



Osmoregulation in the striped catfish *Plotosus lineatus*

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General Abstract

The marine catfish, *Plotosus lineatus* belongs to the family Plotosidae which are unique amongst the teleosts in possessing a specialized extra-branchial salt secreting dendritic organ (DO) and the ability to produce hyperosmotic urine (relative to plasma). Typical marine teleosts use the gill as their primary salt secreting organ and are incapable of producing hyperosmotic urine. My thesis provides new insights into the ion regulatory mechanisms in *P. lineatus* and compares them to those of teleost fishes revealing more of the fascinating physiology of marine catfish which evolved from a freshwater ancestor. My primary focus was to provide the first insights into the molecular osmoregulatory mechanisms of the DO and assess its ionoregulatory role at different salinities [brackishwater (BW) 3‰, seawater (SW) 34‰ or hypersaline water (HSW) 60‰] and through DO ligation experiments. In addition I explored the DO's potential role in ammonia excretion, and addressed the potential compensatory roles of the gill, kidney and gut in ion/osmo-regulation during DO ligation. I focused on Na⁺/K⁺-ATPase, a key ion transport protein, measuring its activity, protein and transcript expression as well as additional key ion transport proteins [Na⁺:K⁺:2Cl⁻ cotransporter (NKCC), cystic fibrosis transmembrane conductance regulator (CFTR), carbonic anhydrase (CA), H⁺-ATPase (VHA) and non-erythroid Rhesus (Rh) glycoproteins (Rhag)]. Furthermore, molecular characterization of the kidney was performed to elucidate the mechanism of production of hyperosmotic urine was performed.

For the first time the molecular osmoregulatory mechanisms of the DO have been determined. I found higher DO NKA activity compare to other organs, with basolateral colocalization with NKCC1, and apical localization of CFTR to acinar gland parenchymal cells. These results are consistent with the previous proposed salt secreting function of the DO and the secondary active Cl⁻ secretory mechanism common to other vertebrate NaCl secreting epithelia (e.g. shark rectal gland, sea bird nasal gland). Thus, supporting the hypothesis of conservation of the NaCl secretory mechanism in vertebrates. In response to HSW acclimation, NKCC1 increased as did DO mass. Although DO specific NKA specific activity actually decreased, the increase in DO mass resulted in significantly higher DO

total NKA activity with HSW. Furthermore, proteomic analysis by immunoblotting and IHC expression of CA, and H⁺-ATPase, and quantitative polymerase chain reaction (qPCR) expression of *slc26a6a* (Cl⁻/HCO₃⁻ exchanger) together indicate a role in acid-base regulation of marine catfish DO. Ligation of the DO in SW acclimated fish, resulted in ionoregulatory impairment as indicated by higher plasma Na and Cl concentrations confirming the DO's role in NaCl secretion. Unlike other teleosts, gill NKA activity was low and not responsive to higher salinity or DO ligation. IHC indicated few NKA-IR ionocytes which rarely expressed NKCC1 and CFTR. These results suggest that the gills of marine catfish are not important for active NaCl excretion. The finding of VHA-IR cells opens the possibility that acid-base regulation maybe the primary ionoregulatory function of the gills. Marine catfish were unable to survive in HSW following DO ligation, and higher stress protein Hsp70 was observed in DO indicating a cellular stress in HSW acclimated fish. All together the physiological data indicated a stress situation and systemic dehydration due to osmotic water lose, resulting in problematic disturbances from internal fluid shifts.

The intestine of marine fishes is important in water uptake to compensate osmotic water losses but drinking seawater adds to NaCl loads. The marine catfish is no different with apical NKCC2/NCC and basolateral NKA expression. The changes in NKA activity, protein and mRNA expression of intestine in *P. lineatus* reveal an important response to ligation regardless of the salinity. Increased NKA activity and protein expression of Hsp70 in the posterior intestine indicate a role in water absorption as main stress of fish.

To explain the hyperosmotic urine observed in Protosidae catfishes, I have observed that the *P. lineatus* renal "chloride cells" have high NKA and NKCC1 expression levels. Kidney NKA activity and NKCC1 protein levels also increase with salinity. In contrast, most teleost fish acclimated to higher salinity decrease NKA activity. *P. lineatus* also has an unusual distal tubule with coiling around an ampullar region. Although DT is present, the absence of apical absorptive NKCC2 expression suggests that it is not involved in absorption as is typical of the DT in teleost fishes. Taken together, it seems the kidney of marine catfish has a physiologically effective role in ion/osmo regulation particularly in HSW environment.

Finally, I also explored the possibility of the DO being involved in nitrogen balance through ammonia excretion. I present for the first time molecular and physiological evidence of apical and basolateral expression of Rhag and Rhbg ammonia (NH₃) transporters, respectively, in DO parenchymal cells by IHC, higher mRNA expression of *rhcg1* in SW and BW compared to HSW, and that DO ligation reduced ammonia efflux rates (J_{Amm}). However, while J_{Amm} of fish increased with salinity, these difference do not correlate with protein or mRNA expression levels of Rh glycoproteins in gills and DO, respectively. It therefore appears that additional ammonia transports such as NHE3 might be involved at higher salinities to facilitated NH₄⁺ fluxes rather than NH₃ via Rh glycoproteins.

In summary, this thesis delivers insight into the evolution of salt regulatory mechanisms under different conditions by confirming a converge evolution with the chondrichthyan and tetrapod salt glands, and establishing the molecular and cellular basis for the unusual production of blood-hyperosmotic urine in the kidney of this vertebrate. This work also clearly establishes the primacy of the DO in ion secretion over the traditional gill ion regulatory role. Although the origin of the DO is unknown, given that it is associated with the renal papillae, and DO parenchymal cells and renal chloride cells show similarities in morphology and transport protein expression, we propose that the DO may have a renal origin much like the relationship between the rectal gland and rectal tissue in elasmobranch fishes.

Resumo Geral

O peixe-gato marinho, *Plotosus lineatus* pertence à família dos Plotosidae que é única entre os teleósteos pois tem um órgão secretor de sal extra branquial denominado órgão dendrítico (em inglês denominado dentritic organ, (DO) e assim referido ao longo desta tese) e a capacidade de produzir uma urina hiperosmótica (em comparação com o plasma). Tipicamente os teleósteos marinhos utilizam as branquias como o principal órgão para secreção de sais e não produzem uma urina hiperosmótica. Nesta tese apresento novos conhecimentos dados acerca dos mecanismos de regulação de iões em *P. lineatus* comparando-os com os semelhantes nos peixes teleósteos, a fim de revelar mais acerca da fascinante fisiologia do peixe-gato marinho. Este que terá evoluído a partir de um ancestral proveniente de água doce. O âmbito deste trabalho é proporcionar alguns dos primeiros detalhes sobre os mecanismos de osmorregulação ao nível molecular no DO e determinar a função deste na regulação de iões em diferentes salinidades [água salobra 3‰, salgada a 34‰ e em solução hipersalina a 60‰, (em inglês e assim denominado ao longo desta tese brackish water (BW), seawater (SW) e hypersaline water (HSW), respectively) e através de uma experiência executando um torniquete no DO. Para isso foquei-me na bomba de sódio-potássio (Na^+/K^+ -ATPase) a qual é uma proteína com funções fundamentais para o transporte de iões, e medindo nesta a sua atividade e expressão ao nível da proteína e do gene assim como outras proteínas essenciais ao transporte de iões como é o caso do co transportador [$\text{Na}^+:\text{K}^+:2\text{Cl}^-$ (NKCC), o gene da CFTR (cystic fibrosis transmembrane conductance regulator), anidrase carbónica (CA), bomba de próton H^+ -ATPase (VHA) e a glicoproteína de Rhesus (Rh) não eritrócito (Rhag)]. Também se procedeu à caracterização do rim ao nível molecular a fim de perceber os mecanismos de produção de urina hiperosmótica.

Pela primeira vez os mecanismos moleculares de osmorregulação no DO foram determinados. Elevados níveis de atividade da NKA foram encontrados no DO comparando a outros órgãos, e caracterizado pela co-localização de NKCC1 e localização apical do CFTR na glândula acinar das células parenquimatosas. Estes resultados são consistentes com anteriores que propunham uma função de secreção de sais ao nível do DO e um mecanismo secundário ativo de secreção

de Cl⁻, comum a outros epitélios onde NaCl é secretado noutros vertebrados (por exemplo na glândula retal dos tubarões e nas glândulas do sal das aves marinhas). Desta forma os resultados aqui apresentados corroboram com a hipótese de conservação dos mecanismos de secreção de NaCl nos vertebrados. Em resposta a ambientes HSW, houve um aumento do NKCC1 assim como da massa do DO. Todavia, NKA no DO diminuiu mas o aumento da massa do DO resultou num aumento da NKA total no DO em HSW. Em acréscimo, análises de expressão da CA e H⁺-ATPase ao nível da proteico por imunomarcagem e imunohistoquímica e quantificação da expressão do gene *slc26a6a* (Cl⁻/HCO₃⁻ exchanger) por reação em cadeia da polimerase (qPCR), sugerem uma função na regulação do acido-base por parte do DO do peixe-gato marinho. O torniquete no DO em peixes aclimatizados em SW resultou na incapacidade de regular níveis de iões o que foi demonstrado pelos elevados níveis de Na e Cl no plasma e assim confirmando a função do DO na secreção de NaCl. Ao contrario de outros teleósteos, a atividade da NKA na brânquia é baixa e não respondeu a meios hipersalinos ou ao torniquete no DO. IHC demonstrou poucas células NKA imunorreativas que raramente expressaram NKCC1 e CFTR. Estes resultados sugerem que a brânquia no caso do peixe-gato marinho não é essencial para a secreção ativa de NaCl. A presença de células imunorreativas à VHA por sua vez sugerem que a principal função da brânquia nesta espécie será a do equilíbrio acido-base. Os peixes-gato marinhos não sobreviveram em HSW após aplicação do torniquete no DO e um aumento das proteínas marcadores de stress Hsp70 foi observado neste órgão o que sugere stresse ao nível celular nos peixes aclimatizados a HSW. Em suma, a informação ao nível fisiológico indica uma situação de stress, desidratação sistémica devido a perda de água por osmose e desta forma resultando em perturbações nos fluidos internos.

O intestino de peixes marinhos tem uma função relevante ao nível da captação de água para assim compensar as perdas de água por osmose. Todavia este mecanismos de ingestão de água em meios marinhos implica um aumento da ingestão de NaCl. O peixe-gato marinho não é diferente dos demais, detendo NKCC2/NCC na zona apical e expressão basolateral de NKA. As alterações da atividade, abundancia de proteína e expressão ao nível do mRNA da NKA no intestino de *P. lineatus* revela uma resposta o torniquete, independentemente da

salinidade do meio. O aumento de atividade da NKA e aumentos da expressão ao nível proteico da Hsp70 no intestino posterior indicam uma função de absorção de água em resposta ao stress sentido pelo peixe.

A fim de explicar a presença de uma urina hiperosmótica observada em peixes-gato da família Protosidae, reparei que as células do cloro renais de *P. lineatus* tem elevados níveis de expressão de NKA e NKCC1. A atividade da NKA no rim e os níveis de proteína referentes à NKCC1 também aumentarem em resposta a um aumento da salinidade. Contrariamente, a maioria dos peixes teleósteos aclimatizados a meios hipersalinos demonstram uma redução na atividade da NKA. Os *P. lineatus* demonstram ainda um túbulo distal for a do comum com um enrolamento em volta da região do ducto. Embora a DT esteja presente, a ausência de expressão de NKCC2 na região apical sugere que não esteja envolvida no processo de absorção como é típico do DT em teleósteos. No geral, o rim do peixe-gato marinho aparenta ter uma função fisiológica ao nível de regulação de iões e osmorregulação em especial em HSW.

Finalmente, também explorei a possibilidade do DO estar envolvido na regulação de compostos azotados como excreção de amónia. Aqui é demonstrado pela primeira vez do ponto de vista molecular e fisiológico a expressão apical e basolateral de transportadores de amónia (NH₃) como o Rhag e Rhbg, respetivamente, nas células parenquimatosas por método da IHC, aumento da expressão de *rhcg1* ao nível do mRNA em SW e BW comparativamente a HSW e que o torniquete no DO reduz a taxa de fluxo de amónia (J_{Amm}). Contudo, enquanto a J_{Amm} dos peixes aumenta com o aumento da salinidade do meio, essa diferença não se apresenta correlacionada com a expressão ao nível proteico ou do mRNA nas glicoproteínas Rh nas branquias e no DO, respetivamente. Desta forma sugere-se que outros mecanismos de transporte de amónia como NHE3 possam estar envolvidos em meios hipersalinos de forma a facilitar os fluxos de NH₄⁺ em vez de NH₃ via glicoproteínas Rh.

Em suma, esta tese apresenta novas informações acerca da evolução dos mecanismos de regulação de sais em diversos ambientes e confirmando um processo de evolução convergente com as glândulas do sal de condrósteos e

tetrápodes e no estabelecimento da base celular e molecular para uma produção pouco usual de uma urina hiperosmótica em relação ao sangue no rim deste vertebrado. Este trabalho estabelece ainda a elevada importância do DO na secreção de iões em relação à tradicional branquia com função de regulação iónica. Embora a origem do DO seja desconhecida, dada a sua associação à papila renal e o facto das células do cloro e as células do parênquima renal demonstram semelhanças morfológicas assim como painel de expressão proteico. Desta forma propomos que o DO possa ter uma origem renal à semelhança da glândula retal e tecido retal dos peixes elasmobrânquios.

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List of Abbreviations

<i>actb</i>	Beta actin gene
<i>atp1a</i>	Na ⁺ /K ⁺ -ATPase α subunit gene
ADP	Adenosine diphosphate
ATP	Adenosine-5'-triphosphate
BSA	Bovine serum albumin
BVA	vacuolar (V-type) proton ATPase B subunit
BW	Brakishwater
CA	Carbonic Anhydrase
Ca ²⁺	Calcium ion
CC	Chloride Cell
cDNA	Complementary DNA
CFTR	Cystic fibrosis transmembrane regulator
Cl ⁻	Chloride ion
ClC	Chloride channel
CO ₂	Carbon dioxide
CT	Cycle threshold
DIDS	4,4'-diisothiocyanostilbene-2,2'-disulfonic acid
DNA	Deoxyribonucleic acid
DMSO	Dimethylsulfoxide
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
FW	Freshwater
H ⁺	Hydrogen ion

H ⁺ -ATPase	vacuolar (V-type) proton ATPase
HCO ₃ ⁻	Bicarbonate ion
Hematocrit	Hct
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IHC	Immunohistochemistry
K ⁺	Potassium ion
kDa	Kilodalton
LDH	Lactate dehydrogenase
Mg ²⁺	Magnesium ion
mM	Milimolar
J _{AMM}	Net ammonia excretion
MRC	Mitochondrion-a rich cell
mRNA	Messenger ribonucleic acid
MS-222	Tricaine methanesulfonate
Na ⁺	Sodium ion
NAD ⁺	Nicotinamide adenine dinucleotide, oxidized form
NADH	Reduced nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
NCC	Na ⁺ :Cl ⁻ cotransporter
NGS	Normal goat serum
NH ₃	Gaseous or unionized ammonia
NH ₄ ⁺	Ionized ammonia or ammonium
NHE	Na ⁺ /H ⁺ exchanger
NKA	Na ⁺ /K ⁺ -ATPase

NKCC	Na ⁺ :K ⁺ : 2Cl ⁻ cotransporter
PAT1	Putative Anion Transporter Cl ⁻ /HCO ₃ ⁻ exchanger
PBS	Phosphate buffered saline
ppt	Parts per thousand
PFA	Paraformaldehyde
PC	Principal cell
qPCR	Quantitative polymerase chain reaction
RBCs	Red blood cells
Rhcg	Non-erythroid Rhesus C glycoprotein
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEI	Sucrose, EDTA, Imidazole buffer
SO ₄ ²⁺	Sulfate ion
SW	Seawater
slc26a6	Putative Anion Transporter 1 (PAT1) Cl ⁻ /HCO ₃ ⁻ exchanger gene
T	Temperature (°C)
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TBS	Tris-buffered saline
TL	Total length
TPBS	Tween-20 in Phosphate buffered saline
TTBS	Tween-20 in Tris-buffered saline

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In these pages that make up my thesis, I am standing here at the end of this journey or beginning of a new one. Representing my hard work over the past years spent on this research; however, it would not have been achieved without the support, collaboration, friendship and dedication of many people during this time.

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This thesis is dedicated to my parents, symbols of dedication and calmness, full of kindness my sisters Saeedeh and Sara, awesome nephew Soroush who is such a miracle in our family life, which has always been extremely supportive of me, providing motivation over the years. And finally to my lovely, patient wife Azin for her dignity and unconditional, nonstop encouragement and support.

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**“It does not matter how slowly you go as long as you do not stop”
Confucius**



Author's declaration

In agreement with the Portuguese law through the article 4th of the “Regulamento Geral dos Terceiros Ciclos de Estudos da Universidade do Porto” of May 11th (GR.02/90/2015), the author states devotion in a major contribution to the conceptual design and technical execution of the work, interpretation of the results and manuscript preparation of the published or under publication articles included in this thesis and presented below.

Publications

Malakpour Kolbadinezhad, S., Hajimoradloo, A., Ghorbani, R., Joshaghani, H and Wilson, J. M. 2012. Effects of gradual salinity increase on osmoregulation in Caspian roach *Rutilus caspicus*. Journal of Fish Biology doi:10.1111/j.1095-8649.2012.03317.x.

Malakpour Kolbadinezhad, S., Coimbra J., Wilson, J. M. 2017. Salinity effects on marine catfish *Plotosus lineatus* osmoregulation (*in preparation*)

Malakpour Kolbadinezhad, S., Coimbra J., Wilson, J. M. 2017. Effect of dendritic organ ligation on marine catfish *Plotosus lineatus* osmoregulation (*in preparation*).

Malakpour Kolbadinezhad, S., Coimbra J., Wilson, J. M. 2017. Molecular characterization of the unique kidney of the marine catfish *Plotosus lineatus* (*in preparation*).

Malakpour Kolbadinezhad, S., Coimbra J., Wilson, J. M. 2017. The dendritic organ of marine catfish *Plotosus lineatus* as an ammonia excretory organ (*in preparation*).

Thesis Aims

The present Doctoral Thesis is focused on “Osmoregulation in the striped catfish *Plotosus lineatus*”.

For the thesis, I address the hypotheses that marine Plotosidae catfishes are unique amongst the teleosts in having an extrabranchial salt gland, the dendritic organ (DO). To assess these hypotheses the main objectives proposed are: the dendritic organ is a salt secreting gland; assessment of ion transport protein expression in the context of the osmoregulatory responses of marine catfish to experimental salinity changes (brackishwater to hypersaline water), molecular, biochemical, morphological and physiological characterization of *P. lineatus* salt regulatory mechanisms (DO, gill, kidney and intestine was also investigated, DO ligation experiments to elucidate its importance and compensatory responses of gill, kidney and/or intestine, molecular study of unique kidney to produce hyperosmotic urine, ammonia excretion of marine catfish and the hypothesis of DO as ammonia excretion organ was tested.

Dissertation organization

The Doctoral Thesis is organized into seven chapters. Chapter one consists on a general introduction where mechanisms in seawater and freshwater osmoregulation; the rapid response and/or acute transition to changing environmental salinity; and background on marine catfish will be provided. Specifically, the general biology of the marine catfish; a perspective on marine catfish which is unique amongst the teleosts in that they possess the DO, an extrabranchial salt secreting organ; a kidney that is unusual in producing a hyperosmotic urine; and why do research on marine catfish? Chapter two covers general materials and methods that are used in most of the following research chapters to avoid repetition. The following four chapters are structured as independent research chapters covering salinity effects (CH3), dendritic organ ligation (CH4), molecular characterization of the kidney (CH5), and DO as an ammonia excretory organ (CH6). The final chapter (CH7) is a synthesis chapter that will integrate the results from the research chapters into a general discussion.

Chapter 1:
General introduction

1. Introduction

1.1 Osmoregulation

As the most basally derived vertebrate group, the fishes are adapted to living in both marine and freshwater aquatic environments, coping with osmotic/ionic challenges of various salinities, pH or ion compositions, in contrast to terrestrial tetrapod animals. The resulting diverse physiologies of the fishes result of natural selection are achieved by adaptation to enormous ranges of differences in these aquatic environments providing important useful information for elucidating the mysteries of the early evolution of vertebrates. In teleost fishes, highly efficient ion/osmoregulatory mechanisms lead to maintenance of body fluid homeostasis, which is necessary for the normal operation of cellular biochemical/physiological processes (Hwang and Lee, 2007, 2011).

Marine teleosts actively hypo-osmoregulate to compensate for passive water loss and salt gain by osmosis and diffusion, respectively, from seawater across their body surfaces (Figure 1) (Marshall and Grosell, 2006). They drink seawater and in the intestine NaCl uptake is used to osmotically drive water absorption in order to address water balance. In addition the kidney produces minimal isoosmotic urine and is the main site of Ca^{+2} , Mg^{+2} , and SO_4^{-2} excretion. The gill is the main organ involved in the excretion of the NaCl load from passive uptake and drinking in teleost fishes via specialized branchial ionocytes called chloride cells or seawater type mitochondrion-rich cells (MRC) (Evans et al., 2005). These cells are rich in mitochondria and have an amplified basolateral membrane with high Na^+/K^+ -ATPase and $\text{Na}^+:\text{K}^+:2\text{Cl}^-$ co-transporter (NKCC) expression and apical cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel expression. In the current model (Figure 2), Na^+/K^+ -ATPase drives the uptake of Cl^- from the blood against its electrochemical gradient via NKCC1 (basolateral isoform). The intracellular Cl^- exits the cell via the apical CFTR Cl^- channel down its electrochemical gradient. Na^+ accumulates in the intercellular space, and exits across a leaky tight junction between neighbouring chloride and accessory cells following its electrochemical gradient.

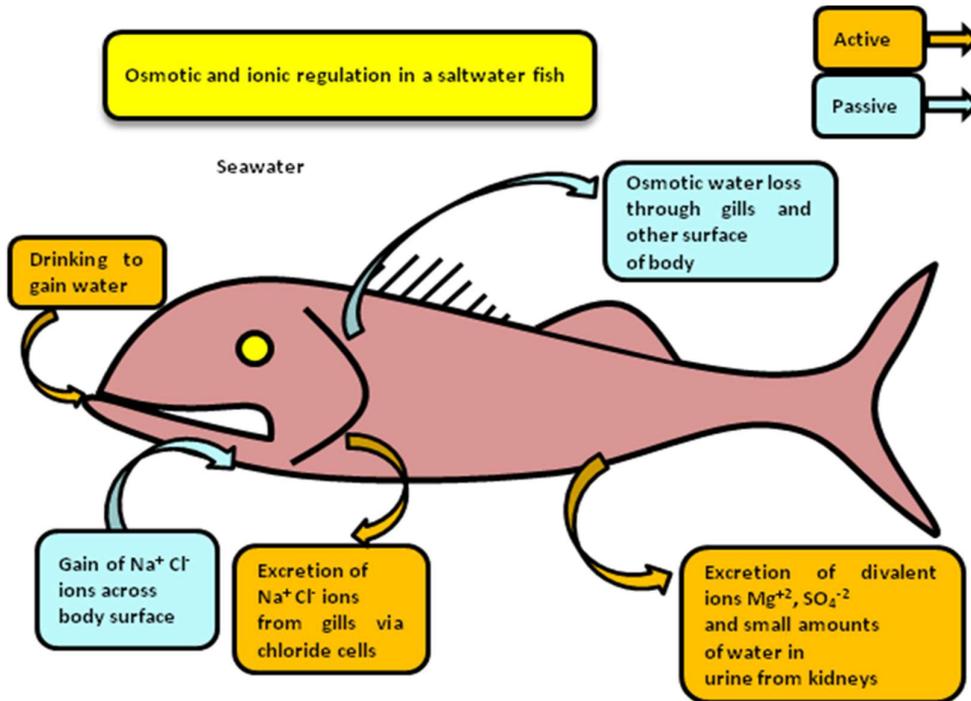


Fig. 1. Osmotic and ionic regulation in a saltwater fish (modified according Beyenbach, 2004)

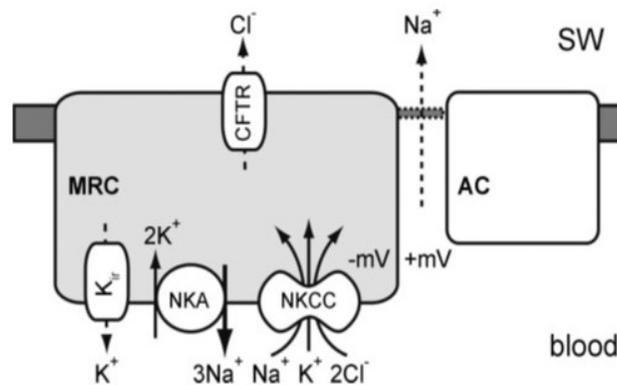


Fig. 2. Working model for the extrusion of NaCl by the marine teleost gill epithelium. The mitochondrion rich cell (MRC) Na^+/K^+ -ATPase (NKA) creates the inward Na^+ gradient to drive uptake of Na^+ , K^+ , and Cl^- from the plasma via a basolateral $\text{Na}^+:\text{K}^+:2\text{Cl}^-$ cotransporter (NKCC); and K^+ is recycled via a K^+ channel (K^+) that helps maintain the membrane potential. Cl^- is extruded across the apical membrane via a Cl^- channel (CFTR) down its electrochemical gradient. The transepithelial electrical potential across the gill epithelium (plasma positive

to seawater) drives Na^+ across the leaky tight junctions paracellularly between the MRC and the accessory cell (AC). (Modified from Evans et al., 2005)

The rapid response and/or acute transition to changing environmental salinity becomes a crucial challenge for avoiding significant internal osmotic disturbances. There are two periods of acclimation for euryhaline teleosts to hyperosmotic environments: a) a crisis period (minutes to hours) involving a rapid increase in gill-ion fluxes, activating exist proteins, water transport and/or other mechanisms (Houston 1959; Wang et al., 2009), and elevated plasma ions and osmolality followed by b) a regulatory period (hours to days onward) including increases of gill NKA activity accompanied by a proliferation and development of MRCs presumably hormonally regulated allowing for increased transport capacity (McCormick and Bradshaw, 2006), increasing net Na^+ and Cl^- efflux and restoring plasma ions balance (Evans et al., 2005; Malakpour Kolbadinezhad et al., 2012).

Fish initiate a drinking response to compensate for the passive osmotic loss of water (Marshall and Grosell, 2006). The gastrointestinal tract facilitates survival by increasing intestinal Na^+/K^+ -ATPase activity and the expression of ion transporters for solute coupled water absorption, increasing the capacity for water absorption(see review by Grosell 2011; Gonzalez 2012; Whittamore 2012). The excess salt from drinking seawater is excreted by the gill chloride cells (Evans et al., 2005).

The renal response to the need for water conservation is a reduction in glomerular filtration rate via reduce the number of filtering glomeruli (Beyenbach and Frömter, 1985; McDonald, 2007) referred to as the 'glomerular intermittency effect' and/or glomerular blood flow (McDonald, 2007) thus lead to the reduction of urine production (Marshall and Grosell, 2006). The kidneys become the primary route to get rid of Ca^{2+} , Mg^{2+} , and SO_4^{2-} derived from intestinal uptake (McDonald and Grosell, 2006; Hickman and Trump, 1969; McDonald et al., 2002; Beyenbach, 2004) . Due to the increased demand for renal MgSO_4 excretion and the need to maintain urine flow rates survival may be limited in hypersaline environments when water loss is critical (see review by Gonzalez 2012). In both freshwater and saltwater fishes, secondary ion transporters necessary to maintain homeostasis are achieved via kidney NKA activity in a crucial role of

provided the driving force (Perry et al., 2003; Marshall and Grosell, 2006). Some marine tetrapods and chondrichthyan fishes have specialized salt glands that excrete a concentrated NaCl solution (e.g., the nasal salt gland secretes salt in marine birds, lachrymal gland in marine turtles, and lingual glands in sea snakes, saltwater crocodiles, rectal gland in elasmobranches (Shuttleworth and Hildebrandt, 1999). The parachymal cells of these glands have similar characteristics (cell structure: large mitochondrion-rich cells with extensive basal lateral membrane folding, shallow 'leaky' tight junctions, and ion transport protein expression: NKA, NKCC1, CFTR and Kir) as teleost branchial chloride cells and the mechanism of NaCl excretion is the same (secondary activity Cl⁻ secretion) (Marshall and Grosell 2006; Holmgren and Olsson, 2011). The numerous origins of this tissue can be assumed to be independent and there are a few hypotheses about the processes underlying the convergent evolution of salt glands across taxa (Babonis and Evans, 2011).

1.2 Marine catfish *Plotosus lineatus*

The marine catfish, *Plotosus lineatus* (Thunberg, 1787) belongs to the order Siluriformes that includes 35 families (Nelson 2006) and is commonly known as the catfish eel, eel-tailed catfish, lined catfish, striped catfish eel, striped eel catfish (<http://www.fishbase.org>). *P. lineatus* exhibits amphidromous characteristics (Pucke and Umminger, 1979) and from an ecological point of view, this is the only catfish species which can be found on coral reefs, entering estuaries or in tide pools (Myers, 1999; Edelist et al., 2012). The vast majority of catfishes are restricted to fresh water and only members of the Ariidae or Plotosidae families are found in marine environments (Nelson, 2006). *Plotosus lineatus* belongs to the Plotosidae, a family consisting of 35 largely freshwater species in 10 genera that are distributed from the Indian Ocean to the western Pacific from Japan to Australia and Fiji (Lanzing, 1967; Nelson, 2006). The phylogeny of the Plotosidae has not been resolved so questions about whether this family has a marine or freshwater origin remain unanswered (Pinna, 2003).

Plotosus lineatus can be recognised by their striped colouration pattern, four pairs of barbels around the mouth (Golani, 2002), and its body shape which tapers to a point posteriorly (<http://www.fishbase.org/summary/4706>). Dorsal and

anal fins are continuous with the caudal fin. It eats mainly benthic invertebrates and algae with larger individuals sometimes eating small fishes. Small black juveniles form dense ball-shaped schools of about 100 fish, while large adults that may be less distinctly striped are solitary or occur in smaller groups of around 20 and are known to hide under ledges during the day (<http://www.fishbase.org/summary/4706>). The species is primarily tropical originally with a wide Indo-Pacific distribution but has been recorded down the east and west coasts of Australia to Sydney, New South Wales and Esperance, Western Australia, respectively (Lanzing, 1967). They are also reported in Mediterranean (Golani, 2002), Persian Gulf, Red sea (Ali et al., 2007).

Plotosus lineatus has invaded the Mediterranean from the Red Sea and was included in the list of 100 worst invasive species (Streftaris and Zenetos, 2006) in the Mediterranean because of its negative health impact due to its single highly venomous serrate spine at the beginning of the first dorsal and at each of the pectoral fins, that cause painful injuries and even be fatal in rare cases (Haddad et al., 2008). However, no deaths have been reported, so far (Edelist et al., 2007). In Taiwan, Hong Kong, and Australia high injury rates have been reported (Isbister 2001; Tam et al., 2007). Furthermore, in the aquarium industry *P. lineatus* has a significant commercial value (Scandol and Rowling, 2007).

1.3 Dendritic organ (DO)

Unique amongst the teleosts are members of the marine catfish family Plotosidae that possess a specialized salt secreting dendritic organ (DO) (Lanzing 1967). The DO is a small fleshy external organ situated on the ventral caudal surface of the fish, posterior to the urogenital papilla and protected by the pelvic fins (Hirota, 1895). It is found in both sexes from early life stages (Lanzing 1967; Laurenson et al., 1993) and contains glandular acini of parachymal cells morphologically similar to salt gland cells suggesting that this organ is also involved in salt transport (Van Lennep and Lanzing, 1967; and Van Lennep, 1968). (Figure 3)

Bloch (1794) provided the first morphological description of the DO and was followed in more detail by Brock (1887) and Hirota (1895) who provided more details on the vascularization with an unusual development of the venous system,

innervation. From its name, the dendritic organ has a symmetrical tree-like branching into irregular lobes with an extensive surface area. The Plotosidae is a predominantly freshwater family while marine and estuarine species possess a DO; however, two species, *P. papuensis*, *Oloplotosus mariae*, that have only been found in fresh water still possess a DO (Lanzing, 1967).

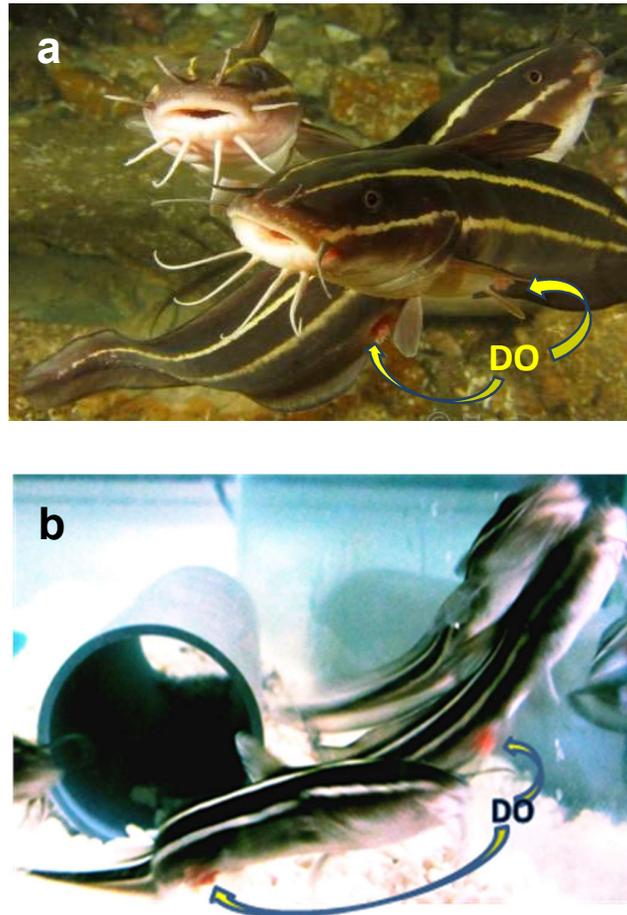


Fig 3. Dendritic organ (DO) of marine catfish *P. lineatus* (a: Striped Catfish at Swansea, New South Wales, 20 February 2011, by Matt Dowse © Matt Dowse; b: CIIMAR, Porto, Portugal, November 2013 by Malakpour Kolbadinezhad)

Van Lennep and Lanzing (1967) made the first detailed electron micrographic study of the DO and identified two potential ion transport cells: principal cells (PC) and clear cells (CC). The PC are large pear-shaped cells with a large ovoid nucleus and have numerous tubular invaginations of the basal and to a lesser extent the lateral plasma membranes that are associated with numerous elongate mitochondria, running in parallel bundles to the apical

cytoplasm. In contrast, the CC are typically smaller, and angular in shape with an irregular nucleus and have an interlacing network tubules that possess a remarkably regular arrangement making up a three dimensional (3D) array communicating with the intercellular spaces similar to the tubular system of teleost chloride cells, and have shorter and fewer mitochondria than PC. Van Lennep (1968) demonstrated that these tubules were continuous with the extracellular space using the lanthanum technique; however, he was unable to demonstrate ATPase activity. Both cell types reach the lumen of the acinus and the apical tight junctions have been shown to be shallow (Van Lennep and Lanzing, 1967).

Taken together, the observations of abundant mitochondria, a tubular system, and 'leaky' apical tight junctions in DO parenchymal cells (Van Lennep and Lanzing, 1967; Van Lennep, 1968) suggest structural and morphological similarity to the salt secretion organs in other vertebrates (Van Lennep 1968; Kirschner 1980). Pucke and Umminger (1979) also demonstrated that DO parachymal cells had high Cl⁻ levels detected with the silver technique, similar to gill chloride cells. Finally Kowarsky (1973) has provided some additional physiologic evidence of the role of the DO in salt regulation in *Cnidoglanis microcephalus* by demonstrating that ligation of the DO impaired plasma ion balance and survival. However, direct evidence is still lacking for a role of the DO in ion regulation.

1.4 Kidney

In SW, hypotonicity of body fluid relative to the environment results in salt entering by diffusion and/or osmotic loss of water across body surfaces (Marshall and Grosell, 2006). Under these conditions, the kidney is primarily involved in conservation/reabsorption of water that is limited to the excretion of excess divalent ions (Ca²⁺, Mg²⁺ SO₄²⁻) in a small volume of isotonic (relative to blood) urine (Marshall and Grosell, 2006; Evans, 2008). Conversely in the FW environment, fish body fluid is hypertonic to the environment, the main challenge of water entering the body via osmosis from permeable body surfaces (gill and skin) requires the compensatory production of a large volume of hypoosmotic

urine accompanied by ion reabsorption such as Na^+ and Cl^- from the filtrate (Marshall and Grosell, 2006; Evans, 2008).

The kidney of marine teleosts generally cannot produce urine that has a higher salt concentration than the blood because of the lack of Henle's loop and collecting duct system as seen in mammalian kidney or countercurrent tubule flow as observed in elasmobranchs and lamprey (McDonald, 2007; Evans, 2008). However, there have been reports of hyperosmotic urine (Fleming and Stanley, 1965; Hickman, 1968; Kowarsky, 1973; Stanley and Fleming, 1964) and McDonald and Grosell (2006) measured urine osmolalities that were at times greater than those in plasma at a range of environmental salinities in the gulf toadfish, *Opsanus beta*. Strikingly, amongst these examples only the Plotosidae catfish *Cnidogobius macrocephalus* has been shown to produce hyperosmotic urine under steady state conditions (Kowarsky, 1973). A study of kidney morphology in *P. lineatus* (Ogawa, 1959) did not suggest anything particularly unusually; however, Hentschel and Elger (1987) noted that the distal tubule has an unusual coiled arrangement and that the collecting tubule has renal chloride cells suggestive of a salt secreting role. In addition, nothing is known about renal ion transport protein expression and thus a molecular investigation of the unique marine catfish kidney is warranted to elucidate the underlying mechanisms of hyperosmotic urine formation.

1.5 Intestine

Drinking is an important component of marine fish osmoregulation to compensate for the passive loss of water by osmosis. The gastrointestinal tract of marine teleost is involved in osmoregulation through the desalination of imbibed seawater by the esophagus accompanied by isosmotic water uptake across the intestine (Usher et al., 1991; Gentz et al., 2011). Solute coupled localised hypertonicity of the lateral intercellular space is the proposed mechanism for intestinal fluid absorption (see reviews by Grosell 2011; Whittamore 2012). This mechanism is referred to as "the standing gradient model" (Larsen and Møbjerg, 2006) and the osmotic and/or ionic local gradients between the gut lumen and the lateral intercellular space are driven by basolateral NKA (see review by Grosell 2011) associating with apical NKCC for

uptake of NaCl (Gregório et al., 2013). Water follows the movement of ions. Increased drinking rate, because of greater fluid loss by diffusion (McDonald and Grosell, 2006), more desalination via esophageal or intestine (Madsen et al., 2014) subsequent more salt loading to the body by ingested SW might be expected by exposing to the HSW of the fish. Thus the intestinal ion and water absorption becoming more important in the later condition (Guffey et al., 2011; McDonald and Grosell, 2006). Since there have not been found any reports of the marine catfish intestines (anterior and/or posterior) physiological role in osmoregulation, in this thesis various salinities and the potential compensatory function in DO ligation period have been addressed.

In seawater fish, the gill is important for acid-base regulation while because of much lower urine flow rates than in freshwater teleosts (Marshall and Grosell, 2005), thus potential very limited renal adjustment of acid-base balance lead to less role compare freshwater fish (Claiborne et al., 1994, 2002, Deigweiher et al., 2008). Generally, the interplay between the intestinal tract and the gills of marine teleost and in contrast between the gills and the kidney of freshwater teleost maintain acid-base balance (see review by Grosell 2011). In a hyperosmotic environment created cellular acid-base disturbances by osmoregulation is unique to marine teleost fish. The inter conversion and transportation of the ions involving as acid-base equivalents (H^+ and HCO_3^-) in enterocytes are required for maintaining pH homeostasis (Taylor et al., 2010).

1.6 Gills

The presence of the specialized salt secreting DO now places a question mark over the ionoregulatory importance of the gill that dominates in other teleosts (Evans et al., 2005). Apart from descriptive morphological studies more than 38 years ago (Pucke and Umminger, 1979), nothing is known about the molecular machinery of the gills in this species. In marine elasmobranches, which also have an extrabranchial salt secreting organ (rectal gland), the gill functions primarily in acid-base regulation and not in ion (NaCl excretion) regulation (Evans et al., 2005). It is possible that in the marine catfish, a similar partition in function is present. This question of the functional role of the gill will be addressed through different salinities acclimations and DO ligation.

The fish gill is also the site of excess nitrogen excretion, in the form of ammonia, and it has been suggested that there is a link of ion regulation to the ammonia excretion pathways (Wright and Wood, 2009). Central to ammonia excretion is the expression of the non-erythroid Rhesus (Rh) glycoproteins that facilitate ammonia transport in fish as NH_3 (Nakada et al., 2007; Nawata et al., 2008; Braun et al., 2009; Wright and Wood 2009, 2012). Furthermore, NKA and NKCC may also be important to ammonium ion (NH_4^+) transport confirmed (Evans et al., 2005; Hwang et al., 2015) because of the similarities in the hydration radius of NH_4^+ and K^+ allowing substitution at transport sites (Randall et al., 1999; Alam and Frankel, 2006). We pose the hypothesis that there is a potential additional physiological role for the DO in contributing to ammonia excretion.

1.7 Aims of thesis

The marine Plotosidae catfishes are unique amongst the teleosts in having an extrabranchial salt gland, the dendritic organ. The central hypothesis of the thesis is that ***the dendritic organ is a salt secreting gland***. To address this hypothesis the following objectives were designed to address the molecular and physiological characterization of this organ as well as its impact on the other iono regulatory organs: the gills, kidney and intestine.

