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Osmoregulation in the sea lamprey, *Petromyzon marinus*

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Author's declaration

In agreement with the Portuguese law though 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

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Ferreira-Martins D, McCormick SD, Campos A, Lopes-Marques M, Osório H, Coimbra J, Castro LFC, Wilson JM (2016) A cytosolic carbonic anhydrase molecular switch occurs in the gills of metamorphic sea lamprey. Sci Rep (*in press*).

Ferreira-Martins D, McCormick SD, Coimbra J, Wilson JM. Parasite-host interactions: osmoregulatory challenges of sea lamprey, *Petromyzon marinus* parasitic feeding on the trout *Salvelinus fontinalis*. (*in preparation*).

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Thesis Aims

The present Doctoral Thesis is focused on “Osmoregulation in the sea lamprey *Petromyzon marinus”.*

For the thesis I address the hypotheses that lampreys evolved similar ion and acid-base regulatory mechanisms as the teleost fishes and feeding in juvenile lampreys has significant osmoregulatory impacts on both lamprey and host. To assess these hypotheses the main objectives proposed are: the identification of lamprey homologues of ion transport proteins; assessment of ion transport protein expression in the context of the osmoregulatory responses of sea lamprey at a number of important points in the lifecycle: freshwater ammocoete larvae, transformers (metamorphic larvae), marine juveniles, and marine and freshwater migrant adults in their spawning run; and to determine the osmoregulatory impact of feeding on both parasite and host. The repertoire of cytoplasmic carbonic anhydrases in the sea lamprey was also investigated where the hypothesis that metamorphosis, which marks a dramatic change in the life style and physiology of sea lamprey, requires changes in cytosolic carbonic anhydrase expression, was tested.

Dissertation Organization

The Doctoral Thesis is organized into five chapters. Chapter one consists on a general introduction where background on sea lamprey will be provided: the general biology of the sea lamprey; a perspective on sea lamprey and evolution of vertebrates; osmoregulatory mechanisms in seawater and freshwater; the effects of feeding on osmoregulation in the parasite and host; the ecological significance; why do research on sea lamprey?; and the cultural significance of sea lamprey. Chapters two, three and four correspond to the experimental work and are structured as independent papers that have or will give rise to separate scientific publications. Within this dissertation, they are free standing, which may lead to a certain degree of repetition throughout the thesis. They are organized according to sea lamprey's life cycle, from ammocoete and through metamorphosis into post-metamorphic juveniles, downstream migration to seawater and initiation of parasitic feeding and finally the upstream spawning migration. In these chapters, key ion transport mechanisms that allow sea lamprey to osmoregulate in their environment at a given life stage are studied at the gene and protein expression levels in addition to other factor such as ion content in plasma, biometric data, cellular and metabolic stress markers, ion transport enzymatic activity, and protein localization.

Tables and figures are numbered sequentially in each chapter with the chapter number integrated in the numbering system. Chapter five consists of an integration of the main results from this thesis that are summarized and analyzed in the overall context of sea lamprey biology and osmoregulation. The bibliography is consolidated at the end of the thesis.

Annexed to the end of this thesis my *Curriculum Vitae* is provided.

General Abstract

The sea lamprey is a living representative of the basal vertebrate lineage and its anadromous populations have a complex life cycle marked by a dramatic metamorphosis from a benthic filter-feeding larvae (ammocoete) into a parasitic juvenile which migrates from freshwater to seawater. After a period that can last up to 2 years, the adults migrate upstream in search of a place in which to spawn. The osmoregulatory challenges sea lampreys face during its migrations are similar to those of most euryhaline fishes, with mechanisms to cope with environmental salinity changes analogous to those of teleost fishes. Nevertheless, some particularities occur at the ultrastructural level and distribution of lamprey epithelial cell types. As it was already mentioned, when in seawater, the sea lamprey adopts a parasitic feeding strategy, where it feeds on the host's body fluids that are generally hypo-tonic compared to the external environment. This in its turn poses another osmoregulatory challenge to the parasite.

The aims of this Ph.D thesis are to provide new insights into the ion and acid-base regulatory mechanisms in sea lamprey and compare them to those of teleost fishes as well as provide the first insights on the molecular osmoregulatory mechanisms of sea lampreys to cope with ion fluctuation due to parasitic feeding. For this we focused on key ion transport proteins [Na^+/K^+-ATPase (NKA), H^+-ATPase (VHA), $Na^+:K^+:2Cl^-$ cotransporter (NKCC), $Na^+:Cl^-$ (NCC), epithelial Na^+ channel (ENaC) and carbonic anhydrase (CA)] during development (metamorphosis), and in response to salinity challenge and feeding.

CAs are important for ion and acid-base regulation by providing an intracellular pool of H^+ and HCO_3^- from CO_2 hydration for exchange with Na^+ and Cl^-, respectively. In addition, it aids metabolic processes and acid-base regulation of individual gill cells that have a high metabolism and generate excessive CO_2 levels. In this thesis a novel CA isoform in sea lamprey (*ca19*) has been identified which is found to be more close related to cytosolic CA isoforms. Results based on the studies on metamorphosis of sea lamprey show for the first time a molecular switch between CA isoforms. Quantitative polymerase chain reaction (PCR) of *ca19* and *ca18* shows that the novel *ca19* levels are highest in ammocoetes and decrease during the seven stages of metamorphosis while *ca18* shows the opposite pattern with the highest levels in post-metamorphic juveniles. Proteomic analysis by immunoblotting using an heterologous CA antibody and two-dimensional electrophoresis (2DE) together with MALDI-TOF/TOF analysis and protein identification approaches corroborated with transcriptomic results. Phylogenetic analysis and synteny studies indicate that both CA genes form one or two independent gene lineages and are

most likely duplicates retained in cyclostomes and were instrumental in the elaboration of CA diversity in vertebrates.

The study on post-metamorphic juveniles, in confirmation of previous reports, demonstrated that sea lamprey were able to withstand a transfer to seawater by down regulating ion uptake mechanisms (NCC, ENaC and V-ATPase E-subunit mRNA expression and kidney NKA activity). Our hypothesis was that feeding on a fairly isosmotic meal relative to its internal milieu, sea lampreys osmoregulation in seawater would be facilitated. Results upon parasitic feeding in seawater demonstrated a decrease in [Cl^-], [K^+] and [Ca^{2+}] levels in plasma and down regulation of branchial NKAα1 and NKCC1 transcript levels, suggesting that a meal that is hyposmotic compared to the external environment compensates for osmotic ion gain through the gills and skin. Increased NKA activity in the anterior intestine indicates a role in nutrient absorption. Heat shock protein 70 (Hsp70) expression was downregulated in the gills of feeding lampreys, which indicates reduction of cellular stress, suggesting habituation to the new life stage set point. The host species (brook trout *Salvelinus fontinalis*) successfully acclimated to seawater. In the hosts 23 attacks were registered, from which ~78% pierced the skin and 17% of those attacks resulted in host death in less than 3 days. Upon parasitic feeding hosts were unable to maintain stable plasma ion levels that increased. Expression of mRNA of NKAα1a in seawater acclimatized non-parasitized hosts was found to decrease as it is no longer necessary for ion uptake, though in the seawater parasitized ones, levels of expression remained similar to those found in freshwater. Based on these results and reduction of blood volume due to parasitic feeding, it is hypothesized that this might be related to cell volume maintenance. Finally, hosts demonstrated loss of appetite when parasitized which contributed to loss of weight, slow growth in length and low condition (K) factor.

Results on upstream spawning adults show that the capacity to restore hypo-osmoregulatory mechanisms is limited and suggested to be related with time spent in contact with freshwater during the upstream spawning run. Upper salinity thresholds of 25 and 17.5 for one week (short term) or two months (long term), respectively, of freshwater acclimation were found. Nonetheless, all salinity challenged groups down regulated FW ion uptake mechanisms (gill transcripts of NCC/*slc12a3* and ENaC/*scnn1* and kidney NKA protein and activity but not transcript). When they reached their salinity limits, lampreys were unable to regulate [Na^+] and [Cl^-] in plasma and intestinal fluid within physiological limits, becoming osmocompromised. Severe anemia was observed (>90% drop in hematocrit) as result of hemolysis. High cytosolic enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST) and LDH in plasma indicated damage to other tissues including liver. Yet, >80% of the short term FW acclimated lampreys did

osmoregulate efficiently with less hemolysis and tissue damage. This osmoregulatory ability correlated with significant up regulation of NKCC1/*slc12a2* transcript levels and the re-emergence of seawater-type ionocytes detected through immunohistochemical NKA-immunoreactivity in gill, the central ionoregulatory organ.

In summary it is proposed in this thesis that loss of the novel ammocoete isoform *ca19* is associated with the loss of the enigmatic ammocoete mitochondrion-rich cells (MRC) and that the increase in *ca18* is triggered by metamorphosis as preparation for the higher activity marine trophic phase of this species lifecycle. We also propose that feeding on an isosmotic meal while in seawater facilitates sea lamprey's osmoregulation in a hypertonic environment. In addition, sea lamprey's parasitic feeding has deep impacts on the host, which adjusts osmoregulatory mechanisms to cope with ion imbalance resulting from body fluid depletion. In the worst case scenarios, severe anemia occurs and hosts die as a direct effect of parasitic feeding. As for the upstream spawning migrating sea lamprey, the work described in this thesis elucidates the molecular and physiological limits during this life stage and the potential return to at least brackishwater in order to search for alternative FW systems in which to spawn.

Resumo Geral

A lampreia marinha é um representante vivo da linhagem mais ancestral dos vertebrados e a sua população anadrómica tem um ciclo de vida complexo, caracterizado por uma metamorfose profunda desde uma larva (amocete) bentónica que se alimenta por filtração, até um juvenil parasita que migra da água doce para a água salgada. Passado um período que pode atingir 2 anos, a lampreia já adulta retorna aos sistemas de água doce e migra para montante em busca de um local apropriado para desovar. Os desafios em termos de osmorregulação que a lampreia marinha enfrenta durante as suas migrações são semelhantes aos da maioria dos peixes eurihalinos, com mecanismos para lidar com alterações de salinidade no ambiente análogos aos dos peixes teleósteos. Contudo algumas diferenças ocorrem ao nível da ultraestrutura e da distribuição de alguns tipos se células epiteliais de lampreia. Como já mencionado, quando presente em água do mar, a lampreia marinha adota uma estratégia de alimentação com base no parasitismo, alimentando-se dos fluidos corporais do hospedeiro que são geralmente hipotónicos em relação ao ambiente exterior. Desta forma surge como mais um desafio à osmorregulação do parasita.

O âmbito desta tese de doutoramento é providenciar novos conhecimentos acerca dos mecanismos de regulação de iões e balanço ácido-base na lampreia marinha tendo como padrão de comparação os peixes teleósteos, assim como proporcionar as primeiras impressões acerca dos mecanismos moleculares de osmorregulação para lidar com possíveis variações de iões devido ao estilo de alimentação parasítica. Para este efeito, focou-se em proteínas com função chave no transporte de iões [Na^+/K^+-ATPase (NKA), H^+-ATPase (VHA), cotransportador $Na^+:K^+:2Cl^-$ (NKCC), cotransportador $Na^+:Cl^-$ (NCC) canal Na^+ epitelial (ENaC) e anidrase carbónica (CA)] durante o desenvolvimento (metamorfose) e em resposta a testes de salinidade e alimentação.

As CAs são importantes do ponto de vista de regulação de iões e balanços ácido-base, fornecendo uma reserva de H^+ e HCO_3^- provenientes da reação de hidratação do CO_2 para as trocas de Na^+ e Cl^-, respetivamente. Além disso, contribui para regulação de processos metabólicos e balanções ácido-base em células individuais da brânquia que apresentam uma metabolismo elevado e geram elevados níveis de CO_2. Nos estudos patentes nesta tese, foi identificada uma nova isoforma de CA em lampreia marinha (*ca19*) que se assemelha mais com as isoformas citosólicas de CA. Os resultados obtidos baseados em estudos sobre a metamorfose das lampreias marinha demonstram pela primeira vez e de um ponto de vista molecular, uma troca entre isoformas de CA. Reação em cadeia da polimerase (PCR) quantitativas efetuadas em *ca19* e *ca18* apontam para maiores níveis de expressão de *ca19* nos amocetes, os quais decrescem no decorrer das

sete etapas de metamorfose e enquanto que a *ca18* demonstra um padrão contrário, com maiores níveis de expressão encontrados em juvenis pós-metamorfose. Análises de proteómica como immunoblotting com anticorpos heterólogos de CA e eletroforese bidimensional (2DE), juntamente com técnicas de MALDI-TOF/TOF e identificação de proteínas corroboraram com os resultados a nível da transcrição. Estudos elaborados sobre a filogenética e sintenia apontam para que ambos os genes de CA formam uma ou duas linhagens independentes de genes que são provavelmente duplicados que foram mantidos nos ciclóstomos e foram essenciais na diversificação de CAs nos vertebrados.

Os estudos elaborados em juvenis pós-metamórficos em acordo com o que estava anteriormente demonstrado, apresentam capacidade para tolerar uma transferência direta para água salgada diminuindo a expressão de mecanismos de captação de iões (expressão do mRNA de NCC, ENaC e V-ATPase subunidade-E e atividade de NKA no rim). A hipótese aqui apresentada é que a alimentação com base numa refeição isosmótica relativamente ao meio interno, facilita o processo osmorregulação na lampreia marinha quando presente em água salgada. Os resultados obtidos em lampreias alimentando-se de forma parasítica demonstraram um decréscimo nos níveis de $[Cl^-]$, $[K^+]$ e $[Ca^{2+}]$ no plasma e diminuição da transcrição de NKAα1 e NKCC1, o que sugere que uma refeição hiposmótica em relação ao ambiente externo contribui com uma compensação para o ganho de iões por osmose através das branquias e do revestimento cutâneo. Níveis elevados na atividade de NKA na região anterior do intestino, sugerem função na absorção de nutrientes. A expressão das proteínas de choque térmico 70 (Hsp70) foi diminuída nas lampreias que se alimentaram, o que poderá indicar redução do stress ao nível da célula o que por sua vez sugere uma habituação ao novo estilo de vida imposto. Em termos dos efeitos na espécie hospedeira (truta-dos-ribeiros *Salvelinus fontinalis*), esta aclimatou-se com sucesso à água salgada. Dos 23 ataques registados durante o período experimental, ~78% perfuraram o revestimento cutâneo do hospedeiro e desses, apenas 17% originaram a morte do hospedeiro em menos de três dias. Quando parasitados, os hospedeiros foram incapazes de manter estáveis os níveis de iões, os quais subiram. A expressão de mRNA de NKAα1a nos hospedeiros aclimatados a água salgada e não parasitados diminuiu uma vez que já não é necessária para captação de iões do meio externo. Contudo em hospedeiros aclimatados a água salgada e parasitados, os níveis de expressão ao nível do mRNA deste gene mantiveram-se semelhantes aos valores de expressão obtidos em água doce. Tendo em conta estes resultados e na redução do volume de sangue no hospedeiro como resultado de parasitismo, é colocado em hipótese que estará relacionado com a manutenção do volume celular. Em conclusão, os hospedeiros quando

parasitados, registaram perda de apetite o que levou a uma redução de peso, crescimento lento a nível do comprimento e fator de condição (K) diminuído.

Relativamente aos resultados dos estudos efetuados nos adultos durante a migração de reprodução para montante, a sua capacidade de recuperar os mecanismos de hipo-osmorregulação demonstrou-se limitada e apontada como dependente do tempo de contacto com a água doce durante a migração. Os limites de salinidade encontrados foram de 25 e 17.5 para uma semana (período curto de aclimatação) e dois meses (período longo de aclimatação), respetivamente. Porém todos os grupos submetidos a um teste de salinidade demonstraram uma redução dos mecanismos de captação de iões típicos de água doce (transcritos branquiais de Na^+:Cl^-, NCC/*slc12a3*, ENaC/*scnn1* e NKA no rim, a nível proteico e de atividade mas não do transcriptoma). Quando atingidos os limites de salinidade tolerados, as lampreias marinhas foram ineficientes na regulação da [Na^+] e [Cl^-] no plasma e no fluido intestinal dentro dos limites fisiológicos, ficando assim osmo-comprometidos. Níveis críticos de anemia foram observados (redução >90% do hematócrito) como resultado de um processo de hemólise. Elevados níveis de enzimas citosólicas alanino aminotransferase (ALT), aspartate aminotransferase (AST) e lactato desidrogenase (LDH) no plasma indicam danos em vários tecidos incluindo no fígado. Contudo >80% das lampreias com uma aclimatação de curta duração em água doce foram capazes de osmorregular eficazmente com menor hemólise e danos a nível dos tecidos. Esta capacidade de osmorregular mostrou-se relacionada com o aumento dos níveis de expressão do transcrito de NKCC1/*slc12a2* e o reaparecimento dos ionócitos de água salgada que foram detetados através de imuno-histoquímica em células imunorreativas à NKA na brânquia, órgão este principal na osmorregulação.

Em forma de resumo, é proposto nesta tese que a perda da nova detetada isoforma *ca19* está associada com o desaparecimento das enigmáticas células ricas em mitocôndrias típicas dos amocetes (MRC) e que o aumento da expressão de *ca18* é despoletado pelo processo de metamorfose como forma de preparação para a etapa trófica marinha (com elevada atividade) do ciclo de vida desta espécie. É ainda aqui proposto que uma alimentação isotónica quando em água salgada, facilita o processo de osmorregulação da mesma num meio hipertónico. Além do já referido, a alimentação parasítica da lampreia marinha apresenta ainda profundos impactos no seu hospedeiro, o qual demonstrou capacidade para reajustar os seus mecanismos de osmorregulação para lidar com o desequilíbrio iónico causado pela perda de fluidos internos. No pior dos casos, ocorre uma anemia grave no hospedeiro que morre como efeito direto do parasitismo. Relativamente à migração para montante para reprodução por parte dos adultos de lampreia marinha, o trabalho aqui presente nesta tese elucida para os limites fisiológicos do ponto de vista molecular durante esta etapa to ciclo de vida e o potencial

para retornar para o oceano ou estuário de forma a procurar novos sistemas de água doce alternativos onde desovar.

List of Abbreviations

2DE	Two dimensional electrophoresis
11-DOC	11-deoxycortisol
18S	18S Ribosomal subunit
28S	28S Ribosomal subunit
aa	Amino acid
AC	Accessory cell
ADP	Adenosine diphosphate
AE	Anion exchanger
ALT/GPT	Alanine aminotransferase
ASIC	Acid-sensing ion channel
AST/GOT	Aspartate aminotransferase
ATP	Adenosine-5'-triphosphate
BW	Brackishwater
CA	Carbonic anhydrase
Ca^{2+}	Calcium ion
CAc	Cytosolic carbonic anhydrase
CC	Chloride cell
cDNA	Complementary DNA
CFTR	Cystic fibrosis transmembrane conductance regulator
CI	Confidence interval
Cl^-	Chloride ion
ClC	Chloride channel
CO_2	Carbon dioxide
CR	Corticosteroid receptor
CT	Cycle threshold
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
ENaC	Epithelial sodium channel
FW	Freshwater
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

H^+	Hydrogen ion
HCO_3	Bicarbonate ion
HSP	Heat shock protein
IEF	Isoelectric focusing
IHC	Immunohistochemistry
IMRC	Intercalated mitochondrion-rich cell
K^+	Potassium ion
kDa	Kilodalton
LDH	Lactate dehydrogenase
Mg^{2+}	Magnesium ion
mM	Milimolar
MR	Mineralocorticoid receptor
MRC	Mitochondrion-rich cell
mRNA	Messenger ribonucleic acid
MS/MS	Tandem mass spectrometry
MS-222	Tricaine methanesulfonate
MYA	Million years ago
Na^+	Sodium ion
NAD^+	Nicotinamide adenine dinucleotide, oxidized form
NADH	Reduced nicotinanide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NCBI	National Center for Biotechnology Information
NCC	Na^+/Cl^- cotransporter
NH_3	Unionized ammonia
NH_4^+	Ammonium ion
NHE	Na^+/H^+ exchanger
NKA	Na^+/K^+- ATPase
NKCC	$Na^+/K^+/2Cl^-$ cotransporter
ORF	Open reading frame
PBS	Phosphate-buffered saline
PC	Pavement cell
PCA	Perchloric acid
PCNA	Proliferating cell nuclear antigen

pI	Isoelectric point
PKA, PKC	Protein kinase A, C
PMF	Peptide mass fingerprint
PP	Protein phosphatase
ppt	Parts per thousand
PVC	Pavement cell
rDNA	Ribosomal DNA
qPCR	Quantitative polymerase chain reaction
RACE-PCR	Rapid amplification of cDNA-ends with PCR
RBC	Red blood cell
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SO_4^{2+}	Sulfate ion
SW	Seawater
T	Temperature (ºC)
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TBS	Tris-buffered saline
TL	Total length
TPBS	Tris phosphate buffered saline
TTBS	Tris-buffered saline
UTR	Untranslated region
UV	Ultraviolet

Table of Contents

List of Tables

List of Figures

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Chapter 1:

General introduction

1.1 General biology of the sea lamprey

The sea lamprey *Petromyzon marinus* Linnaeus 1758 is an anadromous and semelparous (only spawns once before dying) species that migrates twice during its life cycle between freshwater and seawater and then back to freshwater (Fig. 1.1). The larval form (ammocoetes) are microphagous and spend between three to seven years buried in the silt and muddy substrate of rivers and streams before undergoing a period of metamorphosis that lasts approximately three months when they undergo remarkable morphological and physiological changes (Hardisty and Potter, 1971a). From ammocoete to fully transformed juveniles (Fig. 1.2), a total of seven metamorphic stages can be identified, based on external morphological characteristics (Youson and Potter, 1979).

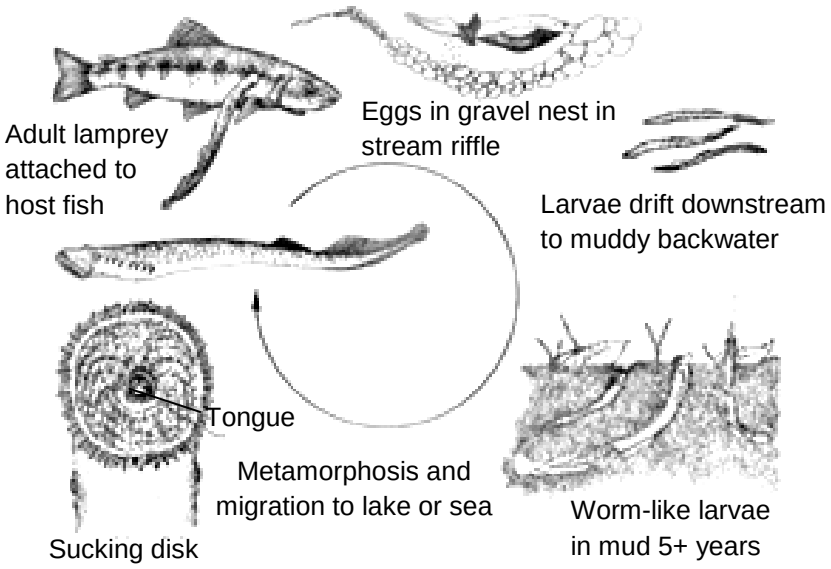

Fig. 1.1 Sea lamprey *Petromyzon marinus* life cycle. Adapted from Hardisty and Potter (1971a).

Fig. 1.2 Sea lamprey *Petromyzon marinus* captured using a Fyke Net during its downstream migration in the Saw Mill River, Montague, Massachusetts, USA.

The ammocoetes are characterized by the lack visual organs and display a dark brownish dorsal coloration that becomes slightly lighter in the ventral area (Fig. 3 a-i/a-ii). From this stage and up until the third stage, the eyes fully differentiate and emerge (Fig. 1.3 b-i/b-ii to d-i/d-ii). Stage four is characterized by the development of the tongue-like piston (Fig. 1.3 e-i/e-ii) followed by stage five, in which marked changes in body coloration occur as a progressive blue darkening of the dorsal area and silvering of the ventral surfaces occur (Fig. 1.3 f-i/f-ii). During stages six and seven (Fig. 1.3 g-i/g-ii), the cornification of the teeth occurs (Fig. 1.3 h-i/h-ii) (Youson and Potter, 1979). By the end of metamorphosis they are completely transformed from a substrate-dwelling, filter feeder into free- swimming animals, capable of parasitic feeding that starts migrating downstream to the sea where they start their marine trophic phase (Fig. 1.3 i-i/i-ii). At this stage, the sea lampreys experience a period of rapid growth and after about two years re-enter freshwater and migrate upstream as adults to find a suitable place to terminally spawn (Beamish, 1980a; Hardisty and Potter, 1971ac).

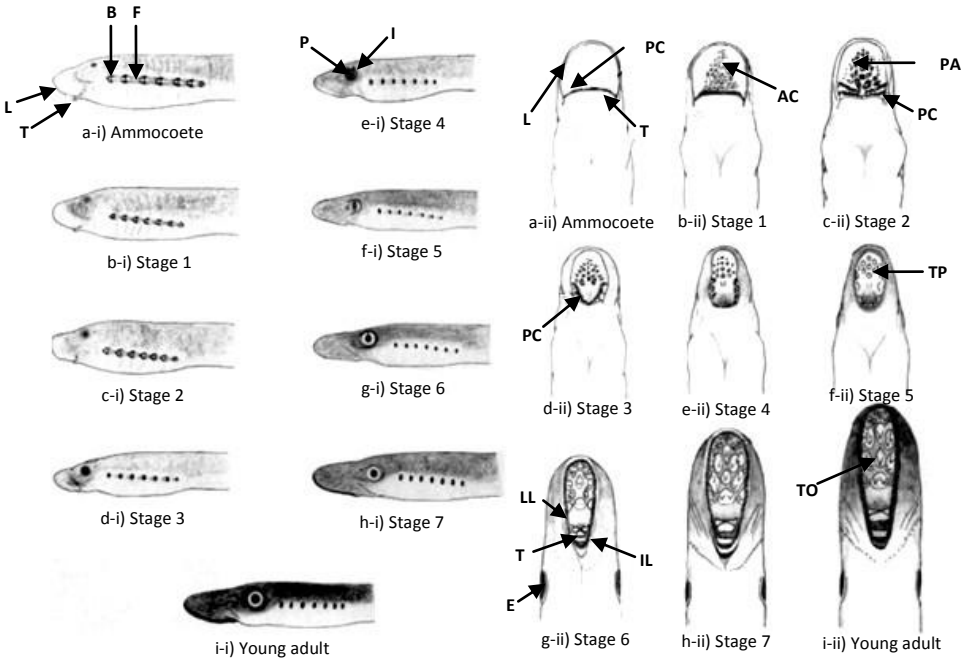

Fig. 1.3 Lateral (a-i to i-i) and ventral (a-ii to i-ii) views of the anterior region of the larval (ammocoete), metamorphic (1 to 7). and young adult stages of anadromous *Petromyzon marinus* L. Abbreviations: AC, anterior oral cirrhi; B, branchiopore; F, furrow; I, iris; IL, infraoral lamina; L, lateral lip of oral hood; P, pupil; PA, papilla; T, transverse lip of oral hood; E. eye; I, infraoral lamina; L, lateral lip of oral hood; LL, longitudinal lingual lamina; P, papilla; PC, posterior oral cirrhi; TL, transverse lingual lamina; TO, teeth of oral disc; TP, tooth precursor. Adapted from Youson and Potter (1979).

1.2 A perspective on sea lamprey and evolution of vertebrates

The sea lamprey is a basal vertebrate (Janvier, 1999) that belongs to the cyclostome superclass (jawless vertebrates) which is contemporarily represented exclusively by the Myxiniformes (hagfishes) and the Petromyzontiformes (lampreys) (Hardisty, 1982). Molecular studies group hagfishes and lamprey in a monophyletic group when constructing phylogenetics based on the 28S (Mallatt and Sullivan, 1998) and 18S (Mallatt and Sullivan, 1998; Stock and Whitt, 1992) rDNA sequences, mitochondrial DNA (Delarbre *et al.*, 2002), and nuclear DNA-coded genes (Kuraku *et al.*, 1999). Despite this, the physiology and morphology of lampreys reassembles to gnathostomatous (jawed) vertebrates more closely than to the hagfishes (Forey and Janvier, 1993; Hardisty, 1982). Together, this results suggest that these two cyclostome groups have been separated for a long time in the evolutionary scale which can date back to more than 500 million years ago, a period that corresponds to the lower Cambrian period of history (Janvier, 1999). In 1932, Homer Smith (Smith, 1932) stated that "*these two groups lead back to a parting of the ways in the evolution of body fluids*".

This statement is pertinent as, to date, hagfishes have only been found in marine habitats (Cholette *et al.*, 1970; McFarland and Munz, 1965) and evidence shows that they remained exclusive to this environment (Lutz, 1975; Robertson, 1974, 1957) as they practically do not osmoregulate (McFarland and Munz, 1965). It has been shown that their internal milieu is essentially iso-osmotic to the marine environment, a situation unique among vertebrates as their sera concentrations of Na^+ and Cl^- approximate those of full-strength seawater (Hardisty, 1979; Robertson, 1974). On the other hand, anadromous species of lampreys such as the sea lamprey *Petromyzon marinus* spend part of their life cycle in freshwater streams (Hardisty and Potter, 1971ab; Hardisty *et al.*, 1989). Lamprey evolved mechanisms of osmorregulation, giving it the capacity to regulate their internal concentration on Na^+ and Cl^-, thus maintaining their internal milieu hyper-osmotic while in freshwater and hypo-osmotic while in marine environments (Beamish *et al.*, 1978; Morris, 1972). Yet, the evolutionary history of lampreys is not yet entirely clear. Based on the fact that the osmolality of seawater is well above the osmolality of lamprey serum, it was believed that lamprey had an ancient freshwater history with the marine parasitic phase emerging late in during their evolution (Hardisty *et al.*, 1989). However, more recently this theory has been questioned when lamprey-like fossils were found in marine deposits dated to the lower Cambrian period (Shu *et al.*, 1999), suggesting that early evolution of

lamprey might have happened in marine environments over 545 million years ago, prior to the time when lampreys started invading freshwater habitats (Janvier, 1999; Shu *et al.*, 1999).

1.3 Osmoregulatory mechanisms in sea lamprey

Due to their high diversity and abundance it is no surprise that the majority of osmoregulation studies have been conducted in teleost fishes (e.g. Karnaky, 1986, 1980; Krogh, 1939; Perry, 1997; Smith, 1932, 1930; Wilson *et al.*, 2000ab; Zadunaisky, 1984). In addition teleosts are found in a wide range of environments from freshwater to brackishwater and seawater with the osmoregulatory challenges they face being similar to that of the anadromous lampreys. Despite the fact that lampreys and teleost fishes are not closely related (see section 1.2. of the present chapter), it is believed that the osmoregulation mechanisms are analogous with the gill and the kidney being the main organs responsible for the active regulation of internal levels of ions (Beamish, 1980b; Hardisty *et al.*, 1989; Morris, 1972; reviewed by Bartels and Potter, 2004). Nevertheless, ultrastructural characteristics and distribution of lampreys epithelial cell types: ammocoete mitochondria-rich cell (MRC), intercalated MRC (IMRC), chloride cell (CC) and pavement cell (PC), differ from those of teleost and close resemble those of certain ion-transporting epithelia in other vertebrates. As previously mentioned, the sea lamprey *Petromyzon marinus* is an anadromous species, where the ammocoete remains in freshwater streams. During this stage they lack the capacity to osmoregulate in hypertonic environments (≥~225 mosmol kg^{-1}) (Beamish *et al.*, 1978; Morris, 1980; Reis-Santos *et al.*, 2008). After going through metamorphosis the juveniles can readily acclimate to full strength-seawater and maintain their internal milieu at ~260 mosmol kg^{-1} (Beamish *et al.*, 1978), showing a remarkable fast capacity to transition from an hyper-osmotic to an hypo-osmotic regulation (Potter and Beamish, 2010). Once the adult lamprey finish its marine trophic phase and embarks on their upstream terminally spawning migration, they lose the ability to osmoregulate in hypertonic environments (Morris, 1958, 1956; Pickering and Morris, 1970).

In the case of anadromous lampreys, changes in the gill epithelium occur during time of migration from freshwater to seawater and vice-versa, that include changes in cell type composition, cellular arrangement and the structure of the tight junctions. In ammocoetes two types of MRCs occur, the ammocoete MRC and the intercalated IMRC, in addition to PC (Bartels *et al.*, 1998). As lamprey undergo metamorphosis the

ammocoete MRC disappear and the chloride cells develop (Peek and Youson, 1979a). By the end of metamorphosis, the gill epithelium of the downstream migrating juveniles possesses chloride cells, pavement cells and intercalated MRC although the latter type disappear at the time of entrance in seawater and do not reappear until the fully grown adult re-enters freshwater for its upstream terminal spawning migration (Bartels *et al.*, 1998). As for the chloride cells, they gradually disappear during the spawning freshwater run (Morris and Pickering, 1976; Morris, 1957). Based on these observations it is believed that the different types of cells reported in the life stages are correlated with the environment's osmolality. This is because intercalated MRC are the only cell type present throughout both freshwater phases, and are absent during the marine phase, thus it is presumed that they plays a crucial role in active ion uptake. In addition, the absence of the ammocoete MRC after the completion of the larval phase suggests that its role must be undertaken by other cell type(s) during the freshwater phases of post-larval life or has a function unique to the larval stage. On the other hand, as chloride cell appear prior to the marine phase and vanish soon after the completion of this phase, it is presumed that it is involved in active ion secretion. Finally, the only cell type that is present on the gill surface throughout the entire life cycle of lamprey is the pavement cell. This would suggest that it plays a role in both fresh and seawater osmoregulatory mechanisms, though there is currently no evidence that this cell type is required for osmoregulation in seawater. Once in freshwater the possession by these cells of a relatively impermeable apical membrane helps protect these animals against an osmotic influx of water across the gill (Bartels and Potter, 2004). Also, pavement cells are flat which minimizes the diffusion distance for gas exchange.

In terms of function in freshwater, the IMRC uptakes Cl^- and secretes H^+, facilitating the uptake of Na^+ through the pavement cells. Contrary, when in seawater, the chloride cells plays a secondary active transcellular transport of Cl^- to provide the driving force for the passive movement of Na^+ by leaky paracellular pathways located between chloride cells (Bartels and Potter, 2004).

1.4 Mechanisms for Freshwater Osmoregulation

As previously mentioned, when in freshwater fish are hypertonic compared to the environment, thus losing ions through their skin and gills and suffering from an osmotic influx of water. In order to overcome this, they actively uptake monovalent ions from the environment across the gill to compensate for ion losses and the kidney is responsible for

the production of a high volume of dilute urine to get rid of the osmotically gained water (Fig. 1.4 A-i) (Marshall and Grosell, 2006). In the gill Na^+ is taken up in exchange for H^+ through an active secretion of H^+ by an electrogenic H^+-ATPase located in the IMRCs and the uptake of Na^+ through the epithelial Na^+ channel (ENaC) in the pavement cells. (Fig. 1.4 A). In order for this mechanism to work, cytosolic carbonic anhydrase (CAc) is essential for maintaining the pool of H^+ to be secreted and Na^+/K^+-ATPase (NKA) in the cell's basolateral membrane with the apical membrane containing ENaC (Ehrenfeld and Klein, 1997; Harvey and Ehrendeld, 1986; Harvey, 1988; Nagel and Dörge, 1996). The presence of H^+- ATPase and ENaC was to be present in the gill epithelium of teleosts using immunocytochemical techniques (Marshall, 2002; Sullivan *et al.*, 1995; Wilson *et al.*, 2000a). Although these studies support the existence of ENaC, no homologs have been found and characterized to date and other channels comparable to the ENaC, such as acid-sensing ion channels (ASICs), are believed to perform its function (Dymowska *et al.*, 2014). In terms of Cl^- uptake, it occurs trough an HCO_3^-/Cl^- antiport (Garcia-Romeu and Ehrenfeld, 1975; Larsen, 1991; Marshall *et al.*, 1997). The cells involved in Cl^- uptake are also characterized by the presence of a Cl^- channel in their basolateral membrane as well as cytosolic carbonic anhydrase (Larsen, 1991) (Fig. 1.4A).

