 mOsmol/kg short of the total osmolality). Although not measured, organic osmolytes such as neutral free amino acids (e.g. taurine and glycine) or small carbohydrates (e.g. myo-inositol) (Fiess et al., 2007) could possibly be an indicator of pathological tissue damage. In contrast to *P. lineatus*, very salinity tolerant species gradual increase plasma ion levels when acclimated to salinities up to about 70-75‰, but thereafter then increase plasma ions in a linear fashion at higher salinities (see review by Gonzalez 2012).

In hypoosmotic conditions, BW, *P. lineatus* are able to maintain plasma Cl^- and osmolality levels but not Na^+ and were better able to regulate Cl^- levels than marine Ariid catfish, which do not have a DO and have higher serum Cl^- levels (Sulya et al., 1960; Pucke and Umminger, 1979). The lower plasma Na^+ and hematocrit suggest a hemodilution but ions were stable.

4.2. Role of gills in salt secretion?

The gill of teleost fishes is typically linked to active ion regulation (Evans, 2008). This is reflected in high levels of NKA, a central driver of ion transport, with dependency of the gill NKA to environmental salinity that may be altered by life history stage, species and experimental conditions in some cases (Evans, 2008; Varsamos et al., 2001; Malakpour Kolbadinezhad et al., 2012). Irrespective of salinity, branchial NKA activity of *P. lineatus* was magnitude lower than DO while unresponsive to HSW acclimation. A similar pattern has been reported in the sharks *Carcharhinus leucas* (Pillans et al., 2005) and *Chiloscyllium punctatum* (Cramp et al., 2015) and ray *Dasyatis sabina* (Piermarini and Evans, 2000) which possess the rectal gland, the extra-branchial salt secreting organ. In elasmobranchs, the gills have a secondary function in ion regulation (Wilson et al., 2002; Evans, 2008). Our results confirm a similarity between gills of *P. lineatus* (Pucke and Umminger, 1979) and elasmobranchs underlining the potential role of DO in salt excretion (Van Lennep, 1968).

The IHC results showed consistency of few branchial NKA-IR cells with NKA activity levels while it was in contrast to observations in most marine teleost fishes (e.g. alewife *Alosa pseudoharengus* Christensen et al., 2012; tilapia *Sarotherodon melanotheron* Ouattara et al., 2009). Restricted few NKA-IR cells to the filament epithelium, were leaving the lamella unimpeded for gas exchange (Evans 2008; Henriksson et al., 2008). Also, co-expressed NKCC1 with NKA-IR cells was very rare to find, although apical CFTR staining was observed in NKA-IR cells. NKCC1 has a key role in the mechanism of secondary active Cl^- secretion and is abundantly expressed in SW type gill ionocytes in teleost fishes (see review by Hiroi and McCormick, 2012). The NKCC1 mRNA expression has been detected in the gills of spiny dogfish *Squalus acanthias* as an elasmobranchs (Xu et al., 1994); however, no immunolocalization of NKCC1 was found in the branchial epithelium of *C. punctatum* (Cramp et al., 2015). This contrasts with the FW stingray *Himantura signifer* where NKCC1 is co-expressed in gill NKA-IR cells following BW (20‰) acclimation (Ip et al., 2013). However, the rectal gland is absent in this species.

4.3. Role of the DO in salt secretion

The notably higher DO NKA specific activity relative to gills, strongly indicates a role for this organ in NaCl secretion. This has also been seen in elasmobranchs with higher rectal gland NKA specific activity compared to their gills (Piermarini and Evans, 2000; Pillans et al., 2005; Cramp et al., 2015). In euryhaline elasmobranchs it has been demonstrated that rectal gland NKA specific activity is higher in SW compared to FW acclimated animals (Piermarini and Evans, 2000; Pillans et al., 2005), although not in response to a moderate HSW acclimation (40‰; Cramp et al., 2015). In *P. lineatus*, DO NKA specific activity was also higher in SW versus BW (3‰) acclimated fish, but unexpectedly was also lower in HSW compared to SW fish. We predicted a similar if not higher NKA specific activity (Cramp et al., 2015). However, considering of the higher DO mass in HSW so that the total DO NKA activity was also higher suggesting an increase in overall capacity. In contrast, *C. punctatum* acclimated to 40‰ did not alter rectal gland size (Cramp et al. (2015). However, larger rectal glands of *D. sabina* (Piermarini and Evans, 1998), *Pristis perotteti* (Gerzeli et al., 1976) and *C. leucas* (Gerzeli et al., 1969; Oguri, 1976) captured in SW compared to FW have been reported. Moreover, in FW stenohaline elasmobranchs the rectal glands are small to vestigial (Thorson et al., 1978).