1- Molecular, biochemical, morphological and physiological characterization of *P. lineatus* salt regulatory mechanisms (DO, gill, kidney and intestine).

2- The physiological significance of the DO in ion regulation will be addressed through the characterization of the responses of the marine catfish to experimental salinity changes (brackishwater to hypersaline water).

i) Determine salinity challenge response from gene to whole animal level.

ii) DO ligation experiments to elucidate its importance and compensatory responses of gill, kidney and/or intestine.

These results will also be used to address the hypothesis that ***the DO has the same conserved secondary active Cl⁻ secretory mechanism found in salt***

secreting organs in other vertebrate groups and that ***DO has a role in ammonia excretion.***

3- RT-PCR based techniques were used to identify *P. lineatus* ion transport protein orthologues and expression quantified by qPCR and protein level expression determined using immunoblotting and immunohistochemistry.

1.8 Organization of the thesis

The Doctoral Thesis is organized into seven chapters. Chapter one consists on a general introduction where mechanisms in seawater and freshwater osmoregulation; the rapid response and/or acute transition to changing environmental salinity; and background on marine catfish will be provided. Specifically, the general biology of the marine catfish; a perspective on marine catfish which is unique amongst the teleosts that possess the DO; a kidney that is unusual in producing a hyperosmotic urine; and why do research on marine catfish? Chapter two covers general materials and methods that are used in most of the following research chapters to avoid repetition. The following four chapters are structured as independent research chapters covering salinity effects (CH3), dendritic organ ligation (CH4), molecular characterization of the kidney (CH5), DO as an ammonia excretory organ (CH6). The final chapter (CH7) is a synthesis chapter that will integrate the results from the research chapters into a general discussion.

1.8 Novel aspects of the thesis

There is a high degree of novelty in the thesis, due to the significance of the possession of the DO to develop a link to converge evolution with the tetrapod lineage in addition to the unusual production of blood-hyperosmotic urine in the kidney of this vertebrate. Non-mammalian vertebrates are generally incapable of urinary concentration. Thus, this work was contributed not only to the elucidation of the basic mechanisms of salt regulation in marine catfish but will also offer us some insight into the evolution of salt regulatory mechanisms under different circumstances. This work may also offer us some insight into the evolution of salt regulation in vertebrates in general. As consistency in form and function of salt

gland have been conserved throughout the evolution of marine vertebrates suggesting that the genetic mechanism leading to the development of this tissue type may also be conserved (Babonis et al., 2009). Thus, studying this system may reveal a mechanism by which these glands have been co-opted from unspecialized gland precursors as reported in other analogous salt glands.

Chapter 2:
Material and Methods

2. Material and Methods

This chapter covers the general methods for animal holding, salinity acclimation, sampling, and analytical techniques. Analytical techniques, include histological analysis, ion quantification,

2.1. Modulation of salinity status and animals collection

Marine catfish *Plotosus lineatus* (~8-13 g) were purchased from Tropical Marine Centre (TMC) Portugal and transported to Laboratory of Ecophysiology CIIMAR (Porto). All fish were acclimatized to laboratory conditions in a 100 L tank with seawater (SW) 34‰, mechanical and biological filtration with aeration and normal photoperiod for three weeks prior to the start of the experiment to avoid any confounding effects of handling stress on osmoregulation (Biswas et al., 2006). Seawater was made up using Instant Ocean® salt. During this period the fish were fed twice daily with diced fish fillets except four days before samplings. Salinity, temperature (range at 26-28°C) and pH (range 7.7–7.9) were measured and fish behaviour was checked daily.

2.2. Salinity acclimation

Three salinity levels were investigated for two weeks [brackishwater (BW) 3‰, seawater (SW-control) 34‰, hypersaline water (HSW) 60‰]. Initially, individuals were transferred to a 30 L tank, in which salinity was changed in a stepwise fashion, from 34 (main tank) to 3 and 60‰ (smaller 30L tanks), by 5‰ per day. Saline media were made from Instant Ocean® salts prepared from a stock solution of 100‰ and diluted to the appropriate salinities. Salinity was reduced by removing water from the smaller tanks and adding an appropriate amount of dechlorinated tap water. Fish were kept in the same tank to decrease handling stress. In order to standardize the water change disturbance between the different groups, a water change of the SW-control group was also conducted. The mortality in these was monitored and moribund fish removed from the experimental tanks. Fish were maintained in the lab and used according to the Portuguese Animal Welfare Law (Decreto-Lei no.197/96) and animal protocols were approved by CIIMAR/UP.

2.3. Sampling

Individual marine catfish were netted and euthanized in a separate smaller tank (1L) with an overdose of ethyl-m-amino benzoate-MS-222 (1:5000, pH 7.5 adjusted with NaHCO₃; Pharmaq UK), weighted (± 0.01 g) and total length (mm)

measured. Blood was collected following caudal transaction using a heparinized capillary tube and was centrifuged at 13000xg using a hematocrit centrifuge (Heraeus Pico 17 Centrifuge, Thermo Scientific) for 5min at room temperature. Haematocrit (Hct) was measured in duplicate to the nearest millimeter then converted to percentage of total blood volume. The isolated plasma was then frozen in liquid nitrogen and kept at -80°C. The following tissues were collected: gill, dendritic organ (DO), kidney, anterior and posterior intestine, liver, heart, brain, muscle, skin and eye then were immediately frozen in liquid nitrogen and stored at -80°C. Gill filaments samples from the second arch on the left side, DO, kidney, and intestine were also excised, immersed in 100µl of ice-cold SEI buffer [sucrose (150 mM), EDTA (10 mM), imidazole (50 mM), pH 7.3] and frozen at -80°C. An additional piece of deskinning epaxial muscle (~1g) was collected into a pre-weighed tube for water and ion analysis. In addition sets of six individuals, blood sampling was done as before (see above), and the body cavity opened by a ventral incision and immersion fixed in 10% neutral buffered formalin (NBF 10%) overnight at 4°C. Fixed tissue was then stored in 70% ethanol at 4°C.

2.4. Histology Analysis

Gill, DO, kidney, and intestine were excised from the fixed carcass and placed into histology cassettes for processing for paraffin embedding. Tissues were dehydrated through an ethanol series, cleared with Clear Rite (Richard Allen Scientific, Kalamazoo MI) and embedded in paraffin (Type 6; Richard Allen Scientific). Sections were cut at 5µm with a Reichert Biocut 2030 microtome and stained with hematoxylin-eosin, Alcian blue (pH 2.5) and/or Periodic Acid Schiff staining protocols. Micrographs were taken with a Leica DFC300FX digital colour camera mounted on a Leica DM 6000 B microscope. Images were imported into Photoshop CS3 to resize and adjust brightness and contrast while maintaining the integrity of the data.

2.5. Ion quantification

One gram of muscle tissue was collected (wet mass) then dried to constant mass at 60°C (dried mass) for the determination of muscle water content [MWC= (wet.mass -dry.mass)/wet.mass].

The dried muscle samples were then digested in five volumes of 65% nitric acid for 3 days. The Na⁺ and K⁺ concentrations were quantified using a flame photometer following dilution in milliQ water (model PFP7; Jenway, Felsted, UK) and expressed as $\mu\text{mol} \cdot \text{g}^{-1}$ wet mass. Plasma samples were also analysed by flame photometry (PinAAcle 900T Atomic Absorption Spectrophotometer; Perkin Elmer Waltham MA). Chloride concentration was measured in plasma samples by reaction with mercuric thiocyanate to form mercuric chloride and free thiocyanate ions. Thiocyanate ions react with ferric ions to form an orange complex of ferric thiocyanate, the absorbance of which is proportional to the amount of chloride in the specimen, and it was measured at 480 nm (Küffer et al., 1975).

2.6. Measurement of Na⁺/K⁺-ATPase activity

The NKA activity was measured according to the microassay protocol of McCormick (1993) with some modifications.

The filaments were thawed and homogenized in SEI buffer containing 0.1% deoxycholic acid at 5800 RPM for 2x15s in a Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) and immediately centrifuged at 15000xg for 5 minutes at 4°C to remove large debris. For the assay, 10 μl of the supernatant were added to 200 μl of pH 7.5 assay mixture [imidazole buffer (50 mM), phosphoenolpyruvate (PEP) (2.8 mM), nicotinamide adenine dinucleotide (NADH) (0.22 mM), ATP (0.7 mM), lactate dehydrogenase (LDH) (4.0 U) and pyruvate kinase (PK) (5.0 U)] including the following salts NaCl (47.2 mM), MgCl₂ (2.6 mM) KCl (21 mM)]. Assays were run in two sets of duplicates, one set containing the assay mixture and the other assay mixture plus ouabain (1 mM, Sigma–Aldrich Chemical Co.; St. Louis MO) to specifically inhibit NKA activity. ATPase activity was detected by enzymatic coupling of ATP dephosphorylation to NADH oxidation measuring at 340nm with a temperature controlled plate reader (Powerwave 340; Biotek, Winooski, VT) and Gen5™ reader control and data analysis software for 10-20 min at 25°C. Total protein concentrations were determined using the Bradford (1976) dye binding assay with a bovine serum albumin (BSA) standard at 600nm and the results are expressed as $\mu\text{moles ADP mg}^{-1} \text{ protein h}^{-1}$.

2.7. Immunoblotting

The tissue samples were homogenized in 50mM imidazole buffer pH 7.5 for 2x15s in a Precellys 24 homogenizer (Bertin Technologies) and immediately centrifuged at 15.000xg for 5 minutes at 4°C. An aliquot of the supernatant was mixed with an equal volume of 2x Laemmli's buffer (Laemmli, 1970), heated for 10 minutes at 70°C and then stored at 4°C. Total protein was measured in the leftover supernatant using Bradford protein assay method with Coomassie Brilliant Blue G-250 (Bradford, 1976) using bovine serum albumin (BSA) as a standard. Protein concentration of the heated samples in Laemmli's buffers was adjusted to 1 $\mu\text{g } \mu\text{l}^{-1}$ using 1x Laemmli's buffer in order to have uniform loading volumes of sample. Samples were loaded at 10-20 μg per well onto 1.5mm thick mini vertical polyacrylamide gels (10% T resolving gels with 4% T stacking gels) using the BioRad MiniProtean III system (BioRad Laboratories; Hercules, CA) and was run at 75V for 15 minutes following a 1 hour run at 150V. Gels were then equilibrated in transfer buffer (48mM Tris, 39 mM glycine, 0.0375% SDS) and bands were transferred to PVDF membranes (Hybond™ ECL; GE Healthcare) using a semi-dry transfer cell (BioRad Trans-Blot SD) for 30minutes at 13V. Membranes were then rinsed in TTBS [0.05% Tween-20 in Tris Buffered Saline (20mM Tris, 500mM NaCl) pH 7.4] and blocked with 10% powdered skim milk in TTBS (blotto) for 1h. Blotto was tapped off and membranes rinsed with TTBS three times for 5 minutes each and were probed with heterologous bovine α -subunits of NKA (αR1) (1:500, Wilson et al., 2007), mouse monoclonal antibody of NKCC1 (T4) (1:200, Tipsmark et al., 2002, Wilson et al., 2004, 2007), a heterologous rabbit anti-bovine cytosolic CA polyclonal antibody (1:2000, Abcam Cambridge UK, Randall, 2014) , V-ATPase B subunit (B2) (1:200, Wilson et al., 2007), Rhesus (Rh) proteins (Rhag, Rhbg) (1: 2000, Nakada et al., 2007) and Heat shock protein (Hsp70) (1:10000 Sigma-Aldrich) diluted in 1% BSA/TTBS, overnight at room temperature in 50 ml falcon tubes using a attached to a rotisserie (LabQuake2; Barnstead International, Dubuque, IA). Then membranes were rinsed with TTBS and incubated for 1 hour with a goat anti-rabbit or anti-mouse IgG secondary antibodies conjugated to horseradish peroxidase, diluted in TTBS (1:50,000). Membranes were rinsed a final time with TTBS (3x 5min) and bands were detected by enhanced chemiluminescence (ECL) using Immobilon Western chemiluminescent HRP substrate (Millipore Corporation,

Billerica, MA U.S.A.). Images were acquired using a luminescent image analyzer Fujifilm LAS-4000 mini and image reader software LAS-4000 version.2.0. Intensity of band signals were quantified using an image analysis software program Multi Gauge v3.1 (FUJIFILM, Tokyo Japan). Membranes were stripped with low pH stripping buffer (25mM Glycine-HCl, 1% SDS, pH 2) for 30 minutes on an orbital shaker, rinsed in TTBS, blocked and reprobred with different antibodies

2.8. Immunohistochemistry

The paraffin serial sections were cut, and collected onto APS (3-aminopropyltriethoxysilane; Sigma Aldrich) coated slides, left to air dry completely and stored in slide boxes. Slides for immunostaining were dewaxed at 58°C for 30min, followed by a series of xylene baths and rehydrated through a descending ethanol series. Sections were then dried at 37°C for 10min, circled with a hydrophobic barrier, liquid blocker (Super PAP pen; Sigma Aldrich).

Antigen retrieval was performed on some sections to improve antibody immunoreactivity (Shi et al. 2011). Some rehydrated sections in TPBS (0.05% tween-20 in 10 mM Na₂HPO₄, 1.8mM KH₂PO₄, 2.7mM KCl 137mM NaCl, pH 7.4) were pretreated with 1% sodium dodecyl sulfate (SDS) in PBS (Brown et al., 1996) for 5min at room temperature, then in tap water to remove the SDS then three times 5min in distilled water (DW) followed by TPBS. Alternatively sections were treated with 0.05% citraconic anhydride (pH 7.3) for 30min at 98°C (Namimatsu et al., 2005). A combination of these pretreatments was also performed on a subset of sections.

All sections were then blocked with 5% normal goat serum (NGS)/1% BSA/0.05 % Tween-20 in PBS (TPBS), pH 7.4, for 20 min, 100µl for each section, and incubated with primary antibody α -subunits of NKA (α R1), NKCC1 (T4), CFTR (R&D systems), Carbonic anhydrase (CA), V-ATPase (B2), and Rhesus (Rh) proteins (Rhag, Rhbg), in 1% BSA/TPBS/0.05% sodium azide, pH 7.3, for 1–2h at 37°C in humidity chamber. Sections were then rinsed in TPBS for 5, 10 and 15min following incubation with secondary antibody goat anti-mouse Alexa Fluor 568 and/or goat anti-rabbit Alexa Fluor 488-conjugated, both diluted 1:500 for 1h at 37°C. Sections were rinsed again as mentioned above except that DAPI (4',6-diamidino-2-phenylindole) in TPBS diluted 1:25000 was included in second

10min rinse step. Coverslips were then mounted with 1:1 glycerol PBS, 0.1% NaN₃. Sections were 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. Optimal exposure settings were predetermined and all images captured under these settings (Reis-Santos et al., 2008).

2.9. Molecular genetics approach

2.9.1. Isolation and quantification of RNA and synthesis of complementary DNA

Gill, dendritic organ, kidney, and intestine, were excised from marine catfish and freeze-clamped in liquid nitrogen. Total RNA was extracted using silica-based columns (Aurum Total RNA mini kit) according to the manufacturer's recommendations (Bio-Rad, Hercules, CA, USA). The bead mill (Precellys 24; Bertin Technologies, Montigny-le-Bretoneux, France) was used for homogenization by using 6400 RPM of two cycles for 15 s with 5 s interval. The Eppendorf MiniSpin Plus (Hamburg, Germany) was used for centrifuging of homogenates for 2 min at 14000 *g* at room temperature and for all spin column steps in the protocol. The DNaseI treatment was performed on-column. A Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) was used to assess the total RNA concentration and purity. Only samples with a λ 260:280 nm ratio between 1.8 and 2.2 were used. Formaldehyde gel electrophoresis (Bio-Rad) (1% agarose/ 2.2M formaldehyde/ MOPS) was performed on denatured 1 μ g samples stained with GelRed (Biotium, Hayward, CA, USA) for determining RNA integrity. Total RNA samples were stored at -80°C. For cDNA synthesis 1 μ g of total RNA was converted to cDNA in a 20 μ l reaction volume (iScript cDNA kit Bio-Rad). Reactions were carried out in a Doppio thermocycler (VWR International Ltd, Lisbon, Portugal) at 25°C for 5 min; 42°C for 30 min; and 85°C for 5 min. Samples were stored at -20°C.

2.9.2. Gene isolation

Consensus primers were designed from a conserved region of β -actin (*actb*, *Sparus aurata*, Santos et al., 1997), Na⁺/K⁺-ATPase α subunit (*atp1a*, *Anguilla anguilla*, Cutler et al., 1995), Cystic fibrosis transmembrane conductance regulator [*cftr* (*abcc7* *Fundulus heteroclitus*, *Petromyzon marinus*, *Anguilla Anguilla*,, , *Oryzias latipes*, Marshall, 2002; Singer et al., 1998; Wilson et al., 2000b; Ferreira-Martins et al., 2016 Wilson et al., 2007, Hsu et al., 2014, cytosolic carbonic anhydrase (*ca17*; *Danio rerio*, Ferreira-Martins et al., 2016), putative anion transporter Cl⁻/HCO₃⁻ exchanger (*slc26a6*, *Danio rerio*, *Tetraodon nigroviridis*, *Anguilla anguilla*, *Xenopus laevis*, *Homo sapiens*, Grosell et al., 2009), non-erythroid Rhesus C glycoprotein (*rhcg*; *Danio rerio*, *Ictalurus furcatus*, *Ictalurus punctatus*,) by multiple sequence alignment (MultAlin, Corpet, 1997). Nucleotide sequences and amplicon sizes of these primers are shown in Table 2.1.

2.9.3. RT-PCR and RT real-time PCR

The PCRs were performed using 0.4 μ l of sample cDNA, 0.08 mM dNTPs, 0.4 mM MgCl₂, 0.4 μ M of each primer and 0.2 U GoTaq® DNA polymerase (Promega, Madison, WI, USA) and 1 μ l of 5 \times Green GoTaq® reaction buffer in 10 μ l reaction volumes for actin PCRs and Phusion Flash (Thermo Fisher Scientific) master mix for the other interested genes. Primers were designed using Primer3 (Rozen and Skaletsky, 2000) and were initially tested for specificity by RT-PCR.

GoTaq reactions consisted of an initial denaturation at 95°C for 2min followed by 30 cycles of: 95°C for 30 s; annealing at 60°C for 30 s; extension at 72°C for 5 s; and ending with a final extension for 5 min at 72°C. The Phusion Flash reactions consisted of an initial denaturation at 98°C for 10 s followed by 35 cycles of: 98°C for 1 s; annealing at 56, 58 or 60°C for 5 s; extension at 72°C for 5 or 10 s; and ending with a final extension for 1 min at 72°C. The PCR products were separated on 2% agarose TBE (Tris-borate-EDTA) gels at 80 V to confirm the size of amplicons. All gels were stained with GelRed and images acquired using a Fujifilm LAS-4000 Mini luminescent image analyzer (Fujifilm, Tokyo, Japan). A DNA ladder 1kB or 100 bp (Bioron GmbH, Ludwigshafen, Germany), depending on the amplicon size, was run on every gel to determine

the size of the amplification products. Single bands of the correct predicted size for each PCR reaction was cut and cleaned using Illustra GFX PCR DNA and Gel Band Purification Kit (GFX column, GE Healthcare, Carnaxide, Portugal) and directly sequenced (StabVida, Oeiras, Portugal). The product was confirmed using tBLASTx. The alignment was done via BioEdit (Version 7.0.9.0; Hall, 1999) and ClustalW was used for sequence assembly. It was not possible to isolate CA transcripts from marine catfish using direct sequencing because of low yield so after cleaning with the GFX kit, PCR product were cloned using the pGEM-T Easy Vector system (Promega) with blue/white colonies in which the positive (white) were grown in LB broth with ampicillin. Plasmids with the correct insert size determined by EcoR1 restriction digest were cleaned using the Illustra plasmid kit (GE Healthcare, Carnaxide Portugal) and were sequenced (StabVida). Partial sequences of *ca17* were isolated.

Relative levels of mRNAs for sodium/potassium ATPase α 1-subunit (*atp1a1/NKA-a*), Cystic fibrosis transmembrane conductance regulator (*cftr*), Carbonic anhydrase (*ca17*), Putative Anion Transporter Cl⁻/HCO₃⁻ exchanger gene (*slc26a6*), Non-erythroid Rhesus C glycoprotein (*rhcg1*) genes were quantified by real-time RT-PCR analysis using SYBR green with an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). Each cDNA sample was diluted 25 times and then 5 μ l added to a reaction mix containing 10 μ l of 2 \times iQ SYBR Green Supermix (Bio-Rad) and 250 nM of each primer in a total volume of 20 μ l. The cycle profile was as follows for the given primers pairs: 95°C for 3 min, followed by 35 cycles of 95°C for 10 s, 56, 58 or 60°C (see supplemental table 1) for 30 s and 72°C for 3-5 s. The generation of a melt curve for every PCR product and preparation of a dilution series has been used to confirm the specificity of the assays and check the efficiency of the reactions, respectively. The *bact*, was used as the reference gene. The analysing of the expression levels of the genes of interest was done based on cycle threshold (CT) values by using the comparative CT method ($2^{-\Delta\Delta CT}$ method) (Livak and Schmittgen, 2001.). Melt curve analysis was performed after each run to confirm single products were amplified. In addition, a subset of samples were run on 2% agarose TBE (Tris-borate-EDTA) gels to confirm the presence of a single amplified product at the predicted correct size.

Table 2.1 Nucleotide sequences and amplicon sizes of primers used in the present study for RT-PCR and qPCR (*actb*, β -Actin; *atp1a1*, Na^+/K^+ -ATPase; *cftr*, Cystic fibrosis transmembrane conductance regulator; *ca17*, Cytosolic carbonic anhydrase; *slc26a6*, Putative Anion Transporter Cl/HCO₃⁻ exchanger gene; *rhcg*, Non-erythroid Rhesus C glycoprotein).

Primer	RT-PCR		qPCR	
	Sequence (5'-3')	Size	Sequence (5'-3')	Size
<i>actb</i>	Fw-GGCCGGACCTACAGACTAC	250	Fw-CCCTCGTGCTGTGCCCATC	108
	Rev-ACCGAGGAAGGATGGCTGGAA		Rev-CTCTTGCTCTGTGCCCTCATCTCC	
<i>atp1a</i>	Fw- GGTATCGCTGGGCTGATGT	632	Fw- CCACCTTAGCCTCTCTGATGAC	183
	Rev- AGGATGGAGTTCCCTCCTGTT		Rev-ATAACCTTGATTCCAGCACTCC	
<i>cftr</i>	Fw- TTTTCTGGTGACAAAGCCCT	188	Fw- TTTTCTGGTGACAAAGCCCT	188
	Rev- ATCGGGAAAGTGCTCGTAGG		Rev- ATCGGGAAAGTGCTCGTAGG	
<i>ca17</i>	Fw- CAGTTCCATTCCATTGGGG	333	Fw- GGGGATCCAGTAATGAGAAAGG	152
	Rev- CAGAGGAGGGTGGTCAG		Rev- CAAGAAGACCCCAACCACAG	
<i>slc26a6</i>	Fw- TGGTGCGGTTTGGATTTGTG	544	Fw- ATCAATCCGAAACCGCTACAG	183
	Rev- ACCAGTTCCTGGTTGCTGTC		Rev- AACAGGAAATCGGAACAGGAA	
<i>rhcg1</i>	Fw- CCACTGTCTCACCCACCTTT	541	Fw- ATTGTGGGAGCCATTACAGC	180
	Rev- AGGCACCTCCCAATAGACCT		Rev- CACAAGTGTCTCTCCCCCTA	

Table 2.2 RT-PCR profiles (*actb*, β -Actin; *atp1a1*, Na^+/K^+ -ATPase; *cftr*, Cystic fibrosis transmembrane conductance regulator; *ca17*, Cytosolic carbonic

anhydrase; *slc26a6*, Putative Anion Transporter Cl⁻/HCO₃⁻ exchanger gene; *rhcg*, Non-erythroid Rhesus C glycoprotein)

Step	RT-PCR	<i>act</i>	<i>atp1</i>	<i>cftr</i>	<i>ca1</i>	<i>slc26a</i>	<i>rhcg</i>
p		<i>b</i>	<i>a</i>		<i>7</i>	<i>6</i>	<i>1</i>
1	Denaturation hot start	95°		98°	98°		
		C	98°C	C	C	98°C	98°C
		2min	10s	10s	10s	10s	10s
2	Denature	95°		98°	98°		
		C	98°C	C	C	98°C	98°C
		30s	1s	1s	1s	1s	1s
3	Anneal	60°		58°	56°		
		C	60°C	C	C	58°C	56°C
		30s	5s	5s	5s	5s	5s
4	Extend	72°		72°	72°		
		C	72°C	C	C	72°C	72°C
		5s	5s	5s	3s	3s	4s
5	Repeat 2-4	30	35	35	35	35	35
6	Final Extend	72°		72°	72°		
		C	72°C	C	C	72°C	72°C
		5min	2min	2min	2min	2min	2min
		n		n	n		

Table 2.3 Real time RT-PCR conditions using iQ SYBR green supermix. *actb*, β -Actin; *atp1a1*, Na⁺/K⁺-ATPase; *cftr*, Cystic fibrosis transmembrane conductance regulator; *ca17*, Cytosolic carbonic anhydrase; *slc26a6*, Putative Anion Transporter Cl⁻/HCO₃⁻ exchanger gene; *rhcg*, Non-erythroid Rhesus C glycoprotein).

Step	<i>actb</i>	<i>atp1a</i>	<i>cftr</i>	<i>ca17</i>	<i>slc26a6</i>	<i>rhcg1</i>
Denaturation	95°C	95°C	95°C	95°C	95°C	95°C
and hot start	3min	3min	3min	3min	3min	3min
Denaturation	95°C	95°C	95°C	95°C	95°C	95°C
	10s	10s	10s	10s	10s	10s
Annealing	60°C	60°C	58°C	56°C	58°C	56°C
	30s	30s	30s	30s	30s	30s
Extension	72°C	72°C	72°C	72°C	72°C	72°C
	5s	3s	5s	3s	3s	4s
Cycle #	30	35	35	35	35	35

Chapter 3:

Salinity effects on marine catfish *Plotosus lineatus* osmoregulation

Malakpour Kolbadinezhad, S., Coimbra J., Wilson, J. M. 2017. Salinity effects on marine catfish *Plotosus lineatus* osmoregulation (*in preparation*)

Abstract

In the present study, we investigated the effect of salinity acclimation [brackishwater (BW) 3‰, seawater (SW-control) 34‰ and hypersaline water (HSW) 60‰] on the osmoregulatory abilities of marine catfish through measurements of blood chemistry, muscle water content (MWC), Na⁺/K⁺-ATPase (NKA) activity and ion transporter expression in gills, dendritic organ, kidney and intestine using immunoblotting (IB), immunohistochemistry (IHC) and PCR. Acclimation to HSW increased plasma osmolality and ions (Na⁺, Cl⁻, Ca²⁺), and hematocrit, and decreased MWC indicating an osmoregulatory challenge. DO NKA activity and protein were significantly higher than other tissues at all salinities; although, NKA activity only increased in kidney and posterior intestine with HSW. However, DO mass was higher in HSW, so although specific NKA activity was actually lower than in SW, total DO NKA activity was higher, indicating higher overall capacity at HSW. BW acclimation resulted in lower NKA activity in gill, kidney and DO. Fish were better able to regulate Cl⁻ levels and the resulting strong ion ratio (SIR) in BW suggests a metabolic acidosis. Elevated Hsp70 levels in DO in HSW indicated a cellular stress response. A strong NKA/NKCC1 immunolocalization was observed in DO parenchymal cells, which was very rare in gills ionocytes. NKCC1 expression was highest in DO at HSW but not detectable by immunoblot in other tissues. CFTR did localize apically to DO NKA-IR cells although not consistently. Taken together, the demonstration of high NKA activity in DO co-expressed with NKCC1 and apical CFTR indicates the presence of the conserved secondary active Cl⁻ secretion mechanism found in other ion transporting epithelia indicating the convergent evolution of salt secreting organs in vertebrates.

3.1. Introduction

Osmoregulatory organs including the gills, kidney and digestive tract are involved in maintenance of body fluid balance as a complex process (Takei and

Hwang, 2016). Gills are the first organ to directly sense external osmotic changes that leading to active uptake (in freshwater) or excretion (in saltwater) of monovalent ions (Na^+ , K^+ , and Cl^-) to maintain plasma osmolality within a narrow range depending on the environmental salinity (Takei and Hwang, 2016). In marine teleosts which are hypoosmotic to the environmental salinity, drinking of seawater leads to the absorption of ingested seawater via intestine with Na^+ and Cl^- (see review Grosell, 2011); however, the role of intestine is minor in osmoregulation of freshwater fishes which are hyperosmotic to the environment (Takei and Hwang, 2016). The critical role of marine teleosts kidney for divalent ion (Mg^{2+} , Ca^{2+} , and SO_4^{2-}) secretion has been reported (Beyenbach, 2004), while in freshwater fishes a large amount of dilute urine it actively secreted to compensate for the large water influx via osmosis across the body surfaces (Beyenbach, 2004; Evans et al., 2008).

In freshwater or seawater, regulation of the osmolality and ion levels of body fluids of fishes is done actively (Edwards and Marshall, 2012). In euryhaline species from freshwater or marine origin plasma osmolalities varied between 160-410 or 235-414 mOsm/kg H_2O , respectively (Freire and Prodocimo, 2007). The effects of changing salinity on plasma osmolality and circulating electrolytes has been reported in a number of euryhaline teleosts (Christensen et al., 2012; Timsak et al., 2008; Outtara et al., 2009; Sardella et al., 2008; Kang et al., 2008; Kato et al., 2005; Bystriansky et al., 2006; Watson et al., 2014; Tait et al., 2017).

The dendritic organ is a small fleshy external organ situated on the ventral caudal surface of *P. lineatus*, in both sexes from early life stages, very close to the urogenital papilla (Hirota, 1895; Lanzing 1967; Laurenson et al., 1993). The parenchymal cells of the DO form glandular acini that are covered by a stratified squamous epithelium (Van Lennep and Lanzing, 1967; and Van Lennep, 1968). Descriptive morphological studies in the gills and DO of *P. lineatus* suggested similarity to the gills and rectal gland of elasmobranchs, respectively (Pucke and Umminger, 1979; Doyle and Gorecki, 1961; Van Lennep and Lanzing, 1967; and Van Lennep, 1968). In addition, the similarity of rectal glands from elasmobranchs and specialized salt glands from marine tetrapods (e.g., the nasal salt gland of marine birds, lachrymal gland of marine turtles, and lingual glands in sea snakes,

saltwater crocodiles) to excrete a concentrated NaCl solution has been reported (Shuttleworth and Hildebrandt, 1999). The characteristics of parenchymal cells of these glands and the mechanism of NaCl excretion is similar as teleost gill chloride cells (secondary activity Cl⁻ secretion) (Marshall and Grosell, 2006; Holmgren and Olsson, 2011). The numerous independent origins of these salt glands leads to the hypothesis of a convergent evolution of salt glands across taxa (Babonis and Evans, 2011).

Since the molecular machinery of the osmoregulatory organs (gill, DO, kidney, intestine) in *P. lineatus* are unknown, for the first time in present study we addressed their molecular mechanisms using a combination of enzymatic analysis, immunohistochemistry, immunoblotting and PCR together with standard osmoregulatory end points in fish acclimated to different salinities. In doing so we also addressed the possibility of a conservation of mechanisms for ion transport in secretory cell similar to other vertebrate salt glands with respect to co-option events.

3.2. Material and Methods

3.2.1. Animals

The purchased marine catfish *Plotosus lineatus* (~8-13 g) from Tropical Marine Centre (TMC) Portugal were transported to Laboratory of Ecophysiology CIIMAR (Porto). All fish were acclimatized to laboratory conditions in a 100 L tank with seawater (SW) 34‰, mechanical and biological filtration with aeration and normal photoperiod for three weeks prior to the start of the experiment to avoid any confounding effects of handling stress on osmoregulation (Biswas et al., 2006). For more details regarding the conditions of holding of fish see Chapter 2.

3.2.2. Salinity acclimation

Three salinity levels were investigated for two weeks [brackishwater (BW) 3‰, seawater (SW-control) 34‰, hypersaline water (HSW) 60‰]. Initially, individuals were transferred to a 30 L tank, in which salinity was changed in a stepwise fashion, from 34 (main tank) to 3 and 60‰ (smaller 30L tanks), by 5‰ per day. Saline media were made

from Instant Ocean® salts prepared from a stock solution of 100‰ and diluted to the appropriate salinities. Fish were maintained in the lab and used according to the Portuguese Animal Welfare Law (Decreto-Lei no.197/96) and animal protocols were approved by CIIMAR/UP. For more details, see Chapter 2.

3.2.3. Sampling

Individual of marine catfish were netted then euthanized in a separate smaller tank (1L) with ethyl-m-amino benzoate-MS-222 (an overdose 1:5000, pH 7.5 adjusted with NaHCO₃; Pharmaq UK), weighted (± 0.01 g) and total length (mm) measured. Blood was collected then centrifuged at 13000xg for 5min at room temperature (Heraeus Pico 17 Centrifuge, Thermo Scientific). Hematocrit (Hct) was measured then converted to percentage of total blood volume. The isolated plasma was then frozen in liquid nitrogen and kept at -80°C. The following tissues were collected: gill, dendritic organ (DO), kidney, anterior and posterior intestine, liver, heart, brain, muscle, skin and eye then were immediately stored at -80°C. Gill filaments samples from the second arch on the left side, DO, kidney, and intestine were also excised, immersed in 100µl of ice-cold SEI buffer and frozen at -80°C. An additional piece of deskinning epaxial muscle (~1g) was collected into a pre-weighed tube for water and ion analysis. In addition sets of six individuals the body cavity opened then immersion fixed in 10% neutral buffered formalin (NBF 10%) then stored in 70% ethanol at 4°C.

3.2.4. Histological Analysis

Gill, DO, kidney, and intestine were excised from the fixed carcass were dehydrated through an ethanol series and embedded in paraffin (Type 6; Richard Allen Scientific). Sections were cut at 5µm (Reichert Biocut 2030 microtome) and stained with hematoxylin-eosin, Alcian blue (pH 2.5) and/or Periodic Acid Schiff staining protocols. Using a Leica DFC300FX digital colour camera mounted on a Leica DM 6000 B microscope micrographs were taken then were imported into Photoshop CS3.

3.2.5. Ion quantification

One gram of muscle tissue was collected then dried at 60°C for the determination of muscle water content (MWC). The dried muscle samples were digested in 65% nitric

acid for 3 days. The Na⁺ and K⁺ concentrations were quantified using a flame photometer (model PFP7; Jenway, Felsted, UK) and expressed as $\mu\text{mol} \cdot \text{g}^{-1}$ wet mass. Plasma samples were also analysed by flame photometry (PinAAcle 900T Atomic Absorption Spectrophotometer; Perkin Elmer Waltham MA). Chloride concentration was measured in plasma samples was measured at 480 nm (Küffer et al., 1975).

3.2.6. Measurement of Na⁺/K⁺-ATPase activity

The NKA activity was measured according to McCormick (1993), Wilson et al. (2007b). After thawing stored samples in 300 μl SEI buffer, final concentration of 0.1% of sodium deoxycholate was added. Fully disintegrated homogenizing done by a motorized pestle homogenizer then were centrifuged (3,200 x g for 30 s at 4°C) and finally samples of 10 μl of supernatant were run in two duplicate sets for the ATPase assay at 340nm with a temperature controlled plate reader (Powerwave 340; Biotek, Winooski, VT) and Gen5™ reader control and data analysis software for 10-20 min at 25°C. One set containing the assay mixture and the other assay mixture plus ouabain (1 mM, Sigma–Aldrich Chemical Co.; St. Louis MO) to specifically inhibit NKA activity. Total protein were determined in the remaining supernatant using the Bradford (1976) at 600nm and the results are expressed as $\mu\text{moles ADP mg}^{-1} \text{ protein h}^{-1}$.

3.2.7. Immunoblotting

The tissue samples were homogenized in imidazole buffer (2x15s in Precellys 24 homogenizer, Bertin Technologies) then immediately centrifuged at 15,000xg for 5 minutes at 4°C. The supernatant was mixed with an equal volume of 2x Laemmli's buffer (Laemmli, 1970), heated for 10 minutes at 70°C and then stored at 4°C. Protein concentration was adjusted to 1 $\mu\text{g} \mu\text{l}^{-1}$ using 1x Laemmli's buffer. Immunoblotting was performed as described in Wilson et al. (2007b) and Reis-Santos et al. (2008). Blots were probed with heterologous bovine α -subunits of NKA (αR1) (1:500), mouse monoclonal antibody of NKCC1 (T4) (1:200), a heterologous rabbit anti-bovine cytosolic CA polyclonal antibody (1:2000, Abcam), V-ATPase B subunit (B2) (1:200) and Heat shock protein (Hsp70) (1:10000 Sigma-Aldrich). Then membranes were incubated for 1 hour with a goat anti-rabbit or anti-mouse IgG secondary antibodies conjugated to horseradish peroxidase (HRP) and the signal were detected by enhanced chemiluminescence (ECL) using Immobilon Western chemiluminescent HRP substrate (Millipore Corporation,

Billerica, MA U.S.A.). Images were acquired using a luminescent image analyzer Fujifilm LAS-4000 mini and image reader software LAS-4000 version.2.0. Intensity of band signals were quantified using an image analysis software program Multi Gauge v3.1 (FUJIFILM, Tokyo Japan).

3.2.8. Immunohistochemistry

Immunofluorescence localization were performed according to Wilson et al. (2007a) and (Reis-Santos et al., 2008). In summary, the paraffin serial sections were cut and dewaxed followed by a series of xylene baths and rehydrated through a descending ethanol series. Antigen retrieval was performed on some sections (Shi et al. 2011) by pretreated with 1% sodium dodecyl sulfate (SDS) in PBS (Brown et al., 1996) then treated with 0.05% citraconic anhydride (pH 7.3) for 30min at 98°C (Namimatsu et al., 2005). All sections were then blocked with 5% normal goat serum (NGS) then incubated with primary antibody α -subunits of NKA (α R1), NKCC1 (T4), CFTR (R&D systems), Carbonic anhydrase (CA) and V-ATPase (B2) following incubation with secondary antibody goat anti-mouse Alexa Fluor 568 and/or goat anti-rabbit Alexa Fluor 488-conjugated. Sections were rinsed DAPI (4',6-diamidino-2-phenylindole) 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.

3.2.9. Molecular genetics approach

2.9.1. Isolation and quantification of RNA and synthesis of complementary DNA

Gill, dendritic organ, kidney, and intestine were excised from marine catfish and freeze-clamped in liquid nitrogen. Silica-based columns (Aurum Total RNA mini kit, (Bio-Rad, Hercules, CA, USA) and Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) were used for extracting and assessing of total RNA concentration (or purity), respectively then were used then were stored at -80°C. The cDNA synthesis was done by converting 1 μ g of total RNA was converted to cDNA (iScript cDNA kit Bio-Rad). Samples were stored at -20°C.

3.2.9.2. Gene isolation

Consensus primers were designed from a conserved region of β -actin (*actb*, *Sparus aurata*, Santos et al., 1997), Na⁺/K⁺-ATPase α subunit (*atp1a*, *Anguilla anguilla*, Cutler et al., 1995), Cystic fibrosis transmembrane conductance regulator [*cftr* (*abcc7* *Fundulus heteroclitus*, *Petromyzon marinus*, *Anguilla Anguilla*, *Oryzias latipes*, Marshall, 2002; Singer et al., 1998; Wilson et al., 2000b; Ferreira-Martins et al., 2016 Wilson et al., 2007, Hsu et al., 2014, cytosolic carbonic anhydrase (*ca17*; *Danio rerio*, Ferreira-Martins et al., 2016), putative anion transporter Cl⁻/HCO₃⁻ exchanger (*slc26a6*, *Danio rerio*, *Tetraodon nigroviridis*, *Anguilla anguilla*, *Xenopus laevis*, *Homo sapiens*, Grosell et al., 2009) by multiple sequence alignment (MultAlin, Corpet, 1997). Nucleotide sequences and amplicon sizes of these primers are shown in Table 2.1 Chapter 2. Pgem-t easy Promega, StabVida, Oeiras, Portugal and BLAST, ClustalX were used for cloning, sequencing and analyzed for sequence similarity, respectively of the correct size of amplification products. The specific primer for Marine catfish were designed by Primer3 (Rozen and Skaletsky, 2000) specifically for 5' and 3' amplification then were initially tested for specificity by RT-PCR (Table 2.2 see Chapter 2). Nucleotide sequences and amplicon sizes are shown in Table 2.1 of Chapter 2.

3.2.9.3. RT-PCR and RT real-time PCR

The PCRs were performed using GoTaq® DNA polymerase (Promega, Madison, WI, USA) and Phusion Flash (Thermo Fisher Scientific) for actin PCRs and other interested genes, respectively (see more details in Chapter 2). Primers were designed using Primer3 (Rozen and Skaletsky, 2000) and were initially tested for specificity by RT-PCR.

The real-time PCR (RT PCR) was done by transcript quantification using SYBR green with an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). A melt curve for every PCR product to confirm the specificity of the assays was generated. Checking the efficiency of the reactions has been done by preparation of a dilution series and the β -Actin was consider as housekeeping gene. The expression levels of the interest genes was analysed based on cycle threshold (CT) values using the comparative CT method ($2^{-\Delta\Delta CT}$ method) (Table 2.3 Chapter 2).

3.2.10 Statistics

Data are presented as means \pm standard deviation (S.D.) or 95% confidence interval. Statistical differences of protein, mRNA expression between groups were determined using one-way ANOVA 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, Fiducial limit was set at 0.05.

3.3 Results:

3.2.1 Osmoregulatory indicators

Plasma and muscle osmoregulatory indicators are presented in the Table 3.3. 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. The resulting plasma strong ion ratio (SIR) was significantly lower in BW fish compared to SW and HSW acclimation. Hematocrit showed a positive correlation with salinity with BW values half of HSW. Acclimation salinity had no effect on plasma K^+ concentrations.