1.5 Mechanisms for Seawater Osmoregulation

In contrast to what happens in freshwater, when in seawater, fish tend to lose water into the environment and in order to overcome this loss, the kidney produces a small amount of highly concentrated urine. In addition a different strategy is used to uptake water from the environment. Like other seawater fishes, lampreys have a drinking mechanism, in which the fish ingests seawater. The water together with monovalent ions is absorbed in the anterior sections of the intestine through osmotic gradients generated by Na^+ and Cl^- taken up by the enterocytes (Pickering and Morris, 1973) and the excess of Na^+ and Cl^- are secreted across the gill (Fig. 1.4 B-i) (Karnaky, 1986, 1980; Loretz, 1995; Smith, 1930). Divalent ions such as Mg^{2+} and SO_4^{2+}, are not taken up in this mechanism (Pickering and Morris, 1973), being excreted in the urine and via defection (Bury *et al.*, 2001; Pickering and Morris, 1973).

For the NaCl to be secreted by the MRC [also known as ionocytes and fist noted by Keys and Willmer (1932)] in the gill of teleosts, three aspects are essential: a driving force that is created by the basolateral NKA; a basolateral $Na^+/K^+/2Cl^-$ co-transporter (NKCC) that allows Cl^- to entry across the basolateral membrane and; the anion channel

homologous to cystic fibrosis transmembrane conductance regulator (CFTR) from mammals (Marshall, 2002; Singer *et al.*, 1998; Wilson *et al.*, 2000b) located in the apical membrane and where Cl^- move its electrochemical gradient into seawater (Fig. 1.4 B-ii). In its turn, the Na^+ is secreted passively via a cation selective paracellular pathway (see review by Evans *et al.*, 1999) located in thin tight junctions between chloride cells and accessory cells, following the Cl^- created electrochemical gradient (Fig. 1.4B) (Ernst *et al.*, 1980; Foskett and Scheffey, 1982; Foskett and Machen, 1985; Karnaky *et al.*, 1977; Sardet *et al.*, 1979).

The NKA is an enzyme that occurs in just about every animal cell and is responsible for the Na^+ uptake across the basolateral membrane. Although it is found present in higher amounts and activity in teleost acclimated to seawater, basal levels of its activity are found in the MRCs of teleosts acclimated to freshwater (Wilson *et al.*, 2000a) which suggest also a role in ion secretion (Wilson *et al.*, 2000b; see review Marshall, 2002). As NKA, $Na^+/K^+/2Cl^-$ cotransporter 1 (NKCC1) increase when teleosts are transferred from freshwater to seawater and during smolting and is colocalized to gill MRCs (Pelis *et al.*, 2001; Tipsmark *et al.*, 2002). However, in lamprey, evidence of NKCC is limited and CFTR-like protein has not yet been described in lampreys. The $Na^+/K^+/2Cl^-$ cotransporter 2 (NKCC2) is an absorptive isoform of NKCC and is mainly expressed in the anterior and mid-regions of the intestine with lower expression also present in the intestine terminal region (see review by Lionetto and Schettino, 2006).

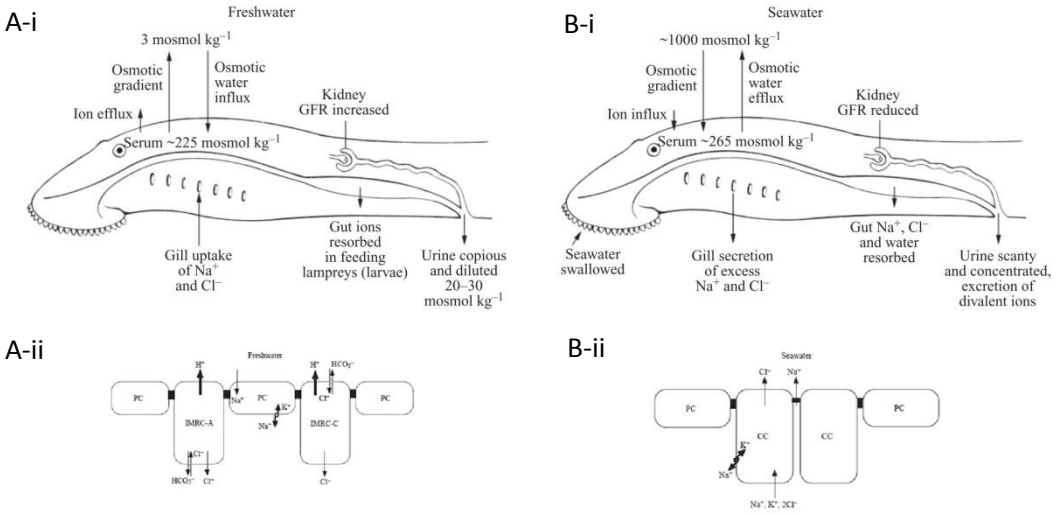

Fig. 1.4 Diagrams showing osmoregulatory mechanisms employed by anadromous lampreys during the freshwater (A) and seawater (B) phases in their life cycles; i-overall scheme; ii-branchial model. Adapted from Bartels and Potter (2004).

1.6 Effects of feeding on osmoregulation in the parasite and host

To date 40 species of lamprey were documented, from which 18 are alleged to be parasitic at some point in their life history (Renaud, 2011). From those, only 9 have a marine trophic phase which includes the sea lamprey *Petromyzon marinus* (Potter, 1980). The sea lamprey count with two distinct ecotypes, anadromous (migrates to the oceans and returns to freshwater systems to spawn) and landlocked (completing its lifecycle in freshwater) (Beamish, 1980a; Hardisty and Potter, 1971a).

The majority of studies on parasite-host interactions with special focus on physiology of feeding in sea lampreys have been performed on landlocked sea lampreys. This because of their devastating effect on Great Lakes fish stocks and the large monetary sums being spent for its control efforts (Hardisty, 2006). Also, information on the feeding habits in the anadromous sea lamprey is limited since they do not have specific choice of hosts (Hardisty and Potter, 1971c; Silva *et al.*, 2014). A recent study by Silva and colleagues (2014) has documented 54 species of fish and marine mammals confirmed as hosts and five more still to be confirmed. Capturing sufficient numbers of sea lampreys while feeding in the ocean is problematic due to their dispersed distribution, solitary nature and lack of targeted fishery (Silva *et al.*, 2014) and the fact that they often detach from host during handling.

1.7 Ecological significance

In addition to its food value to humans, lampreys are key participants in the food chain and have an ecological role at all their life stages. During the ammocoete stage they filter feed, converting algae and detritus into biomass (Sutton and Bowen, 1994; Yap and Bowen, 2003) and they represent a large percentage of stream biomass (Beamish and Youson, 1987). During the post-metamorphic juveniles' downstream migration they serve as food for predatory fishes and birds (Close *et al.*, 2002). When in seawater they become predators and filter feed on a variety of fish species and on occasions marine mammals (Silva *et al.*, 2014). In the Great Lakes, sea lamprey landlocked populations are an invasive species and have had a devastating effect on commercial fish stocks (see SLIS, 1980).

1.8 Why do research on sea lamprey?

The sea lamprey is one of the oldest living groups of vertebrates that has survived at least four of the five mass extinction events documented since the Cambrian explosion (Docker, 2015), and has thus being called a “living fossil”. Since the 1950’s the research work being done on lamprey biology has been increasing. The understanding of lamprey ecology, behavior, and chemical communication has deepened and they are currently used as evolutionary developmental models and in biomedical studies. In addition, the genome of the sea lamprey *Petromyzon marinus* has recently been sequenced and annotated (Smith *et al.*, 2013). For all these reasons it is no twist of fate that everywhere in the world, university students dissect lampreys in comparative vertebrate anatomy classes and develop dissertations related to lamprey biology with an emphasis on conservation of anadromous and control of landlocked populations (Docker, 2015).

1.9 Cultural significance of sea lamprey

In many cultures, lampreys have long been considered a delicacy and have also been used for ceremonial purposes since Roman times. This cultural significance still lives on and lamprey pie is still used by England’s monarchy on special occasions. During her coronation in 1952 and her Silver Jubilee in 1977, Queen Elizabeth II received a lamprey pie as tradition dictates (Renaud, 2011; The Telegraph, 2012). In Portugal the sea lamprey is traditionally eaten during its spawning migration period. Annually prices per kilogram often reach 50€, making it a prime target for poachers (Andrade *et al.*, 2007; Quintella, 2006).

Although importation of landlocked sea lamprey from the Great Lakes into Europe would sound profitable and a solution to control and conservation of the landlocked and anadromous populations respectively, mercury levels of sea lampreys from the Great Lakes are too high to meet European Union standards (Cameron MacEachen *et al.*, 2000).

Chapter 2:

A cytosolic carbonic anhydrase molecular switch occurs in the gills of metamorphic sea lamprey.

Ferreira-Martins D, McCormick SD, Campos A, Lopes-Marques M, Osório H, Coimbra J, Castro LFC, Wilson JM (2016) A cytosolic carbonic anhydrase molecular switch occurs in the gills of metamorphic sea lamprey. Sci Rep (*in press*).

2.1 Abstract

Carbonic anhydrase plays a key role in CO_2 transport, acid-base and ion regulation and metabolic processes in vertebrates. While several carbonic anhydrase isoforms have been identified in numerous vertebrate species, basal lineages such as the cyclostomes have remained largely unexamined. Here we investigate the repertoire of cytoplasmic carbonic anhydrases in the sea lamprey (*Petromyzon marinus*), that has a complex life history marked by a dramatic metamorphosis from a benthic filter-feeding ammocoete larvae into a parasitic juvenile which migrates from freshwater to seawater. We have identified a novel carbonic anhydrase gene (*ca19*) beyond the single carbonic anhydrase gene (*ca18*) that was known previously. Phylogenetic analysis and synteny studies suggest that both carbonic anhydrase genes form one or two independent gene lineages and are most likely duplicates retained uniquely in cyclostomes. Quantitative PCR of *ca19* and *ca18* and protein expression in gill across metamorphosis shows that the *ca19* levels are highest in ammocoetes and decrease during metamorphosis while *ca18* shows the opposite pattern with the highest levels in post-metamorphic juveniles. We propose that a unique molecular switch occurs during lamprey metamorphosis resulting in distinct gill carbonic anhydrases reflecting the contrasting life modes and habitats of these life-history stages.

Key words: ammocoete, ion regulation, ammocoete mitochondrion-rich cell.

2.2 Introduction

The sea lamprey, *Petromyzon marinus*, Linnaeus 1758 is a basal vertebrate characterized by a complex anadromous life cycle. The larvae or ammocoetes are benthic, freshwater filter feeders that undergo a dramatic morphological and physiological transformation into parasitic feeders that migrate downstream to the sea. At the end of the marine trophic phase adults re-enter fresh water and migrate upstream to spawn and then die (Beamish, 1980a; Hardisty and Potter, 1971b).

Carbonic anhydrase is a zinc metalloenzyme, primarily involved in the reversible hydration/dehydration reactions with CO_2, thus involved in CO_2 transport and ionic and acid–base regulation (Henry, 1996). Although carbonic anhydrases are found in all animals, in vertebrates only the α-carbonic anhydrase family is present (Hewett-Emmett and Tashian, 1996; Hewett-Emmett, 2000; Tashian, 1992). In mammals, carbonic anhydrases are categorized according to their subcellular localization. Carbonic anhydrase isoforms 1, 2, 3, 7 and 13 form the functional cytosolic group (Lehtonen *et al.*, 2004) and CA5 orthologues are mitochondrial (Fujikawa-Adachi *et al.*, 1999). The remaining carbonic anhydrase isoforms are membrane-associated with an extracellular orientation (4, 9, 12, 14, 15), secreted (6) or non-catalytic (8,10,11) [reviewed by Gilmour and Perry (2009)].

The teleost cytosolic carbonic anhydrase gene repertoire is notably different from that of mammals (Gilmour and Perry, 2009). Phylogenetic analyses indicate that teleosts retained an ancestral state of a single high activity carbonic anhydrase isoform, in contrast with the carbonic anhydrase gene expansion and functional segmentation in mammals (Gilmour and Perry, 2009). In teleost fishes the carbonic anhydrase 2-like b (blood type) has been found to be mainly expressed in red blood cells (RBC) in zebrafish (Lin *et al.*, 2008) and trout (Esbaugh, 2005; Esbaugh *et al.*, 2004). In contrast, carbonic anhydrase 2-like a has higher expression in gill than kidney and RBC (Esbaugh, 2005; Lin *et al.*, 2008). More recently, cytoplasmic carbonic anhydrases have been cloned and characterized from gills of the Antarctic fishes *Trematomus eulepidotus*, *Trematomus lepidorhinus, Trematomus bernacchii* and *Cygnodraco mawsoni* (Santovito *et al.*, 2012). Cytosolic carbonic anhydrases have also been cloned from blood samples of various non-teleost fishes such as the holostean gar, (*Lepisosteus osseus)* (Lund *et al.*, 2002), the elasmobranch dogfish, (*Squalus acanthias)* (Gilmour *et al.*, 2007), and from cyclostomes such as lamprey, *P. marinus*, (Esbaugh and Tufts, 2006) and the Pacific hagfish, *Eptatretus stouti* (Esbaugh *et al.*, 2009). To date only a single carbonic anhydrase isoform has been characterized in sea lamprey gill tissues and blood (Esbaugh and Tufts, 2006).

For acid-base regulation, fishes rely on metabolic compensation, which involves the exchange of acid-base equivalents directly from their external environment: H^+ and HCO_3^- are exchanged for Na^+ and Cl^-, respectively. Cytosolic carbonic anhydrase is an essential component of this process, providing an intracellular pool of H^+ and HCO_3^- from CO_2 hydration for these exchange processes in ion and acid-base regulatory epithelia such as the gill and kidney (Gilmour and Perry, 2009). Although RBC carbonic anhydrase is central to transport of CO_2 as HCO_3^- in the plasma in most vertebrates, the lack of functionally significant Cl^-/HCO_3^- exchange (band 3 protein) in lamprey RBCs limits transport to intracellular RBC HCO_3^- (Tufts and Boutilier, 1989).

In the present study, we tested the hypothesis that metamorphosis, which marks a dramatic change in the life style and physiology of sea lamprey, requires changes in cytosolic carbonic anhydrase expression. A similar shift has been already documented in amphibian development and has been postulated to be related to distinct life stage strategies and physiological challenges [see review from Tufts *et al*. (2003)]. To address the hypothesis we cloned and sequenced a novel ammocoete carbonic anhydrase orthologue (*ca19*) and together with the previously described carbonic anhydrase (*ca18*) (Esbaugh and Tufts, 2006) elucidated changes during metamorphosis. Expression levels of the carbonic anhydrase lamprey genes were determined at the transcript level using quantitative RT-PCR, and protein level using immunoblotting and further characterized by MALDI-TOF mass spectrometry and molecular modeling. Adaptive changes following salinity exposure that would occur following the normal downstream migration of sea lamprey were also explored.

2.3 Materials and methods

2.3.1 Animals

Petromyzon marinus (L.) ammocoetes were collected by electrofishing from tributaries of the River Minho during the summer and autumn of 2011 and the Fort River MA USA during the summer of 2010. Animals collected from the Fort River MA, USA were sampled in the field [see Reis-Santos *et al*. (2008) for more details] and those from River Minho were transported to the Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Portugal (CIIMAR, UP) and maintained in a large tank with dechlorinated tap water and mechanic and biologic filtration, and provided a sandy silt substrate. Water temperature was maintained at 15°C. Animals were fed with a suspension of yeast twice a week and acclimated to these tank conditions for at least 1

week before experimentation. Animals were treated in accordance with the Portuguese Animal Welfare Law (Decreto-Lei no.197/96) and US Geological Survey institutional guidelines, and animal protocols were approved by CIIMAR/UP and DGV (Ministry of Agriculture) and IACUC.

2.3.2 Metamorphic Series and Salinity Experiment

For the metamorphosis experiment, 16 ammocoetes, nine stage 1, three stage 2, one stage 5, six stage 6, three stage 7 and eight post-metamorphic juveniles staged according to Youson and Potter (Youson and Potter, 1979) were sampled from the Fort River MA, USA. Animals were sampled as described below.

Salinity acclimation of post-metamorphic juveniles was done as described in Reis-Santos and co-workers (2008) using Fort River lamprey.

2.3.3 Sampling

Animals were killed with an overdose of ethyl-m-amino benzoate (MS-222 1:5000 buffered with sodium bicarbonate, pH 7.8 1:5000). Total length (mm), mass (±0.01g) and stage were recorded for each animal. Blood samples were collected from the caudal vessels using a heparinized capillary tube after caudal transection, centrifuged and hematocrit recorded. Separated plasma and RBCs were snap frozen in liquid nitrogen and stored at -80°C. Gill, kidney, anterior intestine, posterior intestine were excised and snap frozen in liquid nitrogen and stored at -80°C for further use.

2.3.4 RNA isolation, quantification and cDNA synthesis

Total RNA was extracted using Aurum$^{(TM)}$ Total RNA Mini Kits according to the manufacture's recommendations (Bio-Rad, Hercules, CA, USA). Homogenization was performed using a bead mill Precellys 24 (Bertin Technologies, Montigny-le-Bretoneux, France) at 6400 rpm for 2 cycles of 15 s with a 5 s interval. Total RNA concentration and purity was determined spectrophotometrically (Nanodrop Thermo Fisher Scientific, USA), integrity assessed by agarose gel electrophoresis and cDNA synthesized from 1µg of total RNA with iScript cDNA Synthesis Kit (Bio-Rad) using a Doppio thermocycler (WVR International Ltd). The cDNA samples were stored at -20°C.

2.3.5 RT-PCR, Real-time RT-PCR and sequencing

PCR reactions were performed using cDNA from gill tissue using either DyNAzyme II DNA Polymerase (Thermo Scientific) or GoTaq® DNA polymerase (Promega, Madison, USA) according the manufacturer's directions. Primers were designed using Primer3 (Rzhetsky and Nei, 1992) and were initially tested for specificity by RT-PCR. Initial PCR reactions were performed using a set of primers designed from zebrafish (*Danio rerio*) carbonic anhydrase mRNA sequence (*zfCA_F1* and *zfCA_R1*). All primers are described in Table 2.1 and cycling profiles in Table 2.2.

Table 2.1 Sets of primers used for RT-PCR, Real-time RT-PCR and RACE-PCR.

ID	Sequence 5'–3'	Reference	GenBank accession no/ ensembl.org
zfCA_F1	CAG TTC CAT TTC CAT TGG GG		BC065611.1
zfCA_R1	CAG AGG AGG GGT GGT CAG		BC065611.1
PmCA_F1	ATA ACG CAG GGT TGC AGA AGG TGA C		
PmCA_F2	GCT TTG ACG AGG CGA AAG ACA AGA G		
PmCA_R1	GGA GAA CCG AGG GGT CGT AGT TCT T		
PmCA_R2	GTC ACC TTC TGC AAC CCT GCG TTA T		
Pm_CA19-F	ATA ACG CAG GGT TGC AGA AG		
Pm_CA19-R	CTT TCA GTG AGC GGA ATG C		
Pm_CA18_F	GCT GAA GCA GTT CCA CTT CC	(Esbaugh and Tufts, 2006)	DQ157849
Pm_CA18-R	CCC TTG CTC CTG ATG ATG TT	(Esbaugh and Tufts, 2006)	DQ157849
Pm_aHb2a–F	CAT GGA TGA CAC CGA GAA GA	(Qiu, 2000)	AF248645

Pm_aHb2a-R	GAC CTG AGC AGG ATG CAA AT	(Qiu, 2000)	AF248645
Pm_aHb9-F	AGA AGC ACG CTC AGG AGT TC		ENSPMAG00000008540
Pm_aHb9–R	AGA GCA GCT GTT CGT TGT CA		ENSPMAG00000008540
Pm_gapdh-F	TGC AAA GCA CGT CAT CAT CTC	(Pancer *et al.*, 2004)	AY578058
Pm_gapdh-R	TTC TCG TGG TTT ACT CCC ATC A	(Pancer *et al.*, 2004)	AY578058
Pm_18S-F	GTA GTT GGT GGA GCG ATT TGT CT	(Stock and Whitt, 1992)	M97575
Pm_18S-R	GGC CGC GTAG CTA GTT AGC A	(Stock and Whitt, 1992)	M97575

Table 2.2 RT-PCR and RACE-PCR cycling profiles: zebrafish cytosolic carbonic anhydrase, zfCA and ; novel cytosolic carbonic anhydrase 19 isoform, *ca19*.

	zfCA	***Ca19***
Reaction type	**RT-PCR**	**RACE**
Denaturation and hot start	94°C	98°C
	30 s	10s
Denature	94°C	98°C
	30 s	1s
Anneal	68°C	68°C
	60 s	5s
Extend	72°C	72°C
	90 s	30s
Repeat cycles	35	45
Final extension	72°C	72°C
	10 min	1min

PCR products were separated on 2% agarose TBE (Tris-borate-EDTA) gels to confirm size of amplicons. All gels were stained with GelRed and images acquired with a Fujifilm LAS-4000 Mini luminescent image analyzer (Fujifilm Tokyo, Japan). A single band of the correct predicted size (327bp) 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 as a cytosolic carbonic anhydrase by tBLASTx. From the resulting sequence two sets of primers were designed for nested RACE reactions; 3'RACE: forward (*PmCA_F1* and *PmCA_F2*) and 5'RACE reverse primer (*PmCA_R1* and *PmCA_R2*). RACE reactions were performed using SMARTer™ cDNA Amplification Kit (Clontech, California, USA) and Phusion Flash (Thermo Fisher Scientific) master mix. Cycling profiles are provided in Table 2.3. The resulting PCR products were isolated and ligated into pGEM-T easy vectors (pGEM®-T Easy Vector Systems, Promega) and

positive colonies were grown in LB broth with ampicillin. Plasmids were isolated using the Illustra plasmid kit (GE Healthcare, Carnaxide Portugal) and were sequenced (StabVida).

Relative levels of mRNAs for sea lamprey *ca18* and *ca19* genes were quantified by real-time RT-PCR analysis using SYBR green with an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). See Ferreira-Martins (2016) for details and Table 2.3 for *ca19* and *ca18* primer pairs. A melt curve was generated to confirm assay specificity and a dilution series prepared to check reaction efficiency. *Gapdh* was used as the housekeeping gene. The comparative cycle threshold (CT) method ($2^{-\Delta\Delta CT}$ method) based on CT values was used to analyze the expression levels of the genes of interest. Random resulting amplicons were run on 2% agarose TBE gel to confirm single amplified product with the expected size.

Tissue distributions of *ca18* and *ca19* in ammocoetes and post-metamorphic sea lamprey were assessed by quantitative RT-PCR (as described above). Blood contamination in the tissue samples was assessed using specific primers designed for sea lamprey hemoglobins that correspond to the major aHb component of this species, *hba2a* (=*aHb2a*) mRNA sequence (Qiu, 2000) and a putative larval hemoglobin *hba9* (=*aHb9*). The transcript levels are indicated as relative mRNA levels per ng RNA. The ammocoete gill tissue was assigned a value of 1.0.

Table 2.3 Real time RT-PCR conditions using iQ SYBR green supermix. Cytosolic carbonic anhydrase-18 , *ca18* (Esbaugh and Tufts, 2006); novel cytosolic carbonic anhydrase-19 isoform, *ca19*; putative larval hemoglobin, *hba9* (Qiu, 2000); hemoglobin PMII, *hba2a* (Qiu, 2000); Glyceraldehyde 3-phosphate dehydrogenase, *gapdh*; 18 subunit ribosomal RNA, *18S.*

	ca18	***Ca19***	***hba9***	***hba2a***	***gapdh***	***18s***
Denaturation and hot start	94°C	94°C	94°C	94°C	94°C	94°C
	3.5 min	3.5 min	3.5 min	3.5 min	3.5 min	3.5 min
Denaturation	94°C	94°C	94°C	94°C	94°C	94°C
	30 s	30 s	30 s	30 s	30 s	30 s
Annealing	60°C	65°C	60°C	60°C	60°C	60°C
	30 s	30 s	30 s	30 s	30 s	30 s
Extend	72°C	72°C	72°C	72°C	72°C	72°C
	30 s	30 s	30 s	30 s	30 s	30 s
Repeat cycles	40	40	40	40	40	40
Melt Curve Analysis: 60°C to 95°C with 1°C increments for30 s each						

2.3.6 Phylogenetic Analysis

Carbonic anhydrase sequences were collected from various genome databases such as Ensembl, GenBank or JGI (Joint Genome Institute) through Blastp searches. We also searched species-specific genome sites (e.g. http://esharkgenome.imcb.a-star.edu.sg/) to complete the screening of carbonic anhydrase gene diversity in vertebrates. Our analysis included all major vertebrate lineages with a total of 63 sequences (Accession numbers and Ensembl codes shown in the Supplemental Table 2.1). An ortholog of CA5 from lamprey was not included in the analysis because it caused long-branch attraction in the tree (not shown). Amino acid sequences were aligned using the MAFFT software 7 with default parameters (Katoh and Standley, 2013). The alignment

was stripped of all columns containing gaps leaving 152 positions for phylogenetic analysis. A Maximum Likelihood tree was constructed at PhyML (http://www.atgc-montpellier.fr/phyml/) protein evolutionary model was calculated in PhyML using smart model selection resulting in LG+G+I. Branch support was estimated using the aBayes method (Anisimova *et al.*, 2011) as implemented in PhyML. Trees were visualized with FigTree (v1.4.2; http://tree.bio.ed.ac.uk/software/figtree/).

2.3.7 Immunoblotting

Gill tissue from the metamorphic series from ammocoete to juvenile stages were analyzed by immunoblotting as described in Reis-Santos and co-workers (2008) with modifications. Ten µg of sample were loaded onto polyacrylamide gels (10% T solving gels; 4% T stacking) and transferred to nitrocellulose membranes (Amersham (TM) Hybond (TM) ECL, GE Healthcare). Following blocking with 5% blotto, membranes were probed with a heterologous rabbit anti-bovine cytosolic CA polyclonal antibody (1:2000, Abcam Cambridge UK)(Randall, 2014) or mouse anti-β-actin monoclonal (1:500; Sigma-Aldrich) overnight at room temperature. Membranes were then rinsed with TTBS (0.05% Tween-20 in Tris Buffered Saline, pH 7.4) 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). Signal was obtained by enhanced chemiluminescence (ECL) with Millipore Immobilon Western chemiluminescent HRP substrate (Millipore Corporation, MA USA). Images were acquired using a luminescent image analyzer Fujifilm LAS-4000 mini and image reader software LAS-4000 version 2.0. Intensity of band signal was quantified using an image analysis software program Multi Gauge v3.1 (Fujifilm Tokyo, Japan). After detection, membranes were stripped with low pH stripping buffer and reprobed with other antibodies.

2.3.8 Two-dimensional electrophoresis (2DE), MALDI-TOF/TOF analysis, protein identification and modeling.

Since both cytosolic carbonic anhydrase isoforms are expressed in RBCs and due to the ease of blood collection we performed proteomic analysis using RBCs. RBC samples (150 µL) from post-metamorphic juveniles and ammocoetes were prepared for two-dimensional electrophoresis as described in Campos and co-workers (2013). The 2DE gels were stained with Coomassie Blue Colloidal (Neuhoff *et al.*, 1988) or transferred to nitrocellulose membranes for 2-D western blotting as previously described in the

immunoblotting methods section. Protein spots of interest identified through 2-D gel carbonic anhydrase probed western blots were excised from gels followed by in-gel digestion using the protease trypsin (Pandey and Mann, 2000). For protein identification samples were analyzed using a 4700 Proteomics Analyzer MALDI-TOF/TOF (AB SCIEX, Foster City, CA, USA) as described in Campos and co-workers (2013). Peptide mass fingerprint (PMF) data was collected in positive MS reflector mode in the range of 700–4000 (m/z) and was calibrated internally using trypsin autolysis peaks. Several of the highest intensity and/or relevant tryptic peaks were selected for MS/MS analysis. Both MS and MS/MS spectra were analyzed using the software GPS Explorer (Version 3.6; ABSCIEX), against a locally stored copy of the UniProt protein sequence database (release 2011_12) using the Mascot search engine (Version 2.1.04) (Campos *et al.*, 2013). Novel Ca19 isoform sequence was manually added to the database. The search included peaks with a signal-to-noise ratio greater than 10 and allowed for up to two missed trypsin cleavage sites. To be considered a match, a confidence interval (CI) of at least of 99%, calculated by AB SCIEX GPS Explorer software, was required.

Petromyzon marinus and *O. mykiss* carbonic anhydrase sequences were submitted to SwissModel (http://swissmodel.expasy.org/) for homology modelling (Arnold *et al.*, 2006; Biasini *et al.*, 2014; Bordoli *et al.*, 2009), crystal structure of human CA2 (2CBA) was uploaded and used as a template. The resulting homology models were submitted to ModEval Model Evaluation Server available at https://modbase.compbio.ucsf.edu/evaluation/ to estimate model quality, both models rendered as accurate. Homology model electrostatic potentials were calculated in PDB2PQR web server (Dolinsky *et al.*, 2004) (http://www.poissonboltzmann.org/). Force field AMBER was selected and pKa's were calculated by PROPKA and assigned using pH 7. Electrostatic potential of the homology models were visualized online in APBS web solver.

Predicted phosphorylated forms of Ca18 and Ca19 were determined *in silico* using NetPhos 3.1 Server (http://www.cbs.dtu.dk/services/NetPhos/; (Blom *et al.*, 2004)) and ProMoST [http://prometheus.brc.mcw.edu/promost/; (Halligan, 2009)].

2.3.9 Statistical analysis

Data are presented as means ± standard error of the mean. Statistical differences in mRNA and protein expression between tissues and life stage groups were determined using two-way ANOVA followed by the *post-hoc* Student-Newman-Keuls (SNK) test. One way ANOVA and SNK tests were performed on ammocoete and juvenile *ca19* and *ca18*

normalized with their respective hemoglobin genes and juveniles exposed to different salinities. The statistic program SigmaPlot 11.0 was used for all analyses (Systat Software, Inc., GmbH, Germany). The fiducial limit was set at $P<0.05$.

2.4 Results

2.4.1 Diversity and evolution of cytosolic CA isoforms in vertebrates

We began by examining the repertoire of carbonic anhydrase sequences in the sea lamprey genome (www.ensembl.org, Pmarinus_7.0). Following that, through PCR we were able to identify a previously unreported carbonic anhydrase gene in this species. The novel carbonic anhydrase sequence was identified in the ammocoete gill. The new transcript is 1786 bp with an ORF of 771 bp and 5' and 3' UTR of 87 and 928 bp, respectively. The transcript codes for a 257 amino acid (aa) protein that has 67.5% aa identity with the published carbonic anhydrase isoform from lamprey and 55.9% to 57.4% and 51.3% to 57.8% with rainbow trout and human Cac (Ca2-like a) and CA2 isoforms, respectively (Supplemental Fig. 2.1). In order to examine possible functional differences in the isoforms, we analyzed the amino acid residues of the active site of the novel lamprey isoform where CO_2 binding and proton shuttling occur (Table 2.4). We showed that the newly sequenced gene was most similar to the existing sea lamprey carbonic anhydrase isoform Ca18 and *O. mykiss* "Cac" with respectively 7 and 8 of 36 aa residue substitutions. This analysis also showed a change at the aa level of a substrate associated pocket at position 207 with a valine (very hydrophobic) substituted for an arginine (hydrophilic) (Table 2.4). In addition, *in silico* analysis performed on carbonic anhydrase cytosolic isoforms showed Ca18 and Ca19 had electrostatic potentials of -8.0 and -2.0, respectively (Supplemental Table 2.3).

Table 2.4 Comparative analysis of carbonic anhydrase putative active site pocket amino acid residues and amino acids whose side chains either project into or border the active site in lamprey (Pma) Ca18 (GenBank; ,AAZ83742.1) and Ca19 (GenBank, ALM25804.1), with rainbow trout (Omy) Cac (GenBank, NP_001166020.1), and human (Hsa) CA1 (GenBank, NP_001729.1) and 2 (GenBank, NP_000058.1) modelled after Tashian *et al*. (1992) and Gilmour *et al*. (2007). The aa were aligned using ClustalW (BioEdit 7.0.9.0). Numbers represent the location of each amino acid relative to alignment with human CA2. * Active site aa residues; Z, zinc binding ligand; +, proton shuttling associated ligand; ~, substrate associated pocket.

	7	29	61	62	64	65	66	67	69	91	92	94	96	106	107	117	119	121	131	141	143	145	192	194	198	199	200	201	202	204	206	207	209	211	244	246
	*	*			*			*			*	*	*	*	*	*	*							*		*	*						*		*	*
					+							Z	Z				Z	~		~	~				~								~	~			
***Hsa* CA2**	**Y**	**S**	**N**	**N**	**H**	**S**	**F**	**N**	**E**	**I**	**Q**	**H**	**H**	**E**	**H**	**E**	**H**	**V**	**F**	**L**	**V**	**G**	**W**	**Y**	**L**	**T**	**T**	**P**	**P**	**L**	**C**	**V**	**W**	**V**	**N**	**R**
***Pma* Ca19**	.	.	.	**S**	.	.	.	**S**	**D**	**G**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**N**	**S**	**A**	.	.	**T**	**P**
***Pma* Ca18**	.	.	.	.	.	.	.	**S**	.	**K**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**F**	**S**	.	.	.	.	.
***Omy* Cac**	.	.	.	.	.	.	.	**Q**	**T**	**K**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**S**	.	.	.	.	.
***Hsa* CA1**	.	.	.	**V**	.	.	.	**H**	**N**	**F**	.	.	.	.	.	.	.	**A**	**L**	.	.	.	.	.	.	.	**H**	.	.	**Y**	**S**	.	.	**I**	.	.

We next undertook phylogenetic analyses to clarify the orthology of the collected lamprey carbonic anhydrase sequences (Fig. 2.1). We also re-examined the evolutionary relationships of intracellular carbonic anhydrase genes in vertebrate species. Our analysis identifies four well-supported clades: CA5, CA7, a third assembly composed of CA1/2/3/13 genes, and a fourth clade with the two lamprey sequences. In support of our findings in the sea lamprey, we also detected the presence of these two sequences in the recently released genome sequence of the Japanese lamprey (Mehta *et al.*, 2013) (Fig. 2.1). CA5 genes are found in all of major vertebrate lineages including chondrichthyans, teleosts and tetrapods. Although a CA5-like sequence was found in the lamprey genome it was not included in the present analysis (see materials and methods). CA7 genes are also found in the basal gnathostome lineage the chondrichthyans. The novel lamprey carbonic anhydrase gene strongly groups with a previously described carbonic anhydrase sequence to form a distinct clade basal to gnathostome CA7 and CA1/2/3/13 (Fig. 2.1). Based on this analysis we rename the sea lamprey carbonic anhydrase described by Esbaugh and Tufts (2006) Ca18 and the novel sequence carbonic anhydrase Ca19. The present analysis also provides some insight into the duplication timing and origin of CA1/2/3/13 isoforms. Despite the poor phylogenetic resolution in some internal clades within the CA1/2/3/13 branch, the combination with genome mapping information of these genes in various sarcopterigyan lineages, allows the proposal that the expansion of the CA1/2/3/13 clade took place after the divergence of coelacanth from tetrapods (Fig. 2.1 and Fig. 2.2). Moreover, we find a further carbonic anhydrase gene unique to amphibians, named CA2b, and an additional Ca3 in birds, named Ca3b.