However, the apparent hypertrophy of the DO may not be adaptive but rather pathological (inflammation or similar). In either case, it is obvious that *P. lineatus* are significantly challenged by HSW and that the DO maybe of limited use under such extreme conditions. The high capacity of *P. lineatus* to move easily between different salinities is suggested due to the observation of a slightly albeit significantly larger DO in BW compared to the SW control fish however, this was not sufficient to increase DO total NKA activity. Since the tissue sampling for the NKA activity measurement had been done after 14 days of acclimation, time course sampling would be applied to have a comprehensive view of NKA activity in the DO of *P. lineatus*.

Strong immunoreactivity co-licalization of NKA and NKCC1 in basolateral tubular system of DO parenchymal cells indicates an ion secretory role in hypo-osmoregulating. In other vertebrate salt secreting organs also the basolateral distribution of NKA and NKCC1 has been demonstrated (Wilson et al., 2002; Lytle et al., 1995; Evans, 2008; Babonis et al., 2009; Babonis and Evans, 2011). In BW, detection of NKCC suggests that maintaining a proportion of active NKCC for cell volume regulation and/or acid-base is important (Gamba, 2005) or it may be present as an inactive non-phosphorylated pool to be quickly activated for an acute response to higher salinity (Flemmer et al., 2010; Christensen et al., 2012).

IHC results of CFTR may reveal the possibility of a different isoform, which cannot be consistently recognized by the monoclonal antibody which is raised against a specific epitope of CFTR (Li et al., 2014). Pucke and Umminger (1979) detected an accumulation of Cl^- ions in the DO epithelium and proposed it was functional in salt secretion. The presence of CFTR in salt glands of reptiles, birds, and elasmobranchs has been confirmed (Shuttleworth and Hildebrandt, 1999) although the antibody used in the present study does not show crossreactivity with elasmobranch (J.M. Wilson personal observations), sea snake (Babonis and Evans, 2011) salt glands or salmonid (S.D. McCormick personal observations) gill CFTRs. Obviously, in future work identifying the putative apical Cl^- channel in DO of *P. lineatus* would firmly establish the presence of the typical ion secretory cell of vertebrate salt glands. Further investigation would be necessary to determine the effect of different salinities (FW to hypersaline) on various isoforms of NKA, their mRNA abundance and likely change with salinities that would be helpful in interpreting the osmoregulatory function of the DO.

4.4. Summary

In summary, the salt secreting function of the DO has been proposed based on physiological, ecological and ultrastructural (Kowarsky, 1973; Lanzing, 1967; Van Lennep, 1968) evidence. Our molecular observation show a strikingly high NKA activity, and localization of NKA, NKCC1 and CFTR in the DO is consistent with the hypothesis suggesting a conservation of rather similar mechanism of ion transporting in secretory cell of vertebrate salt secreting organs (Babonis and Evans, 2011). The gills of *P. lineatus* are unlike those of other marine teleosts and more similar to the gills of elasmobranch fishes in terms of their significances to ion regulation. The unique osmoregulatory strategy of the Plotosidae catfishes amongst the teleosts can be linked to their independent invasion of the marine environment by a FW siluriform ancestor (Lanzing, 1967; de Pinna, 2005). However, in HSW conditions the breakdown of osmoregulatory homeostasis indicated the limitation of this strategy.

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