Muscle water content was significantly lower in HSW acclimated fishes indicating dehydration but was unaffected by BW acclimation. Muscle potassium concentration followed the opposite trend being significantly higher in HSW fish. Muscle sodium content did not differ with salinity, which is reflected in a lower $\text{Na}^+ : \text{K}^+$ ratio in HSW fish. During acclimation, there was mortality (36%) only with HSW acclimation but not in other salinity groups.

3.2.2 NKA activity

In SW marine catfish, specific NKA activity is lowest in gill and posterior intestine, more than three times higher in kidney and anterior intestine and twenty times higher in dendritic organ (Fig. 3.1f). In response to salinity acclimation, similar patterns of NKA activity were detecting in gill and DO with significantly

higher activity in SW acclimated fish compare to both BW and HSW salinities (Fig. 3.1a,e). In both kidney and posterior intestine, NKA activity was significantly higher in HSW, with no differences between SW and BW (Fig. 3.1b,c). No salinity effects were observed in anterior intestine (Fig. 3.1c).

The mass of the DO of SW-control salinity acclimated fish expressed as a percentage of fish body mass was significantly lower compared to BW and HSW salinity. However, in HSW salinity acclimated fish DO mass was greatest at 213% and 243 % of BW and SW-control, respectively (Fig. 3.2a, Table 3.1). The expression of DO NKA activity relative to fish body mass shows that in HSW fish DO NKA was 1.6 and 2.1 fold higher than in SW and BW fishes, respectively.

3.2.3 Western blot

We used antibodies crossreactive with α -subunits of NKA, NKCC1, cytosolic carbonic anhydrase, V-ATPase B subunit and heat shock protein (Hsp70) to determine how salinity affected the abundance of these important transport and stress related proteins in key osmoregulatory organs: gill, kidney, DO, and anterior and posterior intestine.

NKA α subunit expression was detected in all organs of interest as a single band of approximately 100kDa. The relative expression of the NKA α subunit protein was not salinity responsive in gill, intestine or DO (Fig. 3a,c,d,e), but in kidney significantly higher expression was found with HSW (Fig. 3b). NKCC expression was detected only in DO with a pair of prominent immunoreactive bands of 140-260 kDa with some additional higher molecular mass sometimes present as discrete bands. Higher NKCC expression in the HSW salinity acclimated fish relative to BW fish (Fig. 3f). The expression intensities of these bands were approximately 3,6 and 1,9 time greater in HSW acclimated individuals compare to BW and SW-control, respectively.

Regarding use of CFTR antibody the expression was in the predicted molecular mass range as a single band of 160 kDa; however, blots were not clean, and multiple smaller cross-reactive bands were detected, which made

semi-quantification problematic. Because of these difficulties in detecting cross-reactive bands, the antibody was not used in other tissues or for quantification.

Ca17 was detected as an approximately 30 kDa band in all tissues (Fig. 3.4). Relative Ca17 protein expression was significantly lower with HSW in gill and anterior intestine relative to the SW control but not to BW. In contrast, in the DO Ca17 was also significantly lower in HSW but versus BW, with SW values being intermediate (Fig. 3.4e). No detectable differences were found in either kidney or posterior intestine (Fig. 3.4b, f).

The V-ATPase B subunit was found expressed as a ~56 kDa band in gill, kidney, dendritic organ and posterior intestine but not anterior intestine (Fig. 3.5). Three different tissue dependent salinity responsive patterns of expression were observed. The relative protein expression in gill was highest in HSW (Fig. 5a), while in posterior intestine the highest expression was found in BW (Fig. 3.5c). In DO, SW-control showed lower expression than both BW and HSW (Fig. 5d). No detectable change was found in kidney (Fig. 3.5b)

Heat shock protein 70 (Hsp70) protein was found in all of the tissues of interest in our work as a single 70 kDa immunoreactive band (Fig. 3.6). Hsp70 showed significantly higher levels with HSW only in DO relative to the BW with intermediate SW levels (Fig. 3.6e). In BW lower expression of Hsp70 was detected in the gills (relative to SW) and kidney (relative to SW and HSW) (Fig. 3.6a,b). In the anterior intestine, lower expression was found in HSW relative to SW, while no differences with salinity were found in posterior intestine (Fig. 3.6c, d).

3.2.4 Gene expression of *atp1a1*, *ca17*, *cftr*, *slc26a6a*

Using a PCR based approach we identified orthologues of *atp1a1*, *cftr* (*abcc7*), *ca17*, and *slc26a6* in *P.lineatus* from partial sequences. Percentage amino acid identities for each gene compared to channel catfish (*Ictalurus punctatus*), rainbow trout (*O.mykiss*) and zebrafish (*D.rerio*) show a high degree of similarity (Table 3.2). Phylogenetic trees for *P. lineatus atp1a1* show that it is found in the *atp1a1* clade (Appendix Fig X).

Significant effects of salinity were seen in NKA α subunit *atp1a1* mRNA expression levels in DO with a negative correlation with salinity (higher expression in BW compared to HSW) (Table 3.3). No salinity dependent effects were observed in any other tissue.

The HSW acclimated fish had higher *cftr* mRNA expression in anterior intestine but a decrease in DO relative to SW fish (Table 3.3). In kidney, BW acclimation was associated with significantly higher mRNA levels whereas in all other tissue BW was not associated with any significant difference from SW. There were no salinity dependent effects in gill or posterior intestine.

The *ca17* mRNA expression showed higher levels with HSW in the kidney in contrast to lower levels in DO (Table 3.3). In BW the gill showed higher expression but lower expression was detected in DO compares to SW. There were no salinity dependent effects in either anterior or posterior intestine.

The *slc26a6a* was found expressed in all tissues studied. Only in DO did, HSW acclimation show higher *slc26a6a* mRNA expression whereas in the kidney BW acclimation resulted in higher expression (Table 3.3). There were no other salinity dependent effects in gill or intestine.

3.2.5 Immunohistochemistry

3.2.5.1 Gill

The gills of marine catfish have a typical teleost gill organization of filaments with lamellae. In the branchial epithelium strong NKA immunoreactivity (IR) was detected in large isolated ovoid cells throughout the cytoplasm with the exception of the apical region (Fig 3.7). This NKA cellular staining pattern is typical of teleost fish chloride cell or ionocyte tubular system. There were relatively few of these branchial NKA-IR cells which were present in a heterogeneous distribution limited to a few interlamellar regions over the leading edge of the filament and were absent from the lamella. Experimental salinities did not alter the NKA-IR cell

distribution pattern. The secretory $\text{Na}^+:\text{K}^+:2\text{Cl}^-$ cotransporter (NKCC1) expression in gill was rarely detected despite the use of antigen retrieval techniques and positive immunoreactivity in other tissues (DO, kidney and intestine) indicating that species specific immunoreactivity problems were not an issue. The colocalization of NKCC1 in more weakly NKA-IR cells in BW and SW fish are shown in Fig 3.7a,d. Ovoid cells deeper within the filament epithelium showing only NKCC1 staining were observed in HSW (Fig 3.7g). The apical localization of CFTR was detected in some NKA-IR cells with no apparent salinity dependent differences (Fig. 3.7b,e,h). The V-ATPase H^+ -pump was localized in a similar cytoplasmic staining pattern as NKA; however, in separate cells from NKA-IR cells under all acclimation conditions (Fig. 7c,f,i).

3.2.5.2 Dendritic Organ

The DO of marine catfish are external and have branching irregular lobes that are well vascularized. The large parenchymal cells form acini covered by a squamous stratified layer of epithelial cells. The large ovoid to pear-shaped parenchymal cells of the DO generally showed strong NKA and NKCC1 immunoreactivity throughout the cytoplasm indicative of basolateral tubular system staining (Fig. 3.8a,d,g). However, there is a smaller subpopulation of parenchymal cells that are more angular in shape that have noticeably stronger NKA-IR and lack NKCC-IR. Salinity dependent differences in staining were not observed; The apical chloride channel CFTR was only observed once in a SW-control fish and was generally not detectable despite the use of antigen retrieval techniques and positive immunoreactivity in other tissues (gill) indicating that species specific immunoreactivity problems were not an issue (Fig 3.8e). V-ATPase-IR showed rather similar cytosolic localization in of parenchymal cells of the DO ionocytes without salinity dependent differences (Fig. 3.8c,f,i).

3.2.5.3 Intestine

Immunolabeling of NKA in the anterior and posterior intestine of marine catfish acclimated to BW, SW-control or HSW revealed intense staining in the basolateral regions of the intestinal epithelium (Fig. 3.9, 3.10 a,c,f). NKCC2 or

NCC immunoreactivity was detected in apical brushborder of the epithelium in the anterior and/or posterior intestine in all salinity experiments. However, a basal staining was detected in anterior intestine of BW (Fig. 3.9a). CFTR immunoreactivity was detected apically in isolated spindle shaped columnar cells in the epithelium of the anterior and posterior intestine in all of salinity experiment (Fig. 3.9-3.10b,d,g). However, in the posterior intestine a diffuse staining pattern in HSW was also observed (Fig. 3.10h).

In the anterior intestine, V-type H⁺-ATPase is found weakly staining the subapical region of columnar epithelial cells at all salinities although much weaker in HSW (Fig 3.9c,f,i). Staining was not observed in the brush border. Stronger staining is also observed in isolated basal cells in the epithelium and in the lamina propria. In the posterior intestine, apical/subapical expression of V-ATPase pump in SW has been observed while basal cells in the lamina propria of HSW fish and not detected in BW (Fig. 3.10c,f,i).

3.2.6 Histology (goblet cells)

Alcian blue and Periodic Acid Schiff (AB/PAS) staining showed neutral (magenta color) PAS staining, acidic mucin rich (blue) and purple cells indicating combination neutral and acidic glycoconjugates in gill, DO and intestine mucocytes (Fig 3.11, 12).

In the gills neutral, acidic mucin rich (blue) and acidic-neutral (purple) staining goblet cells located at the edge and interlamellar region of filament were detected in all salinities; however, relative abundance varied with salinity. In HSW the distribution of cells was lower compare to other salinities and no acidic mucin rich cells were found while the opposite was observed in BW.

The luminal openings of the glands of the DO ranged from almost completely closed in BW fish to clearly open in HSW fish with a rather unclear border between neighboring cells in the former compare to the latter was detected. In SW acclimated fish, neutral, mixed neutral-acidic mucin cells and apical acidic mucin staining of the cells was more than in HSW and/or BW acclimated fish. Two types of goblet cells in DO at HSW, neutral PAS staining

apically and throughout the cell (presumably type I) and a few cells with no neutral staining in the cell (presumably type II) were detected. .

In anterior and posterior intestine all three types of mucous cells were found in the intestinal epithelium. In anterior intestine distribution of neutral-acidic mucin containing cells was higher while it was acidic mucin cells that dominated in posterior intestine in all of salinities. At HSW, a decreased in the number of mucous cells was found in the intestine.

Table 3.1. Plasma Na⁺, Cl⁻, K⁺, and Ca²⁺ concentrations and osmolality, hematocrit, strong ion ratio (SIR; Na⁺:Cl⁻) and muscle water content (MWC%), Na⁺ and K⁺ concentrations, and Na⁺/K⁺ ratio of *P. lineatus* acclimated to [brackish water (BW) 3‰, seawater (SW-control) 34‰, and hypersaline water (HSW) 60‰]. Salinity difference within a given parameter that do not share the same letter(s) are significantly different from one another.

Plasma	BW (3ppt)	SW-Control (34ppt)	HSW (60ppt)
Na⁺ (mmol l⁻¹)	119.75 ± 16.1 ^a	152.62 ± 14.71 ^b	186.15 ± 40 ^c
Cl⁻ (mmol l⁻¹)	125.5 ± 11.8 ^a	127.8 ± 10.9 ^a	148.7 ± 20.2 ^b
K⁺ (mmol l⁻¹)	4.64 ± 1.70	5.08 ± 0.97	5.05 ± 0.96
Ca²⁺ (mmol l⁻¹)	2.60 ± 0.62 ^a	2.96 ± 0.39 ^a	3.80 ± 1.10 ^b
Osmolality(mOsm)	391.2 ± 112.8 ^a	374.4 ± 20.6 ^a	588.0 ± 108.3 ^b
Haematocrit (%)	15.3 ± 4.2 ^a	23.3 ± 6.3 ^b	29.2 ± 5.4 ^c
SIR (Na⁺:Cl⁻ ratio)	0.96 ± 0.14 ^a	1.20 ± 0.16 ^b	1.2414 ± 0.26 ^b
Muscle			
MWC (%)	86.6 ± 4.5 ^a	87.5 ± 3.7 ^a	77.4 ± 0.9 ^b
Na⁺ (mmol kg⁻¹)	66.9 ± 23.5	64.2 ± 10.7	67.4 ± 15.5
K⁺ (mmol kg⁻¹)	138.5 ± 25.5 ^a	138.6 ± 19.2 ^a	204.0 ± 26.5 ^b
Na⁺:K⁺ ratio	0.48 ± 0.92 ^a	0.46 ± 0.56 ^a	0.33 ± 0.58 ^b

Table 3.2. Comparisons of the amino acid sequence identities of *Plotosus lineatus* Atp1a1, Cfr, Ca17 and Slc26a6 partial sequences with respective orthologues from channel catfish (*I. punctatus*), zebrafish (*D. rerio*) and rainbow trout (*O. mykiss*).

	Atp1a1 (198 aa)		Cfr (43 aa)		Ca17 (111 aa)		Slc26a6 (166 aa)	
<i>I. punctatus</i>	0.949	XP_017312769.1	0.883	XP_017321934.1	0.855	XP_017322776.1	0.801	XP_017323671.1
<i>D. rerio</i>	0.949	Q9DGL6	0.813	NP_001038348.1	0.747	F1R454	0.710	XP_001344243.4
<i>O. Mykiss</i>	0.924	Q6VYM6	0.720	XP_021432274.1	0.756	Q6R4A2	0.536	XP_021422617.1

Table 3.3. Relative mRNA expression of *atp1a1*, Na⁺/K⁺-ATPase; *cftr*, cystic fibrosis transmembrane conductance regulator; *ca17*, cytosolic carbonic anhydrase; and *slc26a6*, putative anion transporter Cl⁻/HCO₃⁻ exchanger gene in the gill, DO, kidney, anterior and posterior intestine tissues of marine catfish *P. lineatus* acclimated to [brackishwater (BW) 3‰, seawater (SW-control) 34‰, hypersaline water (HSW) 60‰]. Data are means ± S.D. (n=3-4). The amounts of mRNAs are normalized to the corresponding *b-actin* abundance from the same sample and the expressed relative to the SW-control group. Different letter indicates a significant difference between salinities, one-way analysis of variance (ANOVA) ($P < 0.05$; see text for details).

Tissue	Genes	BW (3‰)	SW (34‰)	HSW (60‰)
Gill	<i>atp1a1</i>	1.61 ± 0.80	1.00 ± 0.23	0.66 ± 0.10
	<i>cftr</i>	0.97 ± 0.80	1.00 ± 0.66	0.50 ± 0.17
	<i>ca17</i>	1.58 ± 0.15 ^a	1.00 ± 0.23 ^b	1.19 ± 0.37 ^{ab}
	<i>slc26a6</i>	1.04 ± 1.30	1.00 ± 0.58	1.47 ± 1.25
Kidney	<i>atp1a1</i>	1.57 ± 0.89	1.00 ± 0.81	1.62 ± 0.97
	<i>cftr</i>	14.02 ± 12.83 ^a	1.00 ± 0.8 ^b	0.42 ± 0.32 ^b
	<i>ca17</i>	1.60 ± 0.86 ^a	1.00 ± 0.61 ^a	3.00 ± 0.38 ^b
	<i>slc26a6</i>	6.19 ± 4.42 ^a	1.00 ± 0.44 ^b	1.89 ± 2.02 ^b
Ant int	<i>atp1a1</i>	0.84 ± 0.12	1.00 ± 0.64	0.77 ± 0.29
	<i>cftr</i>	1.45 ± 0.55 ^{ab}	1.00 ± 0.57 ^a	2.93 ± 1.23 ^b
	<i>ca17</i>	0.70 ± 0.44	1.00 ± 0.65	0.88 ± 0.24
	<i>slc26a6</i>	1.13 ± 0.20	1.00 ± 0.83	1.46 ± 0.36
Post int	<i>atp1a1</i>	1.44 ± 0.63	1.00 ± 0.40	1.71 ± 0.14
	<i>cftr</i>	0.78 ± 0.42	1.00 ± 0.24	0.84 ± 1.00
	<i>ca17</i>	0.77 ± 0.26	1.00 ± 0.60	0.81 ± 0.55
	<i>slc26a6</i>	0.95 ± 0.78	1.00 ± 0.14	1.20 ± 0.87
DO	<i>atp1a1</i>	1.6 ± 0.80 ^a	1.00 ± 0.22 ^{ab}	0.65 ± 0.10 ^b
	<i>cftr</i>	1.37 ± 0.40 ^a	1.00 ± 0.11 ^a	0.30 ± 0.09 ^b
	<i>ca17</i>	0.65 ± 0.15 ^a	1.00 ± 0.19 ^b	0.44 ± 0.08 ^c
	<i>slc26a6</i>	1.47 ± 0.83 ^a	1.00 ± 0.29 ^b	15.12 ± 2.93 ^c

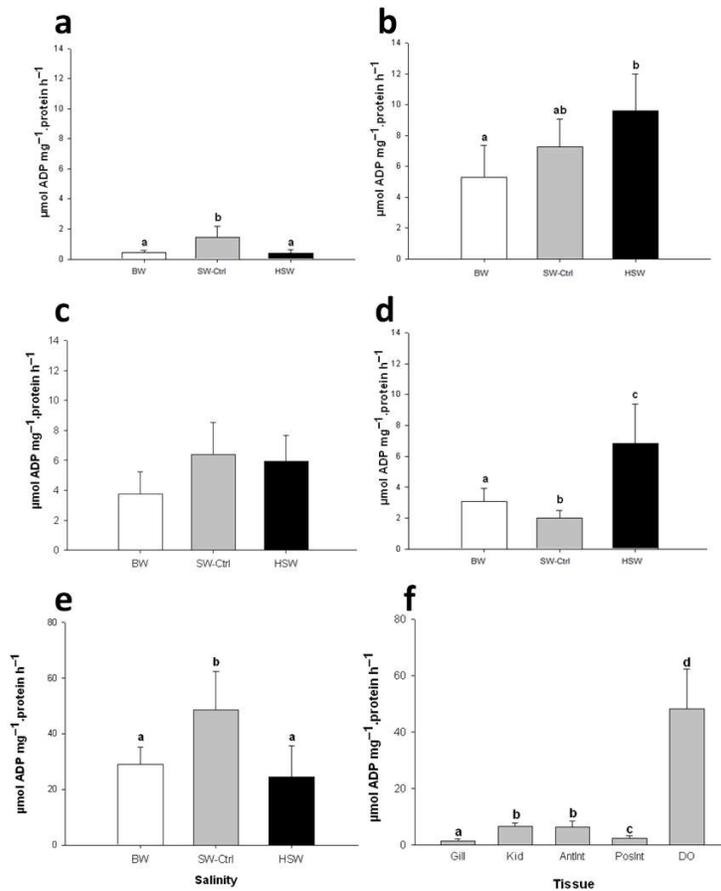


Figure 3.1. Na⁺/K⁺-ATP activity in the gill (a), kidney (b), anterior (c) and posterior intestine (d), and dendritic organ (e) of [brackishwater (BW) 3‰, seawater (SW-control) 34‰, and hypersaline water (HSW) 60‰] acclimated marine catfish *P. lineatus*. A comparison of all tissues in SW acclimated fish is shown in (f). Values are means \pm S.D. (n=5-6). Different lower case letters indicate a significant difference with salinity within each tissue (a-e) or between tissues (f). ($P < 0.05$; see text for details).

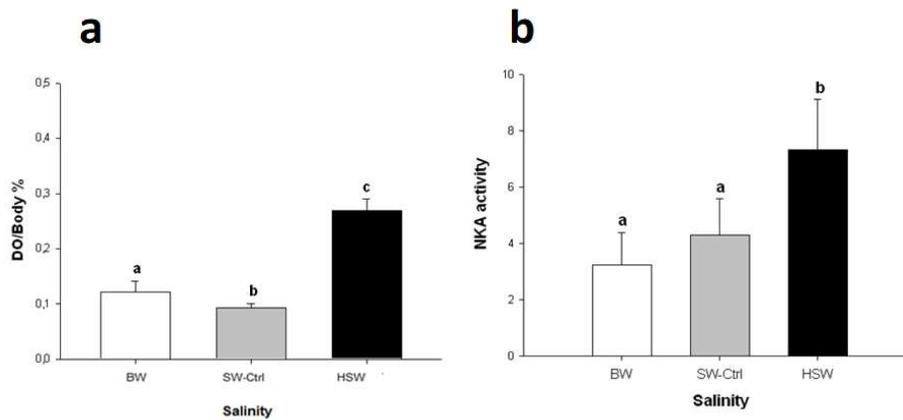


Figure 3.2. The dendritic organ (DO) mass as a percentage of fish wet body mass (a), and the relative expression of DO Na⁺/K⁺-ATPase activity corrected for tissue mass (b) of [brackishwater (BW) 3‰, seawater (SW-control) 34‰, and hypersaline water (HSW) 60‰] acclimated marine catfish *P. lineatus*. Values are means ± S.D. (n=5-9). Different letter indicates a significant difference between salinities ($P < 0.05$).

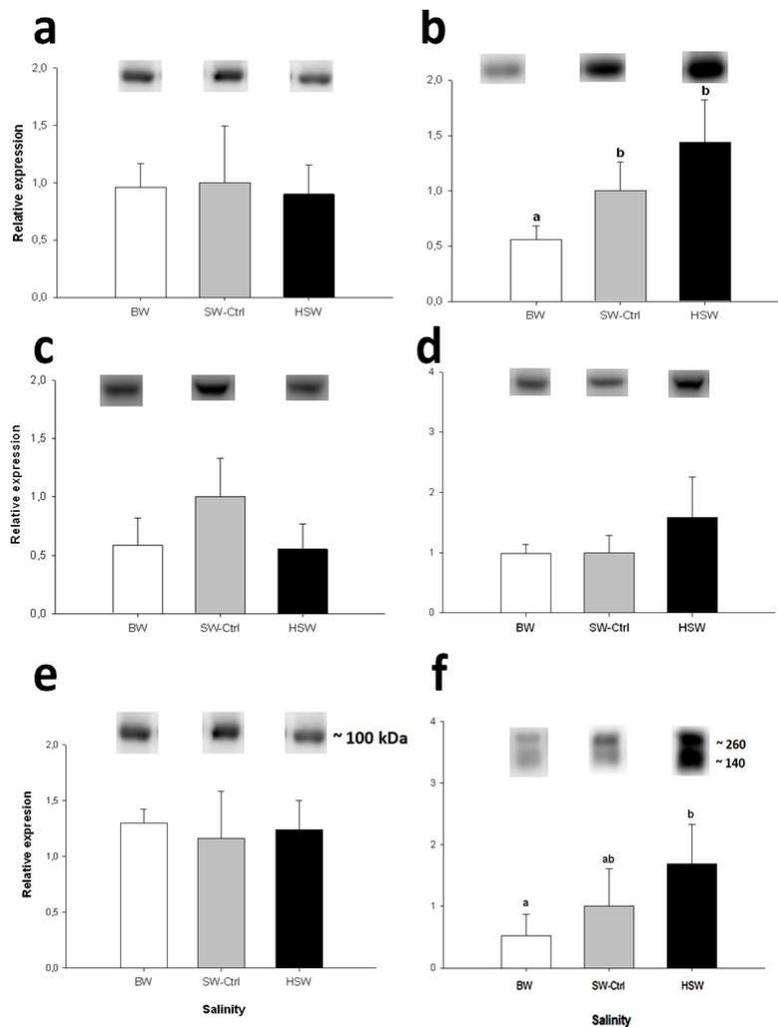


Figure 3.3. Western blot relative expression of NKA α subunit (α R1 antibody) in the gill (a), kidney (b), anterior and posterior intestine (c,d) and dendritic organ (DO) (e) and NKCC protein (T4 antibody) in DO (f) of marine catfish *P. lineatus* acclimated to [brackishwater (BW) 3‰, seawater (SW-control) 34‰, and hypersaline water (HSW) 60‰]. Representative western blots images are shown with the estimated molecular masses of NKA α subunit (~100 kDa) and NKCC1 (~ 140-260 kDa). Values are presented as means \pm S.D of protein abundance (n=5-6). Different letters indicate a significant difference between salinities, one-way analysis of variance (ANOVA) and SNK ($P < 0.05$; see text for details).

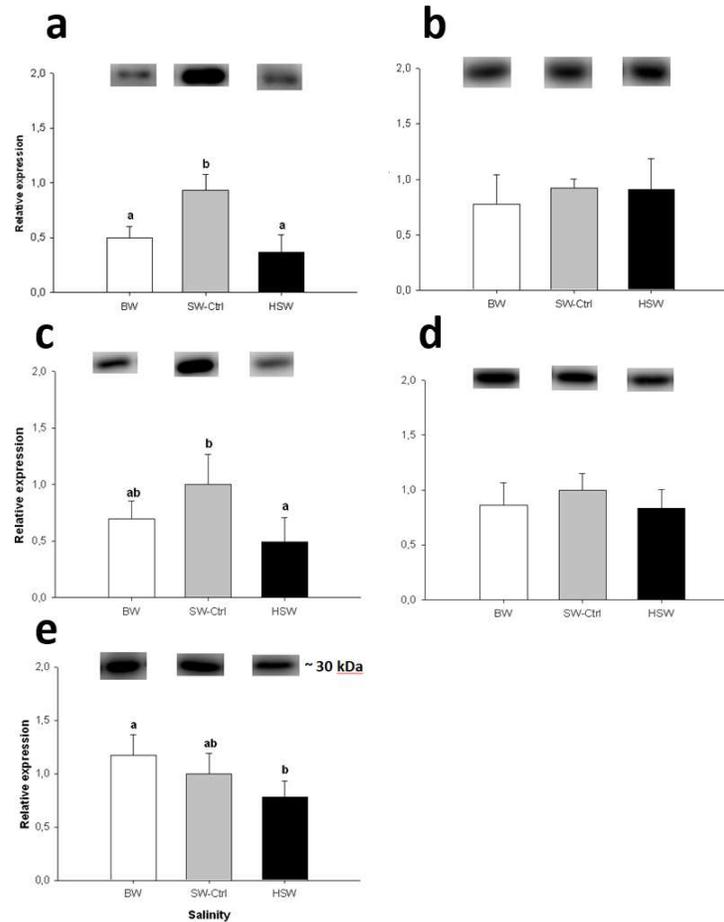


Figure 3.4. Relative expression of cytosolic carbonic anhydrase (Ca17) in the gill (a), kidney (b), anterior and posterior intestine (c,d) and dendritic organ (e) of marine catfish *P. lineatus* acclimated to [brackishwater (BW) 3‰, seawater (SW-control) 34‰, hypersaline water (HSW) 60‰] determined by immunoblotting. Representative images showing band size, intensity and relative molecular mass (~ 30 kDa) of Ca17. Values are means ± S.D of protein abundance (n=5-6). Different letters indicate a significant difference between salinities, one-way analysis of variance (ANOVA) and SNK ($P < 0.05$; see text for details).

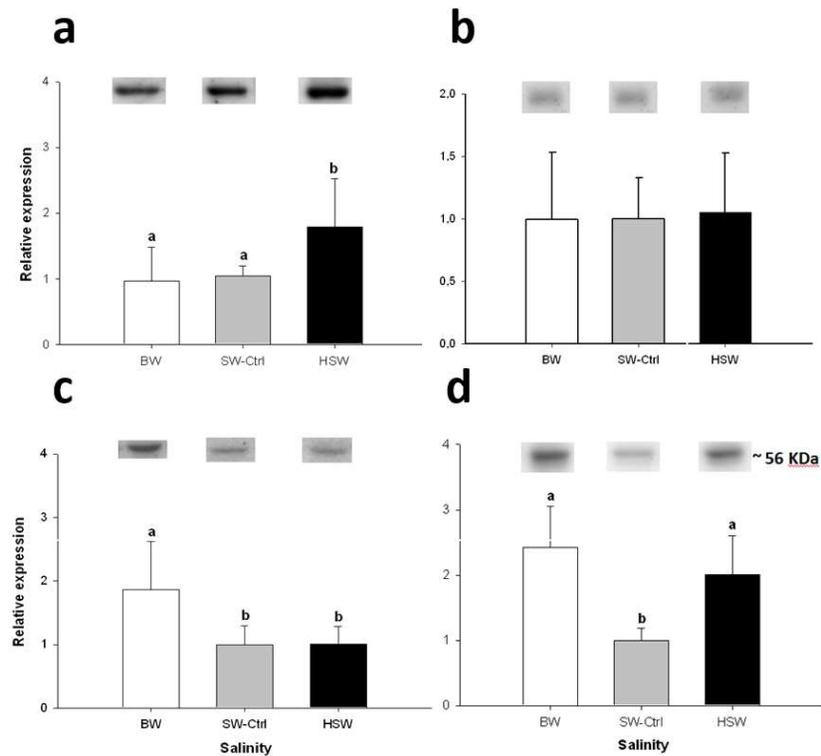


Figure 3.5. Relative western blotting expression of V-ATPase B subunit (B2 antibody) in the gill (**a**), kidney (**b**), posterior intestine (**c**), and dendritic organ (**d**) of marine catfish *P. lineatus* acclimated to [brackishwater (BW) 3‰, seawater (SW-control) 34‰, and hypersaline water (HSW) 60‰]. Representative images showing band size, intensity and relative molecular mass (~ 56 kDa) of V-ATPase B subunit. Values are means \pm S.D of protein abundance (n=5-6). Different letter indicates a significant difference between salinities, one-way analysis of variance (ANOVA) ($P < 0.05$; see text for details).

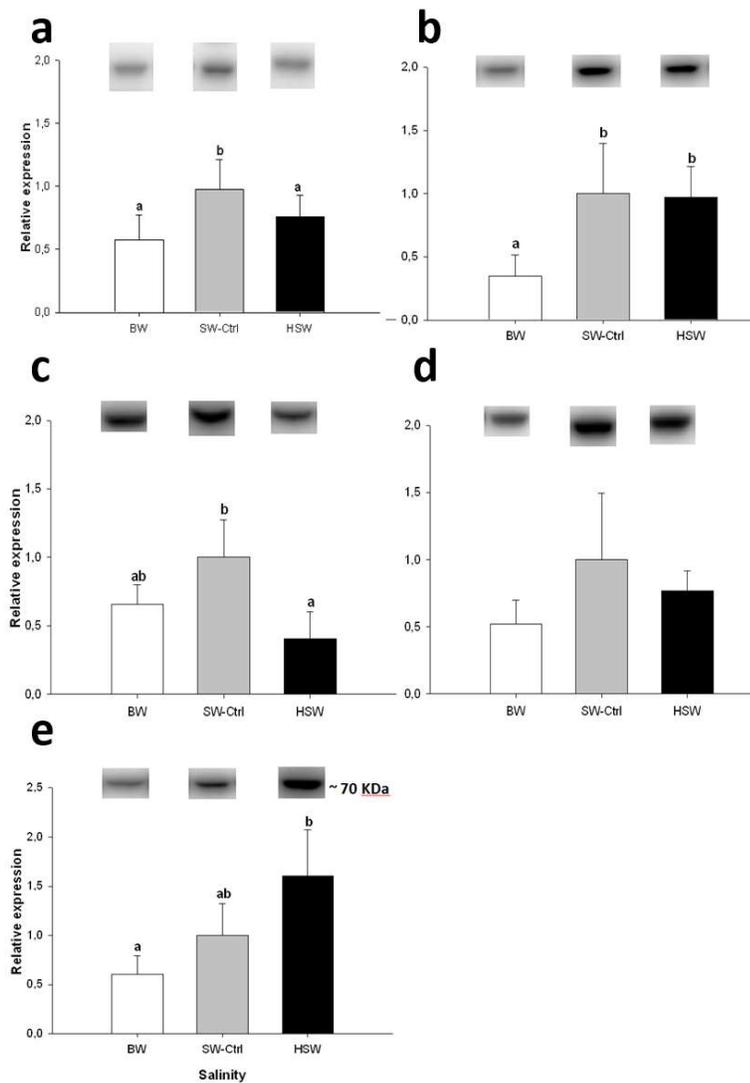


Figure 3.6. Immunoblotting relative expression of heat shock protein 70 (Hsp70) in the gill (a), kidney (b), anterior and posterior intestine (c,d) and dendritic organ (e) of marine catfish *P. lineatus* acclimated to [brackishwater (BW) 3‰, seawater (SW-control) 34‰, hypersaline water (HSW) 60‰]. Images of western blots from single individuals are chosen to represent band size and intensity. Numbers to the right of the western blot image represent molecular mass (kDa) of Heat shock protein (HSP70) ~ 70. Values are means \pm S.D of protein abundance (n=5-6). Different letter indicates a significant difference between salinities, one-way ANOVA and SNK ($P < 0.05$; see text for details).

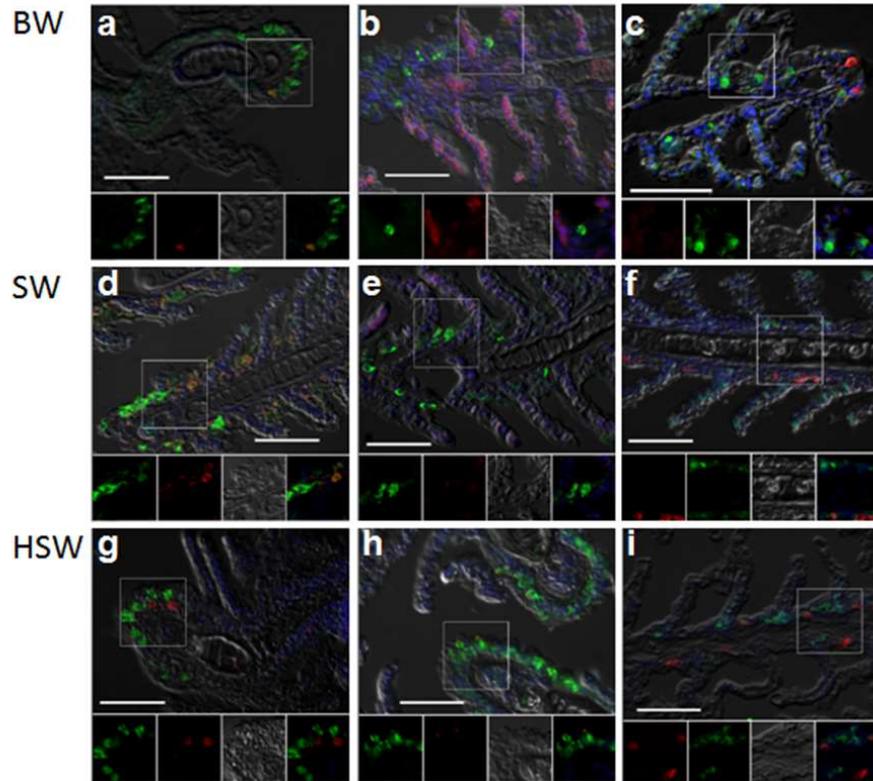


Figure 3.7. Double immunofluorescence localization of Na⁺/K⁺-ATPase (αR1, green **a, b, d, e, g, h**) with NKCC1 (T4, red **a, d, g**) and CFTR (red, **b, e, h**) or Na⁺/K⁺-ATPase (α5, red **c, f, i**) with V-ATPase (B2, green **c, f, i**). Sections were counter stained with DAPI nuclear staining (blue) and overlaid with the differential interference contrast (DIC) images in the gills of marine catfish *P. lineatus* acclimated in brackish water (BW) 3‰ (**a-c**), seawater (SW-control) 34‰ (**d-f**) and hypersaline water (HSW) 60‰ (**g-i**). Scale bar 100 μm in upper panel. See text for details.

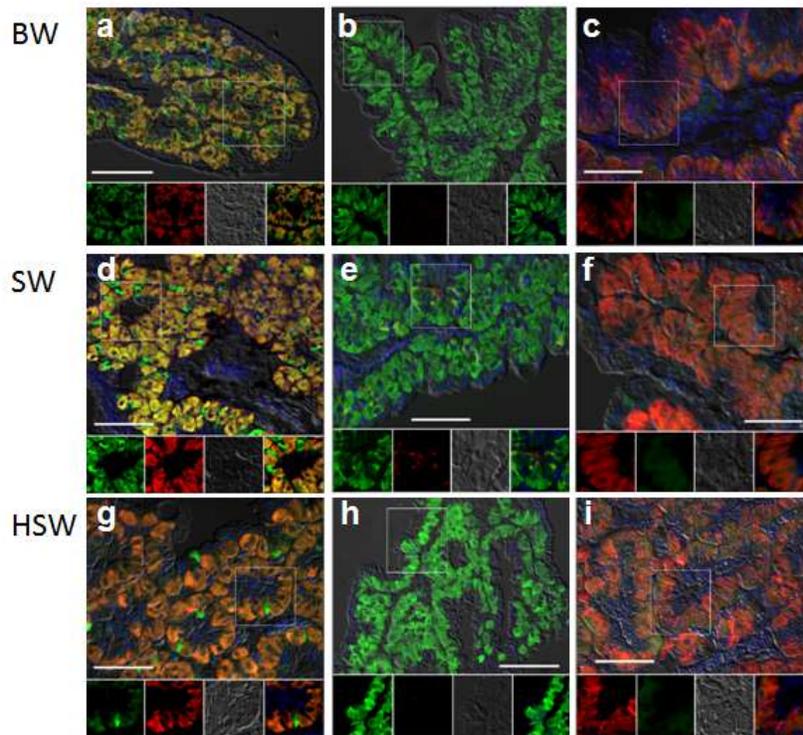


Figure 3.8. Double immunofluorescence localization of Na⁺/K⁺-ATPase (αR1, green **a, d, e, g, h**) with NKCC1 (T4, red **a, d, g**) and CFTR (red, **b, e**) or Na⁺/K⁺-ATPase (α5, red **c, f, i**) with V-ATPase (B2, green **c, f, i**). Sections were counter stained with DAPI nuclear staining (blue) and overlaid with the differential interference contrast (DIC) images in the DO of marine catfish *P. lineatus* acclimated in brackish water (BW) 3‰ (**a-c**), seawater (SW-control) 34‰ (**d-f**) and hypersaline water (HSW) 60‰ (**g-i**). Scale bar 100 μm in upper panel. See text for details.

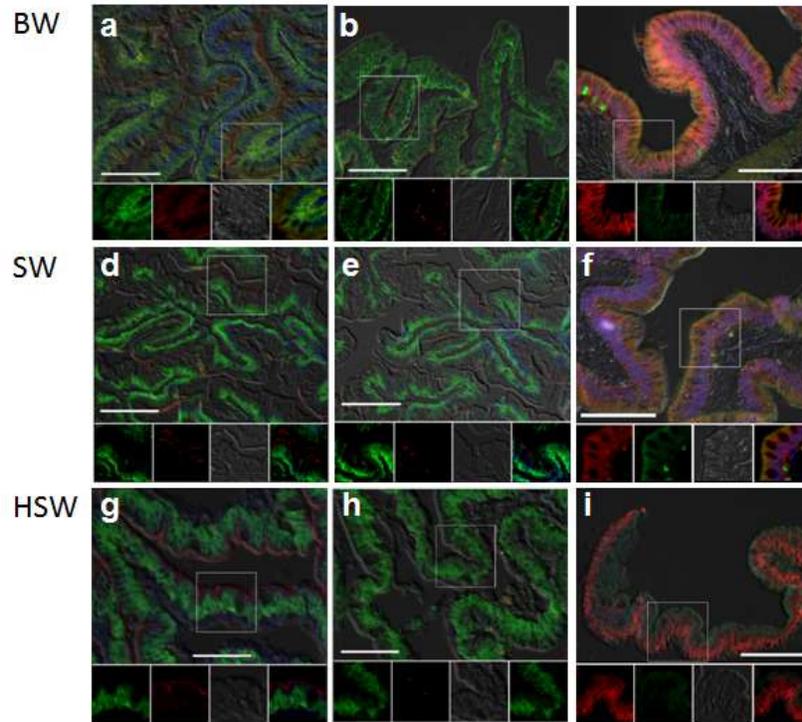


Figure 3.9. Double immunofluorescence localization of Na⁺/K⁺-ATPase (αR1, green **a, b, d, e, g, h**) with NKCC1 (T4, red **a, d, g**) and CFTR (red, **b, e, h**) or Na⁺/K⁺-ATPase (α5, red **c, f, i**) with V-ATPase (B2, green **c, f, i**). Sections were counter stained with DAPI nuclear staining (blue) and overlaid with the differential interference contrast (DIC) images in the anterior intestine of marine catfish *P. lineatus* acclimated in brackish water (BW) 3‰ (**a-c**), seawater (SW-control) 34‰ (**d-f**) and hypersaline water (HSW) 60‰ (**g-i**). Scale bar 100 μm in upper panel. See text for details.