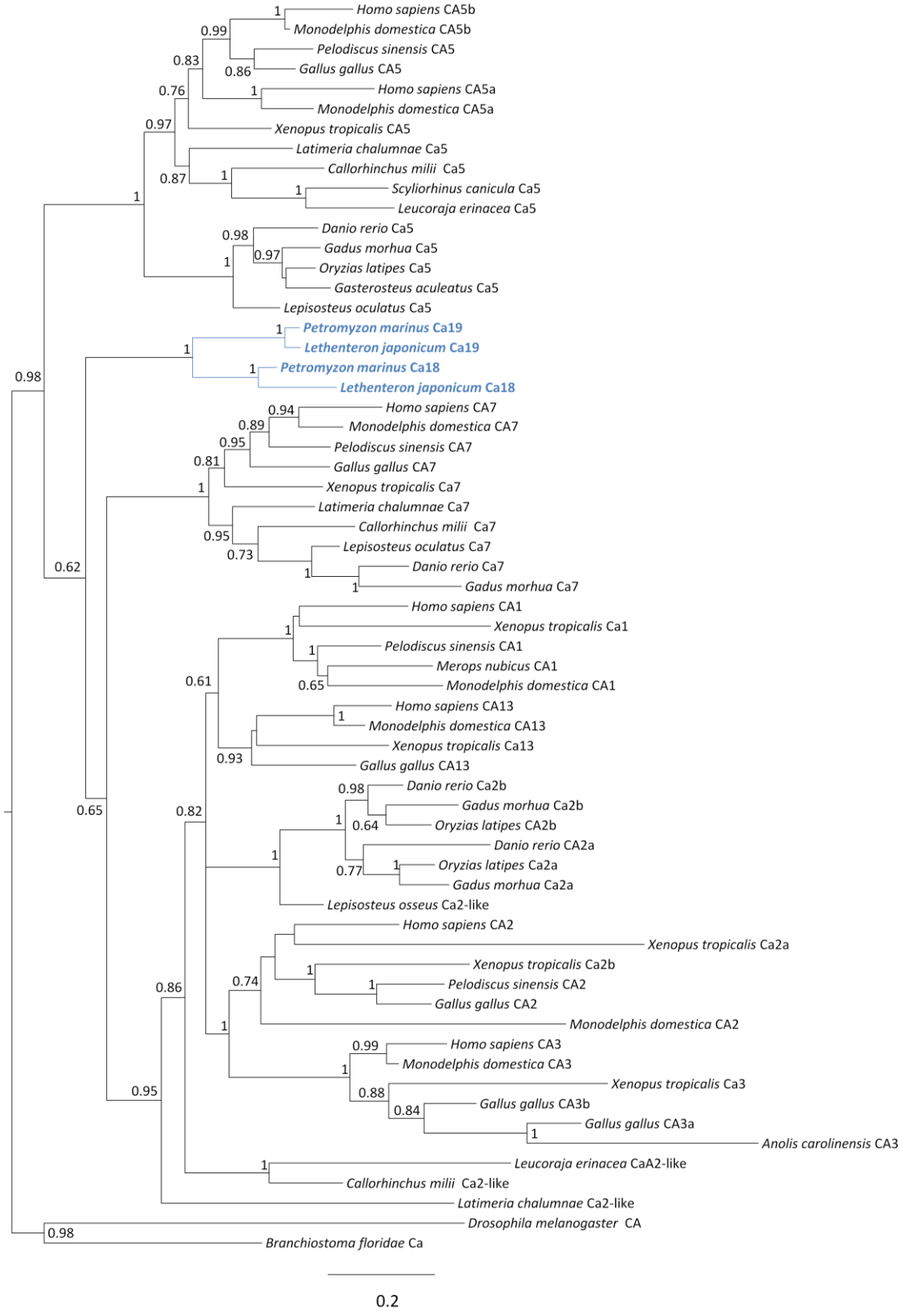

Fig. 2.1 Maximum likelihood phylogenetic tree describing relationships among carbonic anhydrase proteins from representative vertebrate *taxa*. Node values represent branch support using a Bayes algorithm (values below 0.5 are not shown). Accession numbers for all sequences are provided in the supplementary table 2.1.

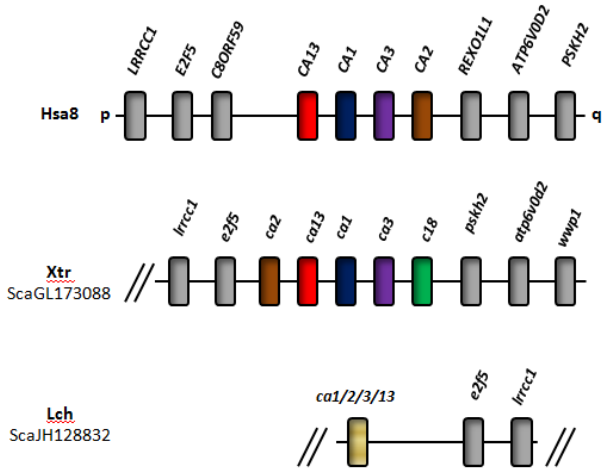

Fig. 2.2 Synteny maps of *CA loci*. Hsa – *H. sapiens*, Xtr- *X. tropicalis* and Lch- *L. chalumnae*.

The finding that the two lamprey cytosolic isoforms group together and are at the base of the gnathostome CA1/2/3/13 and CA7 clades complicates a conclusion over their true orthology. To provide further insights into their origin, we examined the genomic locations of the lamprey carbonic anhydrase genes and compared them with those of their human counterparts (Fig. 2.3). However, this information is available for the lamprey *ca5* and novel cytosolic carbonic anhydrase but not the previously described cytosolic carbonic anhydrase (Esbaugh and Tufts, 2006). The human cytosolic carbonic anhydrase genes localize to genomic regions related by genome duplication, the so-called 2R, at linkage group 3 (Putnam *et al.*, 2008). In the case of the lamprey, we find that the ortholog of the gene close to *ca5* (*heatr3*) maps to human chromosome 16 (Fig. 2.3), providing strong support of its orthology. In contrast, the human orthologues of the genes in the vicinity of the lamprey novel cytosolic carbonic anhydrase do not localize to either chromosome 8 or 16 as would be expected if this gene was a true ortholog of either *CA1/2/3/13* or *CA7*, respectively. However, we find some clues that indicate that the lamprey novel cytosolic carbonic anhydrase might represent a retained paralogue resulting from 2R, but subsequently lost in gnathostomes. In effect, two genes, *c14orf119* and a novel *ccne* gene indicate that this genomic region in lamprey is probably orthologous of a region of the human genome in chromosome 14 which is paralogous to the regions in chromosome 8 and 16 (Fig. 2.3). Considering these observations and the

phylogenetic results, we suggest that the lamprey cytosolic carbonic anhydrases might represent novel carbonic anhydrase gene lineages resulting from genome duplications in vertebrate ancestry, which have been retained uniquely in the cyclostomes lineage. We thus propose calling the previously described (Esbaugh and Tufts, 2006) and novel cytosolic carbonic anhydrases *ca18* and *ca19*, respectively, in line with the naming of new carbonic anhydrases. The recent proposal of a single genome duplication in the vertebrate ancestor would imply a different interpretation of our data (Smith and Keinath, 2015). Thus, additional mapping and phylogenetic data from other cyclostome species should help to clarify these issues. The finding that the two lamprey cytosolic isoforms group together and are at the base of the gnathostome CA1/2/3/13 and CA7 clades complicates a conclusion over their true orthology. To provide further insights into their origin, we examined the genomic locations of the lamprey carbonic anhydrase genes and compared them with those of their human counterparts (Fig. 2.3). However, this information is available for the lamprey *ca5* and novel cytosolic carbonic anhydrase but not the previously described cytosolic carbonic anhydrase (Esbaugh and Tufts, 2006). The human cytosolic carbonic anhydrase genes localize to genomic regions related by genome duplication, the so-called 2R, at linkage group 3 (Putnam *et al.*, 2008). In the case of the lamprey, we find that the ortholog of the gene close to *ca5* (*heatr3*) maps to human chromosome 16 (Fig. 2.3), providing strong support of its orthology. In contrast, the human orthologues of the genes in the vicinity of the lamprey novel cytosolic carbonic anhydrase do not localize to either chromosome 8 or 16 as would be expected if this gene was a true ortholog of either *CA1/2/3/13* or *CA7*, respectively. However, we find some clues that indicate that the lamprey novel cytosolic carbonic anhydrase might represent a retained paralogue resulting from 2R, but subsequently lost in gnathostomes. In effect, two genes, *c14orf119* and a novel *ccne* gene indicate that this genomic region in lamprey is probably orthologous of a region of the human genome in chromosome 14 which is paralogous to the regions in chromosome 8 and 16 (Fig. 2.3). Considering these observations and the phylogenetic results, we suggest that the lamprey cytosolic carbonic anhydrases might represent novel carbonic anhydrase gene lineages resulting from genome duplications in vertebrate ancestry, which have been retained uniquely in the cyclostomes lineage. We thus propose calling the previously described (Esbaugh and Tufts, 2006) and novel cytosolic carbonic anhydrases *ca18* and *ca19*, respectively, in line with the naming of new carbonic anhydrases. The recent proposal of a single genome duplication in the vertebrate ancestor would imply a different interpretation of our data (Smith and Keinath, 2015). Thus, additional mapping and phylogenetic data from other cyclostome species should help to clarify these issues.

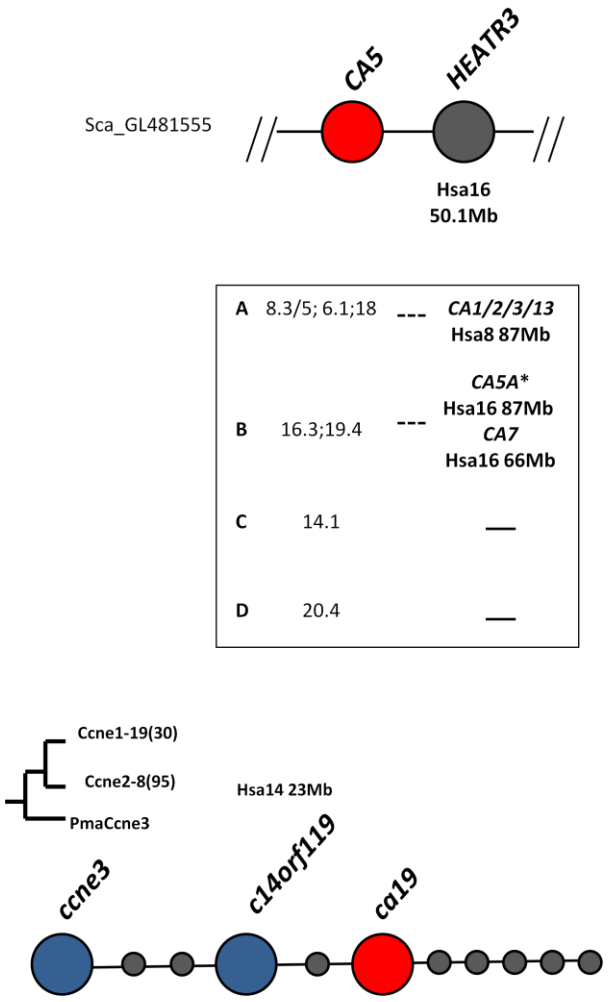

Fig. 2.3 Paralogy analysis of the *CA loci* in the human genome and their comparison to the *ca locus* composition in lamprey [see Putnam *et al.* (2008) for details of chromosome coordinates of linkage group 3]; small grey circles denote genes whose human orthologues are either absent or map to a distinct linkage group.

2.4.2 Tissue distribution

At the mRNA level, the cytosolic carbonic anhydrase isoform *ca19* was expressed in ammocoete gill, blood, and gut (anterior and posterior intestine) at similar levels with significantly lower expression in the kidney (Fig. 2.4a). In post-metamorphic juveniles, RBC had relative higher mRNA expression than the other tissues tested but *ca19* levels were otherwise significantly lower than in any ammocoete tissue tested. The mRNA expression of *ca18* was significantly higher in all post-metamorphic lamprey tissues except kidney (Fig. 2.4b). Blood had the highest mRNA expression levels of *ca18* in post-metamorphic lamprey with no significant differences between the other tissues. In ammocoetes, *ca18* mRNA expression was significantly higher in kidney, with intermediate

expression in gill and blood and the lowest expression in gut (anterior and posterior intestine). RBC's content present in each type of tissue was assessed by the analysis of the corresponding mRNA expression of hemoglobin in tissue samples. Sea lamprey hemoglobin *hba2a (=aHb2a)* showed the highest mRNA expression levels in post-metamorphic juvenile RBC's with similar lower levels (< 1%) in the other tissues (Fig. 2.4c). In ammocoetes, mRNA expression levels of *hba2a* were significantly lower than in postmetamorphic juveniles. In contrast, in ammocoetes the putative larval hemoglobin *hba9 (=aHb9)* mRNA levels were significantly higher in blood (>50-fold) compared to all other tissues (Fig. 2.4d), indicating that blood was unlikely to contribute to observed differences in tissue carbonic anhydrase mRNA levels. The *hba9* mRNA levels were also significantly lower in post-metamorphic juveniles than in ammocoetes (Fig. 2.4d). In addition, *ca19* and and *ca18* expression were calculated using *hba9* and *hba2a* as respective reference genes and tissue mRNA levels expressed relative to RBC groups to further assess RBC contamination (Fig. 2.4e and 2.4f, respectively). In ammocoetes, the gill and gut (anterior and posterior intestine) *ca19* was significantly higher than RBC (and kidney). In post-metamorphic lamprey, *ca18* levels were significantly higher in gill and anterior intestine only. The mRNA expression levels of the reference genes *gapdh* and *18s* were found not to be consistent across the tissues tested (Supplemental Figure 2.2).

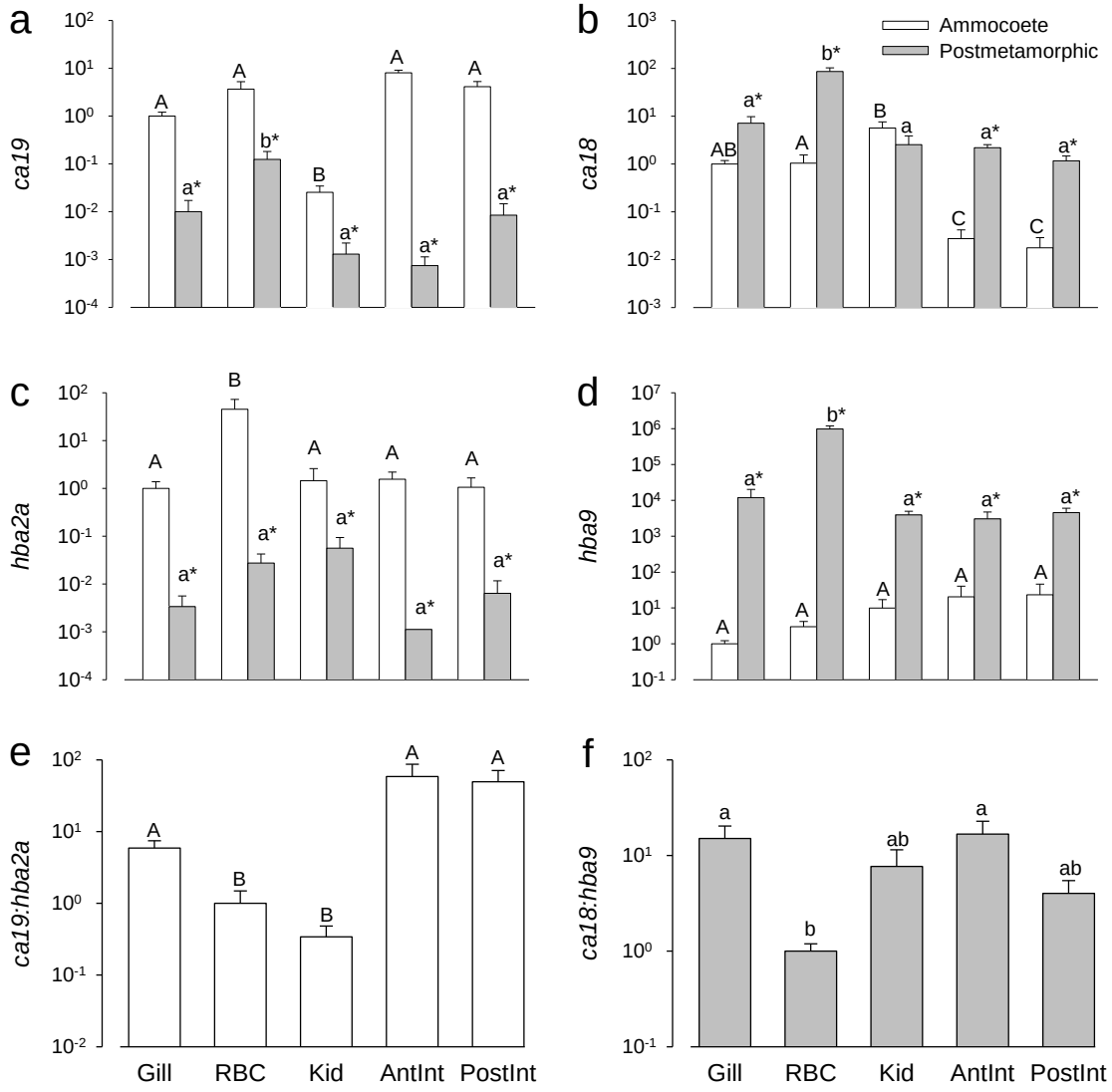

Fig. 2.4 Relative mRNA expression (mean ± S.E.M) of the novel carbonic anhydrase isoform (a) *ca19*, (b) *ca18* (GenBank AAZ83742), (c) putative larval hemoglobin *(hba9)*, (d) postmetamorphic hemoglobin (*hba2a*) in *P. marinus* ammocoete and post-metamorphic juvenile gill, blood, kidney, anterior and posterior intestine determined by qPCR. In the final two panels (e and f) *ca19* and *ca18* are expressed using *hba9* and *hba2a* as respective reference genes and rescaled relative to RBC groups. Tissue expression levels are significantly different if they lack common letters [ammocoetes (uppercase) and juvenile (lowercase)]. Significant differences between ammocoete and juvenile within a given tissue are indicated by an asterisk. Two-way ANOVA and SNK post-hoc test $P<0.05$ (N=4).

2.4.3 Changes in cytosolic carbonic anhydrase mRNA levels during metamorphosis and increasing salinity acclimation of post-metamorphic juveniles

Higher branchial transcript levels of the novel *ca19* isoform were found in ammocoetes compared to post-metamorphic juveniles and adults. Relative mRNA expression levels of sea lamprey *ca18* and *ca19* isoforms in gill tissue were assessed using real time RT-PCR (Fig. 3a,b). The analyses indicated that the *ca19* isoform is almost exclusively expressed in the ammocoete and during the 1st and 2nd stages of metamorphosis. Low levels of expression of *ca18* were found during these stages, which increased significantly from stage 5 onward during metamorphosis. Post-metamorphic juveniles show an increase of *ca18* mRNA expression in response to increased salinity (Fig. 2.6).

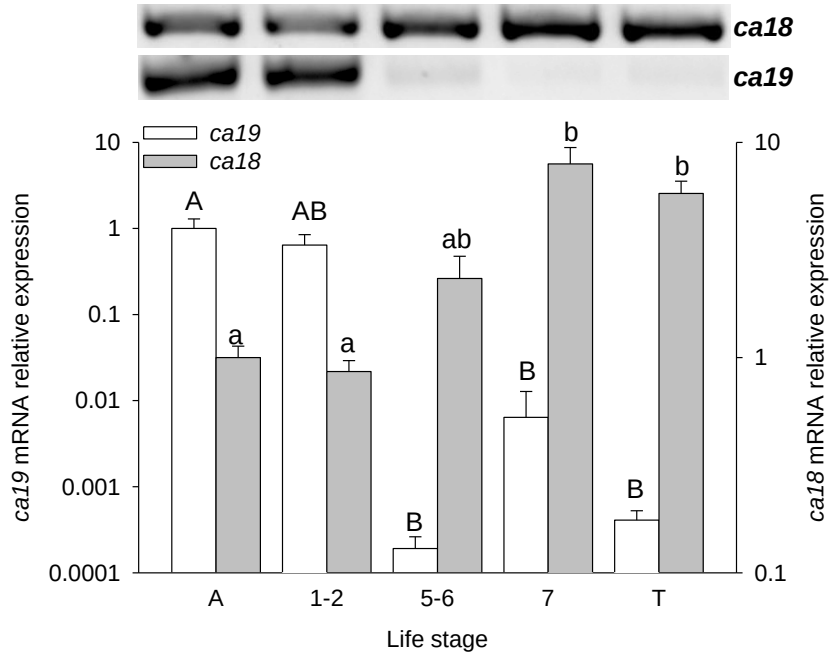

Fig. 2.5 Relative mRNA expression (mean ± S.E.M) of *P. marinus ca18* and *ca19* in ammocoete (A; N=16), metamorphosis stages 1 to 2 (N=12), 5 to 6 (N=5), 7 (N=3) and post-metamorphic (T; N=8). Cropped representative bands from qPCR reactions from the same run are shown above. Changes in *ca18* and *ca19* are analyzed separately and bars with like letters are not significantly different from each other with (in lower and upper case letters, respectively). Two-way ANOVA and SNK post-hoc test, P<0.05.

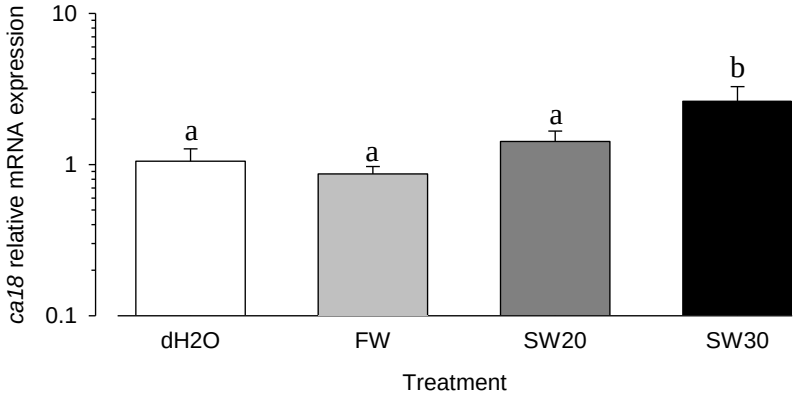

Fig. 2.6 Fold change of *ca18* determined by quantitative real-time PCR (mean ± S.E.M.) in *Petromyzon marinus* post-metamorphic juveniles acclimated to dH_2O (N=3), fresh water (N=5), and salinities of 10‰ (N=2), 20‰ (N=6) and 30‰ (N=6). Bars with like characters are not significantly different from each other.

2.4.4 Immunoblotting and 2-DE analysis

Probing of lamprey gill immunoblots with a heterologous mammalian CA2 antibody revealed the presence of two immunoreactive bands at 27 and 29kDa, respectively (Fig. 2.7). A significant difference in band expression was found in ammocoetes, metamorphic stages 1 to 2 and juveniles. Expression of the 27kDa band was highest in ammocoetes and stages 1-2 and decreased significantly during metamorphosis and was undetectable in post-metamorphic juveniles. In contrast the 29kDa protein band expression was significantly lower in ammocoetes and stages 1-2 and increased significantly at the latter stages of metamorphosis (stage 5-7, post-metamorphic juvenile). Consequently, protein expression of the 27kDa band was significantly greater than the 29kDa band in ammocoetes and early metamorphic stages while no significant differences were found in protein band expression for stages 5 to 7. In post-metamorphic juveniles protein expression of the 29kDa band was significantly greater than the 27kDa band. To confirm the identity of the two bands, a proteomics approach was taken. The 2-DE gel immunoblots probed with the heterologous cytosolic carbonic anhydrase antibody (Fig. 2.8) indicated spots of potential interest. The 2-DE gel spots were examined by MS/MS and database search (Fig. 2.9, Supplemental Table 2.2). High protein identification scores revealed the presence of two Ca18 (Ca18-i, Ca18-ii) in RBC's of ammocoetes and post-metamorphic lamprey (Fig. 2.9A) and six Ca19 spots (Ca19-i, Ca19-ii, Ca19-iii, Ca19-iv, Ca19-v and Ca19-vi) in the RBC's of ammocoetes only (Fig. 2.9B). In agreement with the 1D western blots, the six Ca19 spots corresponded to the 27 kDa band and the Ca18

spots to the larger 29 kDa band. The theoretical pI values for Ca18 and Ca19 were estimated at 5.51 and 6.51, respectively, with a prediction of 20 and 19 probable modifications by phosphorylation each [ProMoST, (Halligan, 2009), (Supplemental Table 2.4], with predicted pI values ranging from 4.64-5.39 and 5.05-6.27. The NetPhos 3.1 Server analysis predicted 27 and 34 potential phosphorylation sites for Ca18 and Ca19, respectively (Supplemental Table 2.5; Supplemental Figure 2.1).

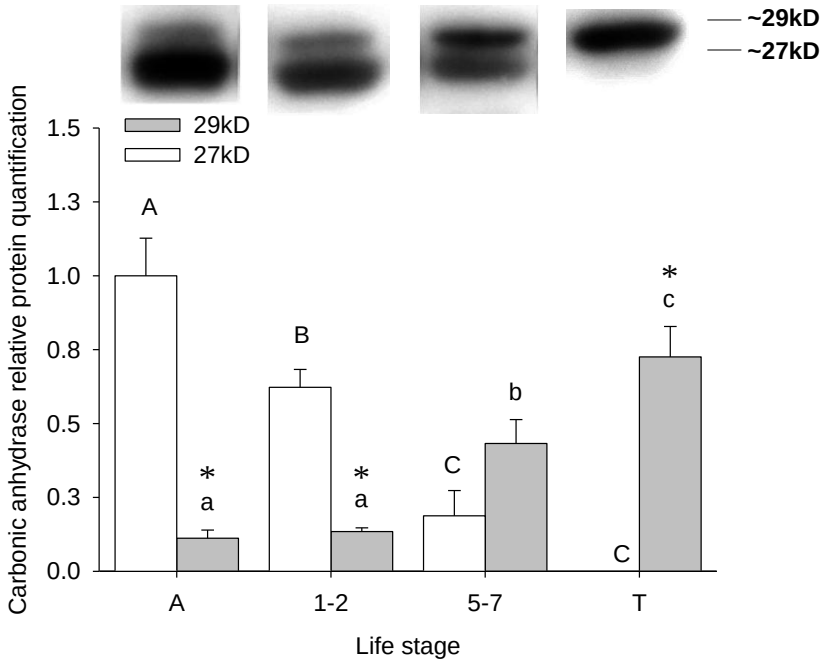

Fig. 2.7 Representative cropped western blot of sea lamprey gill carbonic anhydrase expression (mean ± S.E.M.) using a heterologous cytosolic carbonic anhydrase antibody (1:2000) cross reactivity with 27kD and 29kD bands in a developmental stage specific pattern collected under identical experimental conditions. The developmental series includes ammocoete (N=10); metamorphic stages 1 to 2 (N=20); stages 5 to 7 (N=7); and post-metamorphic juvenile (N=15). Changes in 27kD and 29kD bands are analyzed separately and bars with like letters are not significantly different from each other with (in lower and upper case letters, respectively). Significant differences between 27kD and 29kD bands within a developmental group are indicated by an asterisk. Two-way ANOVA and SNK post-hoc test $P<0.05$.

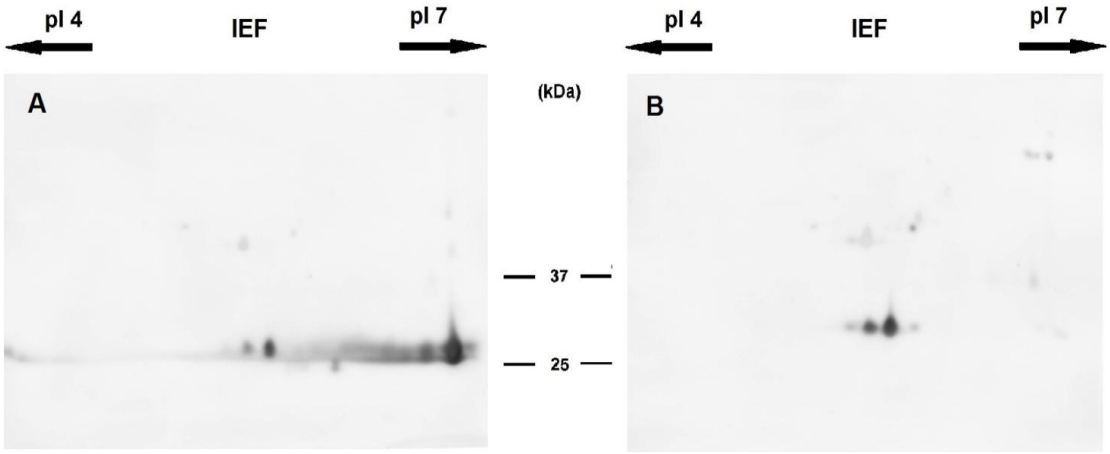

Fig. 2.8 2-DE gel western blotting on erythrocyte samples (150 µg protein) of (A) ammocoete (N=4) and (B) post-metamorphic juveniles (N=4). Proteins were first separated by IEF in a pH gradient from 4 to 7 and further by SDS-PAGE (10%T). Membranes were probed using heterologous cytosolic carbonic anhydrase antibody (1:1000).

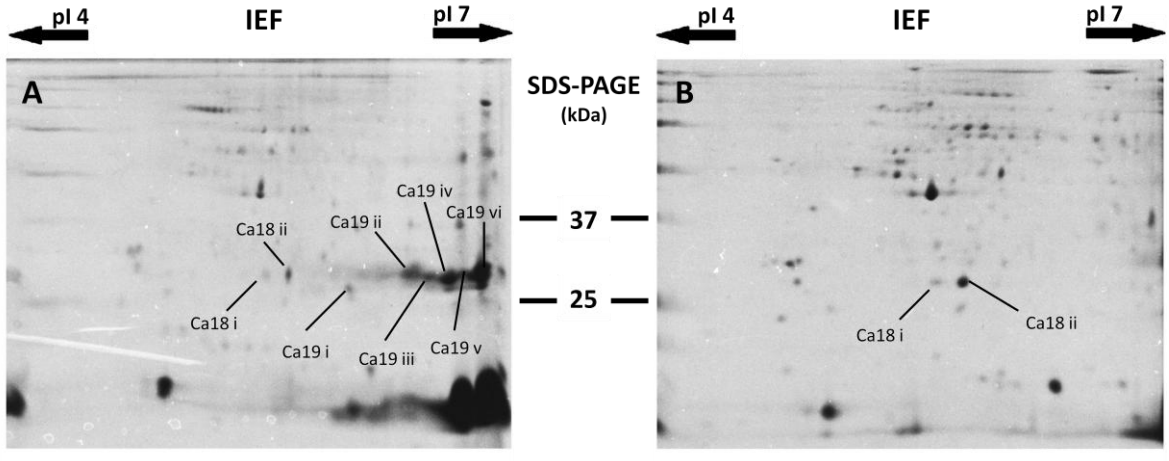

Fig. 2.9 2-DE gels of ammocoete (A) and juvenile (B) red blood cell samples. The gels were each loaded with 150 µg protein. Proteins were first separated by IEF in a pH gradient from 4 to 7 and further by SDS-PAGE (12%T). Gels were stained by Coomassie Blue Colloidal as described by Consoli and Damerval (2001). Spots identified as carbonic anhydrase a (Ca18) and carbonic anhydrase b (Ca19) by MS/MS and database search are indicated.

2.4.5 Discussion

We have identified and characterized a novel cytosolic carbonic anhydrase isoform, *ca19*, which is highly expressed in the gill and RBCs of sea lamprey during its early life history stages. In addition we have compared this novel isoform with the cytosolic carbonic anhydrase (*ca18*) previously described in adult sea lamprey by Esbaugh and Tufts (2006). Our findings indicate a clear and sustained isoform switch during metamorphosis. The *ca19* isoform is expressed during the ammocoete stage and during the initial stages of metamorphosis. In contrast, *ca18* mRNA and protein is expressed at low levels during the larval stage and becomes more highly expressed only during the latter stages of metamorphosis and into the post-metamorphic stages when the sea lamprey is prepared to enter its marine trophic phase (Beamish, 1980a). These results suggest *ca19* isoform may play an important role during the time sea lamprey live in the silty substrates of freshwater streams when the ammocoete is hyperosmoregulating and lives as a filter feeder on a low energy diet.

The carbonic anhydrase gene family has as wide diversity of isoforms and functions among fish groups (Gilmour and Perry, 2009). However, our understanding of carbonic anhydrase diversity, function and distribution is still incomplete when considered in an evolutionary framework. Prior to this study a single high-activity cytosolic carbonic anhydrase isoform (*ca18*) has been found in the sea lamprey, although the evolutionary relationships to other vertebrate carbonic anhydrases is contentious (Esbaugh and Tufts, 2006; Gilmour and Perry, 2009; Gilmour *et al.*, 2007). This isoform was found in a variety of tissues including blood, brain, kidney and gill but absent in muscle, liver and intestine in adult lamprey using Northern blotting (Esbaugh and Tufts, 2006). In our study we have identified a novel cytosolic carbonic anhydrase, isoform 19, which is preferentially expressed in the lamprey's larval stages in tissues such as gill, blood and anterior and posterior intestine. The discrepancy in intestinal expression between the two studies can be explained by the differences in the life history stages and the sensitivity of the techniques that were used. Esbaugh and Tufts (2006) studied adults on their upstream spawning migration, and the digestive system is known to degrade as lampreys do not feed at this stage and die after spawning (Sidon and Youson, 1983). Northern blotting is also less sensitive than PCR based techniques used in the present study (Shi and Chiang, 2005; Wang *et al.*, 1989). In contrast, our finding of *ca18* and *ca19* mRNA expression indicates the presence of cytoplasmic carbonic anhydrases in the gut during larval and post metamorphic stages, when the digestive system is fully functional. In

addition, the hematopoietic tissue in larval lamprey is in the gut associated typhosol (Ardavin *et al.*, 1984; Lanfranchi *et al.*, 1994) and thus high carbonic anhydrase mRNA expression would be expected (Villeval *et al.*, 1985). As for aHb expression, which we used as an indicator of RBC contamination of tissues, our results indicate a switch from *aHb9* to *aHb2a* in the ontogeny of sea lamprey in agreement with a recent report (Rohlfing *et al.*, 2016).

The molecular mass of Ca19 determined *in silico* closely matched the values measured experimentally by gel electrophoresis. This supports our findings indicating changes in protein levels through immunoblotting from gill tissue probed with a heterologous CA2 antibody. The 27kD band shows higher relative protein levels during earlier life-history stages, while during metamorphosis there is a shift in the protein expression of both bands. In the final metamorphic stages the 29kD band is much more intense while the 27kD band decreases and is undetected in fully transformed lampreys. Notably, the predicted molecular masses of Ca19 and Ca18 correspond to the 27 and 29 kDa bands, respectively. However, in order to confirm the reactivity of both isoforms with the heterologous CA2 antibody we utilized an MS/MS analysis approach of the excised 2-DE gel spots. This approach confirmed a match with the previously published carbonic anhydrase sequence (Ca18) by Esbaugh and Tufts (2006) in the RBC's of post-metamorphic lamprey and the carbonic anhydrase sequence identified with this work (Ca19) in RBC's of ammocoetes. The pI of eukaryotic proteins provides insight of their subcellular localization (Weiller *et al.*, 2004) and experimental pI can be applied to distinguish protein isoforms and modifications (Zhu *et al.*, 2005).

In most cases the pI predicted by the databases closely matches the experimentally determined value (Bjellqvist *et al.*, 1994, 1993; Righetti and Bossi, 1997), nevertheless is not uncommon to experimentally observe shifts in the value. These shifts translate protein modifications such as truncations and deletions and more often associated with co - or posttranslational phosphorylations. Phosphorylations play a key role in regulatory mechanisms in the cells (Faux *et al.*, 1996; Gschwind *et al.*, 2004; Hunter, 1995) by replacing neutral hydroxyl groups on serine, threonine or tyrosine residues with phosphate group(s) that are negatively charged (Halligan *et al.*, 2004). As a result, phosphorylation typically induces an acidic shift on pI. Analysis using ProMoST demonstrated that all experimental pI's were found lower than their respective nonphosphorylated theoretical pI value. Additionally, theoretical predictions of pI values using Compute pI/Mw tool according to Gasteiger *et al.* (2005) (ExPASy Server; http://web.expasy.org/compute_pi/) were determined to be 5.47 and 6.23 for Ca19 and Ca20, respectively which are lower than values predicted using ProMoST. Nevertheless this discrepancy seems to be associated with different model applied in the two used

bioinformatic softwares. These differences in the models also likely explain the different predicted number of probable modifications by phosphorylation using ProMoST and NetPhos 3.1 Server softwares.