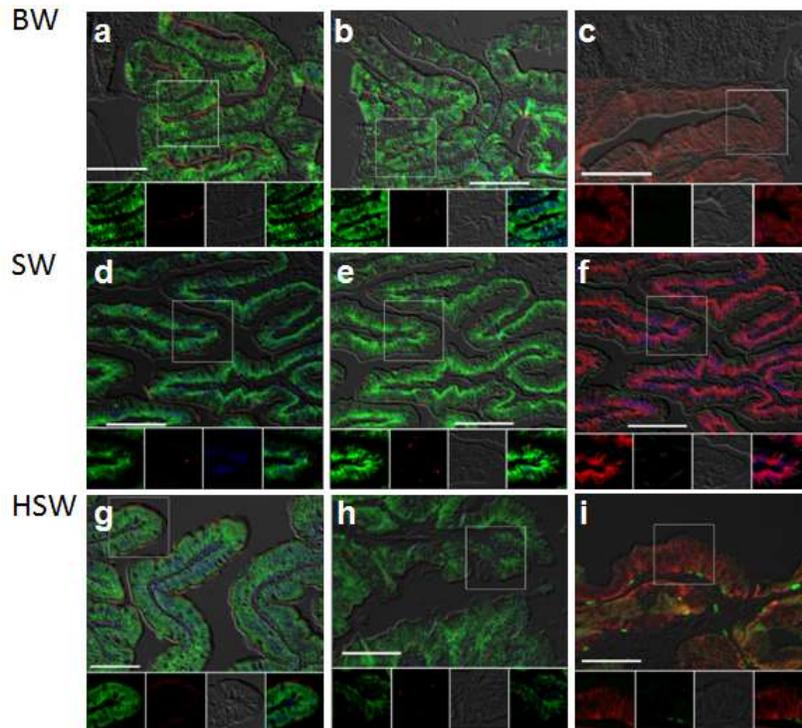


Figure 3.10. Double immunofluorescence localization of Na⁺/K⁺-ATPase (αR1, green **a, b, d, e, g, h**) with NKCC1 (T4, red **a, d, g**) and CFTR (red, **b, e, h**) or Na⁺/K⁺-ATPase (α5, red **c, f, i**) with V-ATPase (B2, green **c, f, i**). Sections were counter stained with DAPI nuclear staining (blue) and overlaid with the differential interference contrast (DIC) images in the posterior intestine of marine catfish *P. lineatus* acclimated in brackish water (BW) 3‰ (**a-c**), seawater (SW-control) 34‰ (**d-f**) and hypersaline water (HSW) 60‰ (**g-i**). Scale bar 100 μm in upper panel. See text for details.

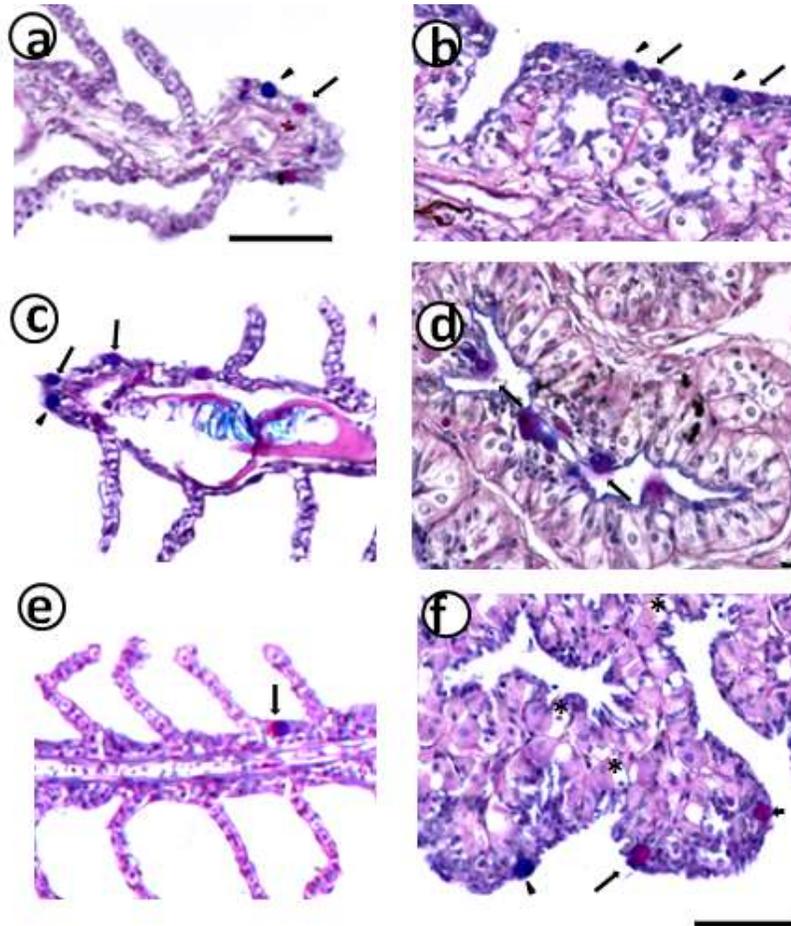


Figure 3.11. Periodic Acid Schiff (PAS)/Alcian blue (pH 2.5) AB/PAS staining of marine catfish *P. lineatus* gills (a, c, e) and DO (b, d, f) acclimated in (a, b) brackish water (BW) 3‰, (c, d) seawater (SW 35‰) and (e, f) hypersaline water (HSW 60‰). The neutral (magenta, short arrow), acid rich (blue, arrowhead) and purple cells (long arrow) are indicating combination neutral and acidic glycoconjugates. Scale bar 100 μ m. See text for details. No neutral staining in cytoplasm (*).

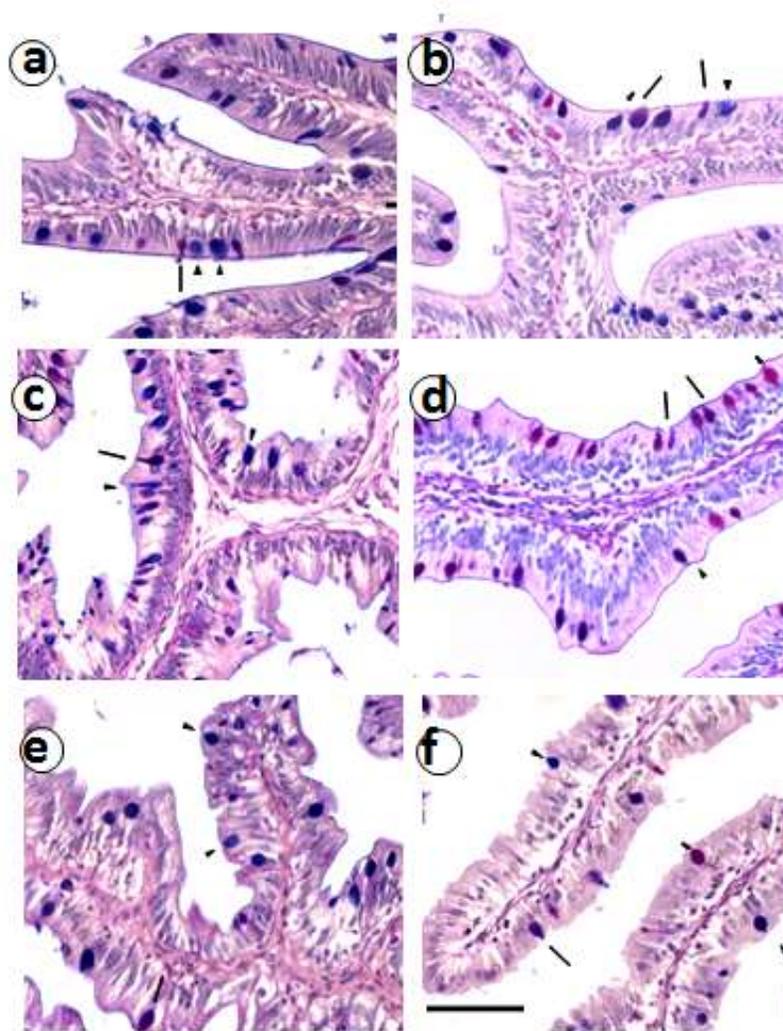


Figure 3.12. Periodic Acid Schiff (PAS)/Alcian blue (pH 2.5) AB/PAS staining of marine catfish *P. lineatus* anterior intestine (**a, c, e**) and posterior intestine (**b, d, f**) acclimated in (**a, b**) brackish water (**BW** 3‰, (**c, d**) seawater (**SW** 35‰) and (**e, f**) hypersaline water (**HSW** 60‰). The neutral (magenta, short arrow), acid

rich (blue, arrowhead) and purple cells (long arrow) are indicating combination neutral and acidic glycoconjugates. Scale bar 100 μm . See text for details.

3.4 Discussion

The marine catfish can osmoregulate across a wide range of salinities (3-60‰) although in HSW some impairment was observed. The dendritic organ of the marine catfish has the molecular machinery for active NaCl secretion using the conserved mechanism of secondary activity Cl⁻ transport with NKA, NKCC and likely CFTR at its core. The gill clearly has a secondary role in ion regulation with few ionocytes and low overall NKA expression. The intestine shows typical attributes of marine teleosts while kidney displays some unique feature that will be explored in more depth in Chapter 5.

3.4.1 Osmo and ionic regulatory responses to salinity acclimation

The observed plasma ion concentrations were in the range of other teleost fish species (see review Whittamore et al., 2012, Table 1). However, in comparison to other studies in Plotosidae, the plasma Na⁺, Cl⁻ and K⁺ of *P. lineatus* in SW-control were less than *P. lineatus* (Pucke and Umminger, 1979) while, Na⁺ was not very different from *Cnidoglanis macrocephalus* (Kowarsky, 1973). In both of these studies osmolality was also higher. These observed 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 (Tipsmark et al., 2008; Sardella et al., 2008; Kang et al., 2008; Uttara et al., 2009; Christensen et al., 2012; Watson et al., 2014; Tait et al., 2017). *P. lineatus* acclimated to HSW had higher plasma osmolality and ions (except K⁺), and hematocrit, and decreased muscle water content (MWC). 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. There are reports from various salinity tolerant species which show gradual increases of plasma ion levels to about 70-75 ppt, then increasing in a more or less linear form at higher salinities (see review Gonzalez 2012).

Plotosus lineatus challenged with BW, or hypoosmotic conditions are able to maintain plasma osmolality and Cl⁻ levels but not Na⁺. *Plotosus* were better able to regulate Cl⁻ levels than Ariid catfish, which does not have DO and have higher serum Cl⁻ levels (Sulya et al., 1960; Pucke and Umminger, 1979). The decrease in plasma Na and hematocrit suggest a hemodilution but muscle water and ions were stable. Reports regarding the effect of lower salinity on MWC from different species vary from showing no effect (Kang et al., 2008; Woo and Chung, 1995) to increased MWC (Jensen et al., 1998; Kelly and Woo, 1999; Kelly et al., 1999; Sinha et al., 2015).

Due to the dominance of the strong ions Na⁺ and Cl⁻ in blood, changes in the Na⁺/Cl⁻ ratio (SIR) has been recommended for indicating acid-base imbalances (Jensen et al., 1998; Sinha et al., 2015). In the present study, the direct measurements of plasma acid-base balance were not done due to the small size of the fish; however, calculations of SIR revealed changes in the plasma levels of weak anions (e.g. HCO₃⁻) and thus acid-base balance. The BW SIR indicates a metabolic acidosis which has also been observed in European sea bass *D. labrax*, reared in lower salinity (Sinha et al., 2015). However, this contrasts with work by Jensen et al. (1998) who have reported a markedly increased plasma SIR following transfer to FW and slight decrease in HSW in *D. labrax*. In *P. lineatus*, HSW had no effect on SIR suggesting no alteration in acid-base status.

3.4.2 Evidence for role of gills in salt secretion?

The gill is typically linked to active ion regulation in teleost fishes (Evans et al. 2005). This is reflected in high levels of NKA, a central driver of ion transport, with dependency of gill NKA to environmental salinity that may be altered by life history stage, species and experimental conditions in some cases (Evans et al. 2005; Varsamos et al. 2001; Malakpour Kolbadinezhad et al., 2012). However, branchial NKA activity of *P. lineatus* was the lowest of the osmoregulatory tissues test, unresponsive to levels of salinity, and an order of magnitude lower than levels in the DO irrespective of salinity. 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 extra-branchial salt secreting organ the rectal gland. In elasmobranchs, the gills have a secondary function in osmoregulation (Burger and Hess, 1960; Wilson et al., 2002). Our results confirm a similarity between gills of *P. lineatus* and elasmobranchs (Doyle and Gorecki, 1961; Pucke and Umminger, 1979) and underline the potential role of DO in salt excretion (Van Lennep, 1968).

The IHC result of few branchial NKA-IR cells was consistent with NKA activity levels and 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). The few NKA-IR cells were restricted to the filament epithelium, leaving the lamella unimpeded for gas exchange (Evans et al., 2005; Henriksson et al. 2008). Also, it was very rare to find NKA-IR cells that co-expressed NKCC1, although apical CFTR staining was observed in NKA-IR cells. NKCC1 is a key component of the mechanism of secondary active Cl secretion and is abundantly expressed in seawater type gill ionocytes in teleost fishes (see review Hiroi and McCormick, 2012). In elasmobranchs, the cDNA expression of NKCC in the gills of spiny dogfish *S. acanthias* (Xu et al 1994); however, in the branchial epithelium of *C. punctatum* NKCC1 could not be localized (Cramp et al., 2015). This contrasts with the freshwater stingray *Himantura signifer* where NKCC1 is co-expressed in gill NKA-IR cells following BW (20ppt) acclimation (Ip et al., 2013). However, the rectal gland is absent in this species. The observation of ovoid cells deep within the filament epithelium which show only NKCC1-IR at HSW are unusual and their potential role has not been determined.

Elasmobranch gills also possess a VHA rich cell that is involved in acid base regulation (Wilson et al. 1997; Piermarini et al. 2001; Tresguerres et al. 2006). Based on our IHC results, this cell type also appears in *P. lineatus*, and under HSW conditions IB results indicated a higher expression level. In killifish, basolateral VHA has also be found in ionocytes (Kato et al. 2003). Thus if the gills of *P. lineatus* have taken on the primary role in acid-base regulation, these cells maybe involved.

3.4.3 Evidence for the role of the dentritic organ in salt secretion?

The higher NKA activity of DO than other ion regulatory organs, notably gills, strongly indicates a role for this organ in NaCl secretion. This is also seen in elasmobranchs with higher rectal gland NKA activity compared to gills (Piermarini and Evans 2000; Pillans et al., 2005; Cramp et al. 2015). It has been demonstrated in euryhaline elasmobranchs that rectal gland NKA specific activity is higher in SW compared to FW acclimated animals but the opposite was observed in *P. lineatus* which was counter intuitive. To explain this discrepancy, if we took into consideration the DO mass which is higher in HSW so that the total DO NKA activity was also higher at HSW suggesting an increase in overall capacity. Larger rectal glands of *D. sabina* captured in SW than FW have been reported by Piermarini and Evans (1998) although no mass difference of rectal gland in *C. leucas* from FW or estuarine-captured were reported by Pillans and Franklin (2004). Also no changes in specific NKA activity of rectal gland has been reported in a few other elasmobranchs acclimated in various salinities (Dowd et al., 2010; Cramp et al., 2015) in. Observation of slightly albeit significantly higher DO/body weight percentage at BW rather than SW-control might indicate to the high capacity of marine catfish to move easily between different salinities, however, this was not sufficient to increase DO total NKA activity. Since the tissue sampling for the NKA activity measurement had been done after 10 days of acclimation, it seems working on time course sampling might be helpful to have a comprehensive view regarding NKA activity of DO in marine catfish.

Strong immunoreactivity of NKA and NKCC1 in parenchymal cells of the DO result in proposing that T4 is for secretory isoform in the DO promoting ion secretion in hypo-osmoregulating of marine catfish. The basolateral distribution of NKA and NKCC in other vertebrate salt secreting tissues has demonstrated (Hazard, 1999; Evans, 2009; Babonis et al., 2009, 2011). Salt secreting function of DO has been proposed physiologically (Kowarsky, 1973), ecologically (Lanzing, 1967) and ultrastructurally (Van Lennep, 1968). Our molecular observation involving either striking high NKA activity or localization of NKA and/or NKCC1 in DO follow the previous hypothesis suggests conservation of rather similar mechanism of ion transporting in secretory cell of vertebrate (Babonis et al., 2011).

Immunoblot (IB) results for NKA α subunit and NKCC (T4) were consistent in molecular weight compared to other vertebrates (Blanco and Mercer, 1998; Lytle et al., 1995, respectively). Finding multiple bands of NKCC might be the result of higher NKCC1 expression and immunoreactivity with either NKCC2 or NCC, reported in different species (Hiroi et al., 2008; Inokuchi et al., 2008; Lorin-Nebel et al., 2006; Christensen et al., 2012; Chew et al., 2015). Alternatively, the lipophilic nature of the NKCC migration through SDS-PAGE gels for IB analysis, or possibly the glycosylated monomer variability and/or different degrees of glycosylation could explain the banding patterns observed (Pelis et al., 2001; Tipsmark et al., 2002; Wu et al., 2003, Kang et al., 2010; Christensen et al., 2012; Kang et al., 2012; Chew et al., 2015). In BW, detection of NKCC suggest that maintaining a proportion of active NKCC for acid-base and/or cell volume regulation 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). Regarding the expected increasing of salt loading as a result of increased drinking and passive uptake (see review Grosell 2011) under HSW conditions, we detected significantly higher protein expression of DO NKCC representing an adaptation to increased salt excretion capacity.

IHC result 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 accumulation of chloride ions in DO epithelium then proposed highly functional in salt secretion. The presence of CFTR in salt glands of birds, elasmobranchs and reptiles have confirmed (Shuttleworth and Hildebrandt, 1999) although the antibody used in the present study does not show crossreactivity with elasmobranch (J.M. Wilson personal observations), or sea snake (Babonins et al., 2011) salt glands or salmonid (S.D. McCormick personal observations) gill CFTRs either. *Cfr* transcript was detected in DO but predicted salinity dependent expression differences were not observed. Obviously, identifying the putative apical Cl⁻ channel in marine catfish DO in future work would confirm the presence of typical iono-secretory cell of vertebrate salt glands.

The inconsistent results of mRNA expression for *atp1a1*, and *cfr* may be related to post-transcriptional (Reilly et al., 2011), post-translational processing or modulation of the NKA kinetic properties by FXYD proteins interacting (Garty and Karlish, 2006; Mahmoud et al., 2000, 2003, 2005; Wang et al., 2008; Tipsmark et al., 2010). Moreover, the quaternary structure of NKA, effecting of shifting in isoform composition and/or phosphorylation state on kinetic of the enzyme pool (McDonough et al., 1990; Hauck et al., 2009; Christensen et al., 2012) might be involved. Obviously, 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 might be helpful to interpret of the osmoregulatory function of DO. The changes in V-ATPase, CA (protein and mRNA expression) in DO of marine catfish together with the PAT1 mRNA (*slc26a6a*) suggest the possible contribution of the DO to acid-base regulation in the marine catfish.

Neutral and acidic mucin staining is found in the DO and there is the possibility of a role for mucus in ion regulation (Handy et al., 1989; Roberts and Powell, 2003; Powel, 2007). In Atlantic salmon *Salmo salar* the greater net efflux of Cl⁻ compare to either Na⁺ or K⁺ has been found (Roberts and Powell, 2003) which may due to differing ion permeability mechanisms (see review Zadunaisky 1984). The highly polyanionic gel of the mucus (Verdugo, 1984) may cause a greater diffusive potential for anions (Cl⁻) while the cations such as Na⁺ or K⁺ may have the potential to be bound by the mucous layer (Zuchelkowski et al., 1985). It seems there is likely a connection between mucous secretion of marine catfish DO and Cl⁻ transport.

3.4.4 Role of the Kidney

Kidney NKA activity is typically responsive to environmental salinity (e.g. Venturini et al., 1992; Kelly and Woo, 1999 Herrera et al., 2009, Tang et al., 2012) although in some species no changes are observed (Fuentes et al., 2005; Laiz-Carrion, Sangiao- Alvarellos et al., 2005; Arjona et al., 2007). In the case of *P. lineatus* kidney NKA activity there was a positive relationship with environmental salinity, whereas in many euryhaline fishes the opposite is observed (Epstein et

al., 1969; Madsen et al., 1994; Kelly and Woo, 1999; Lin et al., 2004; Nebel et al. 2005; Tang et al., 2010, 2012; Yang et al. 2016). This higher NKA activity at lower salinities has been associated with increased urine production and the need to increase ion reabsorption which is driven by NKA (MacDonald 2007) whereas in the case of *P. lineatus* and a few other marine teleosts (Deane and Woo 2004; Herrera et al., 2009; Yang et al 2016) the increased NKA activity could potentially augment active ion secretion. In the case of marine catfish, hyperosmotic urine production has been reported although in the other species this is unknown but well worth investigating further.

In BW higher mRNA expression of *cftr* and *slc26a6a* suggest a functional role of the marine catfish kidney in a regulatory role in Cl⁻ and HCO₃⁻ transport that may be important for addressing the acid-base disturbance indicated by the lower strong ion difference (Jensen et al. 1998). Based on a lack of changes in either CA or VHA, we cannot confirm their involvement.

3.4.5 Role of the intestine

The gastrointestinal tract of marine teleost is involved in osmoregulation through desalination of the imbibed seawater in the esophagus accompanied by NaCl coupled water uptake (Usher et al., 1991; see review Grosell 2011). Drinking rates were not measured in this study although there is ample evidence that shows a positive correlation with salinity (Whittamore 2012). The anterior intestine has higher NKA activity than the posterior intestine but it is only the posterior intestine that is responsive to HSW. In Gulf toadfish *Opsanus beta* higher NKA activity in anterior than posterior intestine was also observed (Guffey et al., 2011). Ruiz-Jarabo et al (2015) have also found significant increasing of NKA activity in posterior region rather than anterior in common galaxias *Galaxias maculatus* at hypersalinity. The intestine has been shown to respond to the increased drinking rate by increasing intestine NKA activity and expression, in addition to a number of key transporters and/or enzymes to coupled water absorption by intestinal epithelium as reported in different species (see review Grosell 2011; Whittamore 2012). There are also reports of variation between anterior or posterior intestine in water absorption and/or ion secretion of marine

teleosts (Gregório et al., 2013; Madsen et al., 2014; Raldúa et al., 2008; Aoki et al., 2003; Kim et al., 2008; 2006; Grosell, 2011).

It seems the ions absorption via NKCC/NCC might be occurring in all of intestine regions as demonstrated by IHC and in agreement with a number of other studies (e.g. Wilson and Castro 2010; Kalujnaia et al., 2007; Esbaugh and Cutler 2016). It has been demonstrated that because of reduced luminal Na^+ , and, therefore, Cl^- concentration along intestine from anterior to posterior (Marshall and Grosell, 2005), the cotransport function of NKCC may be limited so might rely more on $\text{Cl}^-/\text{HCO}_3^-$ exchange to aid water reabsorption through alkalization of the gut and divalent cation precipitation (Taylor et al., 2010; Grosell et al., 2009a; Grosell, 2011). In support, observations were made of yellow-whitish precipitates, particularly in the posterior intestine of fish acclimated either in SW-Control or HSW presumably made of Ca^{2+} and Mg^{2+} carbonates (Grosell, 2011; Madsen et al., 2014). The precipitation of carbonates decreases the osmotic gradient supporting water absorption (Whittamore et al., 2012; Grosell 2011). Finding absolute rates of water absorption in anterior and posterior intestine of marine catfish acclimated in different salinities would help address the relative roles of the two regions to water absorption.

Observation of CFTR in the apical membrane of spindle shaped columnar cells may be address the responsibility of subpopulation of enterocytes for ion (and fluid) secretion as has been reported in Atlantic killifish (Marshall 2002). In sea bream *S. aurata* apical region of the anterior intestinal epithelium showed diffuse staining pattern of CFTR while it was more in the rectum of high salinity fish (Gregório et al., 2013). There are some other studies which did not observe ion or fluid secretion by the intestine (Field et al., 1980; Loretz, 1987a,b, 1995). On the other hand, the observation of higher mRNA expression of *cftr* in the anterior intestine at HSW fish suggests a role in the recycling Cl^- in parallel with the apical $\text{Cl}^-/\text{HCO}_3^-$ exchanger to increase HCO_3^- excretion (Grosell and Taylor, 2007; Taylor et al., 2010). The presumable $\text{Cl}^-/\text{HCO}_3^-$ exchanger is possibly *slc26a6a* (PAT1). Its mRNA expression was detected in intestine but it was not responsive to salinity.

Regarding the localization of V-ATPase at intestine regardless salinity it was generally more obvious in anterior than posterior intestine. It seems the proton pump might have different roles presumably in acid-base regulation and/or involving ion and water transport in marine catfish. In gulf toad fish acclimated in either SW or HSW, H⁺-ATPase showed apical and basolateral of intestine enterocyte plasma membranes (Guffey et al., 2011). Moreover, the apical localization of vacuolar-type (V) H-ATPase in the epithelium of intestine has been reported in different species has also been proposed to aid in carbonate precipitation to maintain water absorption rates (Grosell et al., 2009b; Guffey et al., 2011; Grosell et al., 2007; Grosell et al., 2009a; Gregório et al., 2013; Esbaugh and Cutler, 2016; Cooper et al., 2010).

3.4.6 Cellular stress and salinity

The heat shock proteins (Hsps), which are commonly named stress proteins, are expressed in cells and are involved in maintaining a number of vital cellular processes as part of the cellular stress response (Hightower, 1991; Morimoto and Santoro, 1998; Iwama et al., 2006; Basu et al., 2002). Deane and Woo, (2004; 2011) have shown that salinity can induce a cellular stress response. In DO Hsp70 levels are highest at HSW indicating a cellular stress requiring the activation of stress protein mechanisms to protective action against stress situation (for more details, see review Deane and Woo, 2011). However, given the lower Hsp70 levels in gills, anterior intestine and kidney at one or both salinity extremes suggests less of a stress compare to the DO or a different threshold of salt tolerance.

Chapter 4:

Effect of dendritic organ ligation on marine catfish *Plotosus lineatus* osmoregulation

Malakpour Kolbadinezhad, S., Coimbra J., Wilson, J. M. 2017. Effect of dendritic organ ligation on marine catfish *Plotosus lineatus* osmoregulation (*in preparation*).

Abstract

Unique amongst the teleost, the Plotosidae catfish *Plotosus lineatus* possesses a dendritic organ (DO) which is purported to function as a salt secreting organ, whereas other marine teleosts rely on their gill ionocytes for active NaCl excretion. To address the role of the DO in ionregulation, ligation experiments were conducted in brackishwater (BW) 3‰ and seawater (SW) 34‰ acclimated *P. lineatus* and compared to sham operated fish. Ligation in SW resulted in an osmoregulatory impairment in blood (elevated osmolality and ions, lower hematocrit) and muscle (dehydration). However, SW ligation did not alter gill or kidney Na⁺/K⁺-ATPase (NKA) activity and/or protein expression while a decrease in anterior intestine and increased in posterior intestine were observed but this was not reflected at the protein level. Following ligation in SW, protein levels of carbonic anhydrase (CA) and V-ATPase B subunit (VHAB) were higher in kidney but either lower (CA) or unchanged (VHAB) in other tissues. Heat shock protein (Hsp70) levels were higher with BW ligation but decreased only in posterior intestine. BW-L decreased plasma K⁺ and Na⁺/Cl⁻ ratio (SIR) expressed metabolic acidosis and only lowered gill NKA activity or expression while increased NKA expression in intestine. IB showed increased in interaction between BW and BW-L of CA and Hsp70 or decreased in V-ATPase only in posterior intestine. Immunohistochemistry (IHC) results of NKA α subunit, Na⁺:K⁺:2Cl⁻ cotransporter (NKCC1), CA, V-ATPase B subunit moreover gene expression of *atp1a1*, *ca17*, cystic fibrosis transmembrane conductance regulator (*cftr*) and *slc26a6a* (Cl⁻/HCO₃⁻ exchanger) were detected in all of tissues. Histology results showed more distribution of blue (acidic) mucus cells in gill filament, collecting tubule (CT) of kidney and posterior intestine in SW-CL fish. Taken together, SW-CL fish representing problematic resulting in osmotic disturbance thereby indicating to main role of DO as salt secreting and elucidate no compensatory responses of gill, kidney and/or intestine NKA.

4.1. Introduction

The gill is the central ionregulatory organ in teleost fishes with supporting roles played by the kidney and intestine (Marshall and Grosell 2006; Evans et al. 2005). Branchial mitochondria rich cells (MRCs) or chloride cells, known as ionocytes, are well characterized (Evans et al., 2005; Hwang et al., 2007; Wilson 2011) and function in freshwater and saltwater via actively take up and excretion of monovalent ions (Na^+ , K^+ , and Cl^-) respectively, to maintain plasma osmolality (Takei and Hwang, 2016). In contrast, in the Plotosidae marine catfishes there is ultrastructural (Van Lennep and Lanzing, 1967), histochemical (Van Lennep 1968) and physiological (Kowarsky, 1973) evidence suggesting that their unique dendritic organ is responsible for salt excretion. Kowarsky (1973) has found a significant reduced survival of DO ligated catfish *Cnidoglanism acrocephalus* and increase in plasma Na^+ concentration in hyperosmotic salinities. High Cl^- levels have been demonstrated in DO parachymal cells, similar to gill chloride cells (Pucke and Umminger, 1979). The anatomy of this organ was described first by Bloch (1794) then was followed by Brock (1887) and Hirota (1895). It is a small fleshy external organ situated very close, to the urogenital papilla on the ventral surface of the fish.

This chapter investigated the effect of ligation of the DO on the osmotic and/or ionic regulatory ability and survival of the catfish, *P. lineatus* to elucidate its role in ion regulation and the compensatory responses of the gill, kidney or intestine.

4.2. Material and Methods

4.2.1. Modulation of salinity status and Animals collection

The marine catfish *Plotosus lineatus* (~8-13 g) were purchased from TMC Portugal and transported to Laboratory of Ecophysiology CIIMAR (Porto). Prior to the start of the experiment due to avoid any confounding effects of handling stress on osmoregulation (Biswas et al., 2006), a 100 L tank with seawater (SW) 34‰ and normal photoperiod was used for acclimation of the fish to laboratory conditions for three weeks. Seawater was made up using Instant Ocean® salt. Fish were fed twice daily by the diced fish fillets during this period not fed 4 days before samplings. Fish

behavior, temperature (range at 26-28 °C), pH (range 7.7–7.9) and salinity were checked daily.

4.2.2. Salinity acclimation

Two salinity levels were investigated [brackishwater (BW), brackishwater ligated (BW-L) 3‰, seawater (SW-control), seawater ligated (SW-CL) 34‰] salinity. Initially, individuals were transferred to a 22 L tank (small tank). The salinity was changed by removing water and adding an appropriate amount of dechlorinated tap water in a stepwise fashion from 34 (main tank) to 3, 5‰ per day salinity. Keeping the fish in the same tank was used due to decrease the handling stress and a water change of SW-control group was also conducted in order to standardize fish stress at each salinity change between the different groups. Fish has been checked and dead fish removed from the experimental tanks. Maintaining of fish in the lab was according to the Portuguese Animal Welfare Law (Decreto-Lei no.197/96) and were approved by CIIMAR/UP animal protocols.

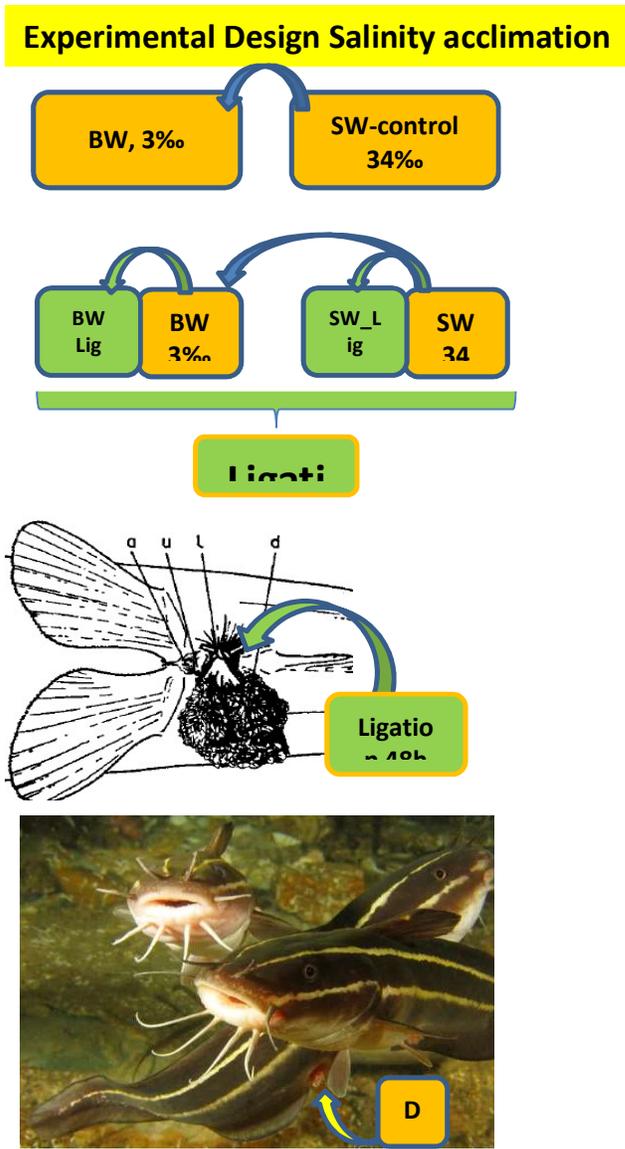


Figure. 4.1 The anal region of *C. macrocephalus*, showing the position of the dendritic organ and ligature in relation to other structures. The pelvic fins are folded anteriorly. a, Anus; u, urogenital papilla; L, ligature; d, dendritic organ leaflets. Kowarskey (1973).

4.2.3. Sampling

Marine catfish were anaesthetized with an overdose of MS-222 (1:5000, pH 7.5 adjusted with NaHCO₃). Blood was collected by using a heparinized capillary tube following caudal transaction, centrifuged at 13000g for 5min (hematocrit centrifuge, Heraeus Pico 17 Centrifuge, Thermo Scientific) at room temperature. The hematocrit (Hct) was measured in duplicate (nearest millimetre) then converted to percentage of total blood volume. The isolated plasma and collected tissues including gill, kidney,

anterior and posterior intestine were then frozen in liquid nitrogen and kept at -80 °C. Blood sampling was done in addition sets of six individuals then the body cavity opened by a ventral incision and 10% neutral buffered formalin (NBF 10%) was used for immersion fixed then stored in 70% ethanol at 4 °C.

4.2.4. Histology staining

Following fixation, tissues were dehydrated through an ethanol series, and embedded in paraffin (Type 6; Richard Allen Scientific). The microtome (Reichert Biocut 2030) was used for sectioning (cut at 5µm) and Periodic Acid Schiff (PAS)/ Alcian blue (pH 2.5) was used as staining protocols. The serial tissue regions were imaged with a digital colour camera (Leica DFC300FX) mounted on a Leica microscope (DM 6000 B). The taken Images resizing, brightness and/or contrast adjustment were done by Photoshop CS3 while maintaining the integrity of the data.

4.2.5. Ion quantification

Wet weight (one gram of muscle tissue) was collected. For obtaining dried weight collected tissue was dried to constant mass at 60 °C then water content (MWC) determined. Nitric acid (65%) five volumes were used for digestion of the dried muscle samples for 3 days. Using a flame photometer (model PFP7; Jenway, Felsted, UK) the Na⁺ and K⁺ concentrations were quantified by flame photometry (PinAAcle 900T Atomic Absorption Spectrophotometer; Perkin Elmer Waltham MA). Chloride concentration of plasma was measured by mercuric thiocyanate reaction forming mercuric chloride and free thiocyanate ions. The latter react with ferric ions to form an orange complex of ferric thiocyanate then the absorbance was measured at 480 nm (Küffer et al., 1975). Plasma samples were also analysed (mOsm kg⁻¹) using freezing-point depression (Melting Point Osmometer, N 961003, Roebling Co.; www.melting-point.buchi.com).

4.2.6. Measurement of Na⁺/K⁺-ATPase

The NKA activity was measured according to the microassay protocol of Wilson et al. (2007b). Gill filament samples from the second arch on left side and other tissues were cut off from the anesthetized fish immersed in 100µl of SEI buffer and frozen at -80 °C. The tissues were thawed then homogenized (Precellysis 24 homogenizer Bertin Technologies at 5800 RPM for 2x15s) in SEI buffer containing 0.1% deoxycholic

acid. Then immediately were centrifuged at 15.000g (5 minutes at 4 °C) to remove large debris. 10µl of the supernatant were added to 200µl of assay mixture (see appendix) while the assay were run in two sets of duplicate, one set containing the assay mixture and the other assay mixture plus a specific inhibit NKA activity, ouabain (1 mM, Sigma–Aldrich Chemical Co.; www.sigmaaldrich.com). ATPase activity was measuring with a temperature controlled plate reader (Thermomax, Molecular Devices) at 340nm for 10-20 min at 25 °C. Total protein concentrations were determined with a bovine serum albumin (BSA) standard by Bradford (1976) dye binding assay at 600nm. The results were expressed as µmoles ADP mg⁻¹ protein h⁻¹.

4.2.7. Immunoblotting

The tissue samples were homogenized by a Precellysis 24 homogenizer, Bertin Technologies) in Imidazole buffer pH 7.5 for 2x15s and immediately centrifuged at 15.000g for 5 minutes at 4 °C. The supernatant was mixed with 2x Laemmli's buffer (Laemmli, 1970) with equal volume of, heated (10 minutes at 70°C) and then stored at 4°C. Leftovers from supernatant were used for protein assays using Bradford method (Bradford, 1976) using BSA as a standard and Coomassie Brilliant Blue G-250. 10-20µg of sample per well was (protein concentration was adjusted to 1 µg µl⁻¹ using 1x Laemmli's buffer) loaded onto 1.5mm thick mini vertical polyacrylamide gels was run. Gels were then equilibrated in transfer buffer and bands were transferred to nitrocellulose membranes (GE Healthcare Amersham TM Hybond TM ECL). Membranes were then rinsed in TTBS and blocked with 10% powdered skim milk in TTBS for 1h. Blotto were probed with heterologous bovine α-subunits of NKA (αR1), NKCC1 (T4), Carbonic anhydrase (CA), V-ATPase (B2), and Heat shock protein (Hsp70), overnight at room temperature in 50 ml falcon tubes using a attached to a rotisserie (LabQuake2). Then membranes were rinsed with TTBS and incubated for 1 hour with a goat anti-rabbit or anti-mouse IgG secondary antibodies conjugated to horseradish peroxidase, diluted in TTBS (1:50000). Membranes were rinsed a final time with TTBS and signal were obtained by enhanced chemiluminescence (ECL) Millipore Immobilon Western chemiluminescent HRP substrate (Millipore Corporation Billerica, MA 01821 U.S.A.). Images were acquired using Fujifilm LAS-4000 mini and image reader software LAS-4000 version.2.0. Intensity of bands signal were quantified using Multi Gauge v3.1 (FUJIFILM).

4.2.8. Immunohistochemistry

The paraffin serial sections were cut and collected onto APS (3-aminopropyltriethoxysilane; Sigma)-coated slides (Reis-Santos et al., 2008), completely dried, dewaxed then rehydrated. Dried Sections were circled with a liquid hydrophobic blocker (DakoPen, Dako DK) following by rinsing in 1% sodium dodecyl sulfate (SDS)/PBS, in tap water then distilled water (DW). The sections were blocked with 5% normal goat serum (NGS) and incubated with primary antibodies α -subunits of NKA (α R1), NKCC1 (T4), Carbonic anhydrase (CA) and V-ATPase (B2) for 1-2h at 37°C in humidity chamber. Sections were then rinsed in TPBS following by incubation with secondary antibody goat anti-mouse Alexa Fluor 568 and/or goat anti-rabbit Alexa Fluor 488-conjugated for 1h at 37°C. Following rinsing as mentioned above except rinsing with DAPI/TPBS diluted 1:25000 in 10min step. Following coverslips were mounted with (DAKO fluorescent mounting media S3023). Sections were viewed on a Leica DM6000 B wide field epifluorescence microscope with a digital camera (DFC340FX, Leica Microsystems, Wetzlar, Germany).

4.2.9. Molecular genetics approach

4.2.9.1. Isolation and quantification of RNA and synthesis of complementary DNA

Gill, kidney, and intestine were excised from marine catfish and freeze-clamped in liquid nitrogen. Total RNA was extracted using silica-based columns (Aurum Total RNA mini kit, (Bio-Rad, Hercules, CA, USA). The Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) was used for assessing of total RNA concentration and purity then were stored at -80°C. 1 μ g of total RNA was converted to cDNA (iScript cDNA kit Bio-Rad). Samples were stored at -20°C.

4.2.9.2. Gene isolation

Consensus primers were designed from a conserved region of β -actin, Na⁺/K⁺-ATPase α subunit, Cystic fibrosis transmembrane conductance regulator (*cftr*), Carbonic anhydrase (*ca*), Putative Anion Transporter Cl⁻/HCO₃⁻ exchanger (PAT *slc26a6*) by multiple sequence alignment (MultAlin, Corpet, 1997). Nucleotide sequences and amplicon sizes of these primers are shown in Table 2.1 Chapter 2. PCR amplification products of the correct size were cloned (Pgem-t easy Promega), sequenced (StabVida, Oeiras, Portugal) then analyzed for sequence similarity (BLAST, ClustalX). Primer3 (Rozen and Skaletsky, 2000) was used for Marine catfish

specific primers designing specifically for 5' and 3' amplification then were initially tested for specificity by RT-PCR (Table 2.2). Nucleotide sequences and amplicon sizes are shown in Table 2.1 Chapter 2.

4.2.9.3. RT-PCR and RT real-time PCR

The real-time PCR (RT PCR) was done by transcript quantification using SYBR green with an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). The generation of a melt curve for every PCR product (confirm the specificity of the assays) and preparation of a dilution series to check the efficiency of the reactions has been used. The β -Actin, was used as the housekeeping gene. Analyzing of the expression levels of the interest genes was done based on cycle threshold (CT) values using the comparative CT method ($2^{-\Delta\Delta CT}$ method) (Table 2.3 Chapter 2).

4.2.10 Statistics

Means \pm standard deviation (S.D.) or 95% confidence interval was used for presenting of the data. Statistical differences of mRNA expression, protein and interaction of salinity and ligation between groups were determined using one-way ANOVA and two-way analysis of variance (ANOVA), respectively followed by the post hoc Student-Newman-Keuls (SNK) test (SigmaPlot 11.0 Systat Software, Inc.) in juveniles exposed to different salinities. Data were log transformed or square root in the case of a failed normality test. Fiducial limit was set at 0.05.