In addition to phosphorylation modifications, multiple Ca19 and Ca20 protein spots in this study as result of a shift in protein pI values could additionally be attributed to acetylation at the N-terminal of the residues sequence. This has already been documented for acidic and neutral protein (Bjellqvist *et al.*, 1993), where the removal of amino group(s) by this process results in an adjustment of the acid-base balance and shifts the protein pI value. Overall, the lower number of experimentally determined Ca18 and Ca19 spots differing in pI value comparing to theoretical predictions and the different number between the detected spots for both isoforms should not be surprising as the lack of differences in experimental pI separated proteins do not exclude the presence of posttranslational modifications. Also, amounts of some phosphorylated forms could be in such low amount that could not be detected by the used method.

Previous phylogenetic analyses have shown that fish cytoplasmic carbonic anhydrase diverged prior to the gene duplication events that gave rise to the mammalian carbonic anhydrase gene cluster (Esbaugh, 2005; Esbaugh *et al.*, 2004; Lund *et al.*, 2002) with the exception of CA7, where orthologues have also been found in fish (Esbaugh and Tufts, 2006). In mammals both high and low turnover carbonic anhydrase isozymes can be found while only high turnover isozymes are present in fish and are catalytically akin to mammalian CA2 (Gilmour, 2010). However, we were able to identify a new cytoplasmic carbonic anhydrase in lamprey. Our findings suggest that cyclostomes retain a unique cytoplasmic carbonic anhydrase set, since both phylogenetics and synteny analysis indicate that *ca18* and *ca19* might not be orthologues of previously described carbonic anhydrase genes in gnathostomes. The examination of the carbonic anhydrase genomic *loci* genes in lamprey and humans supports the hypothesis that duplications in vertebrate ancestry were instrumental in the elaboration of carbonic anhydrase diversity. We hypothesize that the cyclostomes' cytoplasmic carbonic anhydrase genes probably represent retained genome duplicate paralogues. In effect, the lamprey carbonic anhydrase genes might represent genome duplicates of a different origin to gnathostomes. It has recently been put forward that vertebrates underwent one genome duplication, in contrast to the two or three rounds previously proposed (Mehta *et al.*, 2013; Nah *et al.*, 2014), which does not allow us to determine the exact duplication event from which cyclostome *ca18* and *ca19* arose.

An analysis of the Ca19 active site pocket revealed seven and eight amino acid differences from Ca18 (Esbaugh and Tufts, 2006), and rainbow trout "CAc" (Esbaugh, 2005; Esbaugh *et al.*, 2004; Henry *et al.*, 1993; Maren *et al.*, 1980; Tufts *et al.*, 2003),

respectively, both of which have high catalytic rates. In comparisons with the mammalian low turnover CA1 and high turnover CA2, the Ca19 isoform shows twelve and nine amino acid differences from their respective active site pockets. However, for the most part the amino acid differences are predicted to be substitution neutral, and significantly the essential proton shuttle histidine-64 (H) and zinc binding ligand residues (H94, H96, H119) are conserved. However, two of the amino acids that border or have side chains projecting into the active site at positions 91 and 204 show predicted unfavourable substitution ratings (-2 and -3, respectively). At residue 91 conformationally flexible glycine (G) is found in place of hydrophobic isoleucine (I), and at residue 204 polar asparagine (N) is found in place of hydrophobic leucine (L). These and the other neutrally predicted amino acid differences might impart changes in the three dimensional structure (Betts, Matthew J., 2003) and access to the catalytic site potentially impacting Ca19 activity, kinetics and/or inhibitor binding (Marino *et al.*, 2007). Analysis using NetPhos 3.1 Server demonstrated that five possible phosphorylation site correspond to active site pockets of Ca20 (tyrosine-7 (Y), serine-29 (S), S67, threonine-200 (T) and T244 represented in Table 2.1), which are likely to alter the activity of the catalytic activity of the enzyme as demonstrated in rainbow trout by Carrie and Gilmour (2016).

In our analysis of the electrostatic potential of cytosolic carbonic anhydrases in fishes, we find that RBC carbonic anhydrases have values around 1.0 with the exception of zebrafish Ca2-like b, whereas tissue carbonic anhydrases have negative electrostatic potentials that tend to be more variable. Marino *et al*. (2007) observed that both *C. hamatus* and trout tissue type Ca2-like a shared a similar negative electrostatic potential in contrast to RBC Ca2-lika b carbonic anhydrases suggesting a diversification of fish isoforms based more on cell type than species. These differences may reflect the dominate roles of RBC and tissue carbonic anhydrases in blood CO_2 transport, and ion and acid-base regulation, respectively, related to protein interactions. Although both lamprey Ca18 and Ca19 have been demonstrated to be expressed in RBC, they both have negative electrostatic potential values which may reflect the different mode by which lamprey RBCs participate in the convective transport of blood gases (Tufts and Boutilier, 1989).

Cytosolic carbonic anhydrases in the gill are important for whole animal ion and acid-base regulation providing an intracellular pool of H^+ and HCO_3^- from CO_2 hydration for exchange with Na^+ and Cl^-, respectively, as well as for aiding in metabolic processes and acid-base regulation of individual gill cells that have a high metabolism and generate excessive CO_2 levels (Hewett-Emmett and Tashian, 1996). Branchial cytosolic carbonic anhydrases apparently do not have a role in facilitating respiratory CO_2 elimination (Conley and Mallatt, 1988). We propose that loss of the novel ammocoete isoform *ca19* is

associated with the loss of the enigmatic ammocoete mitochondrion-rich cells (MRC) and that the increase in *ca18* is triggered by metamorphosis as preparation for the higher activity marine trophic phase of this species lifecycle.

In ammocoetes, Conley and Mallatt (1988) have localized carbonic anhydrase activity by enzyme histochemistry to the lamellar epithelium and RBCs. Significantly, this lamellar epithelial localization of carbonic anhydrase corresponds to the location of the ammocoete MRCs which are unique to the ammocoete stage and are lost during metamorphosis (Bartels and Potter, 2004; Henry, 1996). This pattern of loss mirrors that of *ca19.* The ammocoete MRCs make up ~60% of epithelial cells covering the gill lamellae and are mitochondrion-rich. The function of ammocoete MRC's is unknown although various hypotheses have been presented that include ion uptake and metabolic waste elimination. The high mitochondrial density of these cells would indicate a high metabolic activity and cytosolic carbonic anhydrase would have a role in modulating cellular acid-base demands (Henry, 1996). A role in ion regulation seems unlikely since Bartels and co-workers (2009) found no morphometric changes with ion poor water challenges and these cells are not present in adult freshwater migrants which also need to hyperosmoregulate.

In addition, we postulate that the environmental conditions ammocoetes live under may shape their carbonic anhydrase expression profile. Ammocoetes live buried in the silt and muddy substrate (Beamish, 1980a; Hardisty and Potter, 1971b) where higher CO_2 levels and humic substances contribute to a more acidic environment (Schindler and Krabbenhoft, 1998). In substrate dwelling sand eel, Behrens *et al*. (2010) have demonstrated that O_2 levels in the surrounding substrate drop and given that CO_2 is eliminated during the exhalation, it would be reasonable to expect that CO_2 levels would be higher. In trout, hypercapnia has been shown to increase branchial carbonic anhydrase (activity, protein and mRNA) (Dimberg and Höglund, 1987; Georgalis, 2006; Nawata and Wood, 2008). While it is purely speculative at this point, the presence of *ca19* during the ammocoete life stages might impart a higher CO_2 tolerance as an adaptation to this environment.

Branchial intercalated mitochondria-rich cells (IMRC), which are expressed in other life history stages, are a more likely candidate for active ion uptake and acid-base regulation (Dimberg and Höglund, 1987; Henry, 1996) and we would propose as a site for the expression of *ca18*. Carbonic anhydrase immunoreactivity has been localized to IMRC using heterologous antibodies in freshwater post-metamorphic *Geotria australis* (Choe *et al.*, 2004) and sea lamprey (Reis-Santos *et al.*, 2008); however, in the latter study weaker immunoreactivity was also found throughout the rest of the gill epithelium. In both IHC studies, a similar apical localization with H^+-ATPase was observed which supports the role of carbonic anhydrase as a provider of an intracellular supply of H^+ for the pump through

the carbonic anhydrase catalysed hydration of CO_2 (Bartels and Potter, 2004; Evans *et al.*, 2005). In addition, results from the salinity acclimation of post metamorphic juveniles revealed an increase in *ca18* mRNA expression suggesting that this isoform plays an important role in adaptation to higher salinity environments as well. However, it should be noted that Reis-Santos *et al.* (2008) did not observe a change in the pattern of CA-immunoreactive cells with salinity acclimation. Nonetheless, increasing expression of *ca18* isoform at the mRNA and protein levels during metamorphosis may reflect the preparation for seawater entry and/or development of the parasitic stage in seawater. In a number of studies on teleost fishes (*Oreochromis mossambicus* (Kültz *et al.*, 1992) *Fundulus heteroclitus* (Scott *et al.*, 2008), *Dicentrarchus labrax* (Boutet *et al.*, 2006)) gill carbonic anhydrase increases with salinity acclimation, although others have found that distribution of carbonic anhydrase does not change [reviewed by (Conley and Mallatt, 1988)]. The high metabolic activity of these ionocytes would require cytosolic carbonic anhydrase to alleviate any cellular acid-base disturbances from endogenously generated CO_2. Such a role for carbonic anhydrase has been demonstrated in the shark salt secreting rectal gland by Shuttleworth and co-workers (2006) which perform the same function as seawater fish gill ionocytes.

2.5 Conclusions

Our study provides insight into the molecular events that occur during the life history of sea lamprey. Specifically we demonstrate a previously unknown molecular switch between carbonic anhydrase isoforms occurring during metamorphosis. We propose that the novel switch from the *ca19* to *ca18* is of functional importance and related to differences in the demands of ion and acid-base regulation and altered metabolic demands from adoption of an active blood-feeding lifestyle. However, clearly this is an area in need of further study, in particular the functional characterization of the catalytic, kinetic and inhibitor binding of Ca19.

2.6 Acknowledgements

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2.7 Author contributions statement

J.M., D.F-M. and L.C. conceived the experiments, D.F-M. and J.M. conducted the experiment(s), D.F-M., L.C., M.L-P., H.O., A.C. and S.M. analyzed the results. All authors reviewed the manuscript.

2.8 Additional information

Genbank assession number for *ca19* KT030772

2.9 Competing financial interests

The authors declare no competing financial interests.

2.10 Supplemental materials

Supplemental Table 2.1 Sequence ID and accession numbers used for phylogenetic study.

Species	Gene	Accession
H. sapiens	CA13	NP_940986
	CA1	NP_001729
	CA3	NP_005172
	CA2	NP_000058
	CA7	NP_005173
	CA5B	NP_009151
	CA5A	NP_001730
M. domestica	Ca13	XP_001366749.3
	Ca1	NP_001028142.1
	Ca3	XP_001366645.1
	Ca2	XP_001376657.2
	Ca5a	XP_007477337.1
	Ca5b	XP_007500931.1
	Ca7	XP_001364411
G. gallus	CA13	XP_003640859
	CA3a	NP_001264339
	CA3b	NP_001264340
	CA2	NP_990648
	CA7	XP_414152
	CA5	XP_414195
M. nubicus	Ca1	XP_008940663.1
A. carolinensis	Ca3	XP_003219614.1
P. sinensis	Ca1	XP_006113045.1
	Ca2	XP_014437144.1
	Ca7	XP_006130611.1
	Ca5	XP_006118785.1
X. tropicalis	Ca13	NP_001072448
	Ca1	XP_002939198
	Ca3	XP_002939197
	Ca2b	NP_001015729
	Ca2a	NP_001072785
	Ca7	AAI21633
	Ca5	NP_001039155
L. chalumnae	Ca2-like	XP_006010309
	Ca7	XP_006004096
	Ca5	XP_005989205

L. osseus	Ca2-like	AAM94169
L. oculatus	Ca7	XP_006641282
	Ca5	XP_006641382.1
D. rerio	Ca2b (Cahz)	NP_571185
	Ca2a (Ca2)	NP_954685
	Ca5	NP_001104671
	Ca7	AAH49309
O. latipes	Ca2b	XP_011484743
	Ca2a	XP_004081218
	Ca5	XP_004069790
G. morhua	Ca2a	ENSGMOG00000015729
	Ca2b	ENSGMOG00000015697
	Ca5	ENSGMOG00000004153
	Ca7	ENSGMOG00000001295
G. aculeatus	Ca5	ENSGACP00000004208
P. marinus	Ca19	KT030772
	Ca18	AAZ83742
	Ca5	ENSPMAP00000010279
L. japonicum	Ca19	JL12788
	Ca18	JL10053
S. canicula	Ca5	ctg95805
C. milii	Ca2-like	AFK10663
	Ca5	XP_007887550
	Ca7	XP_007906064
L. erinacea	Ca5	ctg14021
	Ca2-like	ctg70321
B. floridae	Ca	XP_002594303
D. melanogaster	Ca	NP_523561

Supplemental Table 2.2 Protein identification by MALDI-TOF/TOF analysis. Identification scores obtained with the paragon algorithm from the protein pilot program (*). n.o. = no observation.

Spot #	Protein name	Identified protein and NCBI database reference (1)	Organism	Molecular mass (kDa) (2)	pI (3)	Identification parameters						
						Ident. score (4)	Total ion score (5)	Matched fragmented peptides (6)	sequence coverage % (7)	$M+H^+$ (8)	Peptide sequence (9)	Ion score (10)
Ca18 i	Carbonic anhydrase	tr|Q3Y546|Q3Y546 _PETMA	*Petromyzon marinus*	29	5.47	399	327	3	100	1401.7195 1620.8519 1755.8813	CVLSGGPLPNPYK VDFLDYDPSVLLPK TYSAELHLVHWNSAK	90 92 145
Ca18 ii	Carbonic anhydrase	tr|Q3Y546|Q3Y546 _PETMA	*Petromyzon marinus*	29	5.47	249	224	4	100	837.41 1044.6049 1128.5684 1401.7195	SFAEAANK VTDTLNIIR YKSFAEAANK CVLSGGPLPNPYK	73 29 89 33
Ca19 i	Pma ammocoete carbonic anhydrase	n.o.	*Petromyzon marinus*	27.6	6.23	362	267	3	100	834.4468 1430.66 1725.8707	EQLAAFR QFHFHWGASDAK SYSAELHLVHWNAAK	47 104 116

Ca19 ii	Pma ammocoete carbonic anhydrase	n.o.	*Petromyzon marinus*	27.6	6.23	468	335	2	100	834.4468 1430.66	EQLAAFR QFHFHWGASDAK	48 86
Ca19 iii	Pma ammocoete carbonic anhydrase	n.o.	*Petromyzon marinus*	27.6	6.23	440	333	3	100	834.4468 1430.66 1472.8042	EQLAAFR QFHFHWGASDAK LVCNFRPTQPLK	52 75 62
Ca19 iv	Pma ammocoete carbonic anhydrase	n.o.	*Petromyzon marinus*	27.6	6.23	463	343	4	100	834.4468 1430.66 1472.8042 1725.8707	EQLAAFR QFHFHWGASDAK LVCNFRPTQPLK SYSAELHLVHWNAAK	45 108 46 144
Ca19 v	Pma ammocoete carbonic anhydrase	n.o.	*Petromyzon marinus*	27.6	6.23	457	337	4	100	834.4468 1430.66 1472.8042 1725.8707	EQLAAFR QFHFHWGASDAK LVCNFRPTQPLK SYSAELHLVHWNAAK	47 86 60 144

Ca19 vi	Pma ammocoete carbonic anhydrase	n.o.	*Petromyzon marinus*	27.6	6.23	458	338	3	100	834.4468 1430.66 1472.8042	EQLAAFR QFHFHWGASDAK LVCNFRPTQPLK	44 103 46

1) Accession number of the identified protein from the NCBI database. 2) Protein theoretical molecular mass. 3) Protein theoretical isoelectric point. 4) Identification scores obtained with the algorithms Mowse or Paragon. Minimum scores were 78 and 2 for the Mowse and Paragon algorithms respectively corresponding to a $P<0.05$. 5) The sum of all individual ion scores of the fragmented ions. 6) Different peptides matching the sequence of the identified protein. 7) Percentage of the identified protein sequence covered by the matched peptides. 8) Monoisotopic masses of the fragmented peptides. 9) Sequence of the fragmented peptides. 10) Individual ion scores from the Mowse algorithm respecting the identification of peptide sequences. Values obtained correspond to a $P<0.05$.

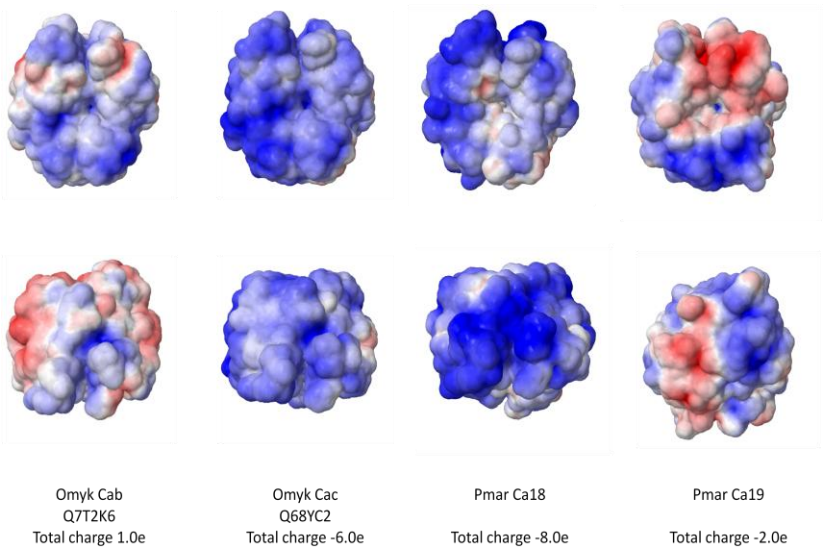
Supplemental Table 2.3 (A) Electrostatic potential of carbonic anhydrase from teleost fishes corresponding to the tissue-cell type (TC; Ca2-like a = Cac) or red blood cell (RBC) type (Ca2-like b =Cahz). (B) Electrostatic potential distribution mapped on the Gaussian-type surface calculated by APBS and visualized with PyMol for trout and lamprey carbonic anhydrases. The potentials range from -5.0kT per proton charge (blue) to 5.0kT per proton charge (red). The structures are graphically depicted looking down the active-site cleft (upper) and in a 180º rotated view (lower).

A

Spp	CA	ID	Total protein charge	Comment
Danio rerio	Ca2-like b	Q92051	0.00	RBC; FW
Oreochromis niloticus	Ca2-like b	ENSONIP00000008038	1.00	RBC; FW
Oryzias latipes	Ca2-like b	ENSORLP00000010477	1.00	RBC; FW
Poecilia formosa	Ca2-like b	ENSPFOP00000002771	1.00	RBC; FW
Xiphophorus maculatus	Ca2-like b	ENSXMAP00000001090	1.00	RBC; FW
Danio rerio	Ca2-like a	Q6PFU7	5.00	RBC; FW
Gasterosteus aculeatus	Ca2-like b	ENSGACP00000019535	1.00	RBC; FW-EUR
Gadus morhua	Ca2-like b	ENSGMOP00000016838	1.00	RBC; SW
Takifugu rubripes	Ca2-like b	ENSTRUP00000020144	1.00	RBC; SW
Tetraodon nigrovidis	Ca2-like b	ENSTNIP00000008780	2.00	RBC; SW-EUR
Petromyzon marinus	ca18		-8.00	RBC/TC; FW-EUR
Petromyzon marinus	Ca19		-2.00	RBC/TC; FW
Onchorhynchus mykiss	Ca2-like b	Q7T2K6	1.00	RBC/TC; FW
Gadus morhua	Ca2-like a	ENSGMOP00000016873	-7.00	TC; SW
Takifugu rubripes	Ca2-like a	ENSTRUP00000029310	-1.00	TC; SW

Gasterosteus aculeatus	Ca2-like a	ENSGACP00000006664	-2.00	TC; EUR
Poecilia formosa	Ca2-like a	ENSPFOP00000009383	-12.00	TC; FW
Xiphophorus maculatus	Ca2-like a	ENSXMAP00000008413	-8.00	TC; FW
Oreochromis niloticus	Ca2-like a	ENSONIP00000008037	-2.00	TC; FW-EUR
Oryzias latipes	Ca2-like a	ENSORLP00000015962	0.00	TC; FW
Tetraodon nigrovidis	Ca2-like a	ENSTNIP00000016652	-5.00	TC; SW
Onchorhynchus mykiss	Ca2-like a	Q68YC2	-6.00	TC; EUR
Chionodraco hamatus	Ca2-like a	P83299	-7.00e	TC; SW

B



Supplemental Table 2.4 Predicted phosphorylation analysis using ProMoST for proteins Ca18 and Ca19. Protein mass in expressed in Daltons (Da); pI, isoelectric point; phosST, phosphorylation serine-threonine; phosY, phosphorylation tyrosine.

Protein	Average mass (Da)	pI	phosST	phosY
Ca18	28795.28	5.51	0	0
Ca18	28795.28	5.39	1	0
Ca18	28795.28	5.27	2	0
Ca18	28795.28	5.17	3	0
Ca18	28795.28	5.07	4	0
Ca18	28795.28	4.98	5	0
Ca18	28795.28	4.91	6	0
Ca18	28795.28	4.83	7	0
Ca18	28795.28	4.76	8	0
Ca18	28795.28	4.70	9	0
Ca18	28795.28	4.64	10	0
Ca18	28795.28	5.39	0	1
Ca18	28795.28	5.27	0	2
Ca18	28795.28	5.17	0	3
Ca18	28795.28	5.07	0	4
Ca18	28795.28	4.98	0	5
Ca18	28795.28	4.90	0	6
Ca18	28795.28	4.83	0	7
Ca18	28795.28	4.76	0	8
Ca18	28795.28	4.70	0	9

Protein	Average mass (Da)	pI	phosST	phosY
Ca18	28795.28	4.64	0	10
Ca19	27456.60	6.51	0	0
Ca19	27456.60	6.27	1	0
Ca19	27456.60	6.08	2	0
Ca19	27456.60	5.91	3	0
Ca19	27456.60	5.75	4	0
Ca19	27456.60	5.59	5	0
Ca19	27456.60	5.44	6	0
Ca19	27456.60	5.30	7	0
Ca19	27456.60	5.17	8	0
Ca19	27456.60	5.05	9	0
Ca19	27456.60	4.95	10	0
Ca19	27456.60	6.27	0	1
Ca19	27456.60	6.08	0	2
Ca19	27456.60	5.91	0	3
Ca19	27456.60	5.75	0	4
Ca19	27456.60	5.59	0	5
Ca19	27456.60	5.44	0	6
Ca19	27456.60	5.30	0	7
Ca19	27456.60	5.17	0	8
Ca19	27456.60	5.05	0	9

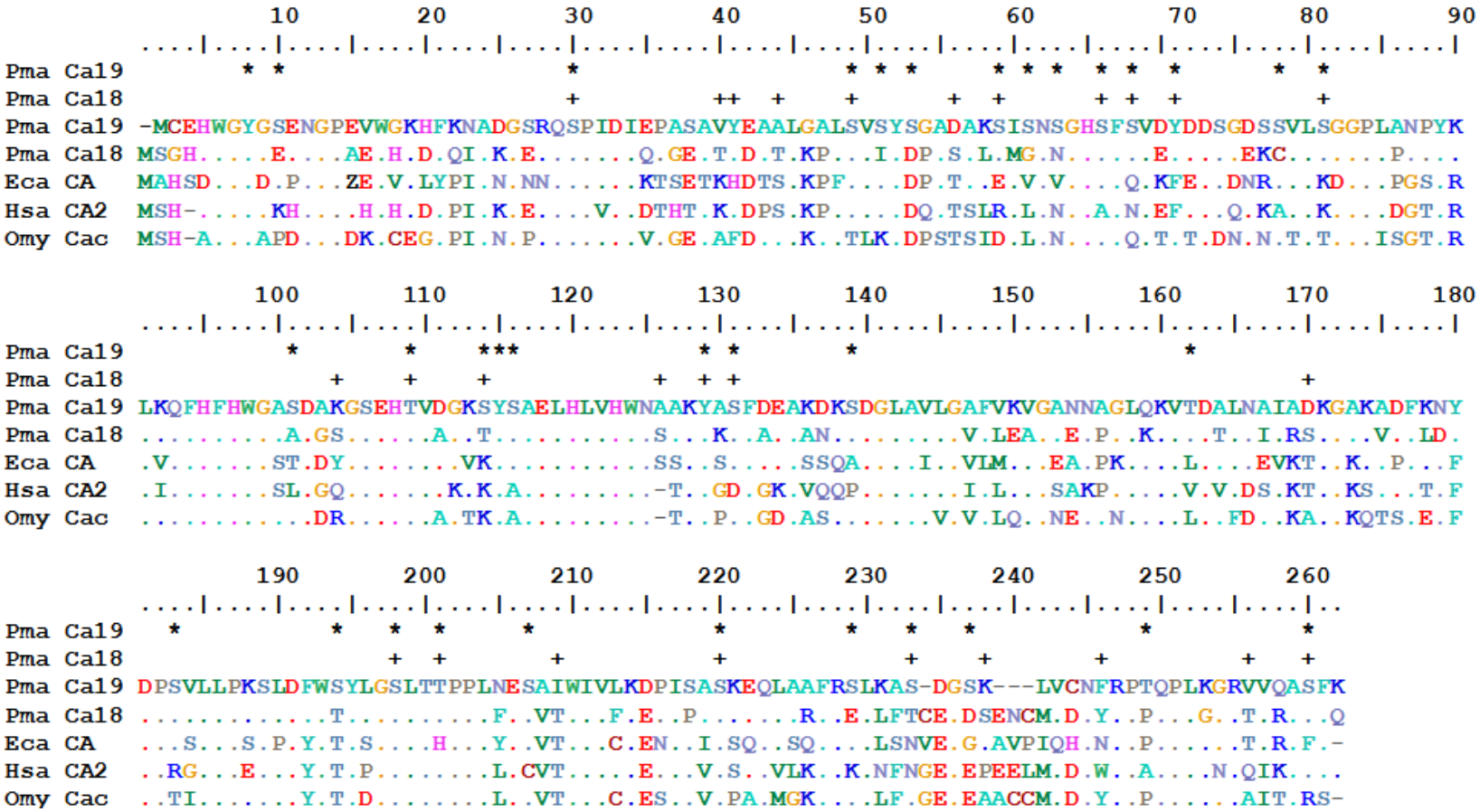
Supplemental Table 2.5 Predicted phosphorylation site analysis using NetPhos 3.1 Server for Ca18 and Ca19. aa, amino acid; cdc2, cell division control 2; CKI, casein kinase 1; CKII, casein kinase 2; DNAPK, DNA-dependent protein kinase; EGFR, epidermal growth factor receptor kinase; INSR, insulin receptor tyrosine kinase; p38MAPK, P38 mitogen-activated protein kinase; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; unsp, unspecific kinase. Predicted phosphorylated aa represented in context centered in the aa motif.

Sequence	aa position	aa	Context	Score	Kinase
Ca18	30	S	GERQSPIDI	0.997	unsp
Ca18	40	T	PGEATYDAT	0.869	unsp
Ca18	41	Y	GEATYDATL	0.531	unsp
Ca18	44	T	TYDATLKPL	0.734	PKC
Ca18	44	T	TYDATLKPL	0.593	unsp
Ca18	49	S	LKPLSVIYD	0.961	unsp
Ca18	49	S	LKPLSVIYD	0.558	PKC
Ca18	49	S	LKPLSVIYD	0.528	PKA
Ca18	56	S	YDPASALSM	0.514	cdc2
Ca18	59	S	ASALSMGNN	0.905	unsp
Ca18	59	S	ASALSMGNN	0.529	cdc2
Ca18	59	S	ASALSMGNN	0.514	CKI
Ca18	66	S	NNGHSFSVE	0.532	cdc2
Ca18	68	S	GHSFSVEYD	0.869	unsp
Ca18	71	Y	FSVEYDDSG	0.961	unsp
Ca18	81	S	KCVLSGGPL	0.622	PKA
Ca18	104	S	AADGSGSEH	0.969	unsp
Ca18	104	S	AADGSGSEH	0.603	CKII
Ca18	109	T	GSEHTVAGK	0.573	PKC
Ca18	114	T	VAGKTYSAE	0.606	PKC
Ca18	126	S	VHWNSAKYK	0.718	PKC

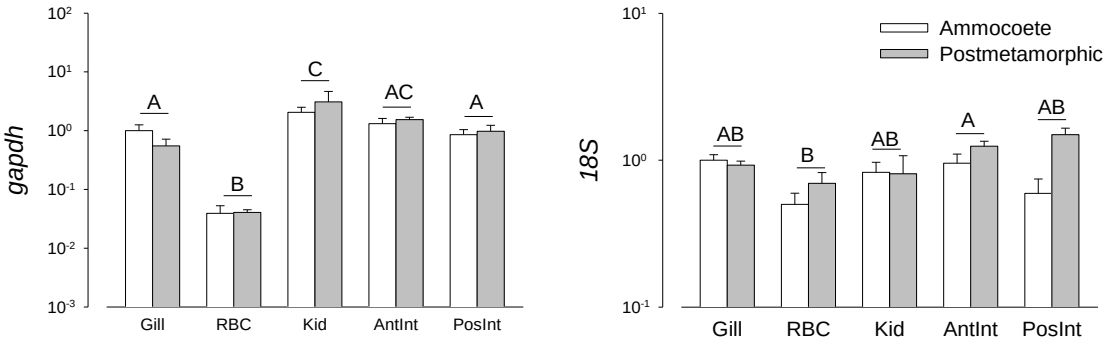
Ca18	129	Y	NSAKYKSFA	0.900	unsp
Ca18	131	S	AKYKSFAEA	0.963	unsp
Ca18	131	S	AKYKSFAEA	0.517	PKG
Ca18	170	S	NIIRSKGAK	0.769	PKC
Ca18	198	S	TYLGSLTTP	0.586	PKC
Ca18	198	S	TYLGSLTTP	0.527	PKA
a18	201	T	GSLTTPPLF	0.584	p38MAPK
Ca18	209	T	FESVTWIVF	0.655	PKC
Ca18	220	S	PIPASKEQL	0.828	unsp
Ca18	233	T	ELLFTCEGD	0.509	CKII
Ca18	233	T	ELLFTCEGD	0.502	CKI
Ca18	238	S	CEGDSENCM	0.705	unsp
Ca18	238	S	CEGDSENCM	0.532	CKII
Ca18	246	Y	MVDNYRPPQ	0.501	EGFR
Ca18	256	T	LGGRTVRAS	0.610	PKC
Ca18	256	T	LGGRTVRAS	0.539	unsp
Ca18	260	S	TVRASFQ--	0.838	unsp
Ca18	260	S	TVRASFQ--	0.540	PKA
Ca19	7	Y	EHWGYGSEN	0.553	unsp
Ca19	9	S	WGYGSENGP	0.526	CKII
Ca19	29	S	GSRQSPIDI	0.998	unsp
Ca19	29	S	GSRQSPIDI	0.511	CKI
Ca19	48	S	LGALSVSYS	0.604	PKC
Ca19	50	S	ALSVSYSGA	0.937	unsp
Ca19	50	S	ALSVSYSGA	0.563	cdc2
Ca19	52	S	SVSYSGADA	0.975	unsp

Ca19	58	S	ADAKSISNS	0.956	unsp
Ca19	58	S	ADAKSISNS	0.566	cdc2
Ca19	60	S	AKSISNSGH	0.678	unsp
Ca19	60	S	AKSISNSGH	0.563	cdc2
Ca19	62	S	SISNSGHSF	0.682	PKC
Ca19	62	S	SISNSGHSF	0.680	unsp
Ca19	65	S	NSGHSFSVD	0.951	unsp
Ca19	67	S	GHSFSVDYD	0.595	unsp
Ca19	67	S	GHSFSVDYD	0.501	CKII
Ca19	70	Y	FSVDYDDSG	0.975	unsp
Ca19	77	S	SGDSSVLSG	0.728	unsp
Ca19	80	S	SSVLSGGPL	0.839	unsp
Ca19	100	S	HWGASDAKG	0.591	PKC
Ca19	108	T	GSEHTVDGK	0.549	PKC
Ca19	113	S	VDGKSYSAE	0.984	unsp
Ca19	113	S	VDGKSYSAE	0.515	cdc2
Ca19	114	Y	DGKSYSAEL	0.502	INSR
Ca19	115	S	GKSYSAELH	0.666	unsp
Ca19	128	Y	NAAKYASFD	0.849	unsp
Ca19	130	S	AKYASFDEA	0.970	unsp
Ca19	130	S	AKYASFDEA	0.582	CKII
Ca19	138	S	AKDKSDGLA	0.672	unsp
Ca19	138	S	AKDKSDGLA	0.540	PKA
Ca19	161	T	LQKVTDALN	0.516	CKII
Ca19	182	S	NYDPSVLLP	0.577	PKC
Ca19	193	S	LDFWSYLGS	0.507	cdc2
Ca19	197	S	SYLGSLTTP	0.686	PKC
Ca19	197	S	SYLGSLTTP	0.591	PKA

Ca19	200	T	GSLTTPPLN	0.584	p38MAPK
Ca19	206	S	PLNESAIWI	0.503	PKG
Ca19	219	S	PISASKEQL	0.833	unsp
Ca19	228	S	AAFRSLKAS	0.553	PKC
Ca19	232	S	SLKASDGSK	0.846	unsp
Ca19	232	S	SLKASDGSK	0.579	PKC
Ca19	235	S	ASDGSKLVC	0.507	cdc2
Ca19	244	T	NFRPTQPLK	0.598	DNAPK
Ca19	244	T	NFRPTQPLK	0.587	PKC
Ca19	255	S	VVQASFK--	0.935	unsp
Ca19	255	S	VVQASFK--	0.807	PKC



Supplemental Figure 2.1 Alignment of *P. marinus* (Pma) sequence obtained from ammocoete gill (Ca19) with published *P. marinus* Ca18 (GenBank, AAZ83742.1), *Equus caballus* (Eca) CA (GenBank, P00917.3), *Homo sapiens* (Hsa) CA2 (GenBank, AAA51908.1), and *Oncorhynchus mykiss* (Omy) Cac (GenBank, AAR99329.1) aa sequences using ClustalW (BioEdit 7.0.9.0). Predicted phosphorylation sites using NetPhos 3.1 Server for Ca18 (+) and Ca19 (*), respectively.



Supplemental Figure 2.2 Transcript level expression of Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) (Pancer *et al.*, 2004) and Small subunit (18S) ribosomal RNA gene (*18s*) (Stock and Whitt, 1992) in *P. marinus* ammocoete and post-metamorphic juvenile gill, red blood cells (RBC), kidney (Kid), anterior (AntInt) and posterior (PostInt) intestine determined by qPCR. Data was analyzed by two way ANOVA followed by SNK post-hoc test (N=4). Tissue expression levels are significantly different if they lack common letters. Significant differences between ammocoete and juvenile within a given tissue are indicated by an asterisk.

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Chapter 3:

Parasite-host interactions: osmoregulatory challenges of sea lamprey, *Petromyzon marinus* parasitic feeding on the trout *Salvelinus fontinalis*.

3.1 Abstract

The sea lamprey, *Petromyzon marinus,* is an anadromous species in which the freshwater ammocoete (larvae) undergoes metamorphosis and the fully transformed juvenile migrates to seawater where it begins parasitic feeding on fish and marine mammal body fluids. Sea lamprey parasitic feeding poses a physiological challenge for the host by inflicting nonlethal wounds, body fluid depletion and also poses an osmoregulatory challenge due to water loss. From the perspective of the sea lamprey, feeding has also impact on osmoregulatory status serving as a source for isosmotic fluid.