4.3 Results:

4.3.1 Osmoregulatory indicators

During acclimation, there was mortality (50%) in SW acclimation ligated fish (48h) but not in sham or BW groups. Attempts at ligation in HSW acclimated fish resulted in 100% mortality so experiments were not pursued further in this group of fishes.

Plasma and muscle osmoregulatory indicators are presented in the Table 1. The seawater ligation [(SW-CL) 34‰] resulted in higher plasma ion concentration including Na^+ , Cl^- and Ca^{2+} , osmolality, hematocrit and muscle Na^+ and K^+ concentrations. Plasma osmolality was more than 70 and 100% higher in 24h48h SW ligated, respectively. The resulting plasma strong ion ratio was significantly lower in

BW fish compared to SW and SW-CL fish. Hematocrit showed a positive correlation with salinity with BW values half of SW-CL (48h). Acclimation salinity had no effect on plasma K^+ concentrations.

Muscle water content was significantly lower in SW-CL (48h) acclimated fishes indicating dehydration but was unaffected by BW acclimation or BW-L ligation. Muscle sodium and potassium concentrations followed the opposite trend being significantly higher in SW ligated fish which is reflected in a respectively higher and lower $Na^+ : K^+$ ratio in muscle of ligated fish. On the other hand, brackish water ligation (BW-L), only resulted in lower plasma K^+ concentrations (Table 1). The $[Na^+]$ and Na^+/Cl^- ratio of plasma and haematocrit showed a significant decrease in BW compare to the SW-control of acclimated fish.

4.3.2 NKA activity

In SW marine catfish, specific NKA activity was lowest in gill and posterior intestine, and more than three times higher in kidney and anterior intestine (Fig. 4.1). In response to SW-CL no significant difference in NKA activity were detecting in gill, or kidney (Fig. 4.1a,b,c) while anterior and posterior intestine NKA activity was 2x lower and 2x higher, respectively than their respective SW sham controls (Fig. 1d). Kidney showed significantly higher NKA activity in SW versus BW acclimated fish, but no ligation effect (Fig. 4.1b). In BW-L NKA activity only increased in the gill (Fig. 4.1a) while sham BW gill was also lower than the corresponding SW sham group (Fig. 4.1a).

4.3.3 Western blot

We used antibodies cross reactive with NKA α -subunits, NKCC1, cytosolic carbonic anhydrase, V-ATPase B subunit and heat shock protein (Hsp70) to determine how DO ligation and salinity affected the abundance of these important gill, kidney, anterior and posterior intestine ion transporters and possible compensatory response of them.

NKA α subunit expression was detected in all organs of interest as a single band of approximately 100kDa. The relative expression of the NKA α subunit protein was not salinity responsive in gill and kidney (data not showed), but in anterior and posterior intestine expression levels were dependent on both salinity and ligation (Fig. 4.2a,b). In anterior intestine, NKA α subunit expression was significantly lower in BW

acclimated fish compared to SW. In SW acclimated fish, both intestinal regions had significantly lower expression following ligation. In contrast the opposite effect of ligation was observed in BW fish following ligation (significantly higher expression). In both regions, the highest levels of expression were present in BW ligated fish. NKCC (T4) expression was not detected in any of the tissues in current experiment (data not showed).

Regarding use of CFTR antibody, a single band of predicted molecular mass range as 160 kDa was expressed; however, multiple smaller cross-reactive bands were also observed thus making it difficult to specifically detect the band of interest, which made semi-quantification problematic. Therefore, the antibody was not use in other tissues or for quantification.

Ca17 was detected as an approximately 30kDa band and interactions between salinity and ligation were detected in all tissues examined (Fig. 4.3). In gill and anterior intestine Ca17 was lower in BW versus SW sham fish but not in kidney or posterior intestine. Relative Ca17 protein expression was significantly lower in gill, anterior and posterior intestine of following ligation in SW fish (Fig. 4.3 a, c, d) while higher expression was found in kidney (Fig. 4.3 b). In BW ligation, significantly higher Ca17 was found only in posterior intestine relative to the BW sham group and no differences in the other tissues (Fig. 4.3 d).

The V-ATPase B subunit (VHAB) was found expressed as a ~56 kDa band in gill, kidney and posterior intestine but not anterior intestine (Fig. 4.4). In gill, VHAB levels were significantly higher in ligated versus non-ligated fish irrespective of acclimation salinity (Fig. 4.4a). In kidney and posterior intestine there were interactions between salinity and ligation. In kidney, ligation resulted in significantly higher VHAB compared to both SW-control and BW-L (Fig. 4.4b). In posterior intestine, the BW sham group had higher VHAB compared to both BW-L and SW controls (Fig 4.4c).

Heat shock protein 70 (Hsp70) protein was found in all of the tissues of interest in our work as a single 70 kDa immunoreactive band (Fig. 4.5). In all tissues Hsp70 were highest in SW versus BW fish (Fig. 4.5a,b). However, in anterior intestine ligation was associated with significantly lower Hsp70 levels but without an interaction with salinity (Fig. 4.5c). An interaction between salinity and ligation was found only in the posterior intestine (Fig. 4.5d). In SW fish, ligation was associated with lower Hsp70

levels whereas the opposite was observed in BW with significantly higher ligation associated expression. SW sham control Hsp70 levels were higher than respective BW shams.

4.3.4 Gene expression of *atp1a1*, *ca17*, *cfr*, *slc26a6a*

Significant differences in NKA α subunit *atp1a1* mRNA expression levels was only found in kidney. Ligation in SW fish was associated with significantly higher expression levels compared to SW controls and BW-L. (Fig. 4.7a).

The *cfr* mRNA expression in gills was not affected by ligation in SW fish, however, BW ligation resulted in significantly higher mRNA levels compared to the BW sham and SW-L group. (Fig. 4.6b). In kidney there was an interaction between salinity and ligation (Fig. 4.7c). BW sham fish had higher *cfr* levels than BW ligated and SW control sham fish. Ligation had no effect in SW fish. Only in anterior intestine difference between BW was higher compare to SW-control (data not shown). IF YOU DON'T SHOW DATA as tables or figures SHOULD PROVIDE SOME NUMBERS HERE.

The *ca17* mRNA levels in gill were significantly lower in SW fish and ligation was also associated with lower levels (Fig. 4.6a). In contrast in kidney, *ca17* levels were higher with ligation independent of salinity (Fig. 4.7b).

The *slc26a6a* mRNA levels was found expressed in all tissues studied. However, only in kidney were significant treatment effects observed. There was an interaction between salinity and ligation. BW sham fish has higher mRNA compared to ligated and SW sham control fish (Fig. 4.7d).

4.3.5 Immunohistochemistry

IHC staining pattern of BW-L and SW-L fish in different tissues of the present experiment were similar to the BW and SW-control acclimated fish; however, a few changes were noted which will be presented in the following section.

4.3.5.1 Gill

The gills of marine catfish have a typical teleost gill organization of filaments with lamellae. In the branchial epithelium strong NKA immunoreactivity (IR) was detected in large isolated ovoid cells throughout the cytoplasm with the exception of the apical

region (Fig 4.8). This NKA cellular staining pattern is typical of teleost fish chloride cell or ionocyte tubular system. There were relatively few of these branchial NKA-IR cells which were present in a heterogeneous distribution limited to a few interlamellar regions over the leading edge of the filament and were absent from the lamella. Experimental salinities and ligation did not alter the NKA-IR cell distribution pattern. The secretory $\text{Na}^+:\text{K}^+:2\text{Cl}^-$ cotransporter (NKCC1) expression in gill was rarely detected despite the use of antigen retrieval techniques and positive immunoreactivity in other tissues (kidney and intestine) indicating that species specific immunoreactivity problems were not an issue. The colocalization of NKCC1 in more weakly NKA-IR cells in BW, SW fish are shown in Fig 4.8a, g. Ovoid cells in filament epithelium showing only NKCC1 staining were observed in BW-L and SW-CL (Fig 4.8d,j). The apical localization of CFTR was detected in some NKA-IR cells with no apparent salinity and/or ligation dependent differences (Fig 4.8b, e, h, k). The V-ATPase H^+ -pump (VHA) was localized in a similar cytoplasmic staining pattern as NKA; however, in separate cells from NKA-IR cells under all acclimation conditions (Fig. 4.8c, f, i, l). Only once was colocalization VHA with NKA-IR ionocytes observed in BW-L (Fig 4.8f)

4.3.5.2 Intestine

Immunolabeling of NKA in the anterior and posterior intestine of marine catfish acclimated to BW or SW with or without DO ligation revealed intense staining in the basolateral regions of the intestinal epithelium (Fig 4.9, 4.10). However, in posterior intestine of BW-L less staining compared to other groups of fish was observed (Fig 4.10d, e). NKCC2 or NCC immunoreactivity was detected in apical brushborder of the epithelium in the anterior and/or posterior intestine in all salinity experiments. However, basal staining in ligated SW fish posterior intestine of fish was observed (Fig 4.10j). CFTR immunoreactivity was detected apically in isolated spindle shaped columnar cells in epithelium of anterior and posterior intestine in all of salinity experiment (Fig 4.10b, e, h, k). However, in posterior intestine a higher staining pattern in SW ligated fish was also observed (Fig 4.10k).

Apical or subapical localization of V-ATPase in columnar epithelial cells of SW ligated fish was observed in anterior intestine (Fig 4.9). In the posterior intestine, apical expression at SW and BW ligated and SW control fish was observed (Fig 4.10j, d, g) while it was not observed at BW in posterior intestine (Fig 4.10c).

4.3.6 Histology (AB-PAS)

Gill Combination of Alcian Blue and PAS (AB/PAS) staining showed a higher number distribution of blue (acidic) mucus cells in interlamellar regions of the gill filament in BW-L individual compare to other groups (Fig. 4.11).

Anterior intestine apical neutral, acid rich (blue) and purple cells indicating combination neutral and acidic glycoconjugates in enterocytes (Fig. 4.12, 4.13) however no differences in distribution of cells have observed. On the other hand in posterior intestine higher number distribution of blue (acidic) mucus cells in ligated fish of brakish water and saltwater have observed (Fig. 4.13)

Table 4.1. Plasma Na⁺, Cl⁻, K⁺, Ca²⁺, osmolality concentrations, hematocrit, and strong ion ratio (SIR; Na⁺:Cl⁻) and muscle water content (MWC) and muscle Na⁺ and K⁺ concentrations, and muscle Na⁺/K⁺ ratio of *P. lineatus* acclimated to [brackishwater (BW), brackishwater ligated (BW-L) 3‰, seawater (SW-control), seawater ligated (SW-CL) 34‰] salinity). Means within a given parameter across treatment groups which do not share the same letter are significantly different from one another (one-way ANOVA, SNK).

Plasma	BW (3ppt)	BW-L (3ppt)	SW-Control (34ppt)	SW-CL24h (34ppt)	SW-CL48h (34ppt)
Na ⁺ (mmol l ⁻¹)	119.75 ± 16.1 ^a	118.65 ± 10.3 ^a	152.62 ± 14.71 ^b		171.1 ± 20.1 ^c
Cl ⁻ (mmol l ⁻¹)	125.5 ± 11.8 ^a	118.1 ± 7.8 ^a	127.8 ± 10.9 ^a		147.7 ± 9.5 ^b
K ⁺ (mmol l ⁻¹)	5.52 ± 0.82 ^a	3.20 ± 1.10 ^b	5.08 ± 0.97 ^{ac}		4.01 ± 0.90 ^{bc}
Ca ²⁺ (mmol l ⁻¹)	2.60 ± 0.62 ^a	2.30 ± 0.7 ^a	2.96 ± 0.39 ^a		3.50 ± 1.20 ^b

Osmolality	391.2 ±	395.0 ±	374.4 ±	549.3 ±	772.3 ±
(mosm kg⁻¹)	112.8 ^a	67.3 ^a	20.57 ^a	72.1 ^b	165.1 ^c
Haematocrit (%)	15.3 ± 4.2	16.6 ±	23.3 ± 6.3 ^c	17.6 ± 4.7	33.7 ± 6.9 ^d
	^a	7.1 ^a		^{abc}	
SIR	0.96 ±	11.9 ±	1.20 ± 0.16		35.3 ± 7 ^a
	0.14 ^a	3.2 ^b	^b		

Muscle

Water content (%)	86.6 ± 4.5 ^a	89.7 ± 4.7 ^a	87.5 ± 3.7 ^a	87.5 ± 3.3 ^a	79.0 ± 3.2 ^b
Na⁺ (mmol kg⁻¹)	66.9 ± 23.5 ^a	58.2 ± 19.7 ^a	64.2 ± 10.7 ^a	97.0 ± 21.3 ^b	74.4 ± 15.8 ^a
K⁺ (mmol kg⁻¹)	138.5 ± 25.5 ^a	121.2 ± 29.4 ^a	138.6 ± 19.2 ^{ab}	148.0 ± 39.1 ^{ab}	174.8 ± 26.5 ^b
Na⁺/K⁺ ratio	0.48 ± 0.27 ^a	0.48 ± 0.15 ^a	0.46 ± 0.56 ^a	0.76 ± 0.21 ^b	0.43 ± 0.17 ^a

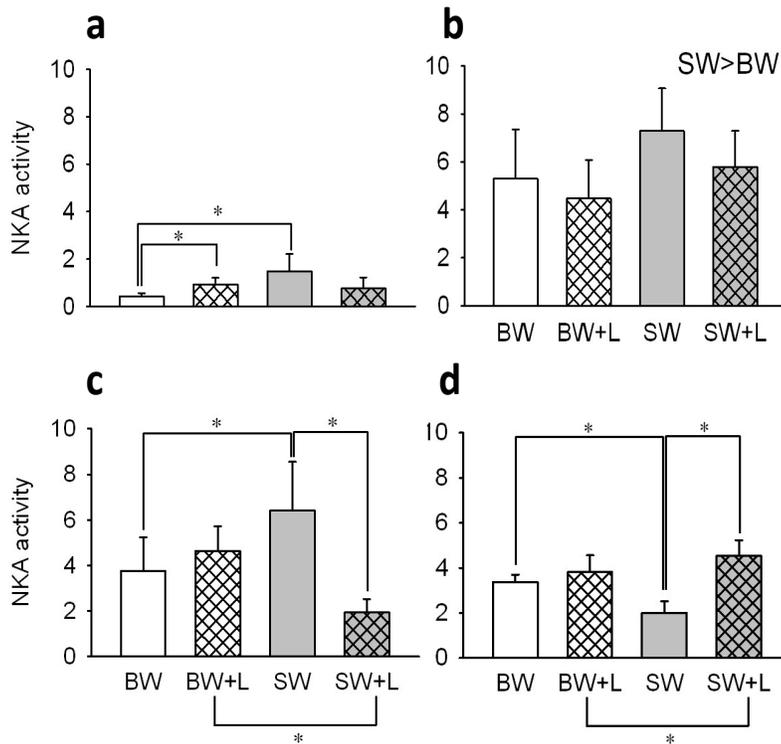


Figure 4.1. Na⁺/K⁺-ATP activity in the gill (a), kidney (b), anterior and posterior intestine (c-d) of marine catfish *P. lineatus* acclimated to [brackishwater (BW), brackishwater ligated (BW-L) 3‰, seawater (SW-control), seawater ligated (SW-CL) 34‰] salinity. Values are means ± S.D. (n=5-6). The asterisks indicate a significant difference between the groups where there was an interaction of salinity and ligation, two-way analysis of variance (ANOVA). Differences between BW and SW, and non-ligation (nL) and ligation (L) are also indicated. (*P* < 0.05; see text for details).

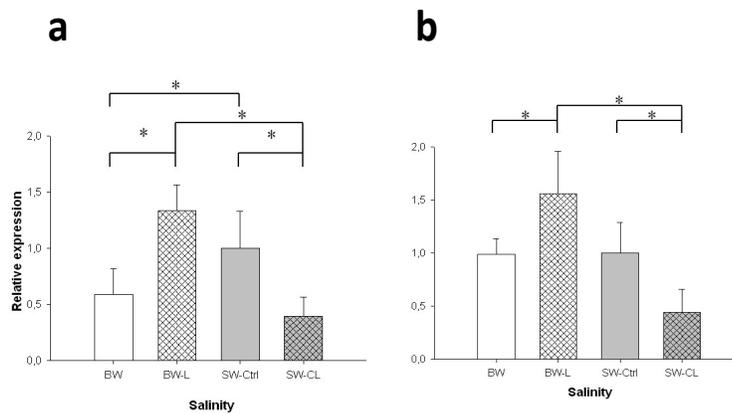


Figure 4.2. Relative expression of NKA α R1 protein in the anterior and posterior intestine (a-b) of marine catfish *P. lineatus* acclimated to [brackishwater (BW), brackishwater ligated (BW-L) 3‰, seawater (SW-control), seawater ligated (SW-CL) 34‰] salinity. Values are presented as means ± S.D of protein abundance (n=5-6). The asterisks indicate a significant difference between the groups where

there was an interaction of salinity and ligation, two-way analysis of variance (ANOVA). ($P < 0.05$; see text for details).

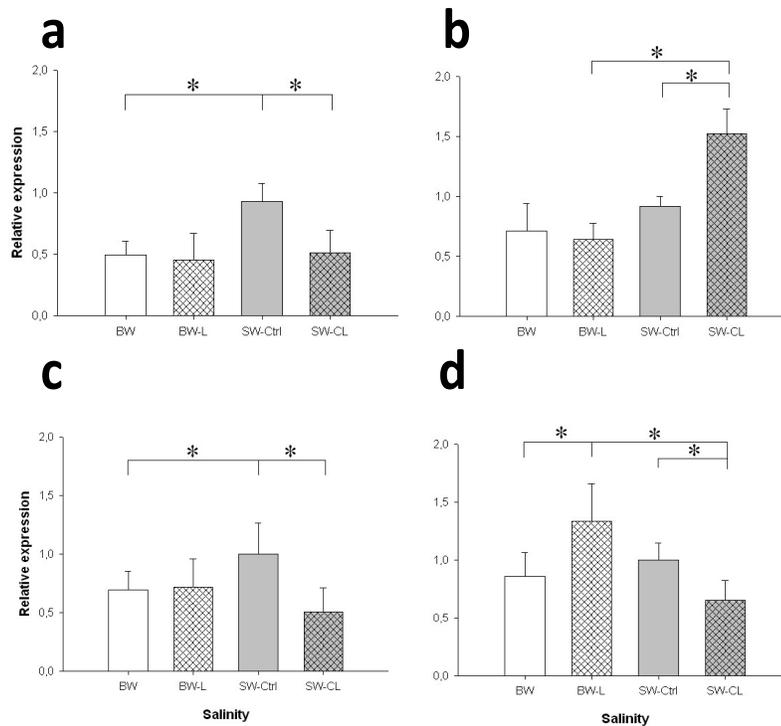


Figure 4.3. Relative expression of cytosolic carbonic anhydrase (CA) protein in the gill (a), kidney (b), anterior and posterior intestine (c-d) of marine catfish *P. lineatus* acclimated to [brackishwater (BW), brackishwater ligated (BW-L) 3‰, seawater (SW-control), seawater ligated (SW-CL) 34‰] salinity. Values are presented as means \pm S.D of protein abundance (n=5-6). The asterisks indicate a significant difference between the groups where there was an interaction of salinity and ligation, two-way analysis of variance (ANOVA). Differences between BW and SW, and non-ligation (nL) and ligation (L) are also indicated. (2-way ANOVA, SNK). ($P < 0.05$; see text for details).

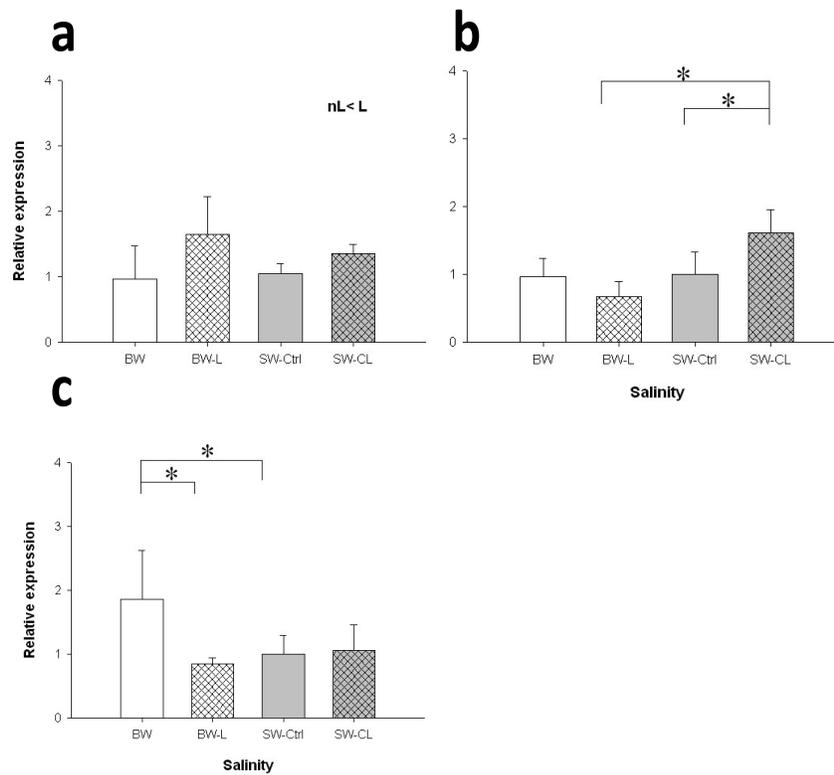


Figure 4.4. Relative western blotting expression of V-ATPase B subunit (B2 antibody) in the gill (**a**), kidney (**b**) and posterior intestine (**c**) of marine catfish *P. lineatus* acclimated to [brackishwater (BW), brackishwater ligated (BW-L) 3‰, seawater (SW-control), seawater ligated (SW-CL) 34‰] salinity. Values are presented as means \pm S.D of protein abundance (n=5-6). The asterisks indicate a significant difference between the groups where there was an interaction of salinity and ligation, two-way analysis of variance (ANOVA). Differences between BW and SW, and non-ligation (nL) and ligation (L) are also indicated. ($P < 0.05$; see text for details).

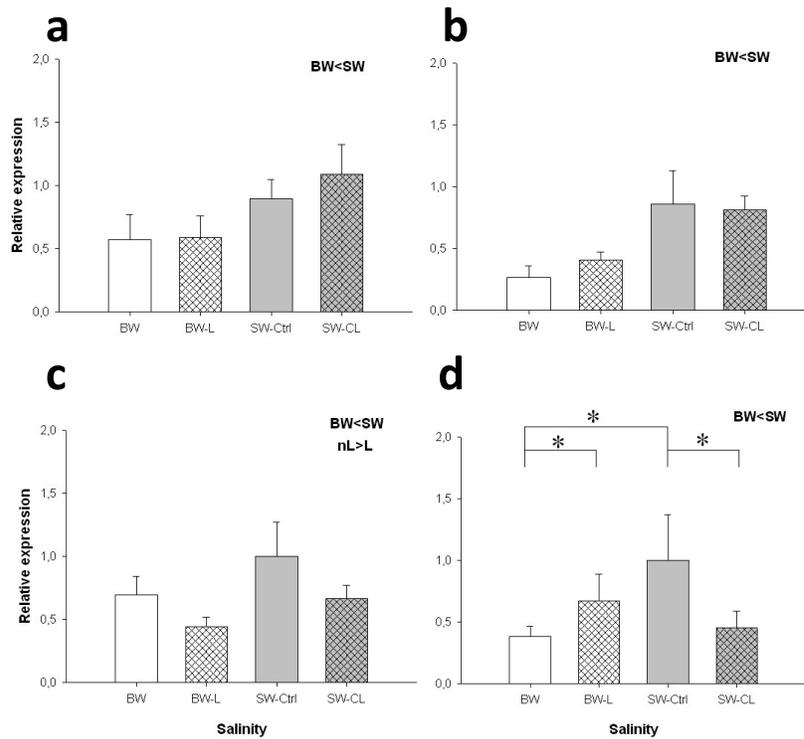


Figure 4.5. Relative expression of heat shock protein 70 (Hsp70) in the gill (a), kidney (b), anterior and posterior intestine (c,d) of marine catfish *P. lineatus* acclimated to [brackishwater (BW), brackishwater ligated (BW-L) 3‰, seawater (SW-control), seawater ligated (SW-CL) 34‰] salinity. Values are presented as means \pm S.D of protein abundance (n=5-6). The asterisks indicate a significant difference between the groups where there was an interaction of salinity and ligation, two-way analysis of variance (ANOVA). Differences between BW and SW, and non-ligation (nL) and ligation (L) are also indicated. ($P < 0.05$; see text for details).

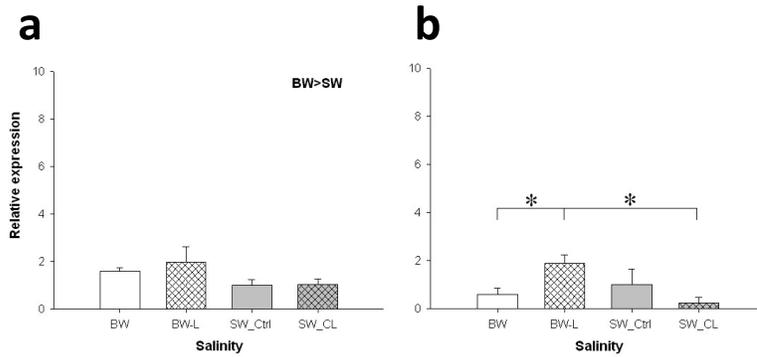


Figure 4.6. Relative mRNA expression of gill *ca17* (a) and *cftr* (b) of marine catfish *P. lineatus* acclimated to [brackishwater (BW), brackishwater ligated (BW-L) 3‰, seawater (SW-control), seawater ligated (SW-CL) 34‰] salinity. Data are means \pm S.D. (n=3-4). The mRNAs expression was normalized to the corresponding *bactin* abundance from the same sample and the expressed relative to the SW-control. The asterisks indicate a significant difference between the groups where there was an interaction of salinity and ligation, two-way analysis of variance (ANOVA). Differences between BW and SW, and non-ligation (nL) and ligation (L) are also indicated. ($P < 0.05$; see text for details).

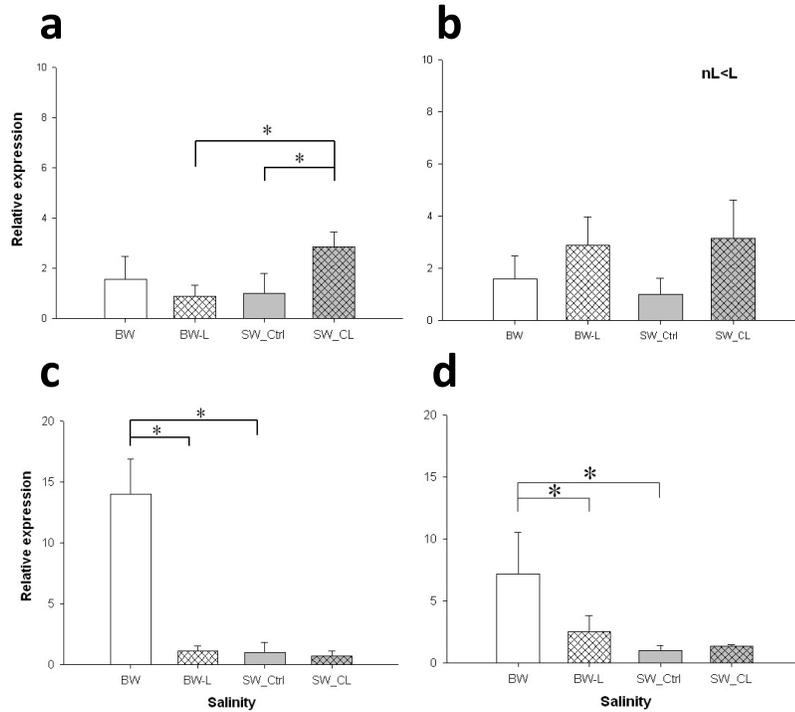


Figure 4.7. Relative mRNA expression of kidney *atp1a1* (a), *ca17* (b), *cftr* (c) and *slc26a6a* (d) of marine catfish *P. lineatus* acclimated to [brackishwater (BW), brackishwater ligated (BW-L) 3‰, seawater (SW-control), seawater ligated (SW-CL) 34‰] salinity. Data are means \pm S.D. (n=3-4). The mRNAs expression was normalized to the corresponding *bactin* abundance from the same sample and the expressed relative to the SW-control. The asterisks indicate a significant difference between the groups where there was an interaction of salinity and ligation, two-way analysis of variance (ANOVA). Differences between BW and SW, and non-ligation (nL) and ligation (L) are also indicated. ($P < 0.05$; see text for details).

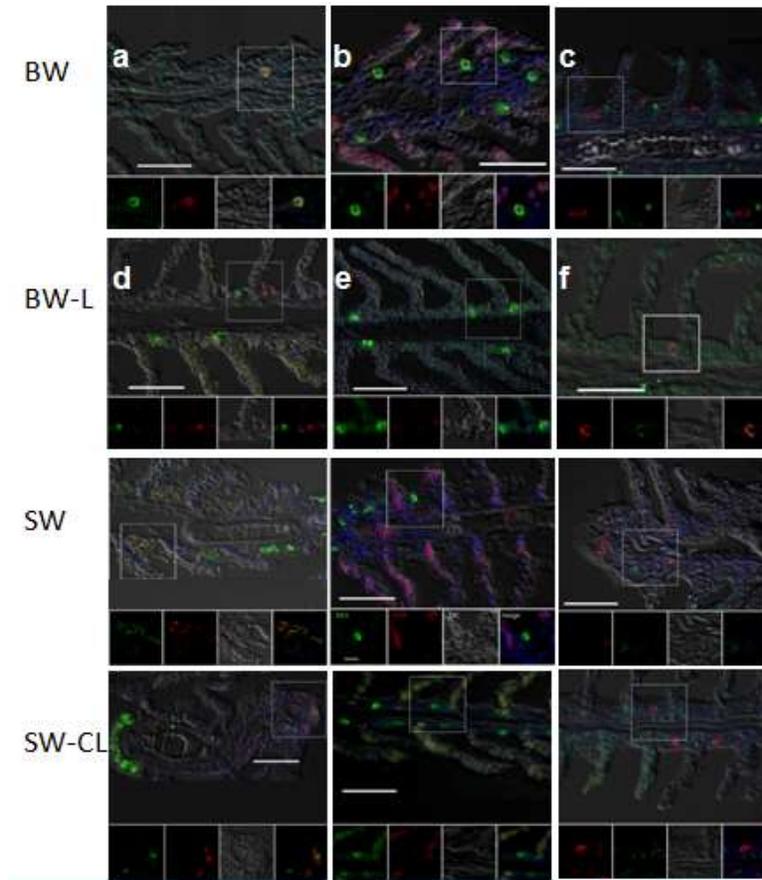


Figure 4.8. Double immunofluorescence localization of Na⁺/K⁺-ATPase (α R1, green **a, b, d, e, g, h, j, k**) with NKCC1 (T4, red **a, d, g, j**) and CFTR (red, **b, e, h, k**) or Na⁺/K⁺-ATPase (α 5, red **c, f, i, l**) with V-ATPase (B2, green **c, f, i**). Sections were counter stained with DAPI nuclear staining (blue) and overlaid with the differential interference contrast (DIC) images in the gills of marine catfish *P. lineatus* acclimated in brackish water (BW) 3‰ (**a-c**), brackish water ligated (BW-L) 3‰ (**d-f**), seawater (SW-control) 34‰ (**g-i**) and seawater (SW-control) ligated (SW-CL) 34‰ (**j-l**). Scale bar 100 μ m in upper panel. See text for details.

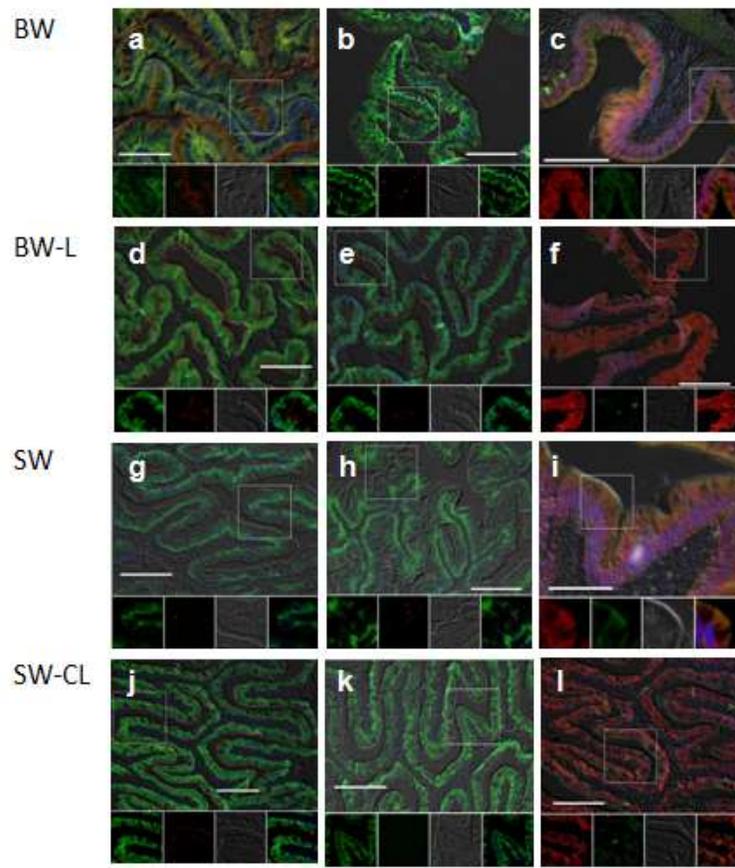


Figure 4.9. Double immunofluorescence localization of Na⁺/K⁺-ATPase (αR1, green **a, b, d, e, g, h, j, k**) with NKCC1 (T4, red **a, d, g, j**) and CFTR (red, **b, e, h, k**) or Na⁺/K⁺-ATPase (α5, red **c, f, i, l**) with V-ATPase (B2, green **c, f, i**). Sections were counter stained with DAPI nuclear staining (blue) and overlaid with the differential interference contrast (DIC) images in the anterior intestine of marine catfish *P. lineatus* acclimated in brackish water (BW) 3‰ (**a-c**), brackish water ligated (BW-L) 3‰ (**d-f**), seawater (SW-control) 34‰ (**g-i**) and seawater (SW-control) ligated (SW-CL) 34‰ (**j-l**). Scale bar 100 μm in upper panel. See text for details.

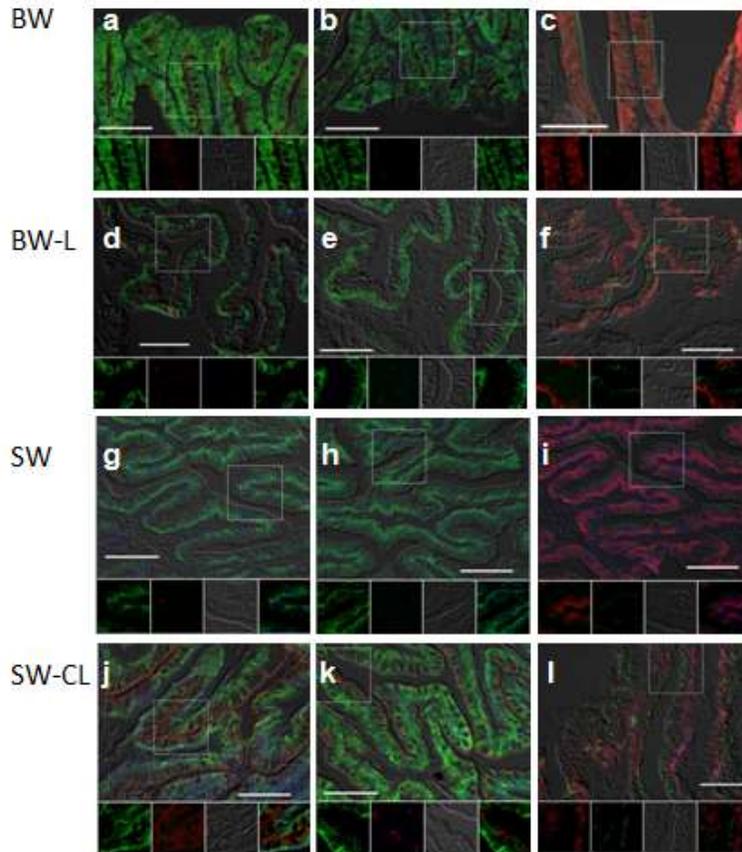


Figure 4.10. Double immunofluorescence localization of Na⁺/K⁺-ATPase (αR1, green **a, b, d, e, g, h, j, k**) with NKCC1 (T4, red **a, d, g, j**) and CFTR (red, **b, e, h, k**) or Na⁺/K⁺-ATPase (α5, red **c, f, i, l**) with V-ATPase (B2, green **c, f, i**). Sections were counter stained with DAPI nuclear staining (blue) and overlaid with the differential interference contrast (DIC) images in the posterior intestine of marine catfish *P. lineatus* acclimated in brackish water (BW) 3‰ (**a-c**), brackish water ligated (BW-L) 3‰ (**d-f**), seawater (SW-control) 34‰ (**g-i**) and seawater (SW-control) ligated (SW-CL) 34‰ (**j-l**). Scale bar 100 μm in upper panel. See text for details.

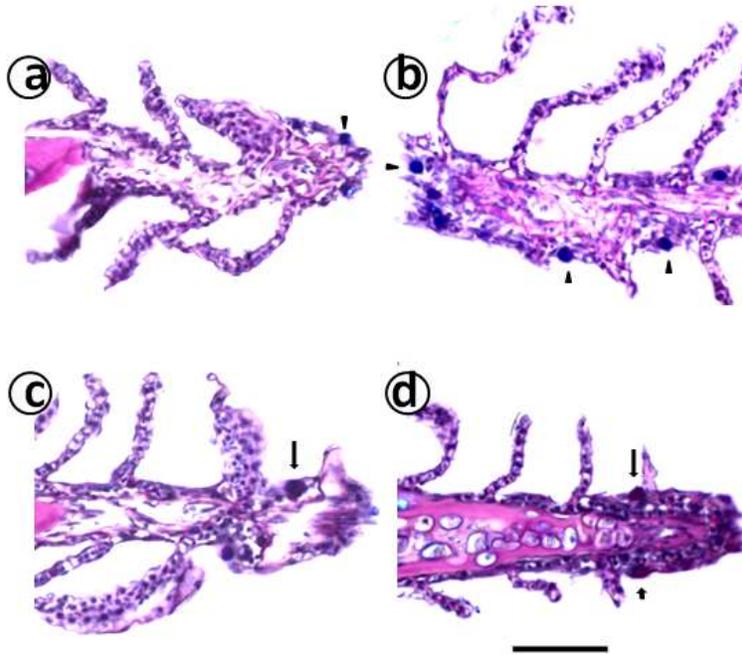


Figure 4.11. Periodic Acid Schiff (PAS)/Alcian blue (pH 2.5) AB/PAS staining of marine catfish *P. lineatus* gill acclimated in (a) brackish water (**BW**) 3‰, brackish water ligated (**BW-L**) 3‰ (b), seawater (**SW** 34‰) (c) and seawater ligated (**SW_CL** 34‰). The neutral (magenta, short arrow), acid rich (blue, arrowhead) and purple cells (long arrow) are indicating combination neutral and acidic glycoconjugates. Scale bar 100 μ m. See text for details.

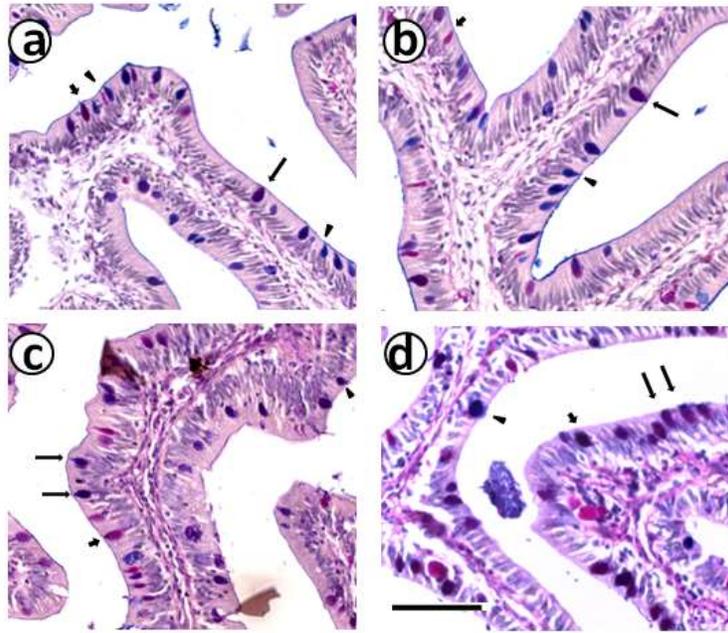


Figure 4.12. Periodic Acid Schiff (PAS)/Alcian blue (pH 2.5) AB/PAS staining of marine catfish *P. lineatus* anterior intestine acclimated in (a) brackish water (**BW**) 3‰, brackish water ligated (**BW-L**) 3‰ (b), seawater (**SW** 34‰) (c) and seawater ligated (**SW_CL** 34‰) (d). The neutral (magenta, short arrow), acid rich (blue, arrowhead) and purple cells (long arrow) are indicating combination neutral and acidic glycoconjugates. Scale bar 100 μ m. See text for details.