To date no studies have been conducted to analyze the parasite-host interaction during the marine life stage of the anadromous sea lamprey. Results show that in seawater lamprey, ion uptake mechanisms were successfully downregulated (NCC, ENaC and V-ATPase mRNA expression) as well as kidney NKA activity. In contrast, ion secretion mechanisms were upregulated (NKAα1 and NKCC1 mRNA expression), except in seawater feeding sea lamprey. This together with the lower ion content in plasma suggest that feeding on a isosmotic body fluids from the host helps compensate for osmotic ion gains through the gills and skin. Also, an increase NKA activity in anterior intestine suggests a role in nutrient absorption. Lower Hsp70 protein in gill suggests reduction in cellular stress.

In the host 23 attacks were registered, from which ~78% pierced the skin. Only 17% of those attacks resulted in host death (<3 days). Parasitized hosts experienced severe anemia, loss of appetite and reduction of condition factor. NKAα1a and NKAα1b mRNA expression (down and up-regulation, respectively) together with plasma ion content and NKA activity demonstrate that brook trout were able to successfully cope with seawater. Parasitic feeding also had an osmoregulatory effects in the host as NKAα1a mRNA which remained high, despite the higher plasma ions levels. It is here hypothesized that NKAα1a is likely playing a role in cell volume maintenance.

3.2 Introduction

Approximately 40 species of lamprey have been described to date (Renaud, 2011) and from those only 18 are believed to be parasitic at some point in their lifecycle (Potter *et al.*, 2015). Of these parasitic species only 9 have a marine trophic phase, which includes the sea lamprey *Petromyzon marinus* (Potter, 1980). However, the sea lamprey has two distinct ecotypes, anadromous (migrates to the oceans and returns to freshwater systems to spawn) and landlocked (completing its lifecycle in freshwater). Lampreys begin their life cycle as ammocoetes (larvae) that live buried in the substrate of streams and filter feed. After this period, ammocoetes undergo a process of metamorphosis and by the time it is complete they emerge from the substrate and in the case of sea lamprey either migrate downstream to the ocean or lakes in anadromous and landlocked forms, respectively. Metamorphosis also marks the transition from filter to parasitic feeding. Following this lifecycle stage, anadromous sea lampreys re-enter freshwater systems and migrate upstream until they find a suitable place to spawn and then die (Beamish, 1980a; Hardisty and Potter, 1971ac).

Most studies on the physiology of feeding in sea lampreys have been performed on landlocked sea lampreys as a result of their devastating effect on Great Lakes fish stocks and the large monetary sums being spent for its control efforts (Hardisty, 2006). On the other hand, information on the anadromous sea lamprey feeding habits is limited since they do not have specific choice of hosts (Hardisty and Potter, 1971c; Silva *et al.*, 2014). Moreover, capturing sufficient numbers of sea lampreys while feeding in the ocean is problematic due to their dispersed distribution, solitary nature and lack of targeted fishery (Silva *et al.*, 2014). In addition, lampreys may often detach from host during handling.

In terms of feeding, observations from Davis (1967) indicate that anadromous sea lamprey in Maine begins feeding in two particular seasons, autumn-early winter and spring. Sea lamprey feed on blood, tissue lysate and other body fluids from their host (Farmer *et al.*, 1975; Gage, 1928; Lennon, 1954), although occasional reports of small fishes (Penczak, 1964) and fish eggs (Bigelow and Schroeder, 1953) have been observed in the intestinal contents of captured lamprey. Landlocked sea lamprey 16-155 g in mass at 10ºC, can ingest from 3 to 30% of their body mass per day. This difference in biology from anadromous sea lamprey to their landlocked ecotype conspecifics could be explained by one or a combination of the following hypotheses: (1) temperature is a key growth factor and if parasitized hosts that opt for warmer waters, lampreys will tend to grow more (Farmer *et al.*, 1977; Swink, 1995, 1993); (2) selection pressure based of size as smaller anadromous marine phase sea lampreys are more susceptible to predation, thus inducing an increase in adult size distribution (Jorgensen and Kitchell, 2005); (3)

requirement of higher biomass to successfully perform the upstream spawning migration (Beamish, 1980a; referred in Jorgensen and Kitchell, 2005); and (4) parasitic feeding on large oceanic hosts with great blood pools results in higher body mass (Jorgensen and Kitchell, 2005). Anadromous ecotype sea lamprey parasitic stage usually lasts from 23 to 28 months (Beamish, 1980a) whilst landlocked ecotype spends from 12 to 20 months at this stage (Hardisty and Potter, 1971a). In laboratory conditions Jorgensen and Kitchell, (2005) were able to demonstrate that landlocked sea lampreys were able to achieve weights of 980 g similar to those found in the anadromous ecotype (Beamish, 1980a) if they were provided the chance to feed on Pacific salmon (≤ 5 kg) for two summers and migrating in spring.

The particular structure of the feeding and respiratory apparatus of the sea lamprey allows for efficient feeding while the adult is attached by its oral disc to a host or the substrate as ventilation of the gills is tidal in which water enters and leaves each gill pouch via the external branchiopore (Hardisty and Potter, 1971a). In order to feed, the sea lamprey starts by opening a wound using its tongue like piston to rasp host skin (Dawson, 1905) and releases both an anticoagulant and cytolytic agent named lamphredin that affects the skeletal muscle, blood vessels, connective tissue, and integument of hosts (Baxter, 2009; Lennon, 1954). Sea lampreys often select the ventral area of hosts near the pectoral fins, which is suggested to be related to the thinner muscular layer, the less dense scaling and blood abundance in this body region (Cochran and Lyons, 2010; Farmer and Beamish, 1973; Farmer, 1980; Nichols and Tscherter, 2011; Silva *et al.*, 2014). The mean red blood cell count in non lethal attacks was 21% lower in hosts (Edsall and Swink, 2001). Later studies performed in trout in freshwater supported these results as hematocrit was reduced from 35.0 to 2.0% in trout parasitized by lamprey and although the percentage of water in the blood increased from 84.5 to 96.4% and plasma osmolality of parasitized trout remained unaltered (Farmer *et al.*, 1975). Landlocked sea lamprey attacks on lake trout showed in 9% of the cases lampreys that were feeding on the host detached from it before its death and 37% of the attacks resulted in the detachment of lampreys before feeding was initiated (Farmer, 1980). The duration of sea lamprey attachment on their host varies from 14 days when host death occurred and up to 83 and 18 days for attachments that did not cause host death and non feeding attacks, respectively (Farmer, 1980). The non-feeding attacks are presumed to be related to satiated lampreys saving energy on locomotion, as swimming performance of lamprey is poor compared to that of teleosts due to the lack of a hydrostatic organ and paired fins to maintain stability (Beamish, 1974).

The survival time of the host fish has been shown to be dependent on its size and on blood loss in relation to blood volume. Fish losing 25% of their blood volume daily,

were able to survive up to 14 days and 2 days, respectively (Farmer, 1980). For this reason, in this study we decided to allow lampreys to attach and feed on brook trout for three consecutive days before sacrificing and sampling both host and parasite. This allowed sufficient time for physiological mechanisms to respond to feeding while minimizing the risk of host death. Also, longer feeding periods would have required larger hosts : lamprey weight ratio of 40:1 to sustain lamprey feeding and host survival (Farmer *et al.*, 1975). In our study, ratio used was 15:1 as larger ratios would implicate host predation on lamprey.

Since most probable hosts of sea lamprey osmoregulate (Silva *et al.*, 2014), their ions content of the internal milieu are well below the levels found in seawater (McCormick, 2013). Therefore, upon parasitic feeding on host hyposmotic body fluids, sea lamprey must compensate for ion levels fluctuation. As for the host, same rule should apply as ions are being drained together with body fluids and need to be reestablished for homeostasis maintenance.

The osmoregulatory mechanisms in sea lamprey are similar to those of teleosts, although difference in the gill at ultrastructural level occur (Bartels and Potter, 2004). When in an hypertonic environment, lampreys resort to a drinking mechanism like other seawater type fishes (Taylor and Grosell, 2009). The Na^+ and Cl^- taken up by enterocytes in the anterior sections of the intestine and following that water is absorbed through osmotic gradients (Pickering and Morris, 1973) and the excess of Na^+ and Cl^- are secreted across the gill (Bartels and Potter, 2004). Divalent ions are eliminated via defecation (Bartels and Potter, 2004). In the gill, the seawater-type ionocytes are the main locations for secretion of excess of monovalent ions (Evans *et al.*, 2005; Ferreira-Martins *et al.*, 2016; Marshall and Grosell, 2006) and have high expression of NKA (Ferreira-Martins *et al.*, 2016; Reis-Santos *et al.*, 2008). NKA is essential for the secondary active Cl^- secretion mechanism (Bartels and Potter, 2004; Evans *et al.*, 2005). The basolateral Na^+/K^+-ATPase creates a favorable electrochemical gradient for Cl^- to enter the cell via Na^+:K^+:$2Cl^-$ cotransporter 1 (NKCC1/*slc12a2*) which then moved out across the apical membrane though a channel homologous of the cystic fibrosis transmembrane conductance regulator (CFTR/*abcc7*) down its electrochemical gradient (Marshall *et al.*, 2002; Singer *et al.*, 1998). In the case of Na^+, it accumulates in the extracellular space and leaks out paracellularly through the leaky tight junctions adjacent to these ionocytes (Bartels and Potter, 2004).

On the other hand, when hyper-osmoregulating the lamprey gill takes up Cl^- which is likely to be performed by epithelial cells with an apical Cl^-/HCO_3^- antiport system, a basolateral Cl^- channel, and cytosolic carbonic anhydrase (CA) (Evans *et al.*, 2005; Marshall, 2002). Reports of immunolocalization of V-ATPase and CA support branchial

mitochondrion-rich intercalated cells (IMRC) as likely ionocytes (Choe *et al.*, 2004; Reis-Santos *et al.*, 2008). As for Na^+ uptake, it is likely achieved via an apical Na^+:Cl^- cotransporter (NCC/*slc12a3*) (Ferreira-Martins *et al.*, 2016) and epithelial Na^+ channel (ENaC/*scnn1*) through indirect coupling with the electrogenic vacuolar H^+-ATPase pump or V-ATPase (Bartels and Potter, 2004; Ferreira-Martins *et al.*, 2016). In teleost fishes such as the brook trout, ENaC is not present and Na^+ uptake is likely performed by an acid-sensing ion channel (ASIC) which has been characterized in other teleost fishes (Dymowska *et al.*, 2014). A cytosolic carbonic anhydrase is essential to create an intracellular pool of H^+s for the V-ATPase and the low intracellular Na^+ levels are maintained by basolateral Na^+/K^+-ATPase. In addition, apical $Na^{+/}H^+$ exchangers have been described as playing a role in Na^+ uptake and acid–base regulation in teleost and elasmobranch fishes as well as hagfish (reviewed by Evans *et al.*, 2005).

The NHE family (*slc9a*) is a multi-gene family and to date a total of nine isoforms have been reported (Brett, 2005) and a β-NHE only found in trout red blood cells (Evans *et al.*, 2005). As NHEs are electroneutral, they rely on the energy created by electrochemical gradients of Na^+ and H^+ to transport ions across the plasma membrane. In addition to Na^+ uptake, NHE isoforms 1, 2 and 3 have been a focus on study in fish acid-base regulation and have been found in both freshwater and seawater acclimated fish (Evans *et al.*, 2005). The NHE1 is suggested to perform as an housekeeping isoform with a role for intracellular pH compensation, particularly in seawater (Edwards *et al.*, 2005). The NHE2 and NHE3 exist in both fresh and seawater acclimated fish and are reported to play a role in systemic acid-base regulation in fish (Perry and Gilmour, 2006) and have been localized to a sub-population of MRCs (Evans *et al.*, 2005).

Rh glycoproteins have been shown to be present in the gill epithelial cells of fish with their main function related to ammonia excretion (reviewed by Wright and Wood, 2009) but also possibly CO_2 excretion (Huang, 2008; Li *et al.*, 2007; Peng and Huang, 2006; Zhou *et al.*, 2003). In fact it has been hypothesized that CO_2 transport was the primary role of Rh (Huang, 2008; Kustu and Inwood, 2006). In vertebrates, the Rh30, Rhag, Rhbg and Rhcg are the four main Rh paralogous genes present (Huang and Peng, 2005). The Rh30 are nonglycosylated and nontransporting and associated with the erythrocyte Rhag complex while the other three have a role in ammonia transport with Rhag usually associated with erythrocytes but occasionally the gills (reviewd by Wright and Wood, 2009). The Rhbg and Rhcg can be found in various tissues including gill, where Rhbg is mostly found localized in the basolateral membrane and Rhcg mostly localized in the apical membrane. The Rhcg is almost exclusively apical and is often coupled with mechanisms for H^+ secretion and Na^+ absorption such as NHE (reviewed by Evans, 2008).

In this study, stress response of freshwater to seawater transition in the fully transformed juvenile sea lamprey and parasitic feeding effect was assessed at the protein levels by immunoblotting targeting the 70-72 kilodalton (kDa) heat shock proteins (Hsp70s). The HSP70s family is part of a very conserved family of stress proteins (Yamashita *et al.*, 2010), which are induced in response to a variety of stress factor as well as metabolic complicacy and act as molecular chaperones with a role in facilitation of synthesis and folding of proteins, protein assembly, secretion, trafficking, and protein degradation (Georgopoulos and Welch, 1993; Hartl, 2002, 1996; Lindquist and Craig, 1988; Morimoto *et al.*, 1990; Pelham, 1982; Wu, 1995; Yamashita *et al.*, 2010).

To date no studies have been conducted to simultaneous evaluate the effects of feeding on the osmoregulatory physiology of both sea lamprey and host from a molecular perspective. The aim of this study is to assess the hypotheses that parasitic feeding on hyposmostic meal compared to the external environment facilitates hyposmoregulation in the juvenile sea lamprey when in seawater. Moreover, we the osmoregulatory effects on the host species when parasitized by sea lampreys were also evaluated. Since it may undergo a loss of ions together with body fluids, thus the need to readjust osmoregulatory mechanisms to this end the first insights at the molecular level (transcript and protein) on this parasite-host interaction in addition to the evaluation of a number of biometric, biochemical parameters and enzymatic activity were made.

3.3 Materials and Methods

3.3.1 Animals

Fully metamorphosed, downstream migrating juvenile sea lampreys (*Petromyzon marinus*) were caught in the Saw Mill River in Montague, Massachusetts, USA, using a fyke net. Brook trout (*Salvelinus fontinalis*) were obtained from the Roger Reed Fish Hatchery, Massachusetts, USA. Animals were transported to the Conte laboratory where they were kept in tanks with a flow-through of freshwater from the Connecticut River. Sea lampreys were not feed as they do not feed during this stage and brook trout were feed *ad libitum* with 3 mm Bio Trout commercial pallets (Bio-Oregon; ME, USA). Natural photoperiod was kept (February-March from western Massachusetts). These conditions

were maintained for a minimum of one week before the start of experiments. Experiments were carried out in accordance with USGS-IACUC guidelines.

3.3.2 Animal Tagging

Animals used in the feeding study were individually marked for identification. Fish were anesthetized using tricaine methanesulfonate (MS-222) according to Summerfelt *et al.* (1990) buffered sodium bicarbonate for tag implantation. Lampreys were tagged using a three color code injected in the caudal fin (Americana Neons Fluorescente Acrilica, Decoart Americana, USA). Brook trout were tagged using 23 mm pit tag as peritoneal implants (Oregon RFID; Oregon, USA). Animals were added to experimental tanks after successful recover from anesthesia. There was no mortality from animal tagging.

3.3.3 Experimental Procedure

Eight 400 liter round fiberglass tanks were setup with biological and, mechanical filtration. Four tanks were filled with 30 ppt seawater prepared by dissolving synthetic sea salt Crystal Sea® Marinemix (Marine Enterprises Int., USA) in dechlorinated tapwater and the remaining tanks with dechlorinated tap water. Tanks were left cycling under these conditions for a minimum of 30 days for nitrifying bacteria to develop in the filter in order to avoid harmful ammonia peaks during the experiments. Water was aerated and UV light sterilized to minimize microbial infections on wounded animals. Twelve brook trout (host; 74.0±11.1 g and 19.7±1.0 cm wet mass and total length, respectively) and 15 juvenile sea lampreys (parasite; 4.9±0.8 g and 16.5±0.9 cm of wet mass and total length, respectively) were added to each freshwater and seawater tank (with replicate tanks). Twelve additional brook trout were kept in a freshwater and a seawater tank as host salinity control groups. Fifteen sea lampreys were kept in similar separate tanks as parasite salinity control groups. Natural photoperiod was maintained, and water temperature was kept at a constant 15±1ºC. Water salinity, temperature, total ammonia and pH were monitored daily. Partial water changes of 20% of total volume were performed every three days. Brook trout were fed daily to satiation and feed mass in container was recorded before and after feeding (±0.1 g). Moribund host fish as result from sea lamprey feeding wounds were removed and sampled and total number of attacks on each host fish was registered daily by individual tracking using PIT tags (brook trout) or color coded tags (sea lampreys).

3.3.4 Sampling

Animals were killed with an overdose of buffered MS-222 (200 mg l^{-1}). Total fish length (mm) and wet mass (±0.01 g) were measured, gender recorded and Fulton's condition factor was calculated (Fulton, 1902; Ricker, 1975). Blood samples were collected from the caudal vessels using a sterile syringe coated with lithium heparin (Sigma-Aldrich) for the brook trout and heparinized glass hematocrit tubes for the juvenile sea lampreys. Samples were then centrifuged at 13,000 xg for 5 min at room temperature in a Micro Hematocrit Centrifuge (Damon/IEC MB. Needham, MA, USA) and hematocrit recorded in duplicate. Plasma and red blood cells (rbc) were separated and gill, kidney, and anterior and posterior intestine samples were also collected. All samples were snap frozen on dry ice and stored at -80ºC. For Na^+/K^+-ATPase activity measurement, gill samples were also collected as described by McCormick (1993).

3.3.5 Ion Quantification

The Na^+ and K^+ concentrations in plasma were quantified using a flame photometer (model PFP7, Jenway) as described by Wilson *et al.* (2007b) and the Cl^- concentration was measured by titration (Chloride Analyzer 925, Corning). For the plasma Ca^{2+} quantification, a commercial kit was used according to manufacturer's instructions (ref# 1001061; Spinreact, Sant Esteve d'en Bas, Spain).

3.3.6 RNA Isolation, Quantification and cDNA Synthesis

Total RNA was extracted using Aurum$^{(TM)}$ Total RNA Mini Kits according to the manufacture's recommendations (Bio-Rad, Hercules, CA, USA). Homogenization was done in a bead mill (Precellys 24, Bertin Technologies, Montigny-le-Bretoneux, France) at 6400 rpm for 2 cycles of 15 s with 5 s interval. Homogenates were then centrifuged for 2 min at 14,000 xg at room temperature (Eppendorf MiniSpin Plus, Germany). On-column DNaseI treatment was performed. A Nanodrop spectrophotometer (Thermo Scientific, USA) was used to assess total RNA concentration and purity. The integrity of the total RNA was assessed by agarose gel electrophoresis (BioRad) using 1.2% formaldehyde agarose gels stained with GelRed (Biotium Hayward, CA, USA). Total RNA samples were stored at -80ºC for further processing. The cDNA was synthesized from 1 µg of total RNA using a High Capacity RNA-to-cDNA Kit in a 20 µl reaction volume (Applied Biosystems,

CA, USA). Reactions were carried out in a Doppio thermocycler (VWR International Ltd) at 25°C for 15 min, 37°C for 120 min; 85°C for 5 s. Samples were stored at -20°C.

3.3.7 RT-PCR and RT- Real-time PCR

Gene specific primers were obtained from published studies (see supplemental table 1) and were initially tested for specificity by RT-PCR. These reactions were performed using 0.4 µl sample cDNA, 2mM $MgCl_2$, 0.2mM dNTPs, 0.5 µM of each primer and 0.025 U GoTaq® DNA polymerase (Promega, Madison, USA) and 4 µl of 5X Green GoTaq® reaction buffer, respectively, in 20 µl reaction volumes. Reactions consisted of an initial denaturation at 94°C for 30 s followed by 35 cycles of: 94°C for 30 s; annealing at 58°C or 60°C (see Table 3.1) for 30 s; extension at 72°C for 30 s, and ending with a final extension for 2 min at 72°C. Resulting products from these reactions were separated on a 2% agarose TBE (Tris-borate-EDTA) gels at 80 V to confirm size of amplicons. Gels were stained with GelRed and images acquired with a Fujifilm LAS-4000 Mini luminescent image analyzer (Fujifilm Tokyo, Japan).

For the sea lamprey, relative levels of mRNAs expression for epithelial sodium channel (*scnn1*/ENaC), sodium-potassium ATPase α1-subunit (*atp1a1*/NKAα1), vacuolar-type H^+-ATPase E-subunit (*atp6v1*E/V–ATPase E), sodium-potassium-chloride cotransporter 1 (*slc12a2*/NKCC1), sodium-chloride cotransporter (*slc12a3*/NCC), and corticosteroid receptor (*cr*) genes were quantified. For brook trout, relative levels of mRNAs expression for sodium-potassium ATPase α1-subunit (*atp1a1a*/NKAa-1a and *atp1a1b*/NKAa-1b), vacuolar-type H^+-ATPase (*atp6v1*b/V–ATPase B subunit), ammonia transporter Rhesus glycoproteins (*slc42a2: rhbg*/Rhbg, *slc42a3: rhcgb*/Rhcg1 and *rhcg1l*/Rhcg2), and sodium-hydrogen exchanger isoform 2 (*slc9a2*/NHE2) (table 3.1) genes were determined. Relative quantification was performed in real-time RT-PCR analysis using SYBR green in an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). The cDNA samples were diluted 50 times and then 5 µl added to a reaction mix containing 10 µl of 2x iQ SYBR Green Supermix (Bio-Rad), and 250 nM of each primer in a total volume of 20 µl. Cycling profile was the following for the given primers pairs: 95ºC for 3 min, 40 cycles of 95ºC for 10 s, 58ºC or 60ºC (see supplemental table 1) for 30 s and 72ºC for 30 s. A melt curve was generated for every PCR product to confirm the reaction's specificity and a dilution series was prepared to check its efficiency. Random resulting amplicons were ran on 2% agarose TBE gel in order to confirm single amplified product with the expected size. The housekeeping genes used were *gapdh* and *β-actin* for lamprey and brook trout, respectively. The comparative CT method ($2^{-\Delta\Delta CT}$ method) based

on cycle threshold (CT) values was used to analyze the expression levels of the genes of interest. Values were then calculated and represented relative to respective species freshwater control group gene expression.

Table 3.1 Primer pairs (sense and anti-sense, respectively) for qPCR with predicted product size, annealing temperature, original gene accession number.

Species	Gene name	GenBank Accession No.	Forward and reverse primer sequences (5'-3')	Product size (bp)	Annealing temperature (ºC)	Reference
Sea lamprey	*gapdh*	AY578058	TGCAAAGCACGTCATCATCTC TTCTCGTGGTTTACTCCCATCA	**72**	**60**	Shifman *et al.*, 2009
	atp1a1	GENSCAN00000136072	CGTGGAATCGTCATCAACAC GCGACAGGATGAAGAAGGAG	**169**	**58**	Ferreira-Martins *et al.*, 2016
	slc12a2	ENSPMAG00000000665	GAGAGGTTTCGCGACAAGAC CGCTCACGAGTAGAACGTCA	**225**	**58**	Ferreira-Martins *et al.*, 2016
	slc12a3	ENSPMAG00000005880	GTCATCACGGTCACCTTCCT ACACCGGAGTGAAATTCTCG	**205**	**58**	Ferreira-Martins *et al.*, 2016
	atp6v1e	ENSPMAG00000008972	GTGAAGGAAGCCATGGAGAA TGGGGTTGACTTTGAAGAGC	**232**	**58**	Ferreira-Martins *et al.*, 2016
	scnn1	ENSPMAG00000007655	GCATCATGGTACACGACCAG AGGCGGAGGAGTAGAGGTTC	**183**	**58**	Ferreira-Martins *et al.*, 2016
	cr	AY028457.1	GTCCCACAAGAGGGTCTGAA GGCCATCATGTCAGGAAACT	**247**	**60**	Ferreira-Martins *et al.*, 2016
Brook trout	*bactin*	AJ438158	CCAACAGATGTGGATCAGCAA GGTGGCAGAGCTGAAGTGGTA	**138**	**58**	Ivanis *et al.*, 2008
	slc9a2	EF446605	TATGGCCATTGTGACCTGTG CAGGCCTCTCCACACTAAGG	**101**	**58**	Ivanis *et al.*, 2008
	atp1a1a	AY319391	CCCAGGATCACTCAATGTCAC CCAAAGGCAAATGGGTTTAAT	**66**	**58**	Kiilerich *et al.*, 2007

atp1a1b	AY319390	CTGCTACATCTCAACCAACAACATT CACCATCACAGTGTTCATTGGAT	**81**	**58**	Richards, 2003
rhbg	EU660221	CGACAACGACTTTTACTACCGC GACGAAGCCCTGCATGAGAG	**173**	**58**	Nawata *et al.*, 2007
rhcgb	DQ431244	CATCCTCAGCCTCATACATGC TGAATGACAGACGGAGCCAATC	**147**	**58**	Nawata *et al.*, 2007
rhcgl1	AY619986	CCTCTTCGGAGTCTTCATC CTATGTCGCTGGTGATGTTG	**88**	**58**	Nawata *et al.*, 2007
atp6v1b	AF140022	TCAGCCTTGGTTGTGAGATG CAACATTGGTGGGAAACAGG	**96**	**58**	Nawata and Wood, 2008
ca2	AY514870	GCCAGTCTCCCATTGACATC CCTGTACGTCCCTGAAATGG	**187**	**58**	Nawata and Wood, 2008

3.3.8 Plasma Lactate, LDH, ALT and AST

Plasma lactate concentration, and the activities of lactate dehydrogenase (LDH), alanine aminotransferase GPT (ALT) and aspartate aminotransferase GOT (AST) were determined using commercial kits according to manufacturer's instructions (ref# 1001330, 41220, 1001170 and 1001160, respectively; Spinreact, Sant Esteve d'en Bas, Spain).

3.3.9 Na^+/K^+- ATPase Activity Measurements

The Na^+/K^+-ATPase activity was measured via a kinetic microassay at 25°C (McCormick, 1993; Reis-Santos *et al.*, 2008) using a BioTek Synergy 2 microplate reader and Gen5™ reader control and data analysis software (Gen5, BioTek Instruments, Winooski, Vt., USA). Samples stored in 300 µl SEI buffer were thawed on ice, sodium deoxycholate was added to a final concentration of 0.1%, and homogenized using a motorized pestle homogenizer until tissue was fully disintegrated. Homogenates were centrifuged at 3,200 x g for 30 s at 4°C and the supernatant used for the ATPase assay. Samples of 10 µl were run in two duplicate sets. In one set ouabain (1.0 mmol l^{-1}) was added to the assay mixture to specifically inhibit Na^+/K^+-ATPase activity. Total protein was measured in the remaining supernatant using a commercial kit according to manufacturer's instructions (Ref# 23225; Pierce™ BCA Protein Assay Kit).

3.3.10 Immunoblotting

Gill samples were homogenized in SEI buffer in a bead mill (Precellys 24) at 6400 rpm for 2 cycles of 15 s with a 5 s interval. Homogenates were then centrifuged for 5 min at 12,000 xg at 4ºC (3K30, Sigma laboratory centrifuges GmbH, Germany). Supernatant was mixed with an equal volume of 2x Laemmli's buffer (Laemmli, 1970), heated for 10 min at 70ºC and then stored at 4ºC. Sample protein concentrations were adjusted to 1 µg $µl^{-1}$ using 1x Laemmli's buffer. Immunoblotting was performed as described in Reis-Santos *et al*. (2008) and Wilson *et al*. (2007b). Blots were probed with mouse anti-β-actin monoclonal (1:500; Sigma-Aldrich), α subunit of the Na^+/K^+-ATPase (αRbNKA and α5 1:1000), heat shock protein (Hsp70 Sigma 1:10000; Sigma-Aldrich), proliferating cell nuclear antigen (PCNA 1:50000), cytosolic carbonic anhydrase (CA2 1:1000), $Na^+:K^+:2Cl^-$ cotransporter (T4 1:500) antibodies and signal was obtained by enhanced chemiluminescence (ECL) with Millipore Immobilon Western chemiluminescent HRP substrate (Millipore Corporation, MA 01821 USA). Images were acquired using a

luminescent image analyzer (Fujifilm LAS-4000 mini) and image reader software (LAS-4000 version 2.0). Intensity of band signal was quantified using an image analysis software program (Multi Gauge v3.1 Fujifilm).

3.3.11 Statistical Analysis

Statistical differences between groups were determined using a one-way ANOVA followed by the post hoc Holm-Sidak test. A Student's t-test was used when suitable for simple comparisons of two group at same salinity (SigmaPlot 11.0 Systat Software, Inc.). Data is show as mean ± standard error of the mean (S.E.M.). Significance value was set at α=0.05.

3.4 Results

3.4.1 Biometric Data

Sea lamprey and brook trout biometric data are presented in Table 3.2. In the sea lampreys that were kept fasting, a lost in body mass occurred in both freshwater and seawater with no salinity effect observed. On the other hand, lamprey in seawater and feeding displayed a positive growth with similar results obtained when length was analyzed. Lamprey kept in shared freshwater tanks with hosts and that did not feed and were excluded from the analysis. Condition factor increased in the seawater feeding lampreys only when compared to freshwater controls. Seawater acclimation resulted in a decrease in hematocrit of ~ 27%, which recovered to freshwater control levels in feeding lamprey.

.

Table 3.2 Sea lamprey (parasite) and brook trout (host), mass change (grams per day), length change (millimeters per day), Fulton's Condition factor (K factor) and hematocrit (%). Sea lamprey groups: freshwater control, FW control (N=7), seawater control, SW control (lamprey N=5; trout N=7), seawater feeding lampreys, SW feeding (N=5) and seawater parasitized brook, SW parasitized (N=6). Values represent mean ± S.E.M. Analysis performed using two-way ANOVA; Values that do not share the same character are significantly different from each other, $P<0.05$

Parasite/ Host	Group	Mass change (g/day)	Length change (mm/day)	K factor	Hematocrit (%)
Sea lamprey	FW control	-0.0107±0.0008 ^{A}	-0.139±0.012 ^{A}	0.116±0.005 ^{A}	23.5±1.1 ^{A}
	SW control	-0.0215±0.0038 ^{A}	-0.247±0.038 ^{A}	0.119±0.006 ^{AB}	17.2±1.4 ^{B}
	SW feeding	0.0144±0.0055 ^{B}	0.005±0.038 ^{B}	0.139±0.007 ^{B}	23.6±2.9 ^{AB}
Brook trout	FW control	1.790±0.213 ^{A}	0.072±0.006 ^{A}	1.270±0.033 ^{A}	37.0±1.1 ^{A}
	SW control	0.762±0.054 ^{AB}	0.033±0.003 ^{B}	1.162±0.022 ^{B}	38.4±1.8 ^{A}
	SW parasitized	-0.088±0.149 ^{B}	0.004±0.007 ^{C}	0.980±0.043 ^{C}	11.6±4.1 ^{B}

In the brook trout, a body mass loss was observed in parasitized trout in seawater. Although both freshwater and seawater control animals were found to gain mass, differences were only found between parasitized trout in seawater and freshwater control. Nevertheless lengths were different in all host groups analyzed with the highest and lowest increase found in the freshwater control and parasitized trout in seawater, respectively. It followed that condition factor also decreased in brook trout after salinity transfer and was more pronounced with lamprey parasitism, which also resulted in severe decreases in the host's hematocrit values.

3.4.2 Tissue Damage Indicators and Ion Levels in Plasma

The presence of tissue metabolic enzymes LDH, ALT and AST in plasma are indicators of tissue damage and data in trout are presented in table 3.3. In the host species, a tendency for plasma LDH increase was observed in seawater; however, only a significant increase of approximately 44 fold was observed in parasitized trout in seawater compared to the freshwater control. No changes were observed in ALT and AST values although this could be masked by high variability, particularly in seawater parasitized trout. Lactate concentration in seawater parasitized trout plasma decrease by 31 and 39% compared to freshwater and seawater controls, respectively (table 3.3). The ion levels in the plasma of lamprey all show an increase after transfer to seawater (table 3.3). However, in feeding sea lampreys, $[Na^+]$ displayed a tendency to decrease as levels were no longer different from freshwater control ones. The $[Cl^-]$, $[K^+]$ and $[Ca^{2+}]$ decreased in feeding lamprey when compared to respective salinity control fasting group, and these ion levels also remained above those found in freshwater starving lampreys. In the brook trout, $[Na^+]$ and $[Cl^-]$ also displayed a tendency to increase upon seawater acclimation but were only significantly higher in parasitized trout when compared to freshwater group. The $[K^+]$ remained unchanged by seawater or parasitism (table 3.3).

Table 3.3 Sea lamprey and brook trout sodium (Na^+), chloride (Cl^-), potassium (K^+) and calcium (Ca^{2+}) content in plasma and brook trout lactate dehydrogenase (LDH), alanine aminotransferase GPT (ALT), aspartate aminotransferase GOT (AST), lactate in plasma. Freshwater control, FW control; seawater control, SW control and seawater feeding or parasitized, SW feeding/parasitized, respectively. Values represent mean ± S.E.M. Analysis performed using an two-way ANOVA; Values that do not share the same character are significantly different from each other, P<0.05.

Species	Group	LDH (U/L)	ALT (U/L)	AST (U/L)	Lactate (mM)	Na^+ (mM)	Cl^- (mM)	K^+ (mM)	Ca^{2+} (mM)
Sea lamprey	FW control					119.5 ± 1.1^{A}	104.6 ± 8.2^{A}	3.3 ± 0.1^{A}	2.7 ± 0.1^{A}
	SW control					150.1 ± 8.7^{B}	142.6 ± 1.0^{B}	5.2 ± 0.1^{B}	5.3 ± 0.1^{B}
	SW feeding					127.8 ± 5.9^{AB}	127.9 ± 2.2^{C}	4.3 ± 0.2^{C}	3.6 ± 0.1^{C}
Brook trout	FW control	24.01 ± 10.36^{A}	1.97±0.22	68.54±3.44	1.92 ± 0.12^{A}	130.9 ± 2.2^{A}	112.3 ± 1.8^{A}	1.88±0.06	
	SW control	268.62 ± 62.77^{AB}	2.479±1.02	77.96±13.6	2.18 ± 0.11^{A}	145.7 ± 3.3^{AB}	121.4 ± 3.3^{AB}	2.33±0.18	
	SW parasitized	1053.16 ± 398.12^{B}	30.70±19.26	166.35±58.31	1.32 ± 0.26^{B}	185.6 ± 15.6^{B}	142.8 ± 9.0^{B}	2.98±0.66	
	FW					0.3	0.1	0.1	0.5
	SW					376.8	445.7	7.8	9.3

3.4.3 Host Feeding

In this study a total of 23 sea lamprey attacks on brook trout were observed and from those 18 resulted in open wounds from which sea lampreys fed (data not shown). As a result four deaths occurred as a result of lamprey attacks in seawater and in only six brook trout did sea lamprey stay attached for the minimum three days for sampling together with their host. Food intake in brook trout was not significantly affected by salinity transfer and from presence of parasite in same tank as appetite in parasitized seawater trout was found to decrease by 56, 49 and 38% when compared to freshwater controls, freshwater non parasitized trout and seawater controls, respectively (Fig. 3.1).

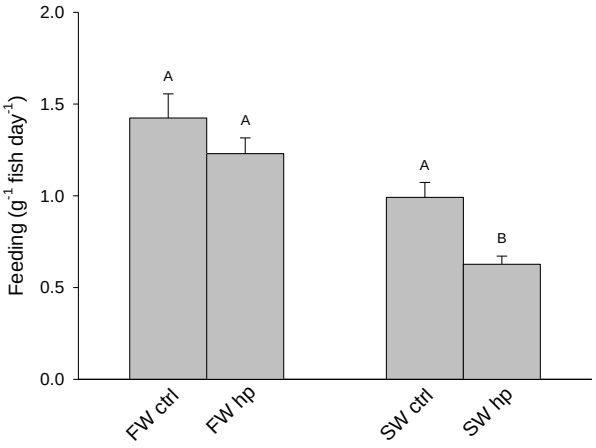

Fig. 3.1 Brook trout feeding (grams per fish per day) in freshwater only host tank, FW control; freshwater host and parasite tank without wounded trout, FW HP; seawater only host tank, SW control; seawater host and parasite tank with wounded trout, SW HP. Values represent mean ± S.E.M. Analysis performed using an ANOVA followed by a Dunn's Method test; Values that do not share the same character are significantly different from each other, $P<0.05$.

3.4.4 Real Time RT-PCR

In the sea lamprey branchial *atp1a1* (Fig. 3.2A) and *slc12a2* (Fig. 3.2B) mRNA expression remained unaltered after lampreys were transferred to seawater and in both genes a decrease in mRNA expression was observed as result of parasitic feeding in seawater. The mRNA expression of *slc12a3* (Fig. 3.2C), *scnn1* (Fig. 3.2D) and *atp6v1e1* (Fig. 3.2E) were lower after lamprey were acclimated to seawater and no significant

change was observed with feeding. The *cr* mRNA expression remained unaltered with any of the conditions tested (Fig. 3.2F).

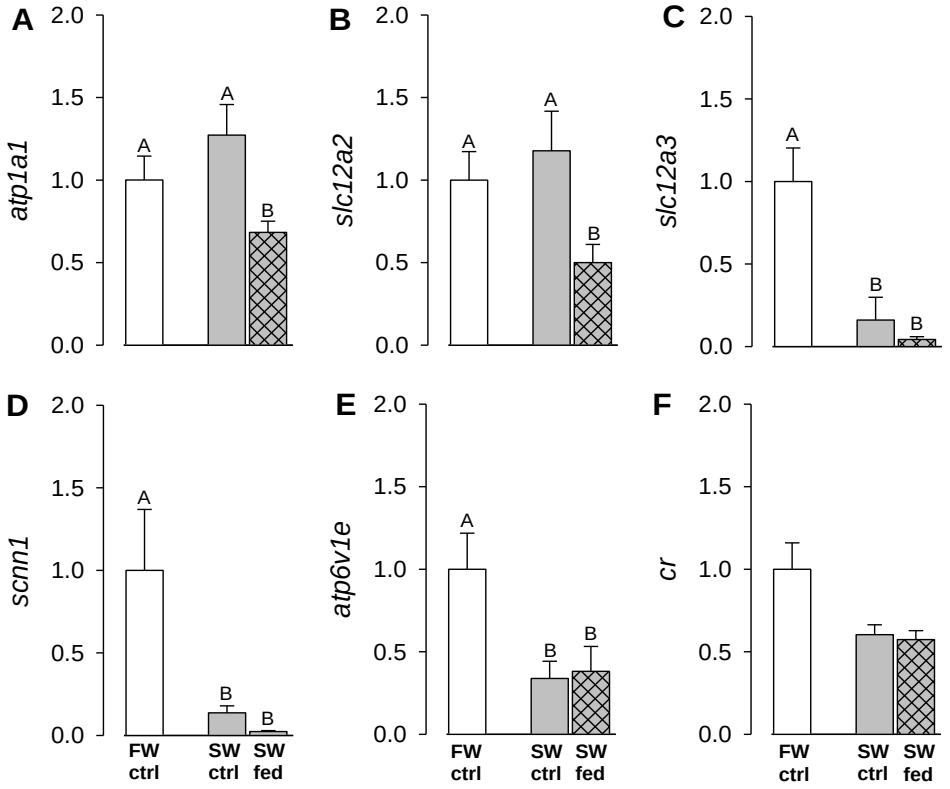

Fig. 3.2 Sea lamprey branchial relative mRNA expression of (A) *atp1a1*, (B) *slc12a2*, (C) *slc12a3*, (D) *scnn1*, (E) *atp6v1e1* and (F) *cr*. Control freshwater acclimated animals, FW ctrl (N=8); control seawater acclimated animals, SW ctrl (N=6); seawater acclimated feeding sea lampreys, SW fed (N=6). Values represent mean ± S.E.M. Values that do not share the same character are significantly different from each other, P<0.05.

In the host species, lower *atp1a1a* mRNA expression was observed in seawater control fish. However, levels of expression in seawater parasitized brook trout were similar to those found in the freshwater group and were significantly greater than seawater controls (Fig. 3.3A). On the other hand, the mRNA expression of the *atp1a1b* was higher in seawater fish and no significant difference was found to be caused from lampreys parasitism compared to control at the same salinity (Fig. 3.3B). As for *slc9a2*, higher levels of mRNA expression were only detected as result of seawater acclimation (Fig. 3.3C). No mRNA expression differences were detected for *rhbg* (Fig. 3.3D), *rhcgl2* (Fig. 3.3E), *rhcgb* (Fig. 3.3F), *atp6v1b* (Fig. 3.3G) or *ca2* (Fig. 3.3H).

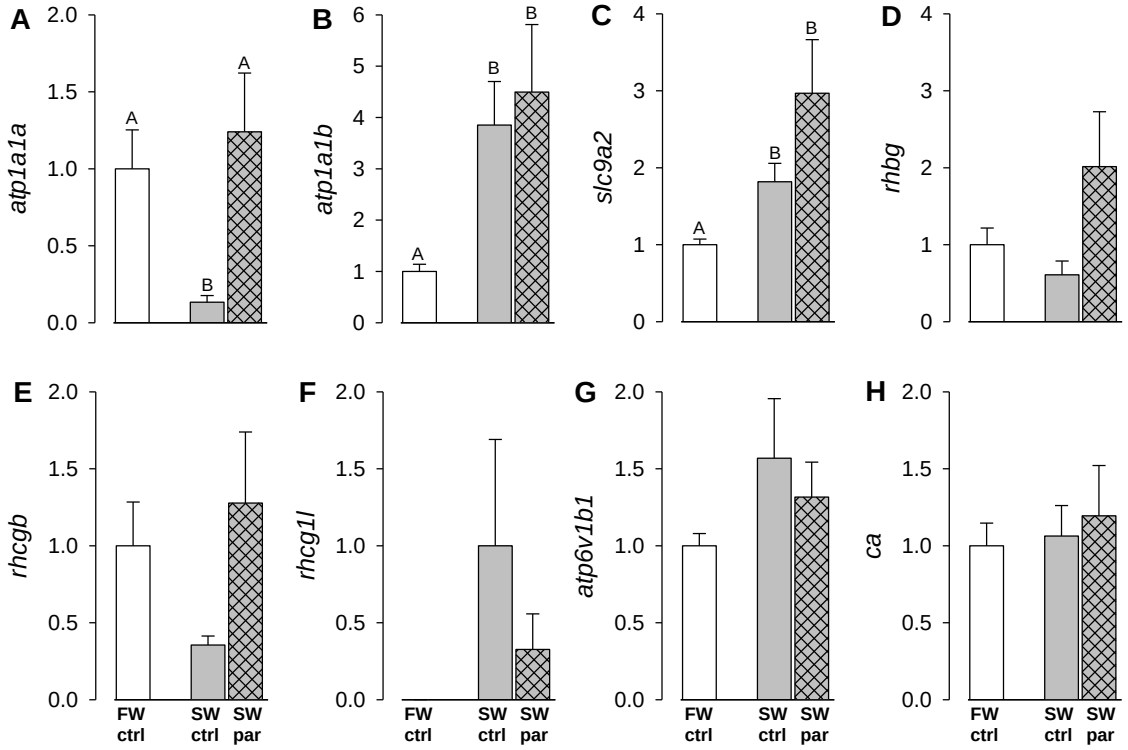

Fig. 3.3 Relative mRNA expression branchial (A) *atp1a1a*, (B) *atp1a1b*, (C) *slc9a2*, (D) *rhbg*, (E) *rhcgb*, (F) rhcgl1, (G) *atp6v1b1* and (H) *ca* in brook trout held in freshwater (FW ctrl), acclimated to seawater (SW ctrl) or parasitized by lamprey in SW (SW par). FW ctrl (N=8; except *slc9a2*, N=7); SW ctrl (N=6); SW par (N=6, except *atp1a1a*, N=5). Values represent mean ± S.E.M. Values that do not share the same character are significantly different from each other. P<0.05.

3.4.5 Immunoblotting

Immunoblotting for the sea lamprey Na^+/K^+-ATPase α subunit using αRbNKA antibody (Fig. 3.4A) resulted in strong single immunoreactive bands in the expected size range (~ 100 kDa). However, no significant differences were observed after seawater acclimation or feeding with either antibody. Similar results were obtained when immunoprobing with α5 antibody (results not shown). Hsp70 immunoprobing detected two distinct bands at 70 (Fig. 3.4B) and 72 kDa (Fig. 3.4C), corresponding to the inducible (Hsp70) and constitutive (Hsc70) isoforms. Hsp70 and Hsc70 expression were not affected by transfer to a hypertonic environment. On the other hand, in seawater feeding juvenile sea lampreys, expression of both isoforms was found to decrease.

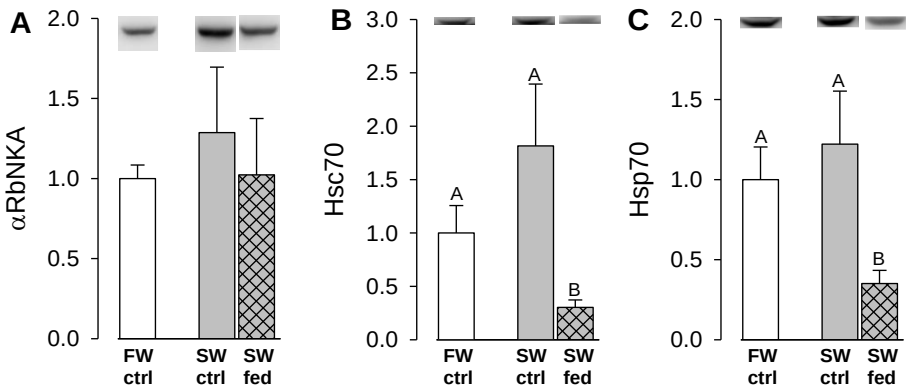

Fig. 3.4 Juvenile sea lamprey branchial relative protein quantification by immunoblotting of (A) Na^+/K^+-ATPase α subunit expression , and (B) constitutive heat hock protein 70 (Hsc70) at 72 kDa and (C) Hsp70 at 70kDa. Groups consisted of freshwater controls, FW ctrl (N=4); seawater controls, SW ctrl (N=4); and feeding seawater juveniles, SW p (N=4). Bars with like characters are not significantly different from each other (one-way ANOVA; P<0.05).

In the brook trout, immunoblotting was performed for NKA, V-type H^+-ATPase B subunit, Hsp70/Hsc70 (70 and 72 kDa bands, respectively), cytosolic carbonic anhydrase (Ca2), NKCC and proliferating cell nuclear antigen (PCNA) (Fig. 3.5). In seawater parasitized brook trout, the NKA α subunit protein expression was found to decrease by 2.7 fold compared to controls at the same salinity. However, no significant differences were found between freshwater and seawater control fish (Fig. 3.5A). The V-ATPase B subunit showed no changes in expression after freshwater to seawater transfer, although in parasitized brook trout protein expression levels decrease by 31.6 and 36.4 fold when compared to freshwater and seawater controls, respectively (Fig. 3.5B). No differences in Hsc70 and Hsp70 (Fig. 3.5C and D, respectively), CA (Fig. 3.5E), NKCC (Fig. 3.5F) or PCNA (Fig. 3.5G) protein levels were found.

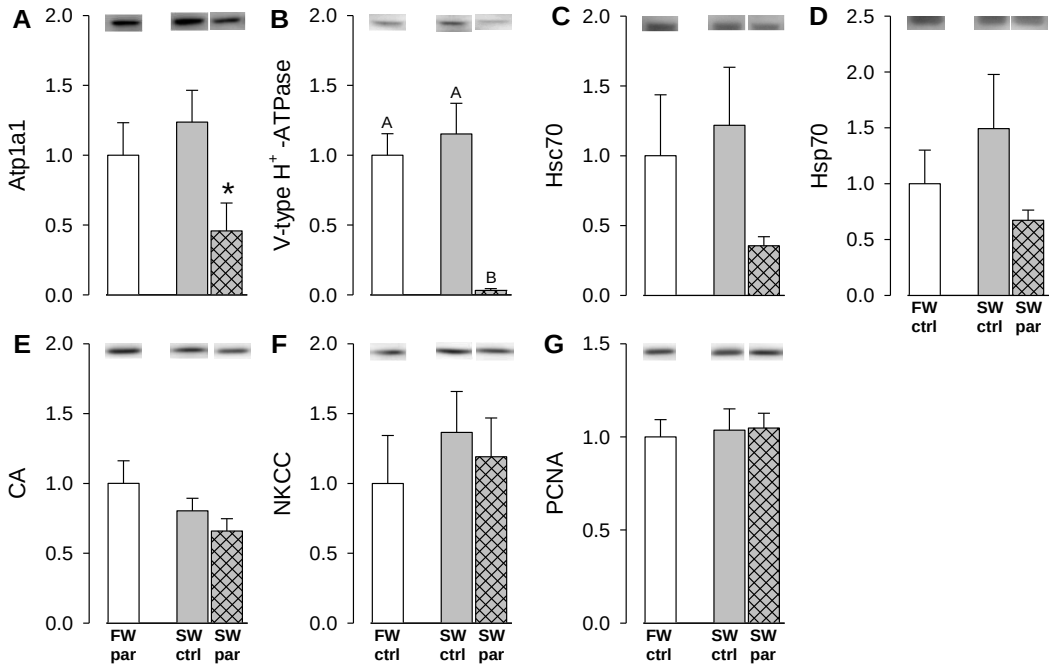

Fig. 3.5 Brook trout branchial relative protein quantification by immunoblotting of (A) Na^+/K^+-ATPase α subunit, Atp1a1 (B) V- ATPase B subunit, heat shock protein 70, (C) Hsc70, (D) Hsp70, (E) carbonic anhydrase CA, (F) NKCC1 and (G) proliferating cell nuclear antigen, PCNA. Control freshwater acclimated animals, FW ctrl (N=8); control seawater acclimated animals, SW ctrl (N=6); seawater acclimated parasitized host, SW par (N=5; except V- ATPase B subunit, Atp1a1 and Hsp70, N=4). Bars with like characters are not significantly different from each other (one-way ANOVA). Differences to respective salinity control groups are represented by an asterisk (*). $P<0.05$.

3.4.6 Na^+/K^+-ATPase Activity

In the sea lamprey, NKA shows no significant differences in activity in the gill with either seawater or feeding. In the kidney of freshwater acclimated sea lampreys, the average NKA activity was 12.712±0.665μmol ADP mg^{-1} protein h^{-1} and lower activity was observed in seawater acclimated lamrey; however, no effect from feeding were observed (Fig. 3.6A). On the other hand in the anterior intestine, NKA activity after acclimation to seawater was similar to that of freshwater (6.629±1.158 and 6.453±0.760 μmol ADP mg^{-1} protein h^{-1}, respectively), though in feeding lampreys in seawater an increase of NKA activity of approximately two fold compared to the freshwater and seawater control groups was observed. Activity levels in the posterior intestine did not differ between groups. Non ouabain sensitive ATPase activity in the kidney of sea lamprey was found to be reduced

by approximately 41 and 34% after transfer from freshwater to seawater and in seawater feeding lampreys (Fig. 3.6C).

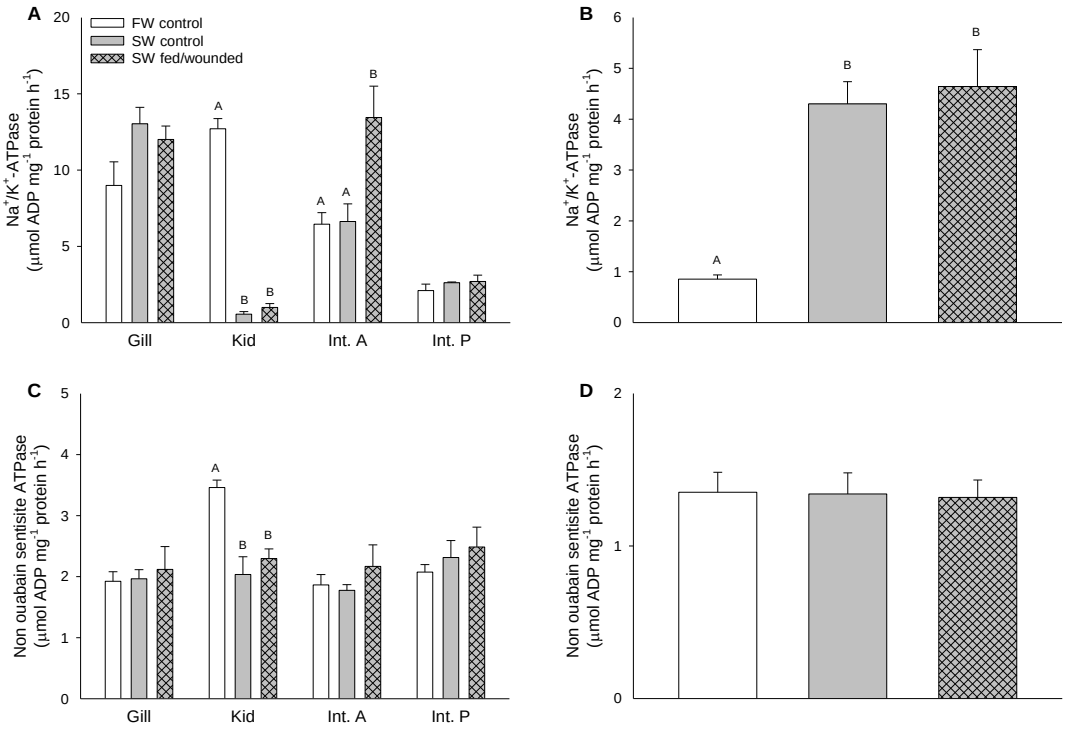

Fig. 3.6 Na^+/K^+-ATPase (A and B) and non ouabain sensitive ATPase (C and D) activities in: gill; kidney, Kid; anterior intestine, Int. A; and posterior intestine, Int P in sea lamprey (A and C) and gill of brook trout (B and D). Freshwater control groups, FW control (N=7, except sea lamprey kidney N=6); seawater control groups, SW control (N=5 for lamprey and N=6 for brook trout) and seawater feeding sea lampreys group, SW feeding (N=5, except for kidney N=4 and gill N=6); or seawater wounded brook trout group, SW parasitized (N=6). Values represent mean ± S.E.M. Analysis performed using one-way ANOVA followed by a Holm-Sidak method test; Bars with like characters are not significantly different from each other. $P<0.05$.

In the brook trout, branchial NKA activity increased approximately five fold after acclimation to seawater for both control and parasitized trout groups (Fig. 3.6B). No change in the non ouabain sensitive ATPase activity was found in the brook trout gill (Fig. 3.6D).

3.5 Discussion

Results from this study demonstrated that sea lamprey successfully acclimated to seawater with slight increase in plasma ions as result of adjustment to new environment. After they began their feeding in seawater, sea lampreys [Na^+], [Cl^-] and [Ca^{2+}] in plasma decreased but remained higher than levels found in freshwater fasting sea lampreys. Only feeding lampreys in seawater grew and fasting sea lampreys in seawater displayed a reduction in the hematocrit value. Transcriptomic analysis demonstrated that *atp1a1* and *slc12a2* expression remained unaltered after acclimation to seawater, though levels of expression decreased in feeding sea lampreys in seawater. The *slc12a3*, *scnn1*, *atp6v1e1* mRNA expression was downregulated after seawater acclimation and was not altered in sea lampreys at same salinity and feeding. Immunoblotting demonstrated a decrease in protein expression of Hsc70 and Hsp70 after sea lampreys began parasitic feeding in seawater and NKA α subunit remained unaltered as effect of feeding or seawater transfer. Nonetheless, a decrease in Na^+/K^+-ATPase and non-ouabain sensitive ATPase activity was observed in the kidney in fasting and feeding sea lampreys in seawater. Higher Na^+/K^+-ATPase was found in the anterior intestine of feeding lampreys in seawater.

In the brook trout, lamprey's attacks to feed on its body fluids only occurred in seawater. Ions levels in the plasma remained fairly unaltered, though when parasitized, [Na^+] and [Cl^-] was found higher compared to freshwater kept brook trout. Nevertheless ion level remained well below those found in the environment. In parasitized brook trout in seawater, a reduction on food intake was observed and animals demonstrated loss of weight and slower growth rates compared to freshwater and seawater controls, which translated in a decrease in their condition factor. A severe drop in hematocrit % was observed in parasitized trout as well as an increase in lactate and change in LDH levels (although not significant for this last one) in plasma. At the mRNA level, an increase in *slc9a2* and *atp1a1b* was observed in both fasting and parasitized brook trout groups in seawater and *atp1a1a* expression was found lower in seawater fasting animals both not in the parasitized ones. At the protein level, V- ATPase B subunit and Atp1a1 were found lower in parasitized brook trout compared to both salinity controls and only compared to controls in seawater, respectively. The Na^+/K^+-ATPase increased in seawater control and parasitized groups.

3.5.1 Salinity and feeding effects on the sea lamprey

In the current study none of the sea lampreys kept in freshwater feed on the host fish, with occasionally attachments occurring but never lasting more than 48 h or causing open wounds. This suggests that the supposed attacks on the host were not for the purposed of feeding but most likely for transport or rest as swimming performance of sea lamprey is weak (Beamish, 1974). Similar behavior has been already documented by Parker and Lennon (1956) and Cochran (1984) and may also explain the non feeding attacks in seawater that were observed in this study where sea lampreys remained attached to the host ≥ 3 d. Jorgensen and Kitchell (2005) hypothesized that sea lampreys occasionally prefer to attach to a small host with the purpose of transport while tracking a larger host. This would be beneficial as a smaller host could fastly decease from severe anemia or infection, while a larger host guarantees a more durable and steady food supply for a longer period of time (Swink, 2003) and without a need to track again new hosts, which is energetically costing for the sea lamprey. In seawater acclimated lampreys that attacked a host and remained feeding for at least 3 consecutive days, an increase in wet mass and length occurred.

Reis-Santos and co-workers (2008) have demonstrated that fully transformed juveniles can withstand direct transfer to seawater without major physiological complications. In our study, hematocrit levels were found to be lower in lampreys acclimated to a hypertonic environment and fasting. On the opposite, in sea lampreys at same salinity and feeding, hematocrit levels remained similar to those found in freshwater held sea lampreys and normal values described in the literature (Reis-Santos *et al.*, 2008). Despite the fact that seawater ionoregulatory mechanisms in post-metamorphic anadromous sea lamprey in freshwater are well developed, blood homoiosmoticity is not maintained when transfers from fresh to seawater occur, though sea lampreys are still osmoregulate efficiently as a new set point is drawn (Beamish, 1980b). It is known that feeding sea lamprey have the ability to osmoregulate from a wide range of salinities and osmolality of young juveniles acclimated up to 16ppt seawater is maintained fairly constant at ~236 mosmol kg^{-1}. On the other hand, when in higher salinities, osmolality has been shown to raise up to 10% in 35ppt seawater (Beamish, 1980b). These types of changes are commonly seen in other euryhaline fishes as the equilibrium set point shifts (Holmes and Donaldson, 1969).

Concentrations of ions in the plasma of seawater acclimated feeding sea lampreys were higher compared to those kept in freshwater (with the exception of Na^+); however, generally values remained lower than those found in the fasted seawater controls. This result suggests that feeding on brook trout body fluids that have lower salts content

compared to seawater, at least partially compensate for ion gain from the environment that anadromous sea lampreys experience through the gills and skin. The excess of monovalent ions is secreted mainly by the gill and the divalent ions by the kidney (Bartels and Potter, 2004; Pickering and Morris, 1970).

Surprisingly, a transfer from a hypo to a hyper-tonic environment did not increase the expression levels of NKAα1 and NKCC1 mRNA. Nevertheless, in feeding juvenile sea lamprey the expression of these genes was found to decrease, supporting the hypothesis that an hypotonic meal relative to the environment would facilitate osmoregulation in this species as suggested by Beamish (1980b). However, NKAα1 expression at the protein level was not different upon seawater acclimation or parasitic feeding in contrast to transcript levels. Our immunoblotting results are in accordance with Reis-Santos and co-workers (2008) immunoblotting using the same antibodies where it was firstly shown in lampreys that seawater ionocytes have high Na^+/K^+-ATPase expression as in teleost fish (Bartels and Potter, 2004; Peek and Youson, 1979a, 1979b). It has been shown that by stages six and seven of metamorphosis, when the sea lamprey is still in freshwater, the seawater type ionocytes have completely developed in the branchial epithelia (Peek and Youson, 1979a, 1979b), thus reflecting the high tolerance of transformers to seawater. In fact, Reis-Santos and co-workers (2008) suggested that this is a common trend among diadromous species as similar branchial increases in numbers of seawater ionocytes that are Na^+/K^+-ATPase rich during downstream migrations had been reported in various species such as salmon (Hoar, 1988; McCormick and Saunders, 1987), American shad (Zydlewski and Mccormick, 1997) and adult eel (Epstein *et al.*, 1967). In the gill, the seawater type ionocytes contain in their cytoplasmic tubular system a sodium-potassium dependent NKA activity (Beamish *et al.*, 1978; Reis-Santos *et al.*, 2008). When in the ammocoete stage, levels of NKA activity remain low and reach their peak of activity after metamorphosis and before entry to seawater to begin their marine trophic phase (Reis-Santos *et al.*, 2008). This demonstrates an enhancing mechanism of hypo-osmotic pre-adaptation as mentioned above. In terms of branchial ion uptake mechanisms, levels of NCC and ENaC and V-ATPase E subunit mRNA were downregulated after a transfer to hypertonic media as these transporters are not being required for ion uptake.

In this study NKA activity in other tissues in the sea lamprey were also determined. In the kidney, higher activity levels in freshwater are associated with ion reabsorption when sea lampreys are hyper-osmoregulating and lower activity after seawater acclimation is expected as lampreys switch from hyper to hypo-osmoregulatory mechanisms and glomerular filtration and urinary flow rates decrease (Rankin, 2002). When in seawater, the sea lamprey drinks seawater which is desalinated in the esophagus and in the anterior sections of the intestine the monovalent ions are up taken

together with water (Bartels and Potter, 2004; Pickering and Morris, 1970). However, in lamprey acclimated to a hypertonic media the intestinal NKA activity remained similar to that found in freshwater lampreys. This suggests that the downstream migrating juveniles have already increased their NKA activity in this tissue for enhancement of seawater tolerance. These results also contrast with the increase in intestinal NKA activity that has been observed in other euryhaline fishes (Fuentes *et al.*, 1997; Seidelin *et al.*, 2000). In this study we have shown for the first time that initiation of the parasitic feeding increases NKA activity in the anterior intestine. As this meal is expected to be hypotonic compared to seawater an increase of NKA activity in this intestinal region would be for nutrient absorption purposes, also allowing for the uptake of Na^+ and possibly other monovalent ions in order to also retain water from the remaining lysate. The Hsp70 has been shown to play a role in cellular homeostasis and to improve the physiological response to external environmental stress effects (Yamashita *et al.*, 2010). Here we observed that juvenile sea lampreys that successfully acclimated to seawater and began parasitic feeding had lower Hsp70 and Hsc70 protein expression compared to control fasting animals at same salinity and in freshwater. It has been demonstrated that upon sudden environmental changes, stress proteins (such as the heat-shock proteins) are induced and a rapid increase in its synthesis occurs in order to help maintaining cellular homeostasis (Yamashita *et al*., 2010). We hypothesize that at the time of downstream migrations, sea lampreys undergo a period of cellular stress due to the switch of habitats and feeding strategies, thus the higher Hsp70 presence. Therefore, the beginning of feeding when in seawater likely marks the time of habituation to this new life stage. When cells are subjected to sudden environmental changes, stress proteins are induced and play a central role in cellular homeostasis.

3.5.2 Effects on the Host

Growth was negative in parasitized brook trout which translated into a significant decrease in condition factor. Parasitized trout hematocrit was reduced ~ 68% compared to control group at same salinity, which demonstrates the impact of lamprey feeding on the host and explains the observed loss of appetite. In the seawater control group, the effects of higher salinity acclimation were observed as animals did not increase in mass as much as the ones acclimated to freshwater with consequent changes in condition factor. Lamphredin is an anticoagulant and cytolytic agent released by lampreys when parasitic feeding, that affects the skeletal muscle, blood vessels, connective tissue, and integument of hosts (Baxter, 2009; Lennon, 1954). Cytosolic enzymes (LDH, ALT, AST) are released

in the blood when tissue is damaged (e.g. Pakhira *et al.*, 2015; Vedel *et al.*, 1998). LDH and ALT are good predictors of muscular damage and AST of liver damage (Casillas *et al.*, 1982; Murray, 1984). Nevertheless these enzymes remained unaltered in parasitized trout plasma. Despite this, a tendency for LDH increase with parasitic feeding which relates with anemia observed in parasitized hosts; however, high variability among individuals masked differences. When a severe drop in hematocrit occurs, plasma lactate would be a good indicator of anaerobic metabolism (Olsen *et al.*, 1992); however, no negative correlation was found and measured lactate levels were actually below those of the salinity control group (1.32 and 2.18 mM, respectively) and freshwater control animals (1.92 mM). A possible explanation for this is the loss of plasma lactate resulting from lamprey feeding. In the plasma, Na^+ and Cl^- concentrations in parasitized trout were higher than those found in trout controls kept in freshwater but not from control group kept in seawater. These results support the hypothesis that parasitized brook trout in seawater were not osmoregulating as efficiently. Concentration of K^+ remained unaltered. Despite the differences in Na^+ and Cl^- concentrations, values remained well below those found in the environment.

In general, after three consecutive days of parasitic attachment, we observed an inability to maintain normal swimming and lost appetite in the brook trout (~ 38% reduction in food intake), which in turn would increase mortality in their natural environment as they would possibly become more susceptible to predation and bacterial infection. In freshwater, where host and parasite shared tanks only two attack marks from sea lamprey were found on brook trout, although we believe this was more related to transport and attachment in order to conservation energy rather than feeding since no skin breakage was achieved before detaching. On the other hand, in seawater host and parasite shared tanks a total of 23 attacks were observed from which ~ 78% resulted in open wounds on the host. In seawater, 17% of host trout mortality occurred before the three days feeding period as a result of blood lost.

Studies on salmonids have shown that many isoforms of the α-subunit of NKA are present in the gill ionocytes (McCormick et al. 2009) with two of them being differentially regulated upon salinity acclimation [NKAα1a and NKAα1b in fish acclimated to freshwater and seawater, respectively (McCormick *et al.*, 2009; Richards, 2003)]. Residual expression of NKAα1b detected in freshwater was expected as it is usually found at lower level and immunolocalized to small filamental ionocytes that are not in direct contact with the environment as they occur below the pavement cells (McCormick, 2013). In the present study, upon acclimation to a hypertonic environment, NKAα1a and NKAα1b mRNA expression were down and up-regulated, respectively, as expected. However, relative expression levels of NKAα1a mRNA in seawater acclimated parasitized brook

trout remained similar to those found in freshwater control. Together, the results on these two isoform of α-subunit of NKA suggest that in parasitized brook trout, NKAα1b is required for seawater tolerance and at the same time NKAα1a is responding to compensate possible cell volume variation.

Hiroi and McCormick (2007) reported an increase in NKA activity with increasing external salinity in non-anadromous brook trout. Similarly, in our work NKA activity increased after transfer from freshwater to seawater reflecting a switch from an hypo to hyper-osmoregulatory strategy with the NKA as the main force for Na^+ and Cl^- secretion in the gill, found in the basolateral tubular system of the seawater type ionocytes (Wilson and Laurent, 2002; Wilson *et al.*, 2000b).

One or several isoforms of NHE are present in gill epithelial cells of seawater fish (Edwards *et al.*, 2005; Evans *et al.*, 2005) and it has been hypothesized that NHE could play a role in gill acid transfers (Edwards *et al.*, 2005). Freshwater hypercapnia studies have shown to induce NHE2 protein expression but limited in seawater hypercapnia (Edwards *et al.*, 2005). Results show NHE2 mRNA was upregulated in fish acclimated to a hypertonic environment, similar to has been observed in other fish (e.g. Liu *et al.*, 2013). Rh proteins are known to be involved with "ammonia" (NH_3 and NH_4^+). Rhag, Rhbg and Rhcg2 have been localized in the pavement cells in the gill lamellae while Rhcg1 has been described in ionocytes that are acid secreting cells (HR cells) (reviewed by Hwang *et al.*, 2011). The Rhcg2 has been proposed to participate in H^+ outward movement through a "acid trapping" ammonia excreting mechanism (Wright *et al.*, 1989; reviewed by Hwang *et al.*, 2011). Nevertheless, in this study, no differences in expression of the Rh protein isoforms were found in brook trout as an effect of seawater acclimation or sea lamprey parasitic feeding, suggesting no differences in ammonia production in the host occurred under these experimental conditions.

3.6 Conclusion

Predator-prey interactions outline behavior, morphology, shape, size and survival of both parties (Alcock, 2001) and parasite-host interactions as the case of sea lamprey and its hosts follows a similar trend. To date limited studies have been conducted on parasitic feeding impacts of anadromous sea lamprey on the marine trophic phase due to its complex life cycle and difficult access to this life history stage. In addition the physiological impact of this feeding strategy on the host's perspective was also assessed.

From the sea lamprey standpoint, in order to counteract the impacts of feeding on a hyposmotic meal while in seawater, sea lampreys decreased mRNA expression of *atp1a1*/NKAα1 and *slc12a2*/NKCC1, which suggests that feeding facilitates hypo-osmoregulation when acclimated to seawater. This is supported by the lower ion content in the plasma of feeding sea lampreys in seawater compared to fasting controls at the same salinity. Moreover an increase in NKA activity in the anterior intestine suggests a role in nutrient absorption and lower protein levels of Hsp's in gill indicates a reduction in cellular stress upon feeding begin at this life stage. Overall, in terms of transition from freshwater to seawater, successfully downregulated of molecular ion uptake mechanisms (NCC, ENaC and V-ATPase) was observed and ion secretion mechanisms were likely already primed for hypo-osmoregulation.

Upon seawater transfer host species, brook trout were able to successfully hyposmoregulate by down and up-regulated NKAα1a and NKAα1b mRNA expression, respectively and increase branchial NKA activity. Ion concentrations in plasma were also maintained and well below the levels found in the environment. Nevertheless, sea lamprey parasitic feeding also has a physiological impact for the host namely in the maintenance of homeostasis. In parasitized brook trout, NKAα1a mRNA remained high with a possible role in cell volume maintenance, though more information at the protein level is needed to confirm mRNA expression. Also, parasitized brook trout displayed a severe anemia and decreased appetite with consequent decrease of condition factor as a result of sea lamprey feeding attacks. Finally, from 23 registered attacks in seawater, nearly 18 were with the purpose of feeding. From the 18 feeding attacks, 17% resulted in the host death in less than 3 days.