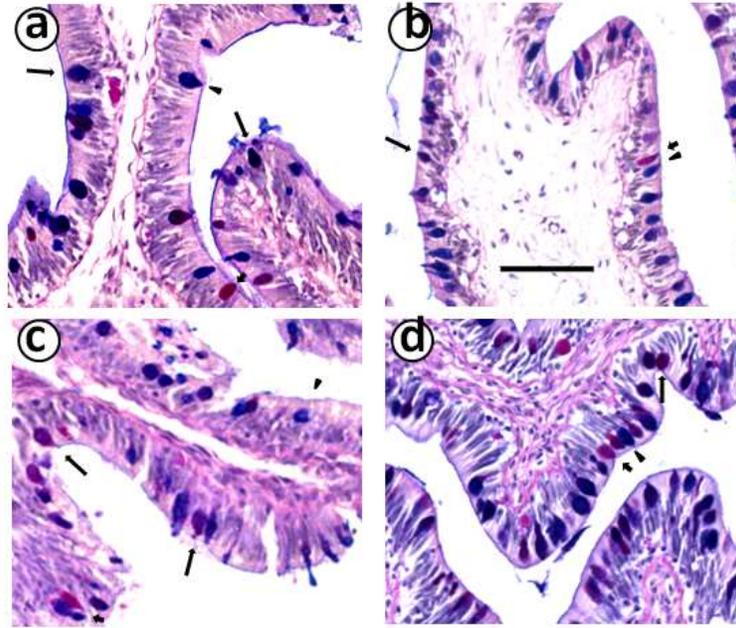


Figure 4.13. Periodic Acid Schiff (PAS)/Alcian blue (pH 2.5) AB/PAS staining of marine catfish *P. lineatus* posterior intestine acclimated in (a) brackish water (**BW**) 3‰, brackish water ligated (**BW-L**) 3‰ (b), seawater (**SW** 34‰) (c) and seawater ligated (**SW_CL** 35‰). The neutral (magenta, short arrow), acid rich (blue, arrowhead) and purple cells (long arrow) are indicating combination neutral and acidic glycoconjugates. Scale bar 100 μ m. See text for details.

4.4. Discussion

4.4.1 Ions and osmolality and acid-base status

Plasma osmolalities of euryhaline species from marine and freshwater origin varied between 160-410 and 235-414 mOsm/kg H₂O, respectively (Freire and Prodocimo, 2007). The observed plasma ions concentrations were in the range of other teleost fish species (see review Whittamore et al., 2012, Table 1). In SW-CL, increase of plasma ions concentration except K⁺, osmolality and hematocrit but not Na⁺/Cl⁻ ratio (SIR) and decreased muscle water content (MWC) was observed. The value of Na⁺ was lower compare to *C. macrocephalus* while osmolality was higher after 48h ligation (Kowarsky, 1973). The latter might be contributed to different conditions of the experiments, or temperature (26-28°C) from Kowarsky (1973, 20°C). Furthermore, increased plasma Ca²⁺ concentration in SW-CL fish may represent the role of DO in Ca²⁺ regulation however needs more molecular evidences.

In our work, SW-CL ligation cause decreased survival, which was similar to Kowarsky (1973). The ligation or excision of rectal gland, for prolonged periods (1 month), in *Squalus acanthias* showed no significant effect on plasma electrolytes in compare to the normal conditions in SW (Burger, 1965; Evans et al., 1982; Evans, 1993; Wilson et al., 2002) or dilute sea water (Burger, 1965). However, regression of the rectal gland tissue in FW adapted sharks compare to SW adapted has been reported by Oguri (1964). Similarity of DO in *P. lineatus* and rectal gland of elasmobranchs has been proposed (Van Lennep and Lanzing, 1967; Van Lennep, 1968; Pucke and Umminger, 1979) however, observation of such instability of SW-CL fish in current study and Kowarsky (1973) may suggest the possibility of existence other approaches in maintenance of ionic-osmotic balance.

4.4.2 Hematocrit, muscle water content and ions

Decreased hematocrit in SW-CL 24h L accompany increased osmolality may lead to obliged water movement then reduction blood hematocrit accordingly. However, considering increased hematocrit at SW-CL 48h L might

be contributed to the stress due to losing of DO and activity to maintenance of ion regulatory status.

Effect of the ligation on MWC may express possible role of DO in water transport directly and/or indirectly. The absence of zonulae occludentes in DO (Van Lennep, 1968) may lead to propose potentially free passage for water or ions from intercellular space to lumen and vice versa. Furthermore, ligation may affect on drinking rate of fish to reduce loaded salt inside the body to avoid of more stresses regarding to salt excretion thereby changing of MWC.

Observation of increased muscle Na^+ and Na^+/K^+ ratio at 24h L followed by a reduction in 48h L where K^+ was however, higher in SW-CL may due to the primary stress of ligation and a compensatory response of fish muscle. The buffer acting of muscle even for short a time has been reported for salt loading of rectal gland ectomised lip shark *Hemiscyllium plagiosum* via showing a significant accumulation of Na^+ (Chan et al., 1967). A slightly higher but not significant muscle Na^+ than sham operated glandless *S. acanthias* has been reported by Wilson et al (2002). Totally, increased muscle ions in SW-CL might be an adaptation strategy in dealing with salt loading in the absence of DO as main salt excretion tissue. However, it seems the latter was not working sufficiently to compensate DO ligation since gradually death of the fish has been observed.

Plotosus lineatus challenged with BW-L,

In BW-L, observation decreased plasma K^+ concentration may be represent role of DO on plasma K^+ regulation, occurring a reduced uptake, rather than increased loss of K^+ presumably via gill (Partridge and Lymbery, 2008; Malakpour Kolbadinezhad et al., 2012). In *C. macrocephalus* (Kowarsky, 1973) BW ligated fish showed a depression in plasma Na^+ levels and no significant change on osmolality however Na^+ was higher compare to the present study while osmolality was lower which may be because of different temperature and/or experimental conditions.

Taken together, it seems due to DO ligation, observed elevated amount of plasma ions and osmolality or hematocrit and in contrary declined MWC thus water lose by osmosis, a systemic dehydration in SW-CL fish representing may

be problematic resulting in a stress situation thus osmotic disturbance thereby indicating to main role of DO in salt excretion.

4.4.3 Metabolic acidosis and alkalosis by ions regulation

Changes in Na^+/Cl^- ratio (SIR) of blood plasma has been recommended for indicating acid-base imbalances (Jensen et al., 1998; Sinha et al., 2015). Since the direct measurements of plasma acid-base balance were not done in current study due to the small size of the fish however calculated, SIR might revealed changes in the plasma level of weak anions (e.g. HCO_3^-) and thus acid-base balance. Observation stability of plasma Cl^- concentration in BW and/or BW-L of fish while SIR was lower compare to other groups express a metabolic acidosis, better regulation of the Cl^- compare to Na^+ in ligated fish thus indirectly represents DO function as Na^+ excretion.

The occurring of “metabolic alkalosis” because of NaCl uptake by gastrointestinal track, after feeding (postprandial period), result in elevated plasma Na^+ (Claiborne, 1997) and thereby elevated intestinal HCO_3^- secretion rates (Taylor and Grosell, 2006) has been suggested in some fish species such as elasmobranch (Wood et al., 2005) and FW trout (Bucking and Wood, 2008; Cooper and Wilson, 2008). Accordingly, detecting increased plasma Na^+ concentration (comparable with Cl^-) and SIR of SW-CL fish compare to BW and BW-L may suggest a metabolic alkalosis. On the other hands, observed declined protein expression of Rhag and/or Rhbg in gill of SW-CL fish may express lower amount of accumulated ammonia inside body presumably blood plasma or less excretion ammonia rate (J_{Amm}) from body to the outside.

4.4.5 Evidence for role of gills in salt secretion?

As previously confirmed, DO but not gill is main organ of ion regulation in marine catfish similar the correlation of gill and rectal gland of elasmobranchs (Piermarini and Evans, 2000; Pillans et al., 2005; Malakpour Kolbadinezhad et al in press). The observation of elevated NKA at BW-L compare to BW fish may express ion regulation without changing in ions concentration such as Na^+ and Cl^- or acid-base regulatory function because of the observation of similar trend in V-ATPase protein expression. Observation of the discrepancies between α

subunit protein, *atp1a1* expression and NKA activity of gills might be related to post translational and/or post-transcriptional modification. It seems ligation has no effect on heterogeneously distribution of detected a few NKA-IR cells in gills filament epithelia. Removing of rectal gland showed no significant change in branchial NKA activity, ionocytes number or ultrastructure in spiny dogfish (Wilson et al., 2002) while change of branchial ionocytes of striped dogfish *Paroderma africanum*, has been reported by Haywood (1975). The lamella role to gas exchange has predictable due to the localization of NKA-IR cells only in filament epithelia (Evans et al., 2005).

The basolateral localization of NKCC1 in gills ionocytes is being used as secondary Cl⁻ secreting (see review Hiroi and McCormick, 2007, 2012). Finding very rare of NKA-IR cells that co-expressed NKCC1, although apical CFTR staining was observed in NKA-IR cells may lead to salt secreting function even somehow as the compensatory mechanisms in the absent of the DO. Secondary function of elasmobranchs gill in osmoregulation (Burger and Hess, 1960; Wilson et al., 2002) due to possessing of rectal gland and similarity between gills of *P. lineatus* and elasmobranchs has confirmed (Doyle and Gorecki, 1961; Pucke and Umminger, 1979, our previous molecular study see Chapter 3).

In SW-CL, it seems a negative correlation between mRNA expression of *cfr* and elevated plasma Cl⁻. In spiny dogfish observation negatively correlated of gill ionocytes numbers with plasma Cl⁻ levels, suggested a role in regulating plasma levels as Cl⁻ elimination (Wilson et al., 2002). Bentley et al (1976) have suggested role of dogfish gill ionocytes in the active Cl⁻ transport. On the other hand, in BW-L observed higher mRNA expression of *cfr* as seen in anterior intestine might lead to more Cl⁻ secretion through gill accompanied the elevated NKA activity however, since plasma Cl⁻ level showed more or less stability in BW-L it seems another regulatory mechanism also could affect on regulation of plasma Cl⁻ level. Considering proposed potential role of DO in Cl⁻ regulation (see more details in Chapter 3) thus gill may reveal a compensatory role, even partly, in Cl⁻ regulation. Moreover, observation of anion exchanger *s/c26a6a* mRNA expression however not effected by ligation, a potential correlation of expressing apical CFTR regarding to facilitated Cl⁻/HCO₃⁻ exchange or HCO₃⁻ secretion (Grosell et al.,

2009b), may be contributed to acid-base regulation in proposed metabolic acidosis and/or ion regulation. However, it needs more evidences.

V-ATPase

Increase trend of V-ATPase protein expression in both BW-L and SW-CL might be a regulatory activity to compensate of ligation. IHC results in SW-CL may indicate V-ATPase is present in only a subpopulation of ionocytes on filament and lack of consistent colocalization with NKA-IR cells which has reported in different species (Catches et al., 2006; Uchiyama et al., 2012; Cramp et al., 2015) and not involving of gills V-ATPase in acid excretion. However, observed colocalization V-ATPase in rare NKA-IR/MR cells in BW-L, which represented increased NKA activity, was similar to the result of Katoh et al., 2003 in *F. heterclitus* where showed higher NKA activities in response to hyposmotic medium. Furthermore, detecting partial sequence of *slc26a6a* may imply the possibility of bicarbonate excretion in gills presenting one of MRCs subpopulation serving as has reported in elasmobranchs (Piermarini et al. 2001; Cramp et al., 2015).

CA

The reversible dehydration or hydration reactions of CO₂ as a crucial catalyzing role of carbonic anhydrase (CA) (see review Marshall and Grosell, 2006; Gilmour and Perry, 2009) and obvious role in ion regulation (Evans et al., 2005; Tresguerres et al., 2006a) has been discussed. Decreased protein expression of CA in SW-CL fish and rather less NKA may serve as a conservation mechanism regarding to the saving of the energy in ligation period. On the other hands, increased mRNA expression of *ca17* in BW-L accompanied elevated NKA activity and higher mRNA expression of *cfr* may be contributed to Cl⁻ secretion or CO₂ excretion through the gill regarding to proposed metabolic acidosis thus acid-base regulatory process. Observed colocalization of CA and NKA-IR cells showed no alter effect of ligation and/or salinity.

Furthermore, Alcian Blue and PAS (AB/PAS) staining showed a higher distribution of acidic mucus cells in interlamellar regions of the gill filament in BW-L fish which may be attributed to compensate the proposed metabolic acidosis of marine catfish at BW-L. Generally, since the potential role of the DO in acid base

regulation has been suggested in our previous study, thus in the absence of DO, it seem gills and other tissues try to compensate the ligation.

4.4.6 Role of the intestine:

The observation higher NKA activity in anterior intestine compare to posterior in SW-control and reversely posterior intestine in SW-CL might be attributed to more functional role of former and latter in steady state and ligated conditions, respectively. Furthermore, lower NKA activity in anterior intestine and decreased protein expression of NKA α subunit, CA in intestine of SW-CL generally may be contributed to less energy consuming in ligation period as adaptation mechanisms.

In SW-CL, observed apical localization of NKCC, V-ATPase on entrocyte cells of posterior intestine compare to SW-control fish may be attributed to Na^+ uptake involving of NHE3 as metabolon and facilitating either drinking rate or coupled water absorption (for more details see review Grosell 2011). Moreover, basolateral localization of NKCC accompanied elevated NKA activity, and more distribution of CFTR compare to SW may represent salt secreting beside of absorbing thus might be result of increasing luminal fluid concentration before reach to kidney. Since, decrease concentration of different ions in lumen fluid from anterior to posterior intestine has been reported in different fish species (Marshall and Grosell, 2006), may be posterior intestine in SW-CL, fish may has role in ion regulation while anterior intestine which showed more expression of V-ATPase via IHC has acid-base regulation. Moreover, AB/PAS staining showed more distribution of acidic mucus cells in posterior intestine of SW-CL fish, which may express compensatory response to absence of the DO.

In BW-L, generally increased of NKA α subunit protein expression has observed in intestine. However, since posterior intestine showed higher protein expression of CA and Hsp70 compare to anterior intestine thus it seems has more active role in BW-L. Furthermore, observations of V-ATPase protein expression only in posterior intestine, which decreased in BW-L, may be contributed to acid-base regulation represent or indirectly represent another source for H^+ presumably NHE.

IHC results of BW-L showed less NKA α subunit expression compares to BW in posterior intestine while NKA activity and/or mRNA expression showed no

significant change thus might express presence of another subunit of NKA or less energy consuming duration the ligation. Observation of apical and/or basolateral localization of NKCC in anterior intestine in BW while BW-L fish showed only apical expression may express an adaptation mechanism in ligation period to salt absorption. Furthermore, apical expression of V-ATPase in BW-L while it was not observed in BW may express acid secretion as compensatory response to metabolic acidosis.

However, observation higher mRNA expression of *cfr* in anterior intestine of BW-L fish may suggest more important role in Cl^- (and fluid) secretion which may increase mucosal Cl^- concentration (Grosell and Taylor, 2007; Taylor et al., 2010) and presumably effect of reducing HCO_3^- secretion rates. The similar trend has also observed in gill in BW-L. Thus, anterior intestine may reveal a compensatory role, even partly, in Cl^- regulation as seen in the gill. Moreover, *slc26a6a* mRNA expression showed no difference in various groups which might work to regulation of generated HCO_3^- by CAs. Regarding the observed dependency of HCO_3^- secretion to exist of CFTR (Garcia et al., 2009) and potential correlation of CFTR regarding to $\text{Cl}^-/\text{HCO}_3^-$ (Grosell et al., 2009b) more research works would be interested in future.

Furthermore, AB/PAS staining showed more distribution of blue acidic mucus cells in posterior intestine of BW-L (similar to SW-CL individuals), which may express the compensatory response to the absence of DO. Taken together it seems in intestine of marine catfish has important role in ligated fish in either BW-L or SW-CL.

Taken together, In BW-L, it seems more distribution of blue acidic mucus cells in interlamellar regions of gills filament, CT of kidney and posterior intestine would be consider as compensatory response to metabolic acidosis.

Heat shock protein (Hsp 70)

Involving of stress proteins named as Heat shock proteins (Hsps) in vital cellular processes has been reported (Morimoto and Santoro, 1998; Iwama et al., 1998, 2006; Basu et al., 2002; Deane and Woo, 2011). In SW-CL, observation less protein expression of Hsp70 in kidney, anterior and posterior intestine may express different threshold of salt tolerance. The observation inverse interaction of SW-control and BW with the ligation groups in posterior intestine may reveal functional role in ligated fish.

Marine Catfish adaptation in SW-CL

It would be logic if fish do the energy conservation in stress conditions. Thus, decreased trend of NKA activity in examined tissues except posterior intestine, decreased protein expression of NKA α subunit particularly in intestine may lead to using less energy thereby production of the ATP and glucose, total O₂ consuming (metabolic rate) thus production of CO₂ thereby required CA which all together may result in energy saving. Since, fasted period was around 14 days in present study, possibly of affecting on energy stores of body such as liver glycogen and/or lipid might be predictable. Stress caused by DO ligation, losing the main organ of ion regulation, and potentially less production of ATP and/or glucose may have synergetic effect thus result in energy store depletion. The latter may cause an anaerobic metabolism as energy fueling in osmotic adaptation (for more details see review Tseng and Hwang, 2008).

Furthermore, observation of inverse interaction of salinity and ligation in protein expression of CA in gill and/or intestine compare to kidney, observed compatibility of NKA activity and protein expression of CA in gill and anterior intestine while posterior intestine and rather kidney showed incompatibility might reveal various roles of tissues in ligation period. It seems kidney of ligated fish may have a role in acid-base regulation due to express increased protein of CA and V-ATPase. On the other hand, observed higher NKA activity in posterior intestine might be related to have more functional role in water absorption. However, since the survival of fish was affected by ligation thus quality of mentioned changes in various organs required more evidences to evaluate. Taken together it seems the compensatory responses of gills, kidney and intestine in SW-CL could work for short period however, they were not sufficient for keeping survival of fish while it was inverse in BW-L.

Chapter 5:

Molecular characterization of the unique kidney of the marine catfish *Plotosus lineatus*

Malakpour Kolbadinezhad, S., Coimbra J., Wilson, J. M. 2017. Molecular characterization of the unique kidney of the marine catfish *Plotosus lineatus* (*in preparation*).

Abstract

The kidneys of Plotosidae marine catfishes are unusual in their ability to produce hyperosmotic urine compared to other marine teleost fishes. In the present study, the effects of different salinities including brackishwater (BW) 3‰, seawater (SW-control) 34‰ and hypersaline water (HSW) 60‰ on *Plotosus lineatus* kidney morphology and distribution of key ion transporters using immunohistochemistry was investigated. Immunohistochemical (IHC) localization of NKA α -subunit shows relatively weak basolateral staining in the proximal tubule I (PTI), distal tubule (DT) and DT ampulla (DTa), and stronger staining in PTII. The collecting tubule (CT) and collecting duct (CD) have very large ovoid cells with colocalization of NKA α -subunit and $\text{Na}^+:\text{K}^+:2\text{Cl}^-$ cotransporter (NKCC1) throughout the cytoplasm. These cells are also strongly eosinophilic. This NKA-NKCC1 staining pattern of renal “chloride cells” indicates a secretory cell type and are attributed to production of unusual hyperosmotic urine. These cells are present at all acclimation salinities. Goblet cells with acidic and neutral mucins are present in the CT/CD and HSW results in the disappearance of neutral mucins. The absorptive NKCC2/NCC were localized apically to cells in both PTI and PTII indicating and absorptive function. Staining was reduced in HSW fish. CFTR was localized apical to PT segments and basolaterally in CT/CD. Carbonic anhydrase was found to weakly staining in all nephron regions while V-type H^+ -ATPase was localized to PTI subapically and PAT1 (Slc26a6) to non-“chloride cells” in the CT. These cells presumably function in acid-base and SO_4^{2-} regulation. Taken together, the kidney of marine catfish has the molecular mechanisms in place for an effective role in ion/osmo regulation particularly in HSW environment.

5.1. Introduction

The primary role of the kidney in marine teleost fishes is the excretion of divalent ions (.eg., Mg^{2+} and SO_4^{2-}) in the face of a continual excess of divalent ions permeating across body surfaces (Hickman, 1968). Furthermore, water conservation is a conflicting demand in this dehydrating environment and thus the kidneys produce small volume of of generally isotonic urine containing excess divalent ions (Marshall and Grosell, 2006; Evans, 2008). Teleost kidney, generally cannot produce hyperosmotic urine because of the lack of zonation and the loop of Henle as seen in mammalian kidney or countercurrent tubules flow as observed in elasmobranchs and/or lamprey (McDonald, 2007; Evans, 2008). Although there have been a few reports of hyperosmotic urine formation in marine teleosts (Fleming and Stanley, 1965; Hickman, 1968; Stanley and Fleming, 1964; Kowarsky, 1973; McDonald and Grosell, 2006). Significantly amongst these examples only the marine catfish *Cnidoglanis macrocephalus* has been shown to produce hyperosmotic urine under steady state conditions (Kowarsky, 1973) which make it an intrigue subject for study.

The Plotosidae kidney belongs to the type II according anatomical classes defined by Ogawa (Ogawa, 1961a; Hickman and Trump, 1969). The nephron segments of marine catfish kidney are similar to FW teleosts including the glomerulus (G), neck (N), proximal tubule I (PTI), proximal tubule II (PTII), distal tubule (DT) and collecting tubule/duct (CT/CD) (Ogawa, 1959; Hentschel and Elger, 1987). The distal tubule of the marine catfish kidney is also unusual in having an ampullar region, enlarged of the tubule just proximal to the start of the CT (Fig 5.1). The distal tubule wraps around the ampullar region. The collecting duct is also populated with large cells similar in morphology to branchial mitochondria-rich cells with a tubular system. These renal 'chloride cells' are found in other catfish species as well as lamprey (Hentschel and Elger 1987). Although the morphology of the marine catfish kidney has been characterized, the molecular information of their kidney is unknown which would be necessary to explain their unusual ability to produce blood-hyperosmotic urine. To this end, histological and immunohistology approaches were taken to address ion transporter distributions in the kidney of marine catfish acclimated to different

salinities. DO ligation was used to help assess the compensatory response of the kidney.

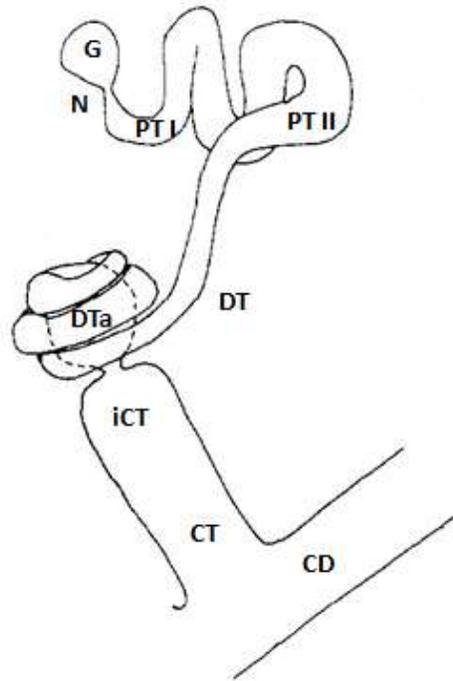


Figure 5.1. Illustration of the *Potosus lineatus* kidney nephron modified from Hentschel and Elger (1987). Glomerulus (G), neck (N), proximal tubule I and II (PTI, PTII), distal tubule (DT), distal tubule ampulla (DTa) and initial collecting tubule (iCT) and collecting tubule (CT) and duct (CD).

5.2 Materials and Methods

5.2.1 Animals

Adult *Potosus lineatus* were acclimated to three different salinities [brackishwater (BW) 3‰, seawater (SW-control) 34‰, hypersaline water (HSW) 60‰] for two weeks. Additional sets of *P. lineatus* acclimated to either BW or SW had their dendritic organs ligated or sham operated and sampled after 48h. Animals were sampled as described in Chapter 2.3.

5.2.2 Histology

Kidneys were excised, and immersion fixed in neutral buffered formalin, dehydrated through an ethanol series, and embedded in paraffin (see Chapter 2.4 for additional details). A Reichert Biocut 2030 microtome was used for cutting sections (5 μ m). The sections were stained with H&E, AB-PAS staining protocols. Micrographs were taken with a Leica DFC300FX digital colour camera mounted on a Leica DM 6000 B microscope. Images while maintaining the integrity of the data were imported into Photoshop CS3 to resize and adjust brightness and contrast.

5.2.3 Immunohistochemistry

The paraffin serial sections were cut and collected onto APS (3-aminopropyltriethoxysilane; Sigma) coated slides (Reis-Santos et al., 2008), completely dried, dewaxed then rehydrated. Antigen retrieval using 0.05% citraconic anhydride and 1% sodium dodecyl sulfate (SDS)/PBS was performed on sections. Sections were then blocked with 5% normal goat serum (NGS) and incubated with α -subunits of NKA (α R1, α 5), NKCC1 (T4), Carbonic anhydrase (CA) and V-ATPase (B2) primary and secondary goat anti-mouse and/or anti-rabbit Alexa fluorophore conjugated antibodies. DAPI was used for nuclei staining. Sections were viewed on a Leica DM6000 B with a digital camera (DFC340FX, Leica Microsystems, Wetzlar, Germany). See Chapter 2.8 for additional details.

5.3 Results

5.3.1 Histology

The kidney of *P. lineatus* does not show zonation. Using the Hematoxylin and Eosin (H&E) staining of marine catfish *P. lineatus* kidney acclimated in seawater (SW 34‰) indicated eosinophilic staining in proximal tubule I (PTI), PTII and collecting tubule/collecting duct (CT/CD) except in distal tubule (DT). In CT/CD large ovoid strongly eosinophilic staining cells were observed (Fig. 5.2). Periodic Acid Schiff (PAS) - Alcian blue (pH 2.5) (AB/PAS) double staining showed strong neck (N) apical acidic mucin staining. Apical PAS staining indicative of brush border was more obvious in PTI than PTII whereas in DT and DT ampullae very weak PAS staining and no AB staining were observed. In the CT/CD strong PAS staining (magenta) glycoconjugates (GCs), Alcian blue acid rich (blue), and purple goblet cells were observed. The latter indicates the presence of combination neutral and acidic mucins (Fig. 5.3). Only HSW acclimation elucidated change in goblet cell staining with the absence of neutral mucin staining (only acidic goblet cells remained). See more details in Appendix 2 Table 2.

The effects of DO ligation on kidney histology reveal a larger nephric space of Bowman's capsule around the glomerular capillaries in BW-L individuals compare to the other treatments observed (Fig. 5.4). With ligation in SW, a smaller lumen size of in ampulla-like end portion of DT (DTa) was observed (Fig. 5.5) compared to sham groups while we observed a larger lumen size with BW-L. The combination of Alcian Blue and PAS (AB/PAS) staining showed a greater distribution of acidic mucin goblet cells in CT/CD with BW-L individuals (Fig. 5.4) while SW-CL showed a reduced distribution of both neutral (magenta) and acidic mucin goblet cell types (Fig. 5.5).

5.2.2 Immunohistochemistry

PTI showed apical NKCC2/NCC localization in BW and SW-control but not HSW (Fig. 5.6, 5.11). Apical and subapical localization of V-ATPase and the cytosolic localization of CA accompanied the basolateral expression of NKA in PTI in BW, and SW-control (Fig. 5.6, 5.9). Apical CFTR staining was observed in some cells in PTI as well as PTII (Fig 5.8). BW acclimation was associated with the absence of this staining (Fig. 5.12). In HSW the localization of CA was similar

to other salinities while basolateral localization of V-ATPase has been observed HSW fish (Fig. 5.11). The PTII showed stronger basolateral expression of NKA than PTI, but weaker apical NKCC2/NCC staining and cytosolic CA staining.

The DT and DTa of BW, SW-control or HSW showed weak basolateral immunoreactivity with NKA but without co-localization with any of the other ion transporters of interest. (Fig. 5.12).

In the CT, robust NKCC1 staining was present throughout the cytoplasm of tubular cells colocalizing with NKA (Fig. 5.6). This staining pattern is consistent with the presence of a basolateral tubular system in these cells. There was also a weak basolateral localization of CFTR, and PAT1 observed in CT/CD in SW-control (Fig. 5.7, 5.10). However, the PAT1 cells were distinct from the NKCC1-NKA IR cells. BW and HSW acclimation did not alter CT/CD staining patterns.

Generally, the IHC staining in ligated BW and SW acclimated fish kidney was more or less similar to other group of fish (BW and SW sham groups). See more details in Chapter 3. Only CFTR in PTI of BW and BW ligated acclimated has not been observed (Fig. 5.13b, e, h, k).

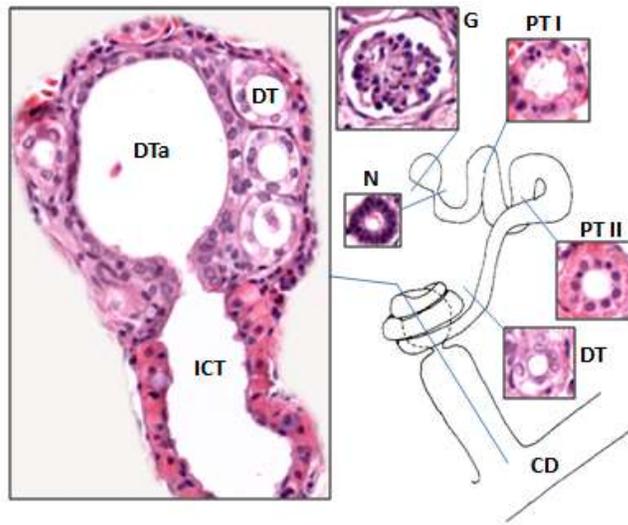


Figure 5.2. Hematoxylin and Eosin (H&E) staining of marine catfish *P. lineatus* kidney acclimated in seawater (**SW** 34‰). Scale bar 100 μm . See text for details. Glomerulus (*), neck (N), proximal tubule I and II (PTI, PTII), distal tubule (DT), distal tubule ampullae (DTa) and initial collecting tubule (ICT). Line drawing modified from Hentschel and Elger (1987).

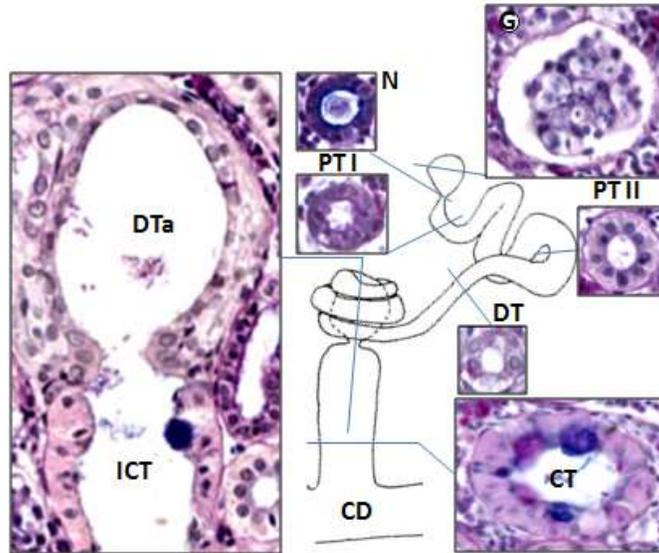


Figure 5.3. Periodic Acid Schiff (PAS)/Alcian blue (pH 2.5) AB/PAS staining of marine catfish *P. lineatus* acclimated kidney seawater (**SW** 34‰). The neutral (magenta), acid rich (blue) and purple cells are indicating combination neutral and acidic glycoconjugates. Scale bar 100 μ m. See text for details. Glomerulus (*), neck (N), proximal tubule I and II (PTI, PTII), distal tubule (DT), distal tubule ampual (DTa) and initial collecting tubule (iCT). Line drawing modified from Hentschel and Elger (1987).

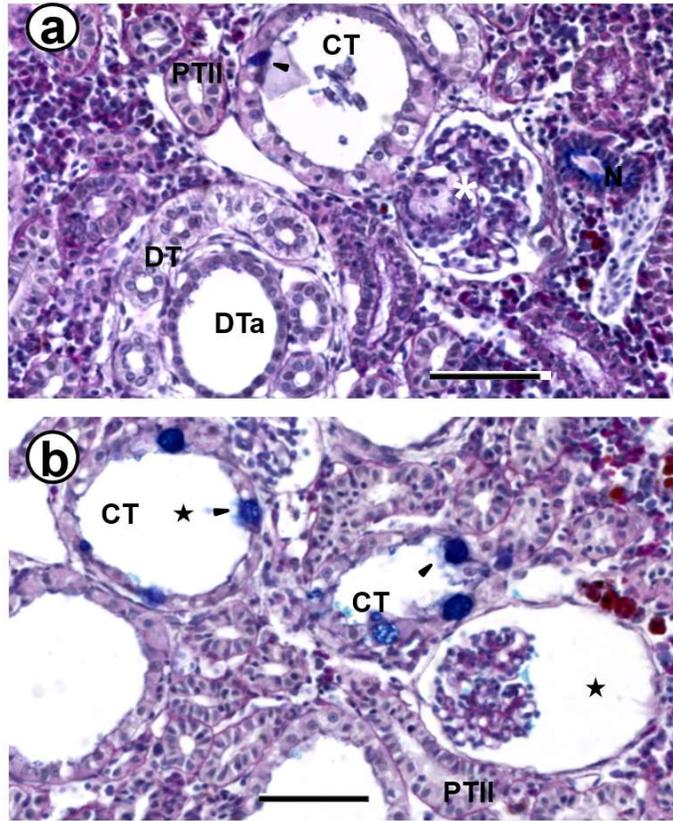


Figure 5.4. Periodic Acid Schiff (PAS)/Alcian blue (pH 2.5) AB/PAS staining of marine catfish *P. lineatus* kidney acclimated in (a) brackish water (**BW**) 3‰, brackish water ligated (**BW-L**) 3‰ (b). The neutral (magenta), acid rich (blue) and purple cells are indicating combination neutral and acidic glycoconjugates. Scale bar 100 μm. See text for details. Glomerulus (white *), neck (N), proximal tubule I and II (PTI, PTII), distal tubule (DT), distal tubule ampula (DTa) and initial collecting tubule (iCT). Scale bar 100 μm. See text for details.

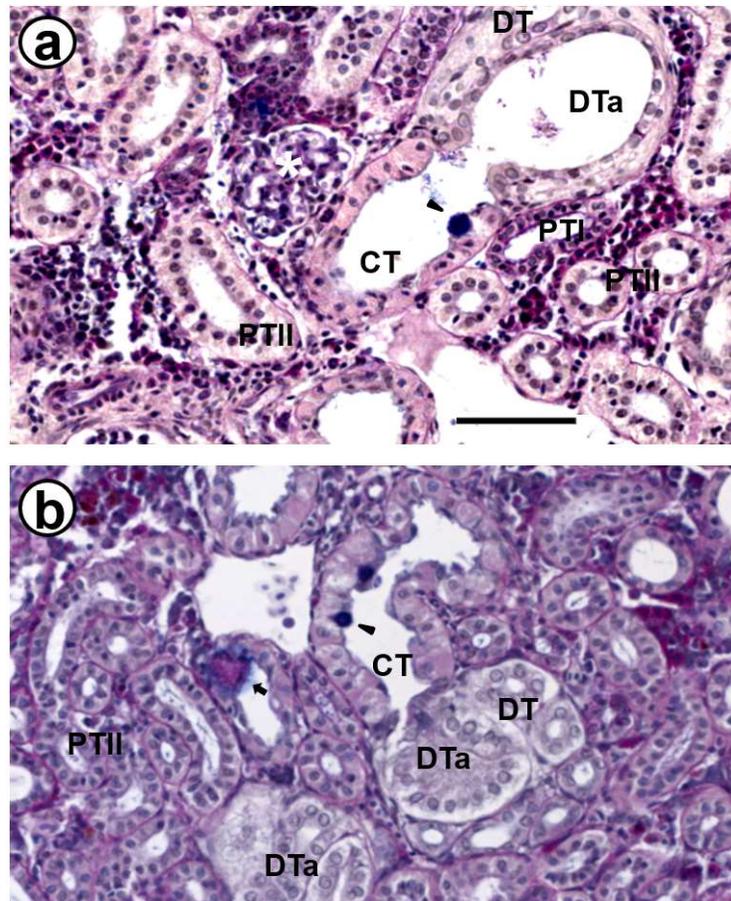


Figure 5.5. Periodic Acid Schiff (PAS)/Alcian blue (pH 2.5) AB/PAS staining of marine catfish *P. lineatus* kidney acclimated in (a) seawater (**SW** 34‰) (b) and seawater ligated (**SW_CL** 34‰). The neutral (magenta), acid rich (blue) and purple cells are indicating combination neutral and acidic glycoconjugates. Scale bar 100 μ m. See text for details. Glomerulus (white *), neck (N), proximal tubule I and II (PTI, PTII), distal tubule (DT), distal tubule ampula (DTa) and initial collecting tubule (iCT). Scale bar 100 μ m. See text for details.

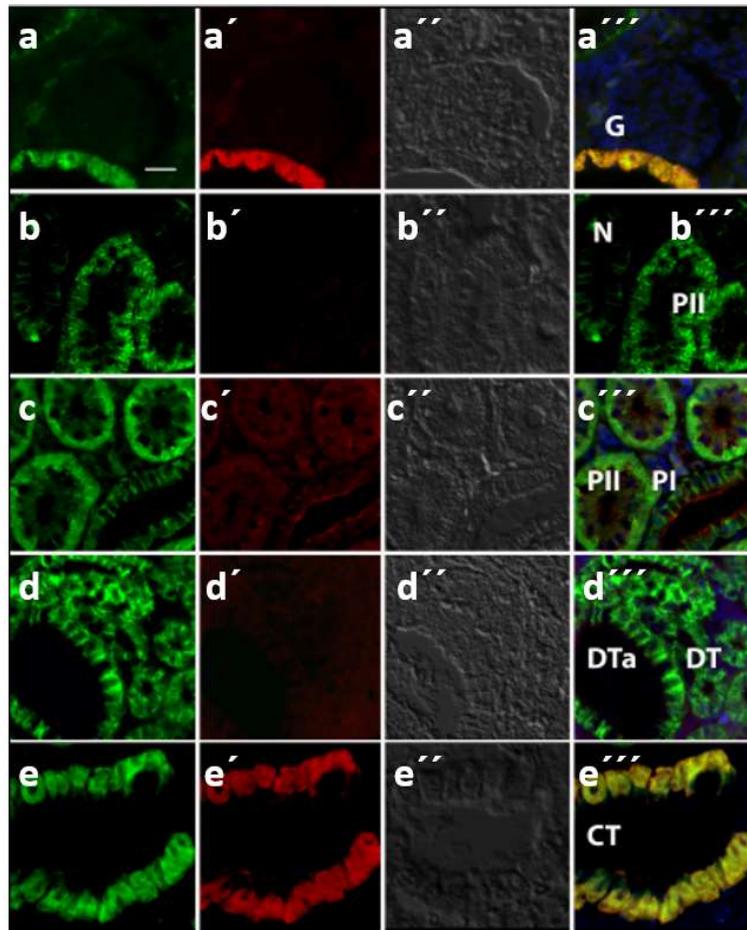


Figure 5.6. Double immunofluorescence localization of Na^+/K^+ -ATPase (**a-e**; αR1 , green) with NKCC1 (**a'-e'**; T4, red) in the nephron of *Plotosus lineatus* acclimated in seawater (SW-control) 34‰. Sections were counter stained with DAPI nuclear staining (blue) and overlaid with the differential interference contrast (DIC) images (**a''-e''**). Scale bar 25 μm in upper panel. See text for details.

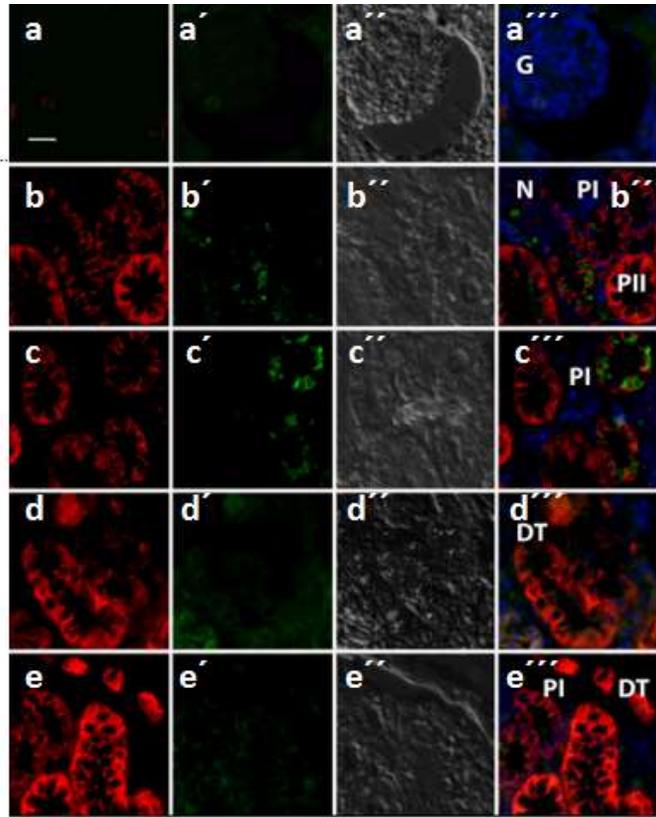


Figure 5.7. Double immunofluorescence localization of Na^+/K^+ -ATPase (**a-e**; $\alpha 5$, red) with VHA (**a'-e'**; VHAB green) in the nephron of *Plotosus lineatus* acclimated in seawater (SW-control) 34‰. Sections were counter stained with DAPI nuclear staining (blue) and overlaid with the differential interference contrast (DIC; **a''-e''**) images. Scale bar 25 μm in upper panel. See text for details.