3.7 Acknowledgements

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Chapter 4:

Effects of salinity on upstream migrating, spawning sea lamprey, *Petromyzon marinus.*

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4.1 Abstract

The sea lamprey, *Petromyzon marinus*, is an anadromous, semelparous species that is vulnerable to endangered in parts of its native range due in part to loss of spawning habitat by manmade barriers. The ability of lamprey to return to the ocean or estuary and search out alternative spawning river systems would be limited by seawater osmoregulatory ability. A reduction in salinity tolerance has been documented in migrants although the underlying mechanisms have not been characterized. This study examines the capacity for marine osmoregulation in upstream spawning migrants by characterizing the physiological effects of salinity challenge from a molecular perspective. Estuarine captured migrants held in freshwater (FW) for approximately one week (short term acclimation) or two months (long term acclimation) were incrementally salinity challenged until loss of equilibrium occurred and upper thresholds of 25 and 17.5, respectively occurred. Regardless of salinity tolerance, all lamprey down regulated FW ion uptake mechanisms (gill transcripts of Na^+:Cl^- cotransporter (NCC/*slc12a3*) and epithelial Na^+ channel (ENaC/*scnn1*) and kidney Na^+/K^+-ATPase (NKA) protein and activity but not transcript). At their respective salinity limits lamprey displayed a clear osmoregulatory failure, and were unable to regulate [Na^+] and [Cl^-] in plasma and intestinal fluid within physiological limits, becoming osmocompromised. A >90% drop in hematocrit indicated hemolysis and higher plasma levels of the cytosolic enzymes ALT, AST and LDH indicated damage to other tissues including liver. However, in short term FW acclimated fish >80% of animals were able to osmoregulate efficiently with less hemolysis and tissue damage. This osmoregulatory ability correlated with significant up regulation of the secretory form of Na^+:K^+:2Cl^- cotransporter (NKCC1/*slc12a2*) transcript levels and the re-emergence of seawater-type ionocytes detected through immunohistochemical NKA-immunoreactivity in gill, the central ionoregulatory organ. This work sheds a light on the molecular and physiological limits to the potential return to seawater for lampreys searching for alternative FW systems to spawn.

4.2 Introduction

The sea lamprey *Petromyzon marinus* (Linnaeus 1758) has an anadromous life history that is characterized by three distinctive stages (Beamish, 1980a; Hardisty and Potter, 1971a). During the ammocoete larval stage sea lampreys are freshwater (FW) benthic stream filter feeders, after which they undergo a dramatic morphological and physiological transformation into parasitic feeding juveniles that migrate to the ocean where they become parasitic feeders. The adults re-enter freshwater, migrating upstream until they find a suitable place to terminally spawn. In Europe sea lamprey populations are declining and facing the threat of extinction as a result of overharvesting of adults and physical loss of spawning and nursery grounds due to construction of manmade barriers (dams and weirs) blocking access to suitable upstream spawning grounds as well as habitat destruction (Almeida *et al.*, 2002b; Close *et al.*, 2002; Renaud, 1997). An understanding of the physiology limitations to cope with these challenges is vital to the management of these threatened or endangered lampreys.

Lampreys are osmoregulators and their spawning migration requires a switch from marine hypo-osmoregulation to FW hyper-osmoregulation (Beamish, 1980b). The gill, kidney and the intestine are the primary organs involved in the active regulation of internal levels of ions using mechanisms proposed to be similar to those of teleost fishes (Beamish, 1980b; Hardisty *et al.*, 1989; Morris, 1972; Bartels and Potter, 2004). In marine environments, fishes drink seawater and excrete excess ions across their gills using seawater-type ionocytes to compensate for osmotic water losses and passive ion gains, respectively (Evans *et al.*, 2005; Marshall and Grosell, 2006). In contrast, in FW fishes actively take up ions using freshwater-type ionocytes to compensate for passive ion losses and produce copious amounts of dilute urine to get rid of osmotically gained water (Evans *et al.*, 2005; Marshall and Grosell, 2006).

The branchial mechanism of NaCl secretion by seawater ionocytes in lampreys is likely by the well characterized secondary active Cl^- secretion mechanism (Bartels and Potter, 2004; Evans *et al.*, 2005). Basolateral Na^+/K^+-ATPase creates the favorable electrochemical gradient for Cl^- to enter the cell via $Na^+:K^+:2Cl^-$ cotransporter 1 (NKCC1/*slc12a2*) and out apically though a channel homologous of the cystic fibrosis transmembrane conductance regulator (CFTR/*abcc7*) (Marshall *et al.*, 2002b; Singer *et al.*, 1998; Jonathan M. Wilson *et al.*, 2000b). Na^+ accumulates extracellularly and leaks out paracellularly through leaky tight junctions associated with these ionocytes (Karnaky, 1986, 1980; Loretz, 1995; Smith, 1930).

In the FW lamprey gill, Na^+ uptake is predicted to be mediated by the epithelial Na^+ channel (ENaC/*scnn1*) through indirect coupling with the electrogenic vacuolar H^+-

ATPase pump (V-ATPase) (Bartels and Potter, 2004). Teleost fishes lack the ENaC and instead Na^+ uptake is via an ASIC Na^+ channel (Dymowska *et al.*, 2014). A pool of intracellular H^+s for the V-ATPase is produced by CO_2 hydration catalyzed by carbonic anhydrase (CA) and low intracellular Na^+ levels are maintained by basolateral Na^+/K^+-ATPase. Cl^- uptake is likely performed by epithelial cells with a Cl^- channel in the basolateral membrane, cytosolic CA (Larsen, 1991), and an apical Cl^-/HCO_3^- antiport system (Garcia-Romeu and Ehrenfeld, 1975; Larsen, 1991; Marshall *et al.*, 1997). Immunohistochemistry of V-ATPase and CA support branchial mitochondrion-rich intercalated cells as likely ionocytes (Choe *et al.*, 2004; Reis-Santos *et al.*, 2008).

Drinking rates have been measured in lamprey, and when in seawater lamprey swallow from 5 to 99 ml/kg/day which is desalinized and absorbed by the gut (Pickering and Morris, 1970). During acclimation to freshwater, drinking decreases to very low levels (Pickering and Morris, 1970; Rankin, 2002). The mechanism of absorption of water across the intestine likely involves an apical $Na^+:K^+:2Cl^-$ cotransporter (NKCC2/*slc12a1*) and $Na^+:Cl^-$ cotransporter (NCC/*slc12a3*) and driven by basolateral Na^+/K^+-ATPase (Cutler and Cramb, 2001; Cutler *et al.*, 1996; Marshall *et al.*, 2002a).

The anadromous sea lamprey upstream spawning migration can be divided in three distinct stages: 1), migration from the ocean to the estuary; 2), pre-spawning holding in the estuary; and 3), upstream movement within rivers and streams to spawning sites (Applegate 1950; Clemens *et al.*, 2010). Since barriers to progress on the spawning migration of lamprey present a potential conservation issue (Close *et al.*, 2002; Lucas *et al.*, 2009; Renaud, 1997), the ability of migrants, which have no fidelity to natal streams (Moser *et al.*, 2015), to search out alternative rivers for spawning may be significant (Kelso and Gardner 2000; Holbrook, 2015; Noyes *et al.*, 2013). However, in a number of studies it has been found that approximately half strength seawater was lethal to upstream migrating anadromous lampreys (Beamish *et al.*, 1978; Galloway, 1933; Morris, 1956). Specifically, Beamish *et al.* (1978) determined the upstream migrant sea lamprey 48h LC50 (lethal concentration to kill 50%) salinity to be approximately 15.2. To date our understanding of physiological constraints in lampreys is poorly studied and understood especially at the molecular level (Moser *et al.*, 2015). Thus, the aim of this study was to determine the physiological effects of salinity challenge in estuarine migrating adult sea lamprey after short (~ 1 week) and long (~ 2 months) term acclimation to FW. The study was focused on the molecular and physiological changes in gill, kidney and intestine in addition to a number of osmoregulatory endpoints in plasma and muscle. In short term FW acclimated salinity challenged fish, osmoregulators and osmocompromised groups were identified by changes in plasma Na^+ and Cl^- ions and hematocrit and subsequently analyzed as separate groups.

4.3 Materials and Methods

4.3.1 Animals

Freshwater migrating adult sea lamprey were collected by artisanal fishermen at the mouth of the River Minho estuary (41.874546 N, -8.849831W) (Araújo *et al*., 2013) using drift trammel nets (Quintella, 2006), during the spring of 2012 and 2013 for the short and long term FW acclimation studies, respectively. The River Minho has a salt wedge type estuary (presence of a vertical halocline) with a salinity ranging from 7-33 and temperature around 14°C. The animals were held in the Aquamuseu in Vila Nova Cerveira in flow through FW tanks and then transported to CIIMAR and maintained in 1000 liter tanks with recirculated (mechanic and biologic filtration) dechlorinated Oporto city tap water at 16ºC. Animals were not fed as they do not feed during this stage of their lifecycle (Larsen, 1980). To minimize the effects of handling stress in the experimentation, animals were acclimated to these tank conditions for at least 1 week before experimentation. Salinity, pH, dissolved oxygen and temperature were monitored daily using a multi-parameter analyzer (HQ40d, Hach Lange, Loveland, USA). Total ammonia was monitored every third day using a commercial kit (04910-NH4/NH3-Test, Sera GmbH, Germany). Animals were treated in accordance with the Portuguese Animal Welfare Law (Decreto-Lei no.197/96) and animal protocols approved by CIIMAR/UP and DGV (Ministry of Agriculture).

4.3.2 Long Term Freshwater Acclimation Salinity Challenge Experimental Procedure

For the first experiment conducted in May-June 2012, a total of 12 sea lampreys (76.4±1.4cm and 840.7±46.3g in total length and wet mass, respectively) were acclimatized to the 1000 liter water system with dechlorinated tap water at 16ºC. Two groups of 4 lampreys were kept in 80 liters tanks with a renewal water flow rate of 6 liters per minute. In one of the tanks, salinity was raised by 5 every two days using natural, filtered seawater at 35 until a brackish water (BW) salinity of 15 was reached. From this point on, salinity was increased by 2.5 every following day to a final salinity of 17.5 (total of 8 days). Preliminary trials indicated this as a salinity limit. The second tanks was set for control animals held in FW that were sampled at the beginning and end of the experimental period (n=3+3). No differences were detected so the control groups were

merged. Water parameters and mortalities were monitored daily. Animals do not feed during this period in their lifecycle (Larsen, 1980; Beamish, 1980a; Hardisty and Potter, 1971ab).

4.3.3 Short Term Freshwater Acclimation Salinity Challenge Experimental Procedure

For the second experiment conducted in March-April 2013, a total of 30 sea lampreys (78.1±0.8cm and 855.3±20.3g in total length and wet mass, respectively) were divided into five group tanks with 6 lampreys. The same experimental procedure as in the first experiment was carried out in four of the tanks until a final salinity of 25 was reached (total of 11 days). Preliminary trials established this as the salinity limit. Six animals were randomly sampled at a salinity of 17.5 [determined upper salinity limit for the first experiment and similar to the limit reported by Beamish *et al.* (1978), Galloway (1933) and Morris (1956)] and the remainder at 25. The remaining tank was set as the control group and held in freshwater and fish were sampled at the beginning and end of the experimental period (n=3+3). No differences were detected so the control group data were merged.

4.3.4 Sampling

Animals were anesthetized with 2-phenoxyethanol (1:2000) and killed by cervical transection. Total fish length (mm) and mass (±0.01g) were measured, gender was determined (presence of testes or ovaries) and Fulton's condition factor was calculated (Fulton, 1902; Ricker, 1975). Blood samples were collected from the caudal vessel using a sterile syringe coated with lithium heparin (Sigma-Aldrich) and centrifuged at 13,000xg for 3 min at room temperature (Pico 17, Heraeus DE). Hematocrit was measured in duplicate to the nearest mm and converted to percentage of total blood volume. Plasma and red-blood-cells (rbc) were separated, snap frozen in liquid nitrogen and stored at -80ºC. The peritoneal cavity was opened ventrally and the gut ligated at anterior and posterior extremities and removed. Gut fluid was collected by draining gut content into Falcon tubes, centrifuged at 13,000xg for 3 min at room temperature and the supernatant was used to quantify ion concentrations. Gill, kidney, anterior, middle and posterior intestine samples were also collected and all tissue samples were snap frozen in liquid nitrogen and stored at -80ºC for further use. One gram of epaxial muscle tissue was also collected to determine water content, and Na^+ and K^+ concentrations. A gill pouch was fixed using 10% neutral buffered formalin at 4°C for 24 h and stored in 70% ethanol at 4°C

for paraffin embedding. For Na^+/K^+-ATPase activity measurement, gill samples were also collected as described by McCormick (1993).

4.3.5 Ion Quantification

Muscle samples were dried to constant mass at 60ºC for water content determination. Dried muscle was then digested in 5 volumes of 65% nitric acid for three days. Na^+ and K^+ concentrations were quantified using a flame photometer (model PFP7, Jenway) as performed by Wilson et al. (2007b). Plasma and gut fluid samples were also analyzed. Chloride concentration was measured in gut fluid, and plasma samples by titration (Chloride Analyzer 925, Corning).

4.3.6 RNA Isolation, Quantification and cDNA Synthesis

Total RNA was extracted using Aurum$^{(TM)}$ Total RNA Mini Kits according to the manufacture's recommendations (Bio-Rad, Hercules, CA, USA). Homogenization was done in a bead mill (Precellys 24, Bertin Technologies, Montigny-le-Bretoneux, France) at 6400 rpm for 2 cycles of 15 s with 5 s interval. Homogenates were centrifuged for 2 min at 14000 RCF at room temperature (Eppendorf MiniSpin Plus, Germany). On-column DNaseI treatment was performed. Total RNA concentration and purity was assessed using a Nanodrop spectrophotometer (Thermo Scientific, USA) and integrity determined by agarose gel electrophoresis (BioRad) in a 1.2% formaldehyde agarose gels stained with GelRed (Biotium Hayward, CA, USA). Total RNA samples were stored at -80ºC. The cDNA was synthesized from 1µg of total RNA with iScript cDNA Synthesis Kit in a 20µl reaction volume (Bio-Rad). Reactions were carried out in a Doppio thermocycler (VWR International Ltd) at 25°C for 5 min; 42°C for 30 min; 85ºC for 5 min. Samples were stored at -20°C.

4.3.7 RT-PCR and RT- Real-time PCR

PCR reactions were performed using 0.4 µl sample cDNA, 2mM $MgCl_2$, 0.2mM dNTPs, 0.5µM of each primer and 0.025 U GoTaq® DNA polymerase (Promega, Madison, USA) and 4µl of 5X Green GoTaq® reaction buffer, respectively, in 20µl reaction volumes. Primers were designed using Primer3 (Rozen and Skaletsky, 2000) and were initially tested for specificity by RT-PCR. Reactions consisted of an initial denaturation at

94°C for 30 s followed by 35 cycles of: 94°C for 30 s; annealing at 58°C or 60°C for 30 s; extension at 72°C for 30 s, and ending with a final extension for 2 min at 72°C.

PCR products were separated on a 2% agarose TBE (Tris-borate-EDTA) gels at 80V to confirm size of amplicons. All gels were stained with GelRed and images acquired with a Fujifilm LAS-4000 Mini luminescent image analyzer (Fujifilm Tokyo, Japan).

Relative levels of mRNAs for epithelial sodium channel (*scnn1*/ENaC), sodium-potassium ATPase α1-subunit (*atp1a1*/NKA-a), vacuolar-type H^+-ATPase (*atp6v1*E/V–ATPase E) , sodium-potassium-chloride cotransporters 1 (*slc12a2*/NKCC1), sodium-chloride cotransporter (*slc12a3*/NCC), corticosteroid receptor (*cr*) 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 50 times and then 5 µl added to a reaction mix containing 10 µl of 2x iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), and 250 nM of each primer in a total volume of 20 µl. Cycling profile was the following for the given primers pairs: 95 ºC for 3 min, 40 cycles of 95 ºC for 10 s, 58 ºC or 60 ºC (see Supplemental Table 4.1) for 30 s and 72 ºC for 30 s. A melt curve was generated for every PCR product to confirm the specificity of the assays and a dilution series was prepared to check the efficiency of the reactions. The *gapdh* was used as the housekeeping gene. The comparative CT method ($2^{-\Delta\Delta CT}$ method) based on cycle threshold (CT) values was used to analyze the expression levels of the genes of interest. Random resulting amplicons were run on 2% agarose TBE gel to confirm single amplified product with the expected size.

4.3.8 Immunofluorescence Microscopy

Immunofluorescence localization of NKA α subunit and V-ATPase B subunit were performed according to Wilson *et al.* (2007a) using a double labeling protocol (Reis-Santos *et al.*, 2008; Wilson *et al.*, 2007a). The antibodies against NKA and V-ATPase were the α5 mouse monoclonal and rabbit BvA1 polyclonal antibodies (Takeyasu *et al.*, 1988; Wilson *et al.*, 2007b).

4.3.9 Plasma Lactate, LDH, ALT and AST

Determinations of plasma lactate, lactate dehydrogenase (LDH), alanine aminotransferase GPT (ALT) and aspartate aminotransferase GOT (AST) were performed

using commercial kits according to manufacturer's instructions (ref# 1001330, 41220, 1001170 and 1001160, respectively; Spinreact, Sant Esteve d'en Bas, Spain).

4.3.10 Gill Na^+/K^+-ATPase Activity Measurement

The Na^+/K^+-ATPase activity was measured via a kinetic microassay at 25°C (McCormick, 1993; Reis-Santos *et al.*, 2008) using a BioTek Synergy 2 microplate reader (BioTek Instruments, Winooski, Vt., USA) and Gen5™ reader control and data analysis software (Gen5, BioTek Instruments, Winooski, Vt., USA). Samples stored in 300 µl SEI buffer were thawed on ice, sodium deoxycholate added to a final concentration of 0.1%, and homogenized using a Precellys 24 bead mill. Homogenates were centrifuged at 14000xg for 5 min at 4°C and the supernatant decanted and used for the ATPase assay and immunoblotting experiments. Samples of 10 µl were run in two duplicate sets. In one set ouabain (1.0 mmol l^{-1}) was added to the assay mixture to specifically inhibit Na^+/K^+-ATPase activity. Total protein was measured by Bradford's method (Bradford, 1976) using bovine serum albumin (BSA) as a standard.

4.3.11 Immunoblotting

The unused supernatant from Na^+/K^+-ATPase activity assay was mixed with an equal volume of 2x Laemmli's buffer (Laemmli, 1970), heated for 10 min at 70ºC and then stored at 4ºC. Protein concentrations were adjusted to 1 µg $µl^{-1}$ using 1x Laemmli's buffer. Immunoblotting was performed as described in Reis-Santos et al. (2008). Blots were probed with mouse anti-β-actin monoclonal (1:500; Sigma-Aldrich) and αRbNKA (1:1000, α subunit of the Na^+/K^+-ATPase) antibodies and signal was obtained by enhanced chemiluminescence (ECL) with Millipore Immobilon Western chemiluminescent HRP substrate (Millipore Corporation, MA 01821 USA). Images were acquired using a luminescent image analyzer (Fujifilm LAS-4000 mini) and image reader software (LAS-4000 version 2.0). Intensity of band signal was quantified using an image analysis software program (Multi Gauge v3.1 Fujifilm).

4.3.12 Statistical Analysis

Statistical differences between groups were determined using one-way ANOVA followed by the post hoc Student-Newman-Keuls (SNK) test. Comparisons between

salinity effect on different tissues was performed using a two-way ANOVA followed by a Holm-Sidak method pairwise multiple comparison (SigmaPlot 11.0 Systat Software, Inc.). Data is show as mean ± standard error of the mean (S.E.M.). Fiducial limit was set at 0.05.

4.4 Results

4.4.1 Hematocrit and Leukocrit

In long term FW acclimated lampreys hematocrit values decreased by 98% with salinity (17.5) challenge, while in the short term FW acclimated lampreys at same salinity levels hematocrits only decreased by 23% (Table 4.1). Short term FW acclimated lampreys challenged with BW-25 which were osmoregulating had a similar 21% decrease whereas in osmocompromised fish hematocrit decreased by 93%. In response to salinity leukocrit levels increased in long term FW acclimated lampreys challenged to BW-17.5. In contrast in short term FW acclimated lampreys leukocrit decreased in BW-25 osmoregulators (Table 4.1).

Table 4.1 Gender (male:female), total length (cm), mass (g), Fulton's condition factor K, hematocrit (%) and leukocrit (%) in long term FW acclimated sea lamprey challenged in freshwater, FW (N=4), 17.5 brackish water (BW) (N=4) and short term FW acclimated sea lamprey challenged in freshwater, FW (N=6), BW-17.5 (N=6), BW-25 osmoregulators (N=14), and BW-25 osmocompromised animals (N=4). Values shown as mean ± S.E.M. Within each experiment, groups that do not share letters are significant different (P<0.05).

FW acclimation	Group	Gender (M:F)	Length (cm)	Mass (g)	Fulton's K factor	Hematocrit (%)	Leukocrit (%)
Long term	FW	2:2	74.6±4.2	758.1±69.3	1.94±0.29	36.7±1.5 [a]	2.1±0.1 [a]
	BW 17.5	2:2	78.1±3.5	923.3±130.8	1.83±0.18	0.6±0.33 [b]	3.8±0.7 [b]
Short term	FW	3:3	78.6±0.5	892.2±18.6	1.84±0.05	45.2±1.2 [a]	2.4±0.3 [a]
	BW-17.5	2:4	78.5±1.5	873.0±44.7	1.81±0.06	34.8±0.5 [b]	1.6±0.3 [ab]
	BW-25 osmoregulating	6:8	78.5±0.9	859.5±25.7	1.77±0.37	35.7±0.9 [b]	1.5±0.1 [b]
	BW-25 osmocompromised	3:1	78.4±2.4	845.4±14.7	1.78±0.13	3.1±1.1 [c]	1.8±0.2 [ab]

4.4.2 Ion Concentrations in Plasma, Muscle and Intestinal Fluid

Plasma Na^+ and Cl^- concentrations increased significantly in all salinity challenged animals in both experiments. Nonetheless only long term FW acclimated sea lamprey challenged to BW-17.5 and non-performing short term FW acclimated lampreys challenged to BW-25 showed increases in these ions that approached those levels found in the environment. Plasma Ca^{2+} concentrations increased three fold in long term FW acclimated salinity challenged migrants and 10-fold in short term FW acclimated salinity challenged osmocompromised animals at BW-25. Plasma K^+ levels remained unaltered in long term FW acclimated salinity challenged animals and a decrease was observed in short term FW acclimated salinity challenged BW-25 osmocompromised animals (Table 4.2).

Table 4.2 Plasma Na^+, K^+, Ca^{2+} and Cl^- concentrations in long term FW acclimated sea lamprey challenged in freshwater, FW (N=4), 17.5 brackish water (BW) (N=4) and short term FW acclimated sea lamprey challenged in freshwater, FW (N=6), BW-17.5 (N=6), and BW-25 osmoregulators, and BW-25 osmocompromised animals (N=4). Values shown as mean ± S.E.M. Within each experiment, groups that do not share letters are significant different (P<0.05). Corresponding water ion concentrations are also listed.

FW acclimation	Group	$[Na^+]$	$[K^+]$	$[Ca^{2+}]$	$[Cl^-]$
Long term	FW	137.7±1.1 [a]	3.2±0.3	1.8±0.5 [a]	96.5±0.8 [a]
	BW-17.5	203.3±1.3 [b]	3.0±0.4	5.8±0.5 [b]	201.3±6.6 [b]
Short term	FW	151.3±3.8 [a]	5.5±0.5 [a]	2.8±0.3 [a]	108.1±1.4 [a]
	BW-17.5	189.2±3.9 [b]	4.0±0.2 [ab]	4.8±0.1 [ab]	141.0±5.6 [b]
	BW-25 osmoregulating	195.0±8.6 [b]	4.6±0.1 [ab]	4.9±0.3 [ab]	148.6±6.7 [b]
	BW-25 osmocompromised	288.7±3.7 [b]	3.3±0.1 [b]	25.9±2.8 [b]	275.5±15.7 [c]
FW		0.5	0.1	0.6	0.1
BW 17.5		237.2	5.4	4.9	286.1
BW 25		350.8	7.3	8.1	421.0

Muscle Na^+ levels were 37-fold higher in long term FW acclimated salinity challenged migrants. For the short term FW acclimated salinity challenged upstream migrating groups, no differences with the FW control group were observed although in the BW-25 acclimated animals there was a significant difference between osmoregulators and osmocompromised fish (Table 4.3). Potassium levels were higher in BW-17.5 sea lamprey from the long term FW acclimated salinity challenge experiment while animals at the same salinity in the short term FW acclimated salinity challenge experiment registered a decrease. The Na^+:K^+ ratio was only increase in BW-17.5 animals from long term FW acclimated salinity challenge experiment. Muscle water content in short term FW acclimated lampreys decreased in BW-17.5 animals and levels decreased further in both BW-25 groups. Intestinal fluid [Cl^-] increased in long term FW acclimated salinity challenged lampreys approaching environmental levels. The same effect was observed in the BW-25 osmocompromised sea lamprey from the short term FW acclimation experiment.

Table 4.3 Muscle concentrations of Na^+ and K^+ (mmol/g wet mass), percentage water and Na^+:K^+ ratio and the intestinal fluid Cl^- concentration (mM) in long term FW acclimated sea lamprey challenged in freshwater, FW (N=4), 17.5 brackish water (BW) (N=4) and short term FW acclimated sea lamprey challenged in freshwater, FW (N=6), BW-17.5 brackish water (N=6), and BW-25 osmoregulators, and BW-25 osmocompromised animals (N=4). Values shown as mean ± S.E.M. Within each experiment, groups that do not share letters are significant different (P<0.05).

FW Acclimation	**Group**	**Muscle**				**Intestinal fluid**
		$[Na^+]$	$[K^+]$	Water content	Na^+/K^+ ratio	$[Cl^-]$
Long term	FW	23.8±5.1 [a]	85.2±17.4 [a]	77.1±0.4	0.28±0.01 [a]	114.8±0.1 [a]
	BW-17.5	889.0±13.4 [b]	179.2±30.2 [b]	75.3±0.9	0.51±0.07 [b]	243.5±2.0 [b]
Short term	FW	58.6±8.7 [ab]	93.0±8.1 [a]	74.4±1.4 [a]	0.63±0.07	117.9±28.7 [a]
	BW-17.5	42.3±4.8 [ab]	56.9±5.2 [b]	68.9±1.0 [b]	0.74±0.02	109.7±9.5 [a]
	BW-25 osmoregulating	36.4±5.0 [b]	73.2±5.7 [ab]	63.9±0.6 [c]	0.50±0.06	121.4±5.1 [a]
	BW-25 osmocompromised	73.9±5.4 [ac]	93.8±12.3 [a]	62.1±0.8 [c]	0.84±0.14	233.9±26.9 [b]

4.4.3 Plasma Lactate, LDH, ALT and AST

Plasma lactate, and activities of LDH, ALT and AST are presented in Table 4.4. In BW-17.5 long term FW acclimated salinity challenged sea lamprey plasma lactate decreased ~3-fold. However, in short term FW acclimated lampreys sea lamprey no differences in plasma lactate were observed except for a significant difference between BW-25 osmoregulators and osmocompromised fish. Plasma LDH activity was only significantly higher (~176-fold) in animals from the short term FW acclimation experiment BW-25 osmocompromised group compared to the FW control. High variability was found in LDH levels in the BW-17.5 challenge animals in the long term FW acclimated lampreys challenge experiment and thus the difference was not significant. Plasma ALT activity was significantly elevated in long term FW acclimated lampreys BW-17.5 and short term FW acclimated BW-25 osmocompromised animals. Plasma AST activity did not show a change in long term FW acclimated sea lamprey groups, although high variation was observed in BW-17.5 group. In short term FW acclimated BW-25 osmocompromised animals AST activity was significantly elevated.

Table 4.4 Lactate, lactate dehydrogenase (LDH), alanine aminotransferase GPT (ALT) and aspartate aminotransferase GOT (AST) in long term FW acclimated sea lamprey challenged in freshwater, FW (N=4), 17.5 brackish water (BW) (N=4) and short term FW acclimated sea lamprey challenged in freshwater, FW (N=6), BW-17.5 (N=6), BW-25 osmoregulators (N=14), and BW-25 osmocompromised animals (N=4). Values shown as mean ± S.E.M. Within each experiment, groups that do not share letters are significant different (P<0.05).

FW Acclimation	Group	Lactate (mM)	LDH (U/L)	ALT (U/L)	AST (U/L)
Long term	FW	8.12±0.43 [a]	172.6±57.2	0±2.0 [a]	165.1±10.5
	BW-17.5	2.73±0.51 [b]	2983.7±1179.7	123.0±57.4 [b]	600.1±246.7
Short term	FW	2.00±0.45 [ab]	8.5±0.8 [a]	1.9±1.4 [a]	61.2±1.6 [a]
	BW-17.5	1.84±0.44 [ab]	10.3±2.2 [ab]	4.5±0.3 [ab]	103.7±20.5 [ab]
	BW-25 osmoregulating	1.00±0.14 [a]	13.0±1.5 [ab]	3.4±0.9 [ab]	94.8±12.9 [ab]
	BW-25 osmocompromised	5.65±3.48 [b]	1493.9±1334.1 [b]	15.6±2.6 [b]	351.6±125.8 [b]

4.4.4 Na^+/K^+ -ATPase Activity

Gill Na^+/K^+-ATPase activities are presented in Fig 4.1A. In the long term FW acclimated sea lamprey, Na^+/K^+ -ATPase activity was highest in the kidney which decreased from approximately 7.2 to 4.1 µmol ADP mg^{-1} protein h^{-1} when animals were acclimated to BW-17.5. Short term FW acclimated sea lamprey challenged to BW-17.5 and BW-25 osmoregulators also display a decrease in NKA activity in the kidney of approximately 7 fold and 3.6 fold, respectively. BW-25 osmocompromised animals displayed a tendency to retain higher NKA activity levels, since levels were not different from the FW group.

In short term FW acclimated lampreys, NKA activity was highest in the anterior intestine. In BW-25 osmocompromised animals NKA activity was significantly lower than the other salinity challenge groups although not the FW control. In contrast, in the posterior intestine of short term FW acclimated lampreys, the BW-25 osmocompromised animals had significantly higher NKA activity than the other groups. No differences in NKA activities in the intestinal regions in long term FW acclimated lampreys were observed. Also in neither experiment were gill NKA activities altered by salinity challenge.

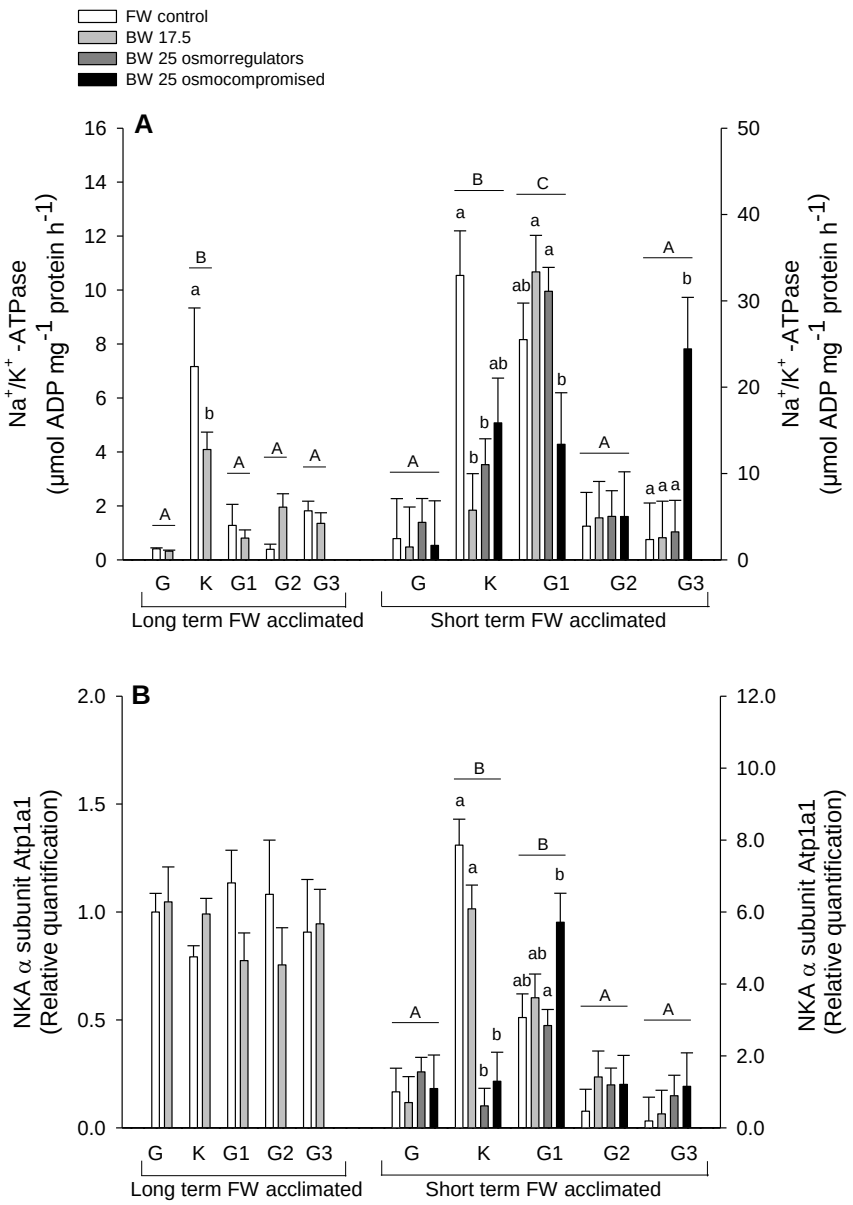

Fig. 4.1 (A) Na^+/K^+ -ATPase activity in: gill, G; kidney, K; anterior intestine, G1; middle intestine, G2; posterior intestine, G3 of long and short term freshwater acclimated adult sea lamprey exposed to salinities of 17.5 and 25. Short term freshwater acclimated sea lamprey freshwater control groups, FW control (N=6, except gill and kidney N=5 and middle intestine N=8); brackish water 17.5 acclimated groups, BW-17.5 (N=6, except anterior intestine N=7); and brackish water 25 which are subdivided into osmoregulating and osmocompromised animals: BW-25 osmoregulating (N=14, except kidney N=13, anterior and posterior intestine N=12 and N=8, respectively); and BW-25 osmocompromised (N=4, except posterior intestine N=3). All long term freshwater acclimated sea lampreys tissue groups were N=4. (B) Representative expression of NKA α1-subunit, Atp1a1 (1:1000). Values are relative to respective freshwater control. Values represent mean ± S.E.M. Different upper case letters indicate significant differences between tissues irrespective of salinity. Different lower case letters denote significant differences with salinity within each tissue. Analysis performed using a two-way ANOVA followed by a Holm-Sidak method test; $P<0.05$.

4.4.5 Immunoblotting

Immunoblotting analyses of NKAα show no changes of protein expression in the long-term FW acclimated sea lamprey. However, in the short term FW acclimated lampreys, higher expression was found in the kidney and anterior intestine. In the kidney, expression was lower in both BW-25 groups. On the other hand, in the anterior intestine, NKA protein levels did not change compared to FW controls; however, BW-25 osmocompromised animals had significantly greater levels than osmoregulators (Fig. 4.1B).

4.4.6 Real Time RT-PCR

Changes at the mRNA level of key ion transport genes (*scnn1*/ENaC, *slc12a3*/NCC, *slc12a2*/NKCC1, *atp1a1*/NKA1a1, and *atp6v1e*/V-type H^+-ATPase E-subunit) and corticosteroid receptor (*cr*) were performed using a real time RT-PCR approach (Fig. 4.2).

The *scnn1* mRNA expression levels were highest in the gill and kidney of long term FW acclimated salinity challenge sea lamprey with lower levels in the intestine. A decrease in relative *scnn1* mRNA expression was found in the gill, kidney and middle and posterior intestine with salinity challenge. In the short term FW acclimated sea lamprey, *scnn1* was highest in the gill and decreased in all salinity groups in this tissue (Fig. 4.2A). There were no changes in the other tissues which had lower starting mRNA levels.

The *slc12a3* mRNA levels were highest in gill in both long and short term FW acclimated lamprey groups and lower in the kidney with no expression found in the intestinal regions. In gill mRNA expression decreased in all salinity challenged animals. In short term FW acclimated lampreys BW-25 challenged osmocompromised animal *slc12a3* mRNA was markedly lower than all other groups (Fig. 4.2B).

The *slc12a2* mRNA expression remained unaltered in the long term FW acclimated sea lamprey although significant differences may have been masked by high variation. In short term FW acclimated lampreys higher levels of *slc12a2* mRNA expression were found in the gill compared to all other tissues analyzed and an increase in expression was found in the gill BW-25 osmoregulators (Fig. 4.2C).

In long term FW acclimated lampreys *atp1a1* mRNA expression was found highest in the anterior intestine in contrast to the lowest levels in the posterior intestine with salinity having no effect on mRNA expression. Short term FW acclimated lampreys

showed higher *atp1a1* mRNA expression in gill, kidney and anterior intestine compared to middle and posterior intestine regions. In BW-25 osmocompromised animals expression increased in all tissues except middle intestine. In BW-17.5 animals *atp1a1* mRNA also increased in anterior intestine (Fig. 4.2D).

V- ATPase E subunit (*atp6v1e*) mRNA expression was highest in the gill of long term FW acclimated sea lamprey with its levels decreasing about 20 fold in animals acclimated to 17.5 BW. No significant differences were observed in the other tissues with salinity challenge. In short term challenge animals the highest *atp6v1e* mRNA expression was observed in gill followed by kidney. In gill the mRNA expression was upregulated in BW-17.5 and BW-25 osmoregulating animals but not BW-25 osmocompromised animals. In contrast, in the kidney, BW-25 osmocompromised animals significantly upregulate *atp6v1e* mRNA expression while in the other salinity groups increases were not significant. Expression in intestinal sections was low and remained unchanged (Fig. 4.2E).

The *cr* mRNA expression was found to be downregulated by salinity in the gill and anterior and middle intestine in the long term FW acclimated lampreys. Expression levels were lowest in kidney and unresponsive to salinity. No changes were found in the short term FW acclimated sea lamprey groups (Fig. 4.2F).

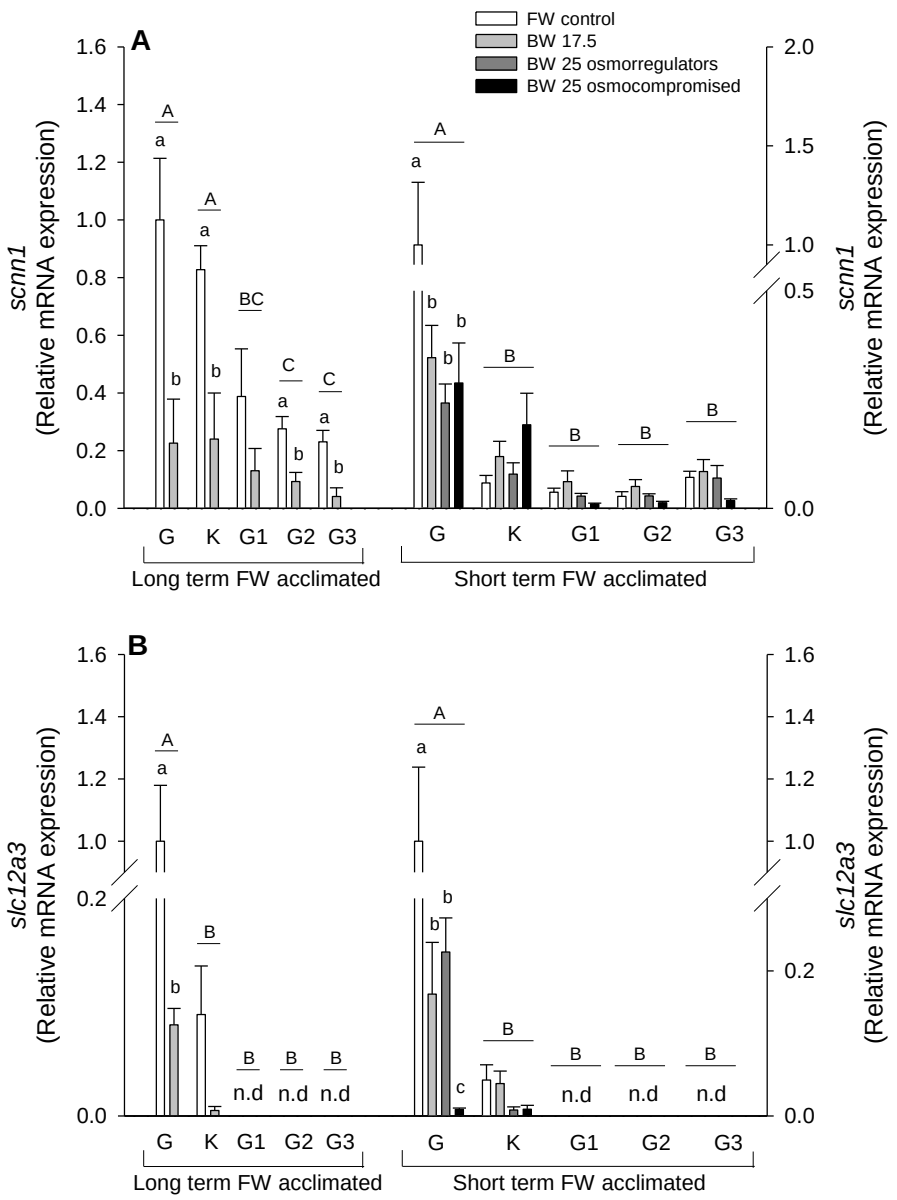

Fig. 4.2 (Continued)

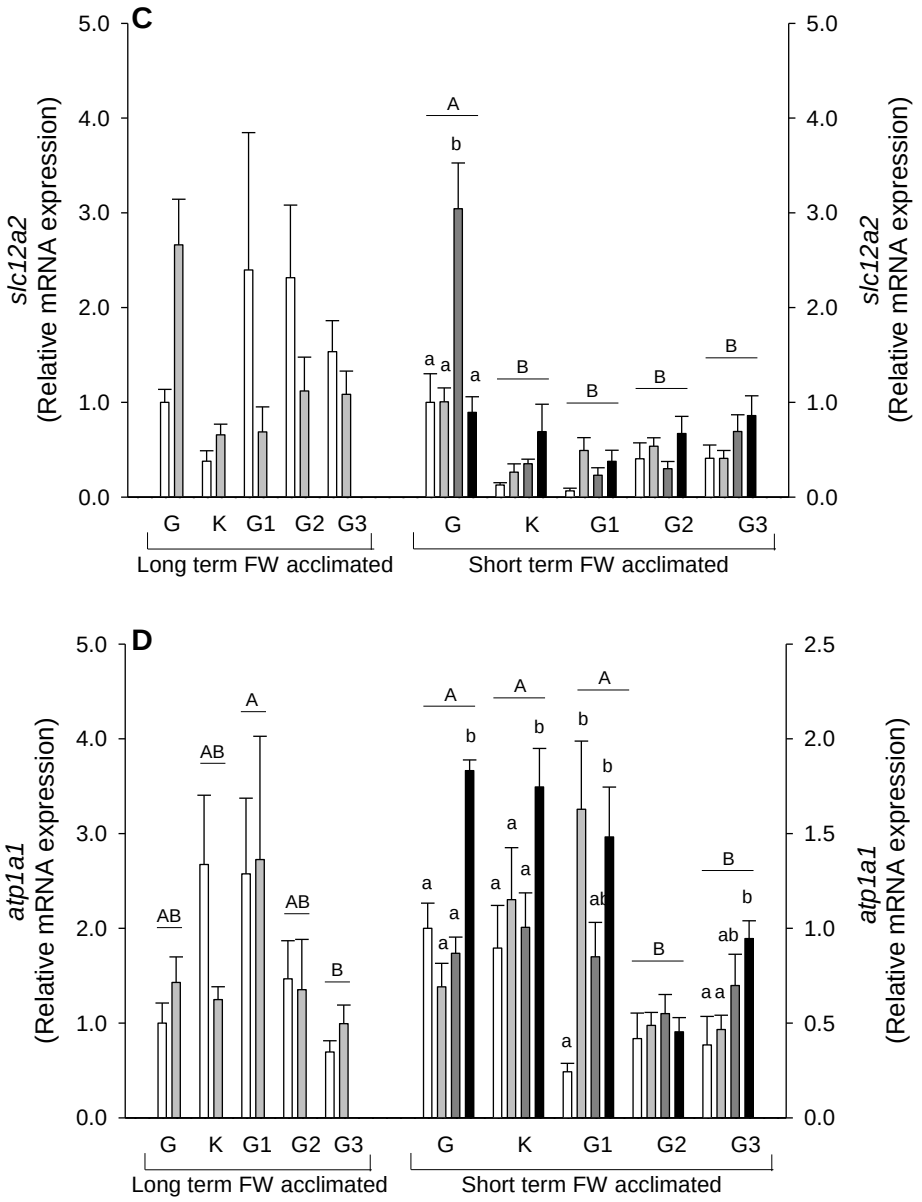

Fig. 4.2 (Continued)

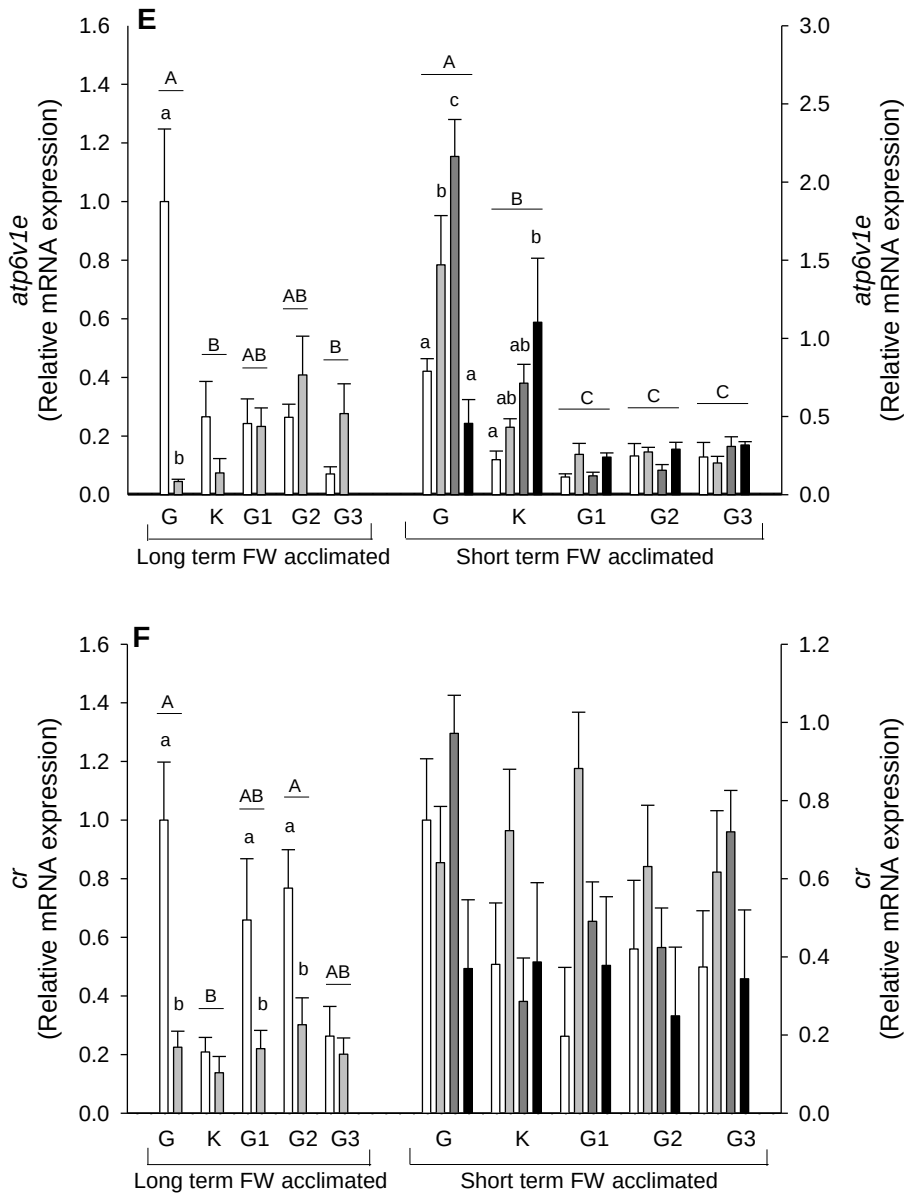

Fig. 4.2 Relative mRNA expression of *scnn1*/ENaC (A), *slc12a3*/NCC (B), *slc12a2*/NKCC1 (C), *atp1a1*/NKA-a (D), *atp6v1e*/V-ATPase subunit E (E) and corticosteroid receptor (F) in gill, kidney, anterior, middle and posterior intestine of long and short term freshwater acclimated sea lamprey in freshwater and brackish water. See Fig. 4.1 caption for details. Values are relative to respective freshwater control.

4.4.7 Immunohistochemistry

Gill sections probed for Na^+/K^+-ATPase (NKA) and V-type H^+-ATPase are shown in Fig 4.3. In sea lampreys acclimated to FW, V-ATPase was immunolocalized to isolated epithelial cells in both the filament and lamellae. Two types of cellular staining pattern were displayed which included whole cell signal and some cells showing labeling

concentrated more on the apical region of these cells (Fig. 4.3A). Strongly immunoreactive cells were also observed in the blood space, which were likely leukocytes. In FW animals NKA immunoreactivity was limited to the basolateral membrane of epithelial cells in the filament but not the lamellar epithelium. When lampreys were acclimated to BW osmoregulators displayed fewer H^+-ATPase immunoreactive (IR) in the lamellae, and a similar immunolocalization pattern was found in the filament epithelium although with few cells with apical immunoreactivity. Under these conditions intense NKA IR was found in clusters of large cells (2-5 cells) in the filament epithelium with labeling present throughout the cell body indicative of SW-type ionocyte tubular system staining (Fig. 4.3B) In BW osmocompromised animals, Na^+/K^+-ATPase IR was weak and limited to the basolateral membrane of cells in the filament epithelium and fewer V-ATPase IR cells were found in the gill with a diffuse cytoplasmic staining pattern (Fig. 4.3C).

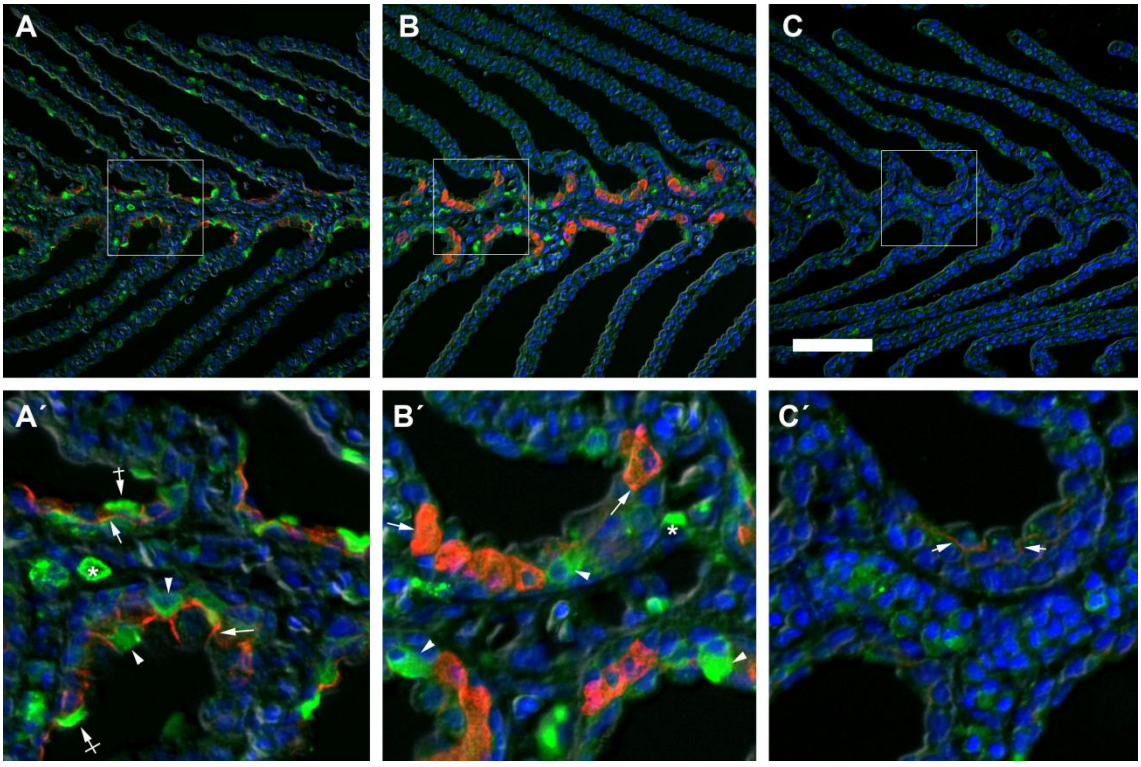

Fig. 4.3 Double immunofluorescence localization of V-ATPase (green) and Na^+/K^+-ATPase (red) with the corresponding merged image overlaid with DAPI nuclear staining (blue) in the gills of upstream migrating lampreys in (A) freshwater, (B) BW-25 osmoregulator and (C) BW-25 osmocompromised. Arrows indicate NKA basolateral immunoreactivity; arrowheads, crossed arrows and asterisks indicate V-ATPase epithelial cytosolic and apical staining and leukocytes, respectively. Scale bar, 100 µm.

4.5 Discussion

In anadromous migratory species that include the sea lamprey, obstructions to passage such as dams and weirs are a major conservation issue (Larinier, 2000; Marmulla, 2001). In Portugal, where this study was conducted, there are dams and other obstructions on the main sea lamprey spawning river systems that result in significant habit loss (Mateus *et al.*, 2012). At best, sea lamprey have been reported to overcome near vertical obstacles 1.5–1.8 m (Scott and Crossman, 2012), but Reinhardt *et al.* (2009) have shown in laboratory experiments that landlocked sea lamprey cannot climb vertically. In cases that fish passage facilities are available, they are not designed with lamprey in mind and are generally not efficient for lamprey passage (Mateus *et al.*, 2012; Moser *et al.*, 2015). Since the sea lamprey is a semelparous species, its ability to reach upstream spawning grounds ultimately determines the success or failure for individuals in this closing step of its lifecycle (Moser *et al.*, 2015). If unable to overcome an obstacle, the sea lamprey has few options: turning back downstream and finding another FW system in which to spawn, spawning in a sub-optimal downstream habitat, or in the worst case scenario dying without spawning. The retreat downstream has been observed in sea lamprey (Applegate, 1950; Kelso and Gardner, 2000; Almeida *et al.*, 2002a; Holbrook, 2015) although these studies are limited to migration in FW. Notably, using telemetry Kelso and Gardner (2000) found 26% of males (39 of 149) with radio transmitters emigrated (migrated downstream out of the rivers they had been released into) and that there were porportionaly more emigrants in rivers with upstream barriers closer to the release point. Holbrook (2015) has also shown that there is a tendency of a small proportion of downstream retreating migrants to keep moving down stream rather than hold or return upstream towards the barrier again. In Pacific lamprey (*Entosphenus tridentatus*), migrants encountering a barrier have been shown to enter downstream tributaries (Noyes *et al*., 2013). It has been suggested that this behavior is linked with the search for migratory cues as no strong directional migration is displayed (reviewed by Moser *et al*., 2015).

In this study, estuarine captured sea lamprey acclimated to freshwater and then tested for salinity tolerance in March and June were unable to acclimate to full strength seawater reaching salinity limits of 25 and 17.5, respectively. This latter value correlates well with previously reported salinity tolerance levels in upstream anadromous migrants [approximately 50% SW; (Beamish *et al.*, 1978; Galloway, 1933; Morris, 1956)]. The short term FW acclimated challenged lampreys could be divided to two groups, osmoregulators and osmocompromised animals which may reflect differences in residency time in the estuary since lamprey may enter the estuary as early as December.

4.5.1 Osmocompromised Animals

The long term FW acclimated lampreys and 18% of the short term FW acclimated lampreys can be classified as being osmocompromised and on the verge of osmoregulatory failure. This was marked by increases in plasma ions with Na^+, Cl^- and Ca^{2+} approaching environmental levels (long term FW acclimated lampreys at BW-17.5) or approximately doubling from FW control fish levels (short term FW acclimated lampreys at BW-25). These values are in reasonable agreement with the Na^+ concentrations reported by Beamish *et al.* (1978) given the high variability they observed in their high salinity treatment (BW-16 and BW-24; $[Na^+]_{pl}$ 177 mM and 226 mM, respectively). As a consequence of these high plasma ion levels, blood hemolysis was observed in the present study with hematocrit decreasing by >90%. Lewis and Ferguson (1966) have demonstrated by performing an osmotic fragility test common in hematology that among premammalian erythrocytes, fish are the most fragile. This crash in blood oxygen carrying capacity would have implication for maintaining aerobic metabolic rates, and osmocompromised animals were observed to be much more sluggish than osmoregulators. Plasma lactate would be an indicator of anaerobic metabolism and has been shown to negatively correlate with hematocrit (Olsen *et al.*, 1992); however, in the current study no correlation was found and changes were not consistent (decrease in long term FW acclimated lampreys and increase in short term FW acclimated lampreys). It should be noted that hematocrit did decrease in osmoregulating fish as well, but by only 20%. Cellular damage was not limited to red blood cells since indicators of liver and muscle damage in fish (Gabriel *et al.*, 2012; Gaim *et al.*, 2015; Kumari *et al.*, 2011) were also increased in plasma (LDH, AST and ALT levels). These cytosolic enzymes are released into the plasma when tissues are damaged (e.g. Pakhira *et al.*, 2015; Vedel *et al.*, 1998). AST is a good predictor of liver damage (Casillas *et al.*, 1982; Murray, 1984) and levels were increased in BW-25 osmocompromised animals. LDH and ALT are released when other tissues are damaged, notably muscle (Kumari *et al.*, 2011). Dehydration was also observed in muscle and accompanied by increases in Na^+ levels. Although, muscle water content values in freshwater in the short term FW acclimation study were lower than literature values of [~77%; (Boutilier *et al.*, 1993; Kott, 1971)], taken together the impacts on muscle correlate with the lethargy observed in osmocompromised animals.

In osmocompromised animals, the emergence of seawater type ionocytes in the gills were not observed. In lamprey, seawater-type ionocytes are readily identifiable by the

appearance of whole-cell NKA immunoreactivity which represents labeling of the tubular membrane system (Bartels and Potter, 2004; Reis-Santos *et al.*, 2008). Unlike in teleost fishes, which have this staining pattern in both FW and seawater ionocytes, in lamprey it is associated only with the seawater type ionocyte (Wilson, 2011). Only the NKA immunoreactivity pattern of basolateral staining in filament cuboidal pavement cells observed in FW lamprey was present in osmocompromised animals (Choe *et al.*, 2004; Reis-Santos *et al.*, 2008). Also no transcriptional upregulation of gill ion transporters NKCC1, and NKA were observed either. Taken together, these results indicate a failure of a hypo-osmoregulatory branchial response in osmocompromised animals.

In the gut, there were generally few changes in ion transporter transcript levels which would indicated an hypo-osmoregulatory response (Li *et al.*, 2014). It has previously been shown that drinking rates decrease upon FW acclimation and thus switching drinking back-on in salinity challenged animals likely limits complete seawater tolerance (Rankin, 2002). Also, the start of their spawning migration and the development of the gonads are associated with atrophy of the gut, and the cessation of feeding (Larsen, 1980). Intestinal fluid Cl^- concentrations doubled in osmocompromised animals when compared to the respective FW control group, from almost iso-ionic with plasma in FW and BW-25 osmocompetent animals. This high intestinal Cl^- suggests that drinking is occurring but that esophageal desalination has failed and thus water absorption is compromised (Loretz, 1995).

4.5.2 Osmoregulators

Since animals challenged in March were able to osmoregulate in salinities of 25, returning to estuarine waters becomes physiologically possible. The upregulation of branchial NKCC1 mRNA in the short term FW acclimated lampreys and the appearance of characteristic NKA-IR seawater-type ionocytes in the gill epithelium indicate a hypo-osmoregulatory response to support the fish's ability to ionoregulate. Morris (1958) has also shown that a small percentage of freshly caught, migrating river lampreys (*Lampetra fluviatilis*) were able to osmoregulate in 50% seawater, osmoregulating similarly to marine teleosts. The kidney would produce low volumes of highly concentrated urine and the water swallowing mechanism would be responsible for compensating the loss of water at the body surface and Cl^- excreted by the SW-ionocytes (Morris, 1958).

4.5.3 Successful Down Regulation of Freshwater Ion Uptake Mechanisms

Regardless of whether lamprey could successfully hypo-osmoregulate in BW or not, all were clearly able to down regulate branchial ion uptake mechanisms (ENaC and NCC). However, the responses of branchial ion pumps NKA and V-ATPase were ambiguous. In the case of the NKA, given its dual importance in both active ion uptake and secretion (McCormick, 1995; Reis-Santos *et al.*, 2008) this is not entirely unexpected. In the case of the V-ATPase which is associated with indirect coupling of Na^+ uptake via the ENaC, decreases in long term FW acclimated lampreys were as expected; however, in short term FW acclimated lampreys transcript expression increased significantly. The significance of this increase is unclear although it may be associated with increased leukocyte infiltration into gill tissue. Also immunohistochemistry indicates that apical expression of V-ATPase is lost suggesting that although present in the gill it is not functional in Na^+ uptake.

In kidney, the switch from hyper to hypo-osmoregulation is associated with a decrease in NKA expression (Lin *et al.*, 2004) and this was apparent in adult sea lamprey (mRNA, protein and activity). In the colon of mammals ENaC is involved in Na^+ reabsorption (Malsure *et al.*, 2014) and in the present study, in the middle and posterior intestine, ENaC transcript expression decreased significantly in long term FW acclimated lampreys, suggesting an adaptive response.

Although 11-deoxycortisol has been reported to be the mineralocorticoid in lamprey (Close *et al.*, 2010), corticosteroid receptor mRNA expression was not affected by salinity in the short term FW acclimated lampreys challenges, although in long term FW acclimated lampreys challenges, down regulation was found in the gill and anterior and middle intestine.

4.6 Conclusions

Given that very little is known about osmoregulatory mechanisms in estuarine and migrating adult lamprey (Moser *et al.*, 2015), the present study should contribute significantly to this knowledge gap. The anadromous spawning migration phase in the sea lamprey life cycle is a time of profound morphological and physiological change. This study demonstrates that they possess the branchial mechanisms for hyperosmoregulation (e.g. ENaC and NCC) and although they can successfully downregulate these mechanisms upon salinity re-exposure following FW acclimation, their ability to conversely up regulate hypo-osmoregulatory mechanisms can be limiting. It has been shown that it is

physiologically possible for lamprey to return to salinities of 25 and although downstream retreat (emigration) has been observed in lamprey that encounter barriers to upstream migration, it remains to be determined whether anadromous lamprey will in fact retreat to the estuarine or coastal waters in search of suitable spawning grounds.

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4.9 Supplementary materials

Supplemental Table 4.1 Primer pairs (sense and anti-sense, respectively) for qPCR with predicted product size, annealing temperature, original gene accession number. Primers with an asterisk (*) were designed with Primer3 and reference sources are given for the remainder.

Gene name	GenBank Accession No.	Forward and reverse primer sequences (5'-3')	Product size (bp)	Annealing temperature (ºC)	Reference
gapdh	AY578058	TGCAAAGCACGTCATCATCTC TTCTCGTGGTTTACTCCCATCA	**72**	**60**	(Shifman *et al.*, 2009)
*atp1a1**	GENSCAN00000136072	CGTGGAATCGTCATCAACAC GCGACAGGATGAAGAAGGAG	**169**	**58**	
*slc12a2**	ENSPMAG00000000665	GAGAGGTTTCGCGACAAGAC CGCTCACGAGTAGAACGTCA	**225**	**58**	
*slc12a3**	ENSPMAG00000005880	GTCATCACGGTCACCTTCCT ACACCGGAGTGAAATTCTCG	**205**	**58**	
*atp6v1e**	ENSPMAG00000008972	GTGAAGGAAGCCATGGAGAA TGGGGTTGACTTTGAAGAGC	**232**	**58**	
*scnn1**	ENSPMAG00000007655	GCATCATGGTACACGACCAG AGGCGGAGGAGTAGAGGTTC	**183**	**58**	
*cr**	AY028457.1	GTCCCACAAGAGGGTCTGAA GGCCATCATGTCAGGAAACT	**247**	**60**	

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Chapter 5:

General Conclusions

5.1 General Conclusions

Overall, the work presented in this thesis reveals more of the fascinating biology of this ancestral cyclostome representative and iconic species, with particular focus on osmoregulation. The studies presented here address the main stages of sea lamprey's life history from the ammocoete stage, through metamorphosis and into the post-metamorphic juvenile stage (chapter two); in fully transformed juveniles transition from freshwater to seawater and initiation of parasitic feeding (chapter three); and finally the upstream spawning migration (chapter four).

The branchial IMRCs in the gill are the main site for ion regulation in this organ. In the case of sea lampreys, Bartels and Potter (2004) suggested that during the ammocoete stage, the sea lamprey possesses a distinct MRC type. The *ca18* was shown to be present in all sea lamprey's life stages while *ca19* was only present in the ammocoete stage and its expression was progressively reduced during metamorphosis until it was not detectable in the post-metamorphic juvenile stage.

After concluding metamorphosis and before entering the ocean, the post-metamorphic juveniles have already acquired the mechanism for seawater tolerance as seawater type ionocytes have already developed by this stage (Reis-Santos *et al*., 2008; chapters two and three of the present thesis). Upon entering seawater, the sea lamprey finds a suitable host and begins its parasitic feeding stage and in this thesis it was demonstrated that feeding on host body fluids facilitates sea lamprey osmoregulation in seawater. This is supported by the down regulation of ion secretion mechanisms and relatively lower plasma ion levels in feeding seawater lamprey. When reaching maturity, the sea lampreys enters a freshwater system and migrates upstream in search of suitable grounds in which to spawn. During their upstream migration, sea lampreys may encounter barriers to migration and therefore may need to move downstream to find new streams to spawn. In the most severe cases, the upstream migrants might need to go back to the estuary or the ocean in search of this alternative spawning river systems and therefore again resort to seawater osmoregulatory mechanisms to cope with the new hyper salinity challenge. Results from this thesis demonstrated that the ability to return to a hyper salinity environments is dependent on the spent time in freshwater during upstream migration. Sea lampreys with short time spent in freshwater retain the capacity to restore seawater type ionocytes and thus osmoregulate efficiently, whilst those with longer periods of time spent in freshwater do not.

To date most studies on various aspects of the biology of sea lampreys have been conducted on the landlocked ecotype populations due to the easy access to large numbers of all life stages and the importance of the efforts to control the populations in the

Great Lakes of North America (Hardisty, 2006). In contrast, the anadromous ecotype populations of sea lamprey are threatened along the eastern coast of North America and western coast of Europe (Docker, 2015). As mentioned in chapter one of this thesis, the anadromous sea lamprey has a complex life cycle (Hardisty and Potter, 1971abc) and the physiological challenges it faces during transitions to and from hypertonic environments are similar to those of euryhaline teleost (Bartels and Potter, 2004; Beamish, 1980ab; Hardisty *et al.*, 1989; Morris, 1972) with analogous mechanisms for hypo and hyperosmoregulation proposed (Bartels and Potter, 2004). In addition to this, as living representatives of the basal vertebrate lineage, they provide insights into the biology of organisms in the past and how evolution took place allowing vertebrates to explore new available ecosystems.

5.2 Initial life stages and preparation for downstream migration

The gill is known to be the main site of ionoregulation in fishes and the presented work demonstrates how the gill of sea lampreys functions in the different life stages and in adaptation to different salinity environments. As ammocoetes the sea lampreys are in freshwater, thus in order to maintain its internal ion balance, the sea lamprey must actively uptake Na^+, Cl^-. K^+, Ca^{2+} and other ions through the gills. In addition, during the larval stage, the sea lampreys live buried in the substrate, which is a microenvironment with distinct characteristics from what this fish experiences during the later life stages. In contrast, after the sea lampreys larvae complete their metamorphosis and start their downstream migration, the osmoregulatory challenges they will face when entering the ocean are reversed and the gill must actively secrete the excess of ions (Bartels and Potter 2004). In chapter two of this thesis, it is shown that a cytosolic carbonic anhydrase isoforms switch is likely to be accompanied by the disappearance of the ammocoete MRC and the emergence of the seawater type ionocytes that grant enhanced tolerance to hypertonic environments as it has already been described by Reis-Santos and co-workers (2008) and also demonstrated in chapter four of this thesis. Results in this thesis suggest that the novel ammocoete carbonic anhydrase isoform *ca19* might play a role in adaptation to the microenvironment in which the ammocoete is subjected to while buried in the substrate. Thus if this is true and *ca19* is demonstrated to be located in ammocoete MRC, it suggests that these particular cells play a role in adaptation to these microenvironments rather than playing a central role in ion regulation.

Overall, in this thesis it is proposed that this switch of isoforms and increase in *ca18* is related to a change of metabolic demands as sea lampreys go from a low energy filter feeding strategy to a high energy parasitic blood-feeding lifestyle and to changing demands for ion and acid-base regulation.

5.3 Osmoregulation during marine parasitic feeding phase

Ahead of entering the ocean, sea lamprey juveniles are fully prepared to cope with the salinity challenge they will face (see chapter one and two of this thesis) and as parasitic feeders, they search for a suitable host on which to feed. Feeding has a strong impact of the physiology of osmoregulating fishes (Marshall and Grosell, 2005) as is in the case of the sea lamprey. In chapter three of the thesis it was demonstrated that sea lamprey were able to successfully acclimate to seawater which involved the downregulation of branchial ion uptake mechanisms (NCC, ENaC and V-ATPase mRNA expression) and kidney NKA activity. Upon parasitic feeding in seawater, sea lampreys downregulated ion secretory mechanisms through NKAα1 and NKCC1 mRNA expression. This, together with lower [Cl^-], [K^+] and [Ca^{2+}] levels in plasma compared to controls at same salinity suggest that feeding on a body fluids meal that is hyposmotic compared to the external environment compensates for ion gain and osmotic water loss through the gills and skin. In addition, an increase in NKA activity in the anterior section of the intestine indicates a role in nutrient absorption. Branchial HPS70 protein expression was found to be downregulated in feeding sea lampreys. Although speculative, it is hypothesized that the higher observed expression in the two salinity control groups could be relate with the period of transition from fresh to seawater and/or fasting effects as sea lampreys do not feed during this stage (Beamish, 1980ab; Hardisty and Potter, 1971ac). Feeding facilitated hyposmoregulation has the additional benefit to the organism of reducing the need to expend as much of its energy osmoregulating which can be used for other purposes such as growth. After a period of parasitic feeding and growth in seawater that can last up to two years (Beamish, 1980b; Hardisty and Potter, 1971ac) sea lampreys find freshwater systems which can be different from their natal streams (Bergstedt and Seelye, 1995) in which to spawn.

5.4 Osmoregulation during upstream spawning migration

To complete the overall look of osmoregulatory aspects of sea lamprey during its different life cycle stage, the osmoregulatory mechanisms in estuarine and upstream migrating adult lamprey were analyzed. Very little is known about this life stage despite the fact that this iconic species is threatened and has high value and economical interest (Andrade *et al.*, 2007; Quintella, 2006).

At the time of the upstream spawning migration the seawater type ionocytes are replaced by the freshwater types, though they lack the ammocoete MRCs (Bartels and Potter, 2004). In chapter four it was established that freshwater acclimated upstream spawning migrants acquire branchial mechanisms for hyper-osmoregulation (e.g. ENaC and NCC) and when challenged to brackishwater, they were able to successfully downregulate these mechanisms. A similar result was observed in chapter three where post-metamorphic juveniles demonstrated high mRNA expression of ENaC and NCC when acclimated to freshwater an expression of these genes was found to be downregulated upon transfer to seawater. In the case of post-metamorphic juveniles, hyper-osmoregulatory mechanisms are found to be upregulated prior to contact with seawater (chapter three; Reis-Santos *et al*., 2008). These results suggest that upstream spawning migrants are able to respond to a hyper-salinity challenge and for that they resort to similar molecular mechanisms to those during their downstream migration to enter the ocean. This indicates that upstream spawning migrant are able to restore the seawater type ionocytes, the same that are already present in the post-metamorphic juveniles and allow them to be directly transferred to an hypertonic environment. Nevertheless, in the upstream migrating sea lampreys the capacity to restore hypo-osmoregulatory mechanisms is limited and appears related with time spent in freshwater during the upstream spawning run. Although downstream retreat (emigration) has been observed (Holbrook, 2015) in the event of lamprey