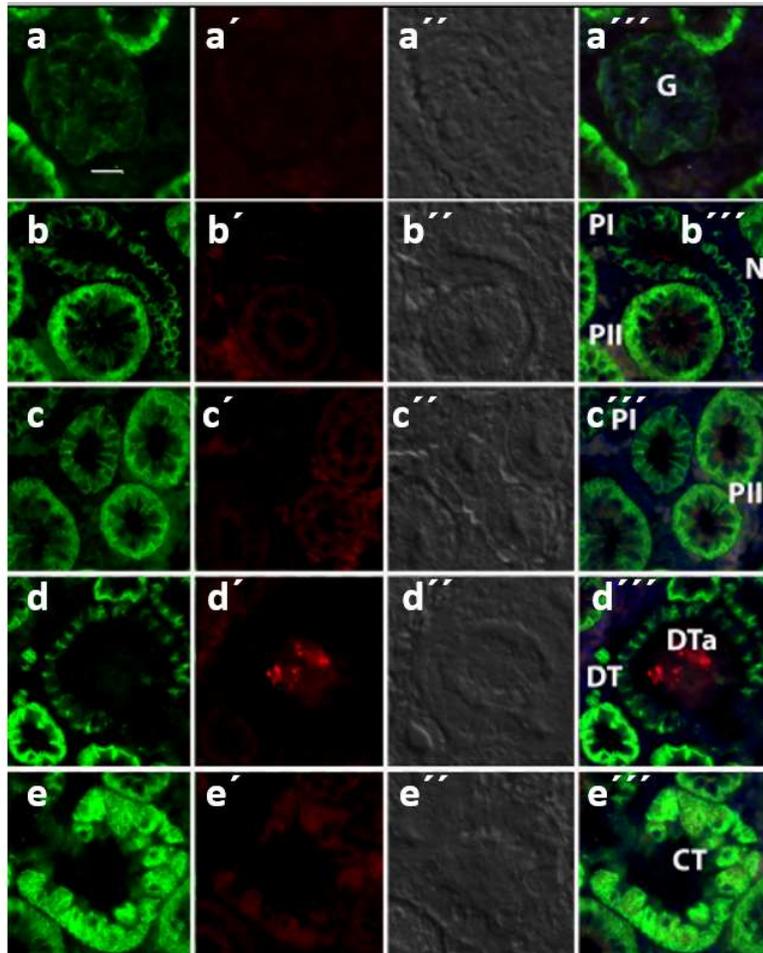


Figure 5.8. Double immunofluorescence localization of Na^+/K^+ -ATPase (**a-e**; αR1 , green) with CFTR (**a'-e'**; red) in the nephron of *Plotosus lineatus* acclimated in seawater (SW-control) 34‰. Sections were counter stained with DAPI nuclear staining (blue) and overlaid with the differential interference contrast (DIC; **a''-e''**) images. Scale bar 25 μm in upper panel. See text for details.

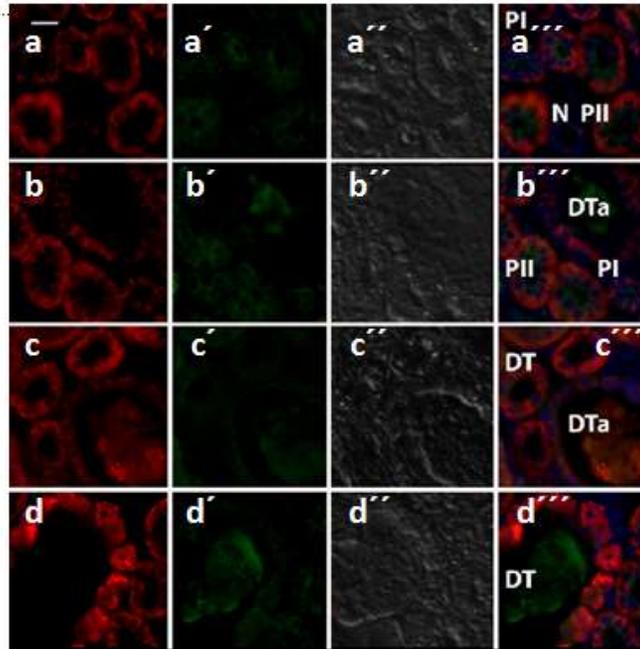


Figure 5.9. Double immunofluorescence localization of Na^+/K^+ -ATPase (**a-d**; $\alpha 5$ red) with Ca17 (**a'-d'**; green) in the nephron of *Plotosus lineatus* acclimated in seawater (SW-control) 34‰. Sections were counter stained with DAPI nuclear staining (blue) and overlaid with the differential interference contrast (DIC; **a''-d''**) images. Scale bar 25 μm in upper panel. See text for details.

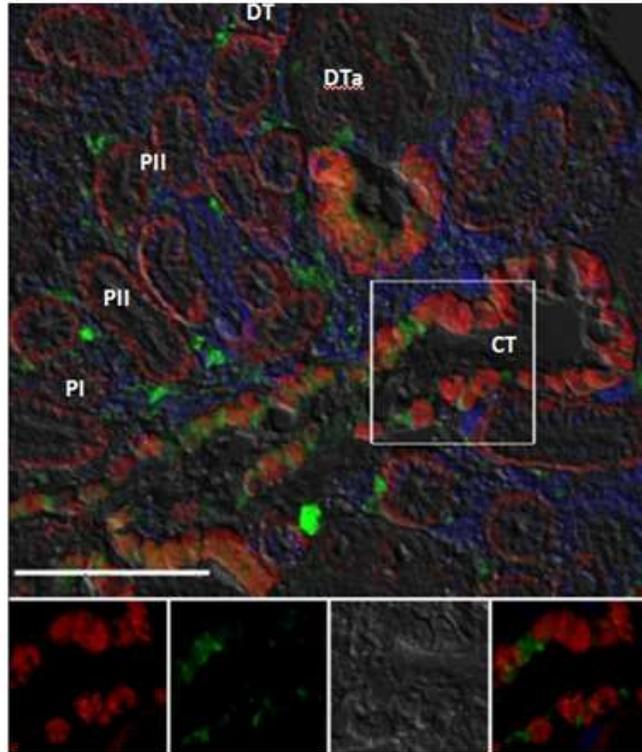


Figure 5.10. Double immunofluorescence localization of Na^+/K^+ -ATPase ($\alpha 5$, red) with PAT1 (green) in the nephron of *Plotosus lineatus* acclimated in seawater (SW-control) 34‰. Sections were counter stained with DAPI nuclear staining (blue) and overlaid with the differential interference contrast (DIC) images. Scale bar 25 μm in upper panel. See text for details.

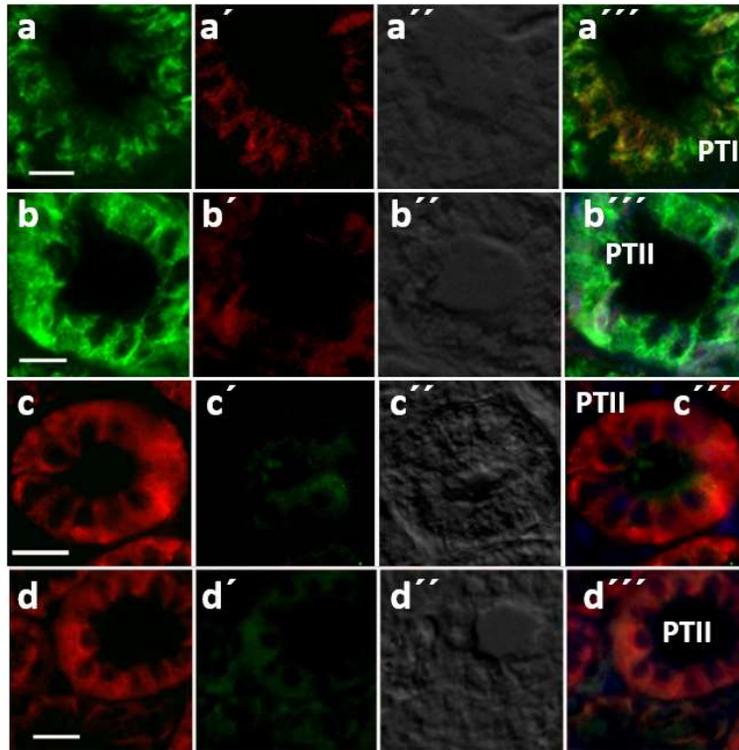


Figure 5.11. Double immunofluorescence localization of Na⁺/K⁺-ATPase (**a-b**; αR1, green) with NKCC1 (**a'-b'**; T4, red) or Na⁺/K⁺-ATPase (**c-d**; α5, red) with Ca17 (**c'**; green) or VHA (**d'**; VHAB green) in the nephron of *Plotosus lineatus* acclimated in hypersaline water (HSW) 60‰. Sections were counter stained with DAPI nuclear staining (blue) and overlaid with the differential interference contrast (DIC) images (**a''-d''**). Scale bar 25μm in upper panel. See text for details.

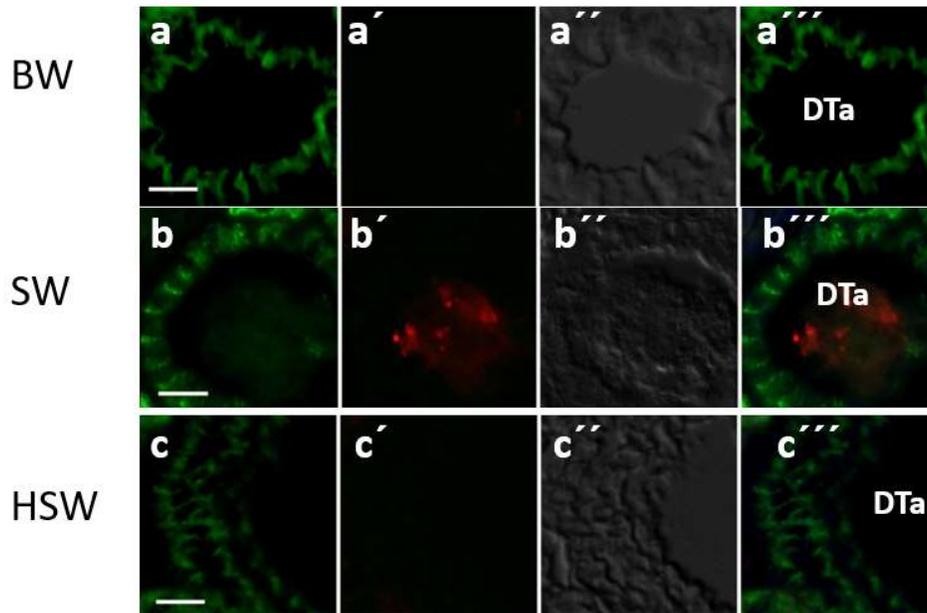


Figure 5.12. Double immunofluorescence localization of Na^+/K^+ -ATPase (**a-c**; αR1 , green) with CFTR (**a'-c'**; red) in the nephron of *Plotosus lineatus* acclimated in brakish water (BW) 3‰, seawater (SW-control) 34‰ and hypersaline water (HSW) 60‰. Sections were counter stained with DAPI nuclear staining (blue) and overlaid with the differential interference contrast (DIC) images (**a''-c''**). Scale bar 25 μm in upper panel. See text for details.

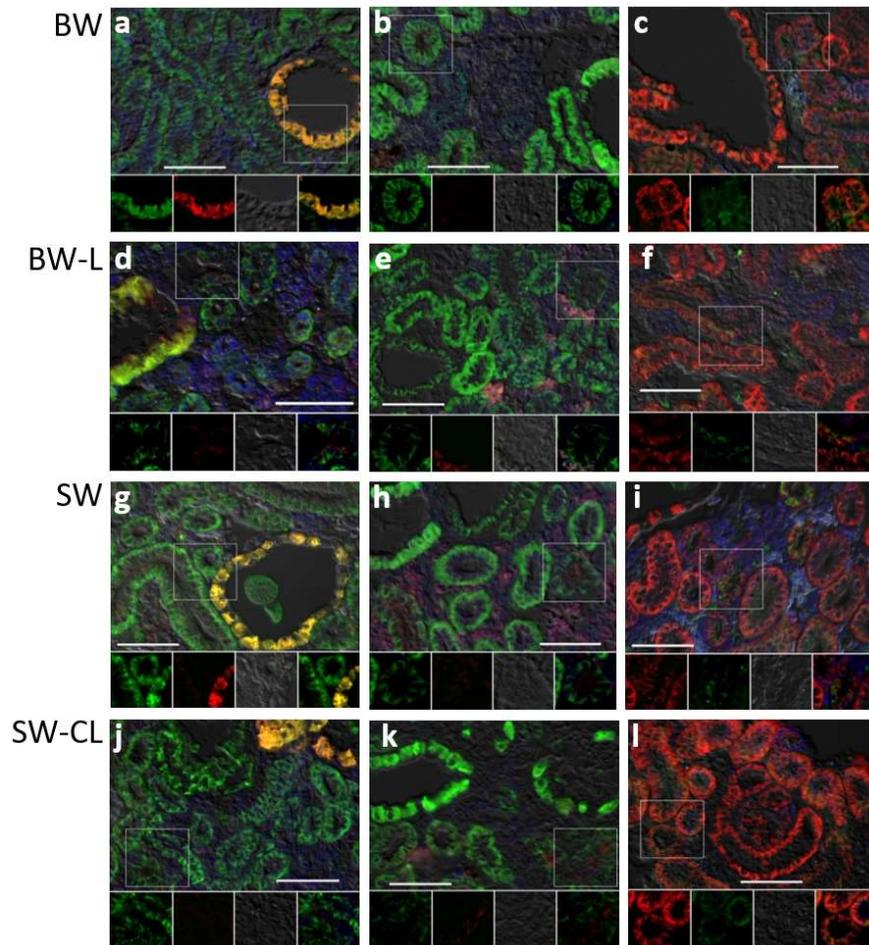


Figure 5.13. Double immunofluorescence localization of Na^+/K^+ -ATPase (αR1 , green **a, b, d, e, g, h, j, k**) with NKCC1 (T4, red **a, d, g, j**) and CFTR (red, **b, e, h, k**) or Na^+/K^+ -ATPase (α5 , red **c, f, i, l**) with V-ATPase (B2, green **c, f, i**). Sections were counter stained with DAPI nuclear staining (blue) and overlaid with the differential interference contrast (DIC) images in the kidney of marine catfish *P. lineatus* acclimated in brackish water (BW) 3‰ (**a**), brackish water ligated (BW-L) 3‰ (**b**), seawater (SW-control) 34‰ (**c**) and seawater (SW-control) ligated (SW-CL) 34‰ (**d**). Scale bar 100 μm in upper panel. See text for details.

5.4 Discussion

5.4.1 Proximal tubule I

In our work, apical localization of NKCC in PTI of BW and SW-control fish indicates the expression of NKCC2/NCC, absorptive isoforms ion absorbing function of the PTI. Apical staining was absent in HSW acclimated fish. The typical absorptive role of PTI regarding to NaCl (actively) and water (passively) has been reported (McDonald, 2007; Evans, 2008; Nishimura and Imai, 1982; Marshall and Grosell, 2006). The apical and/or basolateral localized NKCC in kidney tubules has been reported in dogfish (Biemesderfer et al., 1996), *Dicentrarchus labrax* (Lorin-Nebel et al., 2006), *Fundulus heteroclitus*, *Oncorhynchus mykiss* (Kato et al., 2008) and *Anguilla japonica* (Teranishi et al., 2013). However, the identification the mRNA expression of different isoforms of NKCC might be interesting in future work as has been reported in various other species (Cutler and Cramb., 2002; Tipsmark et al., 2002; Gagnon et al., 2002; Scott et al., 2004; Kato et al., 2008; Hiroi et al., 2005a; Teranishi et al., 2013). The apical localization of CFTR indicates a role of the secondary Cl⁻ secreting for PTI accompanied by absorptive ions function. Regarding the absorptive function of PTI, detecting the pinocytotic invaginations and/or acid phosphatase activity in the apical portion of the epithelial cells (Endo and Kimura, 1984), various number of large granules in PTI epithelial cells (Ogawa, 1959), facilitating the absorption of various materials by possessing the larger lumen diameter than carp and yellowtails which may allow production of slow urine flow (Endo and Kimura, 1984) has been reported. The apical localization of NHE3 in proximal tubule of the rainbow trout suggests an ion absorptive role of proximal tubule (Ivanis et al.,

2008). This may be similar in marine catfish thus the study of localization, protein and mRNA expression of the NHE3 in future work would be interesting. We were unable to find a suitable antibody for the present work.

In PTI, the observation of apical/subapical localization of V-ATPase, apical/cytosolic localization of CA accompanied the basolateral localization of NKA has been observed in PTI of fish kidney at SW and BW potentially HSW may reveal possible role of PTI in acid-base regulation even in different salinity environments. The localization of CA to the cytosol and brush border membranes of proximal tubules in trout and flounder has been reported (Pelis and Renfro, 2004; Georgalis et al., 2006a). The proximal tubule of the teleost fish is thought to be the predominant site for renal acid secretion and/or bicarbonate reabsorption. At least two mechanisms including electroneutral exchanger NHE3 (Hirata et al., 2003; Ivanis et al., 2008a) and V-ATPase pumping (Perry and Fryer, 1997; Perry et al., 2003a; Hirata et al., 2003) have been proposed for acid secretion to the filtrate (Perry and Gilmour, 2006).

Following HSW acclimation ion transport protein localization was more or less similar to the SW-control groups. However, the absent of apical localization of NKCC and V-ATPase may reveal a reduced role of the PTI in ion reabsorption and acid-base regulation at HSW as the main challenge to the marine catfish is salt secretion. The latter may also be related to the observed higher NKA activity in HSW. Taken together it seems the PTI of the nephron is working as either absorptive or secretory segment.

5.4.2 PTII

The PTII of marine catfish kidney nephron shows basolateral localization of NKA, cytosolic CA and apical/subapical CFTR and some NKCC1/NCC. The distribution of basolateral NKA was obviously higher than PTI. The basolateral localization of NKCC has also reported in proximal tubule of European eel (Cutler and Cramb, 2008), and PTII of killifish kidney (Katoh et al., 2008). Moreover, it has been suggested that the driving force for Na^+ , Cl^- , and water secretion can be active transepithelial secretion of Mg^{2+} specially in marine SW acclimated teleosts (Beyenbach and Liu, 1996). So the suggested pathway of salt secretion may occur accompanying Mg^{2+} secretion thus facilitating water secretion because of the production of a reverse osmotic gradient (Beyenbach, 2004). However, we should note the decreased urine rate and/volume in SW fish in comparison with FW fish (McDonald, 2007; Evans, 2008). The abundance of mitochondria in basolateral region of epithelial cell in PTII of the nephron in marine catfish kidney has been confirm by Ogawa (1959) which was also observed in H&E staining work. Furthermore, detecting citric acid cycle enzymes (Hestschel and Meyer, 1982) attributed to the secretion of divalent ions which in marine fish kidney is assumed to be one of main function for ion regulation (Hickman and Trump, 1969). The observation of CA may express the functional role of the PTII in acid-base regulation of marine catfish kidney via H^+ , HCO_3^- transportation. Furthermore, the observation of greater expression of CA in PTII than PTI of marine catfish kidney suggests a more important role of the PTII in acid-base regulation.

5.4.3 Distal tubule (DT):

The DT is present in FW teleosts as a diluting segment (Hickman and Trump, 1969; Beyenbach, 2004) and is thus absent in the majority of truly marine species (Hickman and Trump, 1969). However, trout (Nishimura and Imai, 1982), Anguillidae (Ogawa, 1968a) and marine catfish Plotosidae (Ogawa, 1959) has been reported to retain the DT. The DT of *P. lineatus* has a unique coiling or wrapping of the early portion, around the ampulla like end portion of DT (Hentschel and Mayer, 1987) which seems to insulate the last portion of the DT from the sinuses of the portal system. The DT ampulla does not have a single cilium project into the tubular lumen. The role of renal portal system is to allow the continuation of tubular function when glomerular blood flow is reduced (in SW) particularly in aglomerular species, and may result in reduced urine production/urine flow rate (potentially via the effect of catecholamine hormone system, McDonald, 2007). Because of this insulation the tubular function of the end of DT is questionable. The absence of the basolateral interdigitation, detected as short infoldings of the lateral/basal cell membranes which were greatly reduced in the ampulla-shaped end portion of the DT and the observation of very shallow apicobasal zonula occludens (Hentschel and Mayer, 1987) maybe express permeability to water that results in the increase of the concentration of the urine.

According to our result, apart from weak basolateral expression of NKA in DT, no other transporter was detected in appreciable amounts. The lack of apical NKCC2/NCC localization would argue against the typical role of the DT as a diluting segment (Hickman and Trump, 1969; Evans, 2008). There are some results of IHC in DT of other species such as in killifish and trout, basolateral and apical localization of NKA and NKCC2, respectively (Katoh et al., 2008),

pufferfish, apical NaCl cotransporter (NCC; Katoh et al., 2011), and tilapia, basolateral kidney specific Cl⁻ channels, OmClC-K (Miyazaki et al., 2002).

The apical fingerprint-like microridges of DT which may be suspected to improve mucus adherence and spread (reviewed by Abaurrea-Esquisoaín and Ostos-Garrido, 1996a) and also an adaptation owing to reduce of surface area (Endo and Kimura, 1984) has also been reported. Furthermore, the apical part of DTa showed the faint blue staining of AB/PAS which maybe the presence of acidic mucin potentially aiding sulphate secretion. If the latter is true the marine catfish reveals a unique feature of divalent ion secretion to urine may result to help to form hyperosmotic urine. However, we need more physiological and molecular evidence. Taken together, it seems that the DT of marine catfish may does not appear to be involved in an absorptive function typical of the DT in other teleosts.

The histological observation in our study showed two morphological changes in the unique ampulla like end portion of DT (DTa) (Hentschel and Elger, 1987). In SW-CL, a smaller lumen size of DTa compare to other groups was observed while it was larger in BW-L. Respectively they may be contributed less and more volume of water transport leading to formation of hyperosmotic and hypoosmotic urine, respectively. Kowarsky (1973) found lower concentrations of urine Na⁺ and osmolality following BW-L (4ppt) and formation hypoosmotic urine in *C. macrocephalus*.

5.4.4 CT/CD:

The existence of renal chloride cells in both FW and SW catfish has been reported (Komuro and Yamamoto, 1975; Hentschel and Meyer, 1982; Hentschel and Elger, 1983). In our work, detecting high protein expression (IB) and/or colocalization of NKA α -subunits and NKCC throughout the cytoplasm of CT in the kidney of marine catfish individuals in all of salinities by IHC might be attributed to the production of the unusual hyperosmotic urine in the marine catfish. However, we did not detect significant changes in the protein expression of α -subunits of NKA by IB which might be attributed to post-transcriptional regulation of protein expression and/or protein stability (Lee et al., 2016).

The typically shared model of the reabsorptive function of the DT and CT regarding the Na^+ , Cl^- , and K^+ has been proposed within several groups of teleost species (Hickman and Trump, 1969). The apical localization of NKCC in CT of both killifish and trout (Kato et al., 2008) and apical localization of NCC in eel (Teranshi et al., 2013) has been reported. However, in *P. lineatus* the IHC result do not indicate apical expression of NKCC rather strong basolateral localization.

According to the Diamond theory, 1960s (occurring locally occluded area which can attract water locally) it has been proposed potentially two ways of water movement from inside the cell by possessing the invagination of plasma membrane or between the cells by paracellular space. The former may occur in the epithelial which has MRCs. Furthermore, the possessing a large amount of MRCs in the gills (Evans, 2005), DO cells and CT of the marine catfish kidney has also been confirmed in our works thus can make them a potential site of the water movement against an osmotic gradient. Considering the structurally study in nephron of marine catfish kidney by Hentschel and Elger (1987) which showed

that CT possess different types of cells including ionocytes, pavement, basal and the goblet cells moreover moderately deep zonula occludens and zonula adherens have also been observed in the ionocytes and the IHC result of our work may reveal the role of the CT epithelium in water absorbing from the filtered urine tend to create an hyperosmotic urine. The high osmotic gradient of the produced urine can be result in the absorption of higher amount of water to the urine, in spite of the necessity of keeping vital water inside the body of the fish. On the other hand, since water conservation is critical in SW teleosts, the observation of high basolateral expression CFTR may create a 'local occluded area' between the epithelial cells as a compensatory mechanism and/or a physiological adaptation in marine catfish. In Atlantic salmon different types of TJ proteins have been detected in the gills, intestine and kidney. The latter showed an increase in two types of the TJ proteins (tricellium and claudin-3) during SW acclimation (Tipsmark and Madsen, 2012). However, the latter interpretation needs to be confirmed molecularly, with the study on possible existence of various types of TJ and AQPs proteins, their expression and localization in different tissues of the marine catfish in future work. The reabsorption of monovalent ions by apical NKCC accompany by basolateral NKA and Cl⁻ channel may work together leading to firstly an increase in divalent ions concentration in the produced urine, secondly creating locally high osmotic area between the cells to absorb the water.

The PAT1 *slc26a1* and *slc26a6a* has been identified in renal tubule of rainbow trout and pufferfish acclimated in SW (Katoh et al., 2006, 2009), and Slc26a1 localized to the basolateral membrane localization in proximal tubule of eel kidney (Nakada et al., 2005; Watanabe and Takei, 2011b). The dual

expression of Slc26a6a either in apical or basolateral of CT and also in a separate cell which show only the Slc26a6a expression in SW-control of marine catfish may express the role of the CT in Cl^- and HCO_3^- (SO_4^{2-}) regulation. The apical membrane of localized Slc26a6a may secrete SO_4^{2-} into the lumen of renal tubule, in exchange for Cl^- , as has been shown in the eel kidney in SW (Watanabe and Takei, 2011a). It has been proposed that in SW eels SO_4^{2-} fluxed into the body could be excreted by the different tissues including the gills, skin and digestive tracts as mucus. Furthermore, using AB/PAS staining has shown positive staining throughout the cytoplasm and somewhat apical acidic mucin staining of epithelial cells of CT which indicates mucopolysaccharid and acidic (potentially sulphate) mucus secretion by the CT. Moreover, the sparsely distribution of a few purple and blue goblet cells, representing combinations neutral and acidic mucins, respectively has been observed in CT/CD of marine catfish in different salinities. However, the observation of only acidic mucocytes in CT in HSW acclimated marine catfish suggests a role of the kidney CT in bicarbonate and/or sulphate secretion. However, the latter needs more details to be confirmed. We didnot test the protein expression and/or localization of Slc26a6a in BW or HSW thus it seems the proposed test would be interesting in future work.

AB/PAS staining identified more large acidic mucous cells in CT/CD of BW-L fish suggesting the need to secrete more acidic mucus in BW-L. The possible acid mucus secreting (presumably involving SO_4^{2-}) to produce the hyperosmotic urine has been proposed in SW-control or HSW fish in Chapter 3, which is in constrast with formation of hypoosmotic urine in BW-L observed by Kowarsky (1973). However, the possibility of greater water transporting due to the

observation of larger lumen of Bowman's capsule around the glomerular capillaries and/or larger lumen size of DTa in BW-L fish might compensate for the divalent ion secretion and finally diluted urine to a hypoosmotic excretion. However, the latter hypothesis will require additional molecular evidence to be confirmed.

5.4.5 Summary of characteristics of the nephron of marine catfish kidney that might be involved in the formation of hyperosmotic urine

i) Presence of renal "chloride cells" in the CT which can have main role in salt secretion as indicated by strong NKA and NKCC1 location thus contributing to the formation of hyperosmotic urine. The latter needs to be confirmed by measurement of the urine formation in future work.

ii) Presence of the unique DT feature by showing the coiling of the early portion around the ampulla like end portion of DT. Moreover, the absence of the basolateral interdigitation, detecting short infoldings of the lateral/basal cell membranes which were greatly reduced in the ampulla-shaped end portion of the DT (Hentschel and Mayer, 1987), all together may express permeability to the water leading to increase the concentration of the urine. In addition the absence of apical NKCC2/NCC suggests lack of the traditional DT function in reabsorption.

iii) Presence of light apical acidic mucus staining in DT and more obviously in CT by AB/PAS. Furthermore, representing Alcian blue positive mucous cells particularly in HSW which may express the acidic (potentially sulphate) mucus secretion.

Regarding the production of hyperosmotic urine compare to the blood which observed in *Cnidoglanis*, Hickman and Trump, 1969 proposed an explanation which could be involved a) the secretion of divalent ion, b) reduced volume of glomerular filtrate, c) high tubular impermeability to water and d) possibility of sodium secretion into the tubules. In our work, the apical localization of slc26a6a in CT and also acidic mucous cells with the possible role in SO_4^{2-} secretion might agree with a). Regarding b), possibly reduced glomerular filtration decreases the urine volume might be homogenous processes. Unique structural features of DT in *Plotosus* explained by Hentschel and Elgar (1987) or the observation of abundant secretory renal chloride cells in CT/CD and their potential role in producing local osmotic area (see above) and moreover proposed in salt secretion might be related to the suggestion c) or d), respectively.

Measurement of urine sodium and osmolality in BW or SW of *Cnidoglanis* by Kowaresky, (1973) showed that at salinities of 4 and 11‰ both urine sodium and osmolality were lower than plasma however when they exceed salinity over 20‰ the concentration of sodium and osmolality was higher than plasma. The latter suggests that by increasing salinity higher amounts of sodium and other ions, possibly divalent, could be found in marine catfish urine. It seems measurement of different ions concentration such as Na^+ , Cl^- , K^+ , Ca^{2+} , Mg^{2+} and SO_4^{2-} in urine can be interested in future work to have more clear interpretation of producing hyperosmotic urine by marine catfish. This was not done in the present study because of the small size of the animals.

Chapter 6:

The dendritic organ of marine catfish *Plotosus lineatus* as an ammonia excretory organ

Malakpour Kolbadinezhad, S., Coimbra J., Wilson, J. M. 2017. The dendritic organ of marine catfish *Plotosus lineatus* as an ammonia excretory organ (*in preparation*).

Abstract

In the present study, we investigated the potential role of the marine catfish dendritic organ (DO) in ammonia excretion by examining the effect of salinity [brackishwater (BW 3‰), seawater (SW 34‰) and hypersaline water (HSW 60‰)] and DO ligation on ammonia excretion and ammonia transporter expression. In gill, immunohistochemistry (IHC) showed an apical localization of Rhesus-associated glycoprotein (Rhag) in some NKA immunoreactivity (IR) cells limited to a few interlamellar regions of the filament and both apical and basolateral membranes of pillar cells in all salinities or with DO ligation. In DO, apical and subapical localization of Rhag and Rhbg were found in the parenchymal cells of the acini. In the kidney an apical localization of Rhag in proximal tubule I (PTI), basolateral in proximal tubule II (PTII) except HSW and throughout the cytoplasm of collecting tubule (CT) cell were observed all salinities. DO ligation in BW fish resulted in kidney lacking apical and/or basolateral localization of Rhag in PTI and/or PTII, respectively. Apical staining of Rhag at SW-CL of posterior intestine was observed but unresponsive to salinity or ligation. Immunoblotting (IB) of Rhag and Rhbg was found only in the gills with levels were inversely correlated with salinity. Ligation was associated with the lowest Rhag levels in SW fish gill. The expression of *rhcg1* mRNA was detected only in gills and DO. HSW was associated with the lowest expression and ligation was without effect on expression levels. Ammonia flux rates (J_{Amm}) were significantly lower in BW acclimated fish but were not affected by ligation. However, SW ligated fish has significantly lower J_{Amm} . Taken together these results indicate that the DO potentially has a physiological role in ammonia excretion in addition to salt secretion. The salinity depend effects indicate Rh glycoproteins, a NH_3 transport mechanism dominates at lower salinities while a yet to be defined NH_4^+ transport mechanism (e.g. NHE3) is important at higher salinities.

6.1. Introduction

The gills of the fish are proposed as the main organ of ammonia excretion in both of FW and SW environments and are linked with ion regulation (Wright and Wood, 2009). The non-erythroid Rhesus glycoproteins have been found to function as NH_3 channels to facilitate ammonia excretion in fishes and other vertebrates (Wright and Wood 2009). In the proposed model of ammonia excretion Rhag localized in red blood cell and pillar cell membranes facilitating the movement of ammonia to the gill epithelium where it diffuses through a basolateral Rhbg and finally apical membrane Rhcg1 and/or Rhcg2 to cross into the environment (for more details see review Wright and Wood, 2009, 2012).

Recently, in the gills of *A. testudineus* a transport mechanism in active NH_4^+ and Na^+ excretion in fresh water and seawater, respectively has been reported including Na^+/K^+ -ATPase (NKA), $\text{Na}^+:\text{K}^+:2\text{Cl}^-$ cotransporter 1a (Nkcc1a) and cystic fibrosis transmembrane conductance regulator Cl^- channel (CFTR) (Ip et al., 2012a; Loong et al., 2011; Ip et al., 2012b). Because of the similarities in the hydration radius of NH_4^+ and K^+ allowing substitution at transport sites (Randall et al., 1999; Alam and Frankel, 2006) thus NKA and NKCC may also be important to ammonium ion (NH_4^+) transport (Evans et al., 2005; Hwang et al., 2015). Alternatively, the Na^+/K^+ exchanger (NHE) has been shown to function as a $\text{Na}^+/\text{NH}_4^+$ in zebrafish (Ito et al. 2014) providing another avenue for ammonia transport.

In the present study initial observations of Rhag and Rhbg expression in DO lead us to propose the hypothesis that there is a potential additional physiological role of this organ in ammonia excretion. We investigated the effects of salinity and DO ligation on ammonia excretion rates and transporter expression levels using immunohistochemical, immunoblotting and PCR based approaches.

6.2 Materials and Methods

6.2.1 Experiments

6.2.1.1 Salinity experiment

Plotosus lineatus were acclimated for two weeks in their respective salinity tanks [brackishwater (BW) 3‰, seawater (SW-control) 34‰, hypersaline water (HSW) 60‰] where they were fed twice daily with diced fish fillets. Ammonia flux measurements were done as described in section 6.2.1.3 and terminally sampled.

6.2.1.2 Ligation experiment

Fish were fasted for 4 days and then anaesthetized with 1:10 000 MS222. The dendritic organ was ligated using suture thread in BW and SW acclimated fish as described by Kowarsky (1973) in *Cnidogobius macrocephalus*. In preliminary experiments, ligated *P. lineatus* in HSW did not survive and ligations were not pursued. Control fish were anaesthetized and sham ligated. Ammonia flux measurements were done as described in section 6.2.1.3 and terminally sampled.

6.2.1.3 Ammonia flux measurements

Food was withheld for 4 days prior to the start of ammonia flux measurements where six fish were transferred to individual 2 L glass aquaria (water volume set to 1 L) with the salinity matching the acclimation salinity. The experimental aquaria were shielded with black plastic to minimize visual disturbance and fitted with individual air-stones for aeration. To measure ammonia excretion rates, initial water samples (duplicate 2 mL) were taken followed by 0, 1, 2, 3, 6, 9, 12 h and final water sample collection after 24 h. For ammonia analysis, water samples were acidified with concentrated HNO₃ (2 µl per 1 ml water sample converting all ammonia to NH₄⁺ to avoid NH₃ loss through volatilization) and immediately frozen (-30 °C) for later analysis of total ammonia concentration.

6.2.2 Sampling

Fish were killed with an overdose of MS-222 (1:5000), and gill, kidney, intestine and DO were excised and either fixed in 10% neutral buffered formalin for 24h and stored in 70% ethanol, or frozen in liquid nitrogen and stored at -80°C.

6.2.3 Analysis

6.2.3.1 Immunohistochemistry

Fix tissue was processed for immunohistochemistry as described in Wilson et al. (2007). Sections of paraffin embedded tissue will probed with Rh glycoprotein antibodies developed against fugu Rhcg1, Rhcg2, Rhbg, and Rhag (Nakada et al. 2007) using indirect immunofluorescence. Sections were double labeled with the mouse monoclonal NKA antibody $\alpha 5$ (ref). Secondary antibodies used were goat anti-rabbit Alexa 488 (Invitrogen). Slides were viewed with a Leica DM6000B photomicroscope.

6.2.3.2 Western blotting

Frozen tissue was thawed in SEI buffer and homogenized with a bead homogenizer (Precellys24) and prepared for immunoblotting as described in Wilson et al. (2007) using a BioRad mini-protean 3 setup and semi-dry transfer apparatus. Protein was measured using the Bradford method. PVDF membranes were probed with Rh glycoprotein antibodies described in section 6.2.3.1 and detected by ECL using a FujiFilm LASmini documentation system.

6.2.3.3 PCR and phylogenetic analysis

RNA was isolated from tissue using BioRad Arum columns, and converted to cDNA using an iScript kit using 1ug of total RNA (BioRad). PCR reactions were conducted using PhusionFlash master mix (Thermo Scientific) and a VWR thermal cycler (Table 2.X; 2.X). Isolated PCR products were sequenced (Stabvida) and bioinformatic analysis performed (tblastx, clustalx). A neighbor-joining tree was constructed using MEGA 7. Quantitative PCR was performed.

6.2.3.4 Ammonia

Water total ammonia nitrogen (TAN) concentrations were measured colourimetrically using a quantitative microplate technique using the salicylate–hypochlorite method, modified from Verdouw et al. (1978) (for details see Moreira-Silva et al., 2009). Ammonia excretion rates J_{Amm} were calculated from the change in ammonia concentration over the initial 6 h of the flux period, which was found to be linear, and expressed in $\mu\text{mol TAN/g/h}$.

6.2.3 Statistics

Data are presented as means \pm standard deviation (S.D.) or 95% confidence interval. Statistical differences of protein, mRNA expression and interaction of salinity and ligation between groups were determined using one-way ANOVA and two-way analysis of variance (ANOVA), respectively followed by the post hoc Student-Newman-Keuls (SNK) test (SigmaPlot 11.0 Systat Software, Inc.) in juveniles exposed to different salinities. In the case of a failed normality test, data were square root or log transformed. Fiducial limit was set at 0.05.

6.3 Results:

6.3.1 Immunohistochemistry

In the gills there was an apical localization of Rhag detected in some NKA-IR cells. These NKA-IR cells were present in a heterogeneous distribution limited to a few interlamellar regions over the trailing edge of the filament and were absent from the lamella (Fig. 6.1). There were no apparent salinity dependent difference in Rhag localization or NKA-IR distribution. Rhag protein was also found in both apical and basolateral membranes of pillar cells and red blood cells (RBCs) in all salinity.

In the dendritic organ, there was an apical localization of Rhag in the parenchymal cells of the acini (Fig. 6.2), while Rhbg had a subapical localization in these cells. The parenchymal cells possess strong NKA immunostaining throughout the cell indicative of tubular system basolateral localization which was observed in different salinities.

In the kidney segments, there was an apical localization of Rhag in PTI at all salinities (Fig. 6.3), basolateral in PTII except HSW and throughout the cytoplasm of CT. In the gill, the distribution of IR-NKA cells with apical localization of Rhag showed no salinity and/or ligation dependency in gills while the apical and/or basolateral localization of Rhag in PTI and/or PTII, respectively was not detected at BW-L fish (Fig. 6.3d). In the posterior intestine, there was an apical

localization of apical Rhag expression in enterocytes of posterior intestine at SW-CL fish (Fig. 6.4).

6.3.2 Western blot

Heterologous antibodies immunoreactive with fugu Rhesus (Rh) proteins Rhag, and Rhbg were used to determine how salinity and/or ligation affected the abundance of these proteins in key osmoregulatory organs: gill, kidney, DO, and anterior and posterior intestine. Immunoreactive with fugu Rhcg1 and Rhcg2 antibodies was not observed in *P. lineatus* tissues.

The Rhesus glycoprotein ammonia transporters Rhag and Rhbg were found only in gill as ~47.5 kDa bands (Fig. 6.5). Expression of both Rhag and Rhbg proteins was significantly lower with HSW acclimation; however, with BW acclimation Rhag was significantly higher (Fig. 6.5a). No difference between BW and SW Rhbg was observed (Fig. 6.5b). In the ligation experiment, an interaction between salinity and ligation was detected in Rhag but not Rhbg levels (Fig. 6.6). In the case of Rhag from the ligation experiment, BW levels were again significantly higher than in SW controls, and at both salinities, ligation was associated with lower Rhag levels.

6.3.3 Gene expression of *rhcg1*

Using a PCR based approach we were able to isolate a partial sequence of an Rhcg orthologue from *P. lineatus* gill which was 504 bp (168 aa). The construction of a phylogenetic tree using the Neighbor-joining method reveals that this sequence clusters with Rhcg1 orthologues from other teleosts (Fig 6.6). *P. lineatus* Rhcg1 has sequence homology of 86.3%, 76.9% and 78.1% with *Ictalurus punctatus* Rhcg (XP_017341435.1), *Danio rerio* Rhcg1 (AAM90586.1), and *Oncorhynchus mykiss* Rhcg1a (ABD92924.1), respectively. The expression of *rhcg1* mRNA was detected in the gill and DO only. However, there were no significant change with salinity in gill; although, in DO lower expression was found in both BW and HSW acclimated fish (Table 1). There were no significant change with salinity and ligation in gill in the DO ligation experiment (Table 1).

6.3.4 Ammonia flux data

In control *Plotosus lineatus* in seawater, the net total ammonia excretion (J_{Amm}) was $368 \mu\text{mol kg}^{-1} \text{h}^{-1}$ (Fig. 6.8). Ammonia flux rates were significantly lower in BW acclimated fish but were not affected by HSW. In the ligation experiment there was a significant interaction between acclimation salinity (BW and SW) and treatment (control, sham, and ligation) (Fig 6.9). In SW fish, J_{Amm} was significantly higher in sham operated fish, with ligated fish having significantly lower J_{Amm} compared to SW control fish. There were no significant treatment difference in BW fish. In comparisons between BW and SW fish within each treatment group, J_{Amm} was significantly higher in SW control and sham operated fish but not ligated fish.

Table 1. Real-time PCR expression of *rhcg*, non-erythroid Rhesus C glycoprotein in the gill and DO tissues of marine catfish *P. lineatus* acclimated to different salinities [Salinity Exp: brackishwater (BW) 3‰, seawater (SW-control) 34‰, hypersaline water (HSW) 60‰] or ligated in BW and SW [Ligation Experiment]. Data are means \pm s.d. (n=3-4). The amounts of mRNAs are normalized to the corresponding *b-actin* abundance from the same sample and the expressed relative to the SW-control group. Different letter indicates a significant difference between salinities, one-way analysis of variance (ANOVA) ($P < 0.05$; see text for details).

Salinity Exp	BW	SW-Control	HSW
Gill	1.06 \pm 0.35	1.00 \pm 0.27	1.04 \pm 0.56
DO	0.28 \pm 0.10 ^a	1.00 \pm 0.25 ^a	0.04 \pm 0.01 ^b

LigationExp.	BW	BW-L	SW-Control	SW-CL48h
Gill	1.06 \pm 0.35	0.85 \pm 0.06	1.00 \pm 0.27	0.31 \pm 0.31

Figures

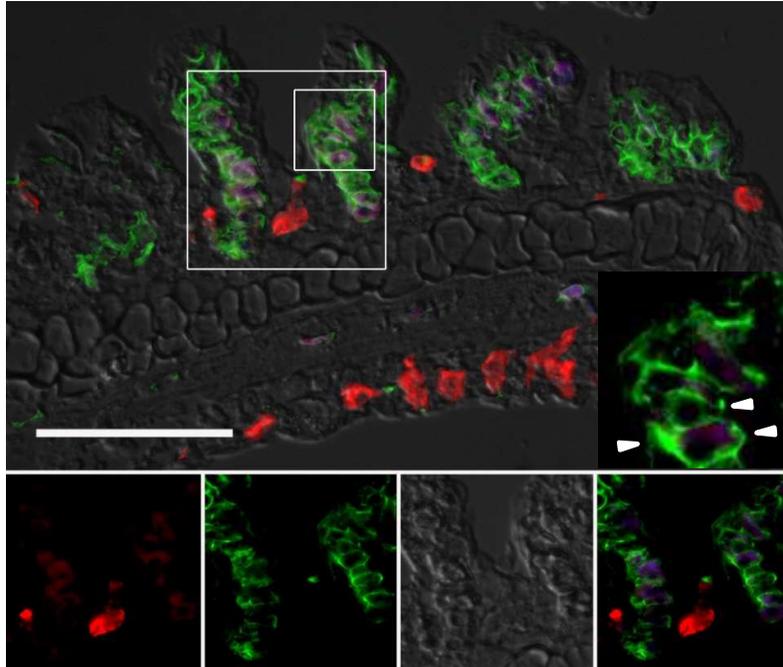


Figure 6.1. Double immunofluorescence localization of Na⁺/K⁺-ATPase (α 5, red) with Rhag (green) the gills of marine catfish *P. lineatus* acclimated in seawater (SW-control) 34‰. Sections were counter stained with DAPI nuclear staining (blue) and overlaid with the differential interference contrast (DIC). Arrowheads indicate to apical and basolateral membranes of pillar cells. Scale bar 100 μ m. The inset picture is 4X.

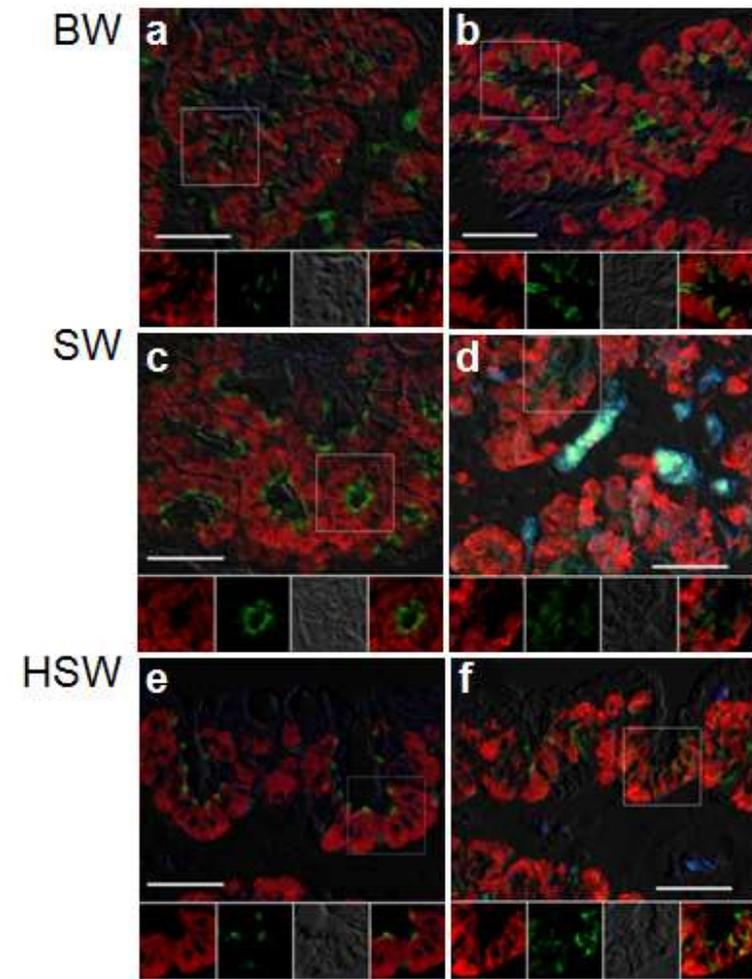


Figure 6.2. Double immunofluorescence localization of Na⁺/K⁺-ATPase (α5, red a-f) with Rhag (green a, c, e) and Rhbg (green, b, d, f) in dendritic organ of marine catfish *P. lineatus* acclimated in brackish water (BW) 3‰ (a-b), seawater (SW-control) 34‰ (c-d) and hypersaline water (HSW) 60‰ (e-f). Sections were counter stained with DAPI nuclear staining (blue) and overlaid with the differential interference contrast (DIC) images. Scale bar 100 μm.

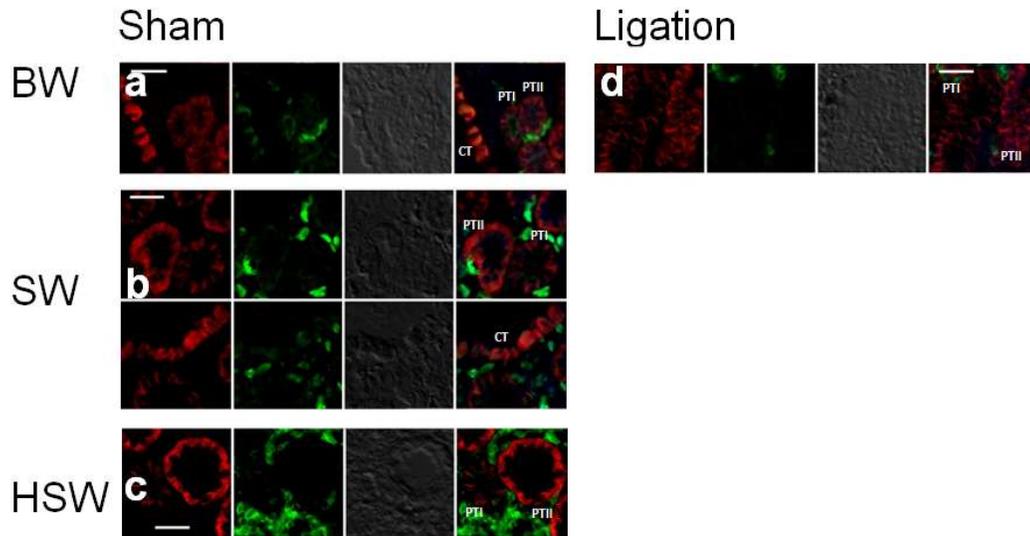


Figure 6.3. Double immunofluorescence localization of Na⁺/K⁺-ATPase (α5, red) with Rhag (green) in kidney of *P. lineatus* acclimated in brackish water (BW) 3‰, (a), seawater (SW-control) 34‰ (b) and hypersaline water (HSW) 60‰ (c). Sections from BW ligated fish are also included (d). Sections were counter stained with DAPI nuclear staining (blue) and overlaid with the differential interference contrast (DIC) image. Abbreviations: PT proximal tubule, N neck, CT collecting tubule. Scale bar 100 μm.

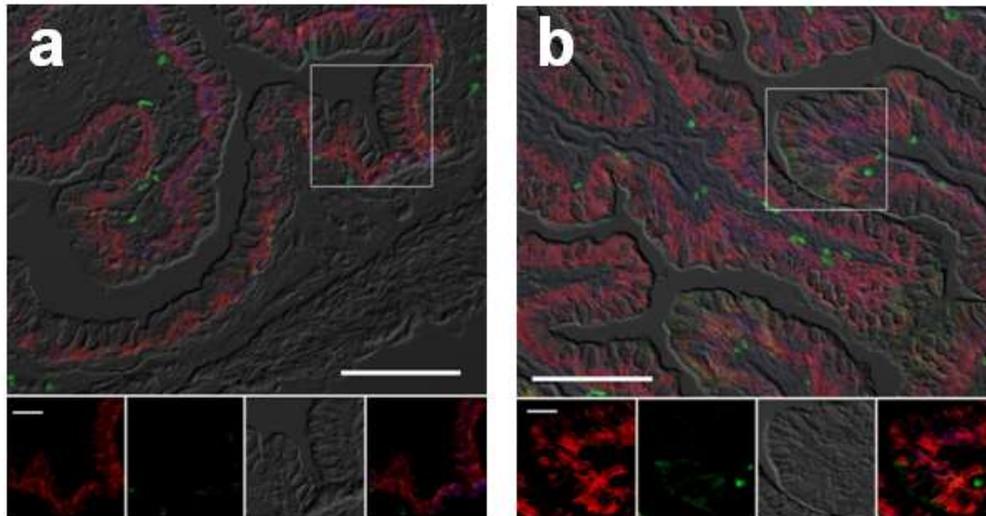


Figure 6.4. Double immunofluorescence localization of Na⁺/K⁺-ATPase (α5, red) with Rhag (green) in posterior intestine of *P. lineatus* acclimated in seawater (SW-control) 34‰ (a) and (SW-control) ligated SW-CL ligated fish. Sections were counter stained with DAPI nuclear staining (blue) and overlaid with the differential interference contrast (DIC) image. Scale bar 100 μm.

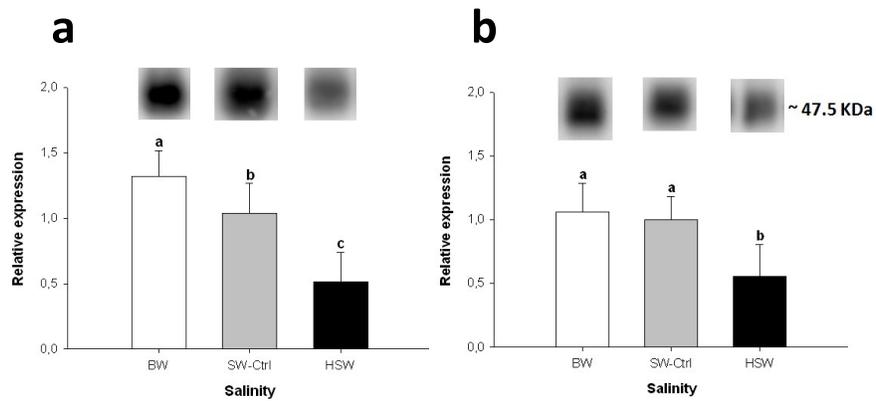


Figure 6.5 Relative expression of Rhesus (Rh) protein Rhag (a) and Rhbg (b) in the gill tissue of marine catfish *P. lineatus* acclimated to [brackishwater (BW) 3‰, seawater (SW-control) 34‰, and hypersaline water (HSW) 60‰]. Representative images showing band size, intensity and relative molecular mass (~ 47.5 kDa) of Rhag and Rhbg. Values are means \pm S.D of protein abundance (n=5-6). Different letter indicates a significant difference between salinities, one-way analysis of variance (ANOVA) and SNK ($P < 0.05$; see text for details).

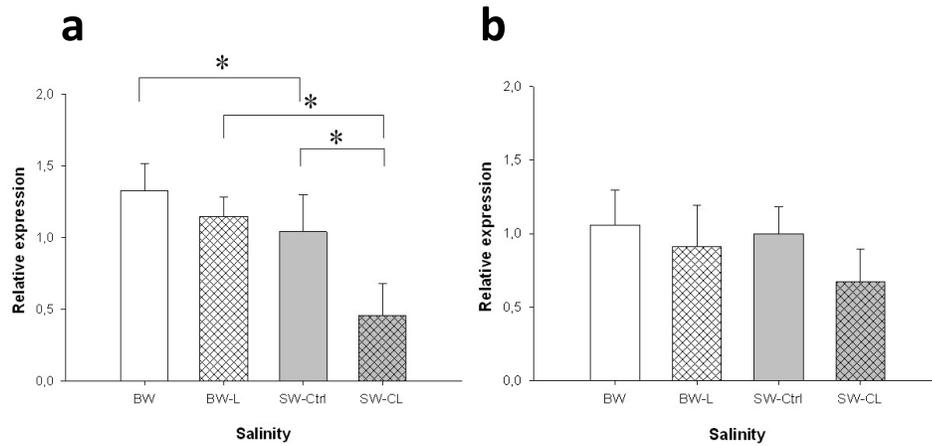
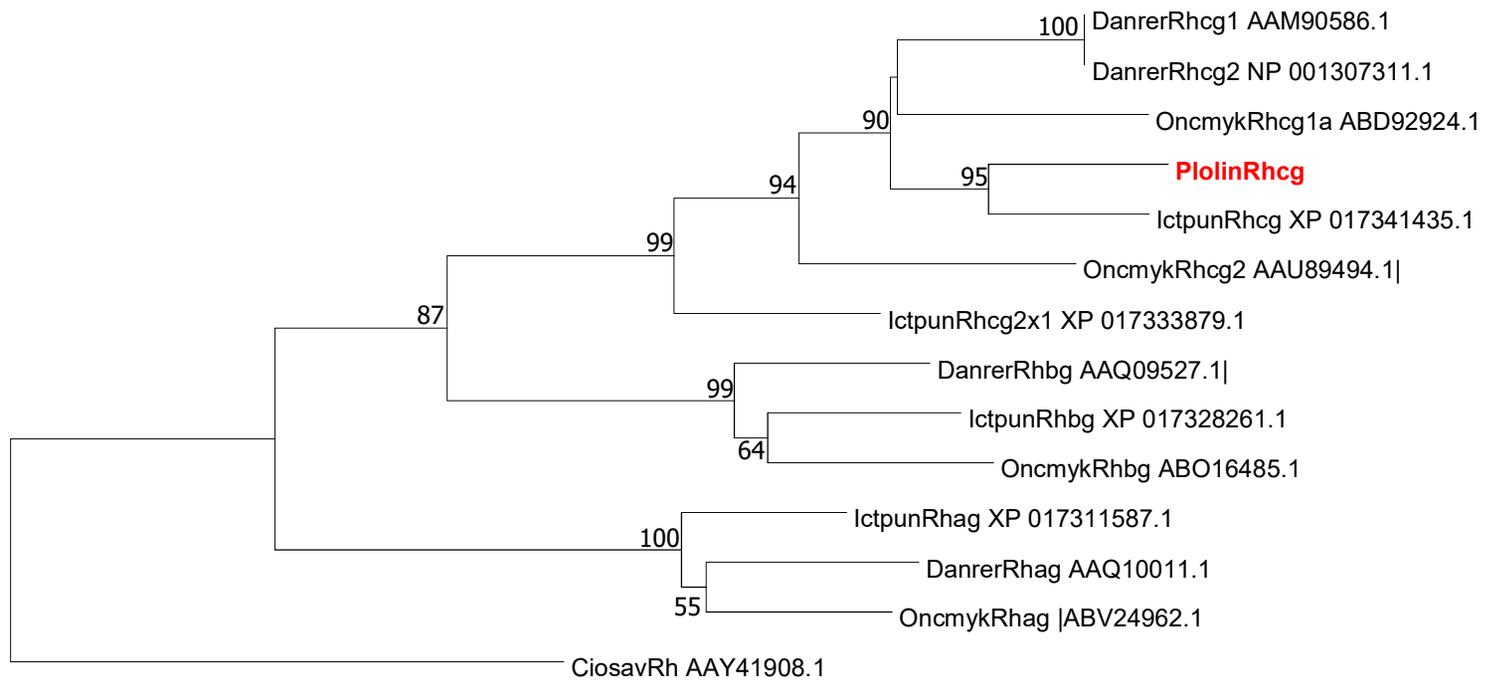


Figure 6.6. Relative expression of Rhag (a) and Rhbg (b) proteins in the gill of marine catfish *P. lineatus* acclimated to [brackishwater (BW), brackishwater ligated (BW-L) 3‰, seawater (SW-control), seawater ligated (SW-CL) 34‰] salinity. Values are presented as means \pm S.D of protein abundance (n=5-6). The asterisks indicate a significant difference between the groups where there was an interaction of salinity and ligation, two-way analysis of variance (ANOVA). ($P < 0.05$; see text for details).



0.050

Figure 6.7. Rooted phylogenetic tree of Rh glycoprotein homolog sequences. The tree was constructed with the neighbor-joining method with 1000 bootstrap trials. The optimal tree with the sum of branch length = 1.95154907 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 14 amino acid sequences with Genbank accession number provided. All positions containing gaps and missing data were eliminated. There were a total of 145 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [4].

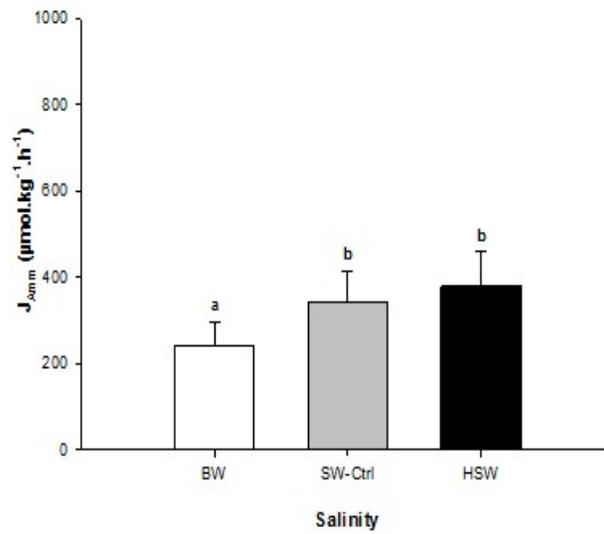


Figure 6.8. Net ammonia excretion rates ($\mu\text{mol TAN kg}^{-1} \text{h}^{-1}$) of marine catfish *P. lineatus* acclimated to [brackish water (BW) 3‰, seawater (SW-control) 34‰, hypersaline water (HSW) 60‰]. Data are means \pm s.d. (n=5-6). Different letters indicate a significant difference between salinities. One-way ANOVA and SNK ($P < 0.05$; see text for details).

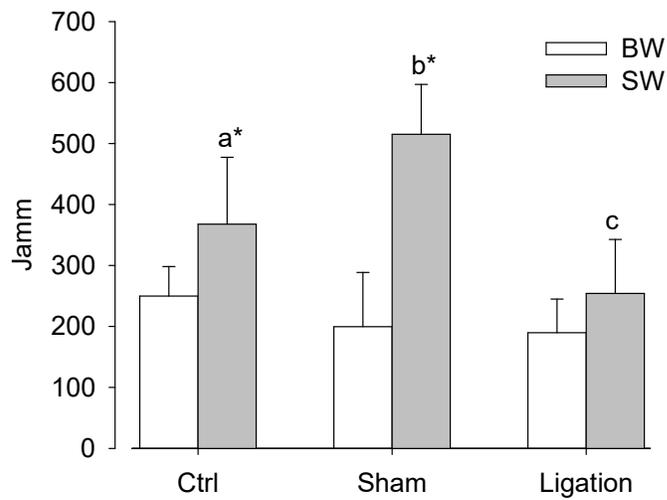


Figure 6.9. Ammonia excretion rates ($\mu\text{mol TAN kg}^{-1} \text{h}^{-1}$) of marine catfish *P. lineatus* acclimated to seawater (SW) or brackish water (BW), and either sham operated (Sham) or had the dendritic organ ligated (Ligation). Data are means \pm s.d. (n=5-6). Different letter indicates a significant difference between groups. Two-way analysis of variance (ANOVA) with SNK post-hoc test ($P < 0.05$; see text for details).

6.4 Discussion

From our original observations of respective apical and basolateral Rhag and Rhbg localization in the DO parenchymal cells, we proposed the hypothesis that the DO has a probable physiological role in ammonia excretion. According to the proposed model for ammonia excretion in fish gill, ammonia diffuse into the epithelial cell via basolateral Rhbg and out across the apical membrane via Rhcg1 and/or Rhcg2 (see review Wright and Wood, 2009). The observation of apical localization of Rhag in DO NKA-IR cells in the present study is not very usual, and there are a growing number of reports of this localization pattern (Claiborne et al., 2008; Braun et al., 2009; Chen et al., 2017). Ammonia excretions rates positive correlate with salinity in *P. lineatus*. Unexpectedly, salinity effects on ammonia excretion rates do not correlate with Rh glycoprotein expression levels. In fact an inverse relationship is observed suggesting other ammonia transporters are likely involved. However, in supported of this additional function of the DO in nitrogen balance, we find that DO ligation decreases ammonia excretion.

Interestingly Rh glycoproteins were detected through IHC, but not IB in DO. However, we were able to observe expression of *rhcg1* mRNA in DO that was higher in SW and/or BW compare to HSW thus all together presenting a hypothesis of an extra physiological role for DO in ammonia excretion besides solely salt secreting. Recently, mRNA expression of *rhbg* has been found in rectal gland and gill of spiny dogfish shark; however, the cellular distribution of ammonia transporters is of yet still to be determined (Nawata et al., 2015a).

It has been proposed that the substitution of NH_4^+ and K^+ at transport sites because of similarity in the hydration radius raises the possibility of NKA being involving in ammonia excretion (Randall et al., 1999; Alam and Frankel, 2006; Nawata et al., 2010a). With this in mind, the observation of high expression of NKA α -subunits and NKCC1 in DO in our previous experiment (Chapter 3) makes the DO as probable tissue, which has a potential capacity for ammonia excretion.

The observed apical localization of Rhag and considering $\text{NH}_4^+/\text{Na}^+$ model (see review Wright and Wood, 2009), the presence of an apical V-ATPase pump or Na^+/H^+ exchanger (NHE3) is required to maintain the NH_3 gradient to drive

ammonia efflux. The absence of the detection of apical V-ATPase in gill or DO (see Chapter 3) suggests the alternate mechanism of NHE3 is likely present (Wright and Wood, 2009; Ip and Chew 2010). To date, we have been unable to confirm this by IHC. On the other hand, the role of CFTR in anion (HCO_3^- , Cl^-) excretion in gill ionocytes of *Anabas testudineus* but not NHE3 has been proposed (Ip et al., 2012b; Chen et al., 2017). Observations of CFTR/*cftr* in *P. lineatus* gills and DO (see Chapter 3) thus presents the possibility of this mechanism being involved in a similar process. However, functional evidence is required to substantiate this hypothesis.

Furthermore, Rh glycoproteins were identified also in CO_2 movement (Perry et al., 2010). The possible dual role in Rhag expression of erythrocytes responding differentially to high CO_2 and ammonia has been suggested (Nawata and Wood, 2008; Nawata et al., 2010). Rhag expression is present in red blood cells and lamellar pillar cells in *P. lineatus* and other fishes (Nakada et al., 2007b; Wood et al., 2013) contributing to the facilitated diffusion of NH_3 and/or CO_2 .

Observations of Rhag protein in epithelial cells of kidney tubules including PTI, PTII and CT in all of salinities may represent renal tubule involvement in ammonia regulation in marine catfish. Recently expression of Rh glycoproteins have been shown in kidney of zebrafish, *Danio rerio* (Nakada et al., 2007), mangrove rivulus, *Kryptolebias marmoratus* (Cooper et al., 2013) and common carp, *Cyprinus carpio* (Wright et al., 2014). Apical localization of Rhag and CFTR (see Chapter 3) in PTI in contrast to the basolateral localization of both in PTII and/or CT, and the detection of mRNA expression of *cftr* and *slc26a6a* (see chapter 3) all together suggest the involvement of kidney renal tubules in ion secreting and/or absorption, acid-base and ammonia regulatory (see more details in Chapter 3).

The results of ligation experiment in both salinities regarding the interaction between acclimation salinity and ligation reveals the effect on J_{amm} particularly in SW thus confirmed proposed role of DO in ammonia excretion. Considering SW, the observed higher J_{amm} in sham operated fish at SW-CL may be due to recovery times of fish that could be affected by the stress of ligation, temperature differences of anesthesia and recovery water (Aguiar et al., 2002; Stehly and Gingerich, 1999) or drug dosage (Hseu et al., 1997, 1998; Ross, 2001).

Salinity dependence of ammonia excretion appears species dependent as trout, catfish and goldfish showed lower J_{amm} with higher salinity while the opposite is observed in sturgeon and striped bass (Altinok and Grizzle, 2004; Medeiros et al., 2015). Although Wood and Nawata (2011) have observed the inverse relationship in trout (SW>FW). However, significant relationships between salinity and toxicity have not been observed in fishes (Randall and Tsui, 2002; Wood, 1993). In our experimental observations there was lower J_{Amm} of fish in BW accompanied paradoxically by higher Rh glycoprotein expression in gills. The decrease and increase permeability of NH_4^+ and NH_3 , respectively has been reported in toad fish acclimated to low salinity (5‰) compared to SW (Evans et al., 1989). Thus Rh glycoproteins that function as NH_3 may have a more dominate role at lower ionic strengths whereas NH_4^+ transport, possibly linked by NHE3 (Ito et al. 2014) dominated at higher salinities. In addition, there is likely an accumulation of ammonia (T_{Amm}) inside the body presuming in blood plasma indicating a metabolic acidosis. Similar results of low ammonia excretion rates and plasma ammonia accumulation have been reported in European sea bass at BW (Sinha et al., 2015). Conversely in HSW higher J_{amm} versus the observed lower protein or mRNA expression of Rh glycoproteins in gills and DO, respectively might further reflected the dominance of NH_4^+ (NHE3) versus NH_3 (Rh glycoproteins) transport mechanisms.

Observing higher Rhag protein expression in gill at BW or BW-L may express associated role of Na^+ uptake via the gill, according $\text{Na}^+/\text{NH}_4^+$ exchange complex model via NHE3 (Tsui et al., 2009; Wright and Wood, 2009). According to the ionoregulatory hypothesis (Zimmer et al., 2014) ontogeny of branchial Na^+ uptake has been proposed which can occur as ammonium excretion potentially in trout to eliminate of lethal metabolic ammonia. Furthermore, increased mRNA expression either in *cftr* or *ca* at BW-L in our previous experiment, may thus indicate a role of CFTR in anion (HCO_3^- , Cl^-) excretion (Ip et al., 2012b; Chen et al., 2017). However, the distribution of IR-NKA cells with apical localization of Rhag showed no salinity and/or ligation dependency in gills. The *in vivo* investigation of J_{Amm} separately in gill, kidney and intestine would be interested in future works (Zimmer et al., 2014).

Lack of apical and/or basolateral localization of Rhag in PTI and/or PTII, respectively at BW-L fish, which was converse to other groups, may express effect of the ligation on ammonia excretion capacity of kidney that needs more details regarding the involving of kidney in ammonia excretion particularly in ligated fish. Furthermore, the observed apical Rhag expression in entocytes of posterior intestine at SW-CL fish may be attributed to the Na⁺ uptake of intestine according Na⁺/NH₄⁺ exchange complex model as a metabolon (Tsui et al., 2009; Wright and Wood, 2009) and reveal involvement of the intestine in ammonia excretion in ligated fish as a compensatory response. Furthermore, possibility of intestine involving in ammonia excretion has reported (Chew et al., 2009, 2010; Wilson et al., 2013).

7. General Discussion:

Overall, the work presented in this thesis reveals more of the fascinating biology and physiology of marine catfish of the Plotosidae family that is unique amongst the teleosts in possessing the DO, with particular focus on osmoregulation. The studies presented here address the effect of different salinities on marine catfish osmoregulation (CH3); effect of dendritic organ ligation and interaction with different salinities (CH4); molecular characterization of the unique kidney (CH5); and address the potential role of the DO of marine catfish as an ammonia excretory organ (CH6).

7.1 The dendritic organ and ion regulation

The gill is known to be the main site of ionoregulation in fishes; however, the presentation of our molecular observations including higher DO NKA activity compare to other tissues, basolateral colocalization of NKA and NKCC1, and the apical localization of CFTR confirm previous hypothesis that the DO has a salt secreting function based on indirect physiological, ecological and ultrastructural evidence (Kowarsky, 1973; Lanzing, 1967; Van Lennep, 1968, Pucke and Umminger, 1979). Our results also support the hypothesis of a conservation of rather similar mechanism of secondary active Cl⁻ ion transport in secretory cell of vertebrates (Hazard, 1999; Evans, 2009; Babonis et al., 2009, 2011). We found two types of parenchymal cells one large ovoid to pear-shaped showed strong NKA and/or NKCC1 immunoreactivity throughout the cytoplasm, and a second smaller subpopulation of cells that are more angular in shape and have noticeably stronger NKA-IR and lack NKCC-IR which confirmed characterization of two types of glandular cells, the principal cell (PC) and clear cell (CC), respectively reported by Van Lennep and Lanzing (1967) based on TEM observations. However, interpretation of the special role of clear cell needs more work. Furthermore, the finding of protein and IHC expression of carbonic anhydrase, V-ATPase, and mRNA expression of *slc26a6a* suggest that the DO may also be involved in acid-base regulation. However, more work is needed to clarify this role of the DO.

Plotosidae marine catfish can be found in a wide range of saline environments from hypersaline to brackish water throughout the Indo-Pacific (Lanzing 1967). At HSW, elevated plasma osmolality and/or ions, decreased survival of the fish and muscle dehydration all together indicate a systemic dehydration due to water loss by osmosis, and disturbances from internal fluid shift. This is problematic for the fish, resulting in a stress situation particularly in DO as indicated by elevated stress protein Hsp70 levels. Furthermore, the data of DO mass and/or expression of NKA activity relative to fish body mass suggest increasing of overall capacity to overcome the ionoregulatory challenge of hypersalinity.

However, BW acclimation represents the plasticity/capacity of marine catfish to easily movement between various salinities as euryhaline species in the lower end of the salinity spectrum. We found indirectly that fish had experience of metabolic acidosis predicted from a lower Na^+/Cl^- ratio (SIR) (Cameron and Iwama 1989) as has been observed in European sea bass *D. labrax*, reared in lower salinity (Sinha et al., 2014). However, this contrasts with work by Jensen et al. (1998) who have reported a markedly increased plasma SIR following transfer to FW and slight decrease in HSW in *D. labrax* indicative of metabolic alkalosis and acidosis, respectively.

The shifting to a secondary role of the gills of *P. lineatus* in osmoregulation due to the presence of the DO as the primary salt secreting organ represents similar relationship between the gills and rectal salt gland of elasmobranchs (Burger and Hess, 1960; Kirschner 1980; Wilson et al., 2002). Furthermore, branchial ionocytes that are present may be primarily involved in acid-base regulation similar to elasmobranchs gill, presented by two subpopulations of MRCs serving in base (bicarbonate) and acid excretion in the gills have reported in sharks (Piermarini et al. 2001; Choe et al. 2005; Tresguerres et al. 2007; Cramp et al., 2015). In the case of *P. lineatus* basolateral VHA rich ionocytes have been identified which may possess an apical $\text{Cl}^-/\text{HCO}_3^-$ exchanger for base secretion as in elasmobranchs.

Since the DO is external organ, ligation was an effective non-invasive way to examine loss of function. It provided valuable evidence about the importance of the DO as well as the compensatory responses of the other osmoregulatory organs (gill, kidney and intestine). We found that the ligation of SW acclimated fish increased ions and osmolality of plasma while it has negative effect on survival, MWC and generally osmoregulatory capacity of fish. Notably fish could not survive without the DO in HSW. From the loss of the DO through ligation, a compensatory response from the other osmoregulatory organs was predicted. However, ligation in SW did not alter gill or kidney NKA expression while, a decrease and increase were observed in anterior or posterior intestine, respectively. In general, the intestine was also the most responsive in ion transporter expression indicators but the patterns of change indicated a complex response that will require measurements of drink rates and ion and water flux rates to interpret properly. Taken together, it seems due to DO ligation, is problematic resulting in a stress situation thus osmotic disturbance thereby indicating to main role of DO in salt secreting and the compensatory responses of gills, kidney and/or intestine in SW-CL could work for short period however, they were not sufficient for continued survival of fish while, in BW where the demands for osmoregulation differed, survival was not negatively impacted. It follows that the species of Plotosidae catfishes without a DO are more commonly associated with freshwater (Lanzing 1967).

7.2 Renal mechanisms for hyperosmotic urine formation

Earlier observations by Kowarsky (1973) demonstrated that the Plotosidae catfish could produce hyperosmotic urine in contrast to typical marine teleost fishes (MacDonald 2007) although the mechanism has not been addressed. The finding of renal chloride cells in the collecting tubule (CT) and collecting duct (CD) that highly express NKA and NKCC1 indicate a strong NaCl secretory function in this segment. Our result, showed in HSW kidney NKA activity and relative protein expression increased which might indicate to providing much driving force to increase water reabsorption thus a decrease in urine

production and/or to ion transport. IB result showed no detectable change in CA and V-ATPase while *ca* mRNA expression showed an increase. In BW, marine catfish can be involved in Cl⁻ and HCO₃⁻ regulation due to observation of increased level of *cfr* and *slc26a6a* mRNA expression.

According IHC results PTI of nephron worked as either ions absorptive or secretory segment while it was only ion secreting in PTII. Interestingly, DT might not be involved in ion absorption as diluting segment which has reported in various species (Marshall and Grosell, 2006; Evan, 2008). Detecting high protein expression (IB) and/or colocalization of NKA α -subunits and NKCC1 throughout the cytoplasm of CT/ CD in all of salinities might be attributed to produce unusual hyperosmotic urine. The potential role of kidney segments in acid-base regulation except DT may be predictable. Taken together, it seems the kidney of marine catfish has a physiological effective role in ion/osmo regulation particularly in HSW environment. In addition, nephron glomerulus space and DTa lumen size increased in BW-L suggesting higher glomerular filtration rates and in SW-CL the opposite changes all together indicating water transport adaptation of ligated fish.

Characteristics of nephron in marine catfish kidney, which might be involved in formation of hyperosmotic urine:

i) Possessing confirmed MRCs throughout the cytoplasm of CT which can have main role in salt secreting thus result in formation of hyperposmotic urine. The latter needs to be confirmed by measurement of the urine formation activity in future work.

ii) Possessing unique DT feature by showing DTa which, showed basolateral localization of NKA and/or NKCC. The latter is according the accepted model of salt secreting epithelial cells. Moreover, the absence of the basolateral interdigitation, detecting short infoldings of the lateral/basal cell membranes which were greatly reduced in the DTa (Hentschel and Mayer, 1987), all together may express permeability to the water leading

to increase the concentration of the urine. In summary, function of salt secreting instead being diluting segment as evidenced in other teleosts (Beyenbach, 2004).

iii) AB-PAS Showed light apical blue staining (acidic mucus) in DTa and more obvious in CT. Furthermore, representing blue mucus cells particularly in HSW which may express the acidic (potentially sulphate) mucus secreting.

iv) (Endo, 1989) reported a specific glomerular vasculature of *P. lineatus* by showing a thick afferent arteriole that may have role as a filtration barrier and in contrary exceptionally two thin efferent arterioles. Former and the latter can cause the high filtration pressure in glomerulus of the capillaries (Guyton, 1986) which may serve as reduced glomerular filtration thus possibly decrease urine volume in SW and/or HSW thereby if the divalent ion secretion and/or water reabsorption occur in the different segments of the marine catfish kidney nephron all together may be involved in formation of hyperosmotic urine.

Regarding the production of hyperosmotic urine compare to the blood which observed in *Cnidogobius*, Hickman and Trump (1969) proposed an explanation which might be involved (i) "secretion of divalent ions" thus apical and basolateral expression of *slc26a6a* in CT and also in a separate cell in SW-control of marine catfish may express role of the CT in Cl^- and HCO_3^- (SO_4^{2-}) regulation in our study and also blue mucus cells probably role in SO_4^{2-} secretion might be accordingly. (ii) "Reduced volume of glomerular filtrate" which possible reduced glomerular filtration due to a specific glomerular vasculature of *P. lineatus* (Endo, 1989) thus decrease urine volume might be homogenous processes. (iii) "High tubular impermeability to water" which can be confirmed via unique structural feature of DT and DTa in *Plotosus* (Hentschel and Elgar, 1987) and our molecular observation results. (iv) "possibility of Na^+ secretion into the tubules" which confirmed via structural observation of high MRCs in CT (Hentschel and Elgar, 1987) and potential role in producing local osmotic area (Perry, 2011) to water absorption, moreover our

molecular observation proposing salt secreting of CT due to high protein expression and localization of NKA and/or NKCC.

Measurement urine Na⁺ and osmolality in BW or SW of *Cnidoglanis* by Kowaresky (1973) showed at 4 and 11‰ both urine Na⁺ and osmolality were lower than plasma however by the exceed salinity over 20 ‰ the concentration of Na⁺ and osmolality was higher. The latter means by increasing of salinity higher amount of Na⁺ and other ions possibly divalent could be found in marine catfish urine. It seems measurement of urine different ions concentration such as Na⁺, Cl⁻, K⁺, Ca²⁺, Mg²⁺ and SO₄²⁻ can be interested in future work to have more clear interpretation of producing hyperosmotic urine by marine catfish.

7.3 Role of the DO in ammonia excretion?

Gills are accepted as the main site of ammonia excretion in fishes (Wright and Wood, 2009); however, in the present work we demonstrated that DO of marine catfish *P. lineatus* may have an extra physiological role in ammonia excretion. The latter hypothesis was supported by the observation of apical and subapical expression of the ammonia transporters Rhag and Rhbg, respectively in DO parenchymal acini by IHC, expression of *rhcg1* mRNA. Although Rhag is generally associated with RBCs and endothelial (pillar) cells, the observation of an apical localization of Rhag in NKA-IR cells is observed in the gills of *P. lineatus* as well as other species (Chen et al. 2016) does not make this localization in DO too surprising. Apical or basolateral localization of Rhag in PTI and II, respectively except HSW and BW-L, moreover throughout the cytoplasm of CT in kidney nephron in all of salinities and apical staining of Rhag in posterior intestine at SW-CL fish all together presented the involving of kidney and posterior intestine in ammonia regulation even in ligated fish. IB of Rhag and Rhbg was found only in the gills with less vs high expression at HSW and BW, respectively not for Rhbg. The interaction between salinity and ligation was detected in Rhag associated with lower Rhag levels it

may reveal adaptation mechanism to handle the eliminating of excess ammonia. The expression of *rhcg1* mRNA was detected only in the gills and DO via lower expression with salinity changing in the latter while no dependency to salinity and/or ligation interaction was found in gills. Heterologous Rhcg antibodies did not work in *P. lineatus* so for future work species specific antibodies need to be develop.

7.4. Origin of the dendritic organ

Overall, this thesis contributes not only to an increase our knowledge regarding basic osmo and/or salt regulation mechanism in the unique marine catfish but will also offer us some insight into the evolution of salt regulatory mechanisms under different circumstances. Thus, it can also be applied to develop a link to converge evolution with the tetrapod lineage in addition to the unusual production of blood-hyperosmotic urine in the kidney of this vertebrate. Conserved form and function of salt gland throughout the evolution of marine vertebrates suggesting the conservation of the genetic mechanism leading to the development of this tissue type (Babonis et al., 2009). Thus, co-option from unspecialized gland precursors as reported in other analogous salt glands may have been revealed by studying this system

Compared to the salt secreting organs of other vertebrates, the dendritic organ is different because it is external and it is unknown what gland or organ may have been coopted. The DO is juxtaposed to the urogenital papilla so it might therefore have arisen from skin, the digestive tract (rectal tissue), urinary system or reproductive system. The latter seems unlikely even though glandular tissue may be present because the DO is present in both males and females. Skin is a possibility because cutaneous ionocytes have similar characteristics of DO parenchymal cells (high NKA, NKCC, CFTR) (Cooper et al. 2013; Hiroi et al. 2008). Teleost digestive tract is aglandular with the exception of the stomach (Wilson and Castro 2010) which differs from the chondrichthyan fishes from which the rectal gland is derived (Loretz 1987). It is also tempting to speculate that the

renal system may have given rise to the DO since renal chloride cells show striking similarities to DO parenchymal cells (Henteschel and Elger 1987; Chapter 5).

7.5. Final statements and future directions

Marine catfish have a specialized salt secreting organ, the DO, that is marked by profound differences in their morphology and physiology that evolved independently from other teleost lineages. The studies in this thesis illustrate how these mechanisms respond during salinity acclimation and/or DO ligation, although the control mechanisms that regulate various osmoregulatory responses, allowing movement between environments has not been address and clearly research needs to be done in this area. The DO is also present in Plotosidae that are found only in fresh water (Lazing 1967). It would enlightening to explore the function of the DO in these fishes where ion secretion is not necessary. Is it a vestigial organ like the rectal gland is in some freshwater elasmobranch fishes (Ballantyne and Robinson 2010)? Might it have taken up a secondary function like ammonia excretion?

In order to better understand the mechanisms that promote these changes, future studies are needed to assess endocrine control of marine catfish osmoregulatory mechanisms and to better understand the potential role of NKCC/NCC, CA, V-ATPase, Na⁺/H⁺ exchanger, Cl⁻/HCO₃⁻ exchanger, Na⁺/HCO₃⁻ cotransporter (NBC) proteins in ion and acid-base regulation between salinities. The work on the marine catfish juveniles allowed the first insights into the osmoregulatory challenges and mechanisms inherent to adaptation. The nervous system control of secretion and abundant neurotransmitter vasoactive intestinal peptide (VIP) in salt gland of crocodilians, birds and elasmobranches rectal gland have been proposed (Cramp et al., 2007; Hildebrandt, 2001). Detecting such neurotransmitters and the phosphorylation state of CFTR, NKA and NKCC proteins (Babonins et al., 2011) in DO of marine catfish at different salinities would be interesting in future work. Furthermore, the measurement of urine ions and osmolality in different salinities, the molecular responses of marine catfish gills, DO,

kidney and/or intestine to acid-base perturbation, respiratory acidosis (hypercapnia 1%CO₂) and/or hypoxia in order to elucidate regulatory function are also needed.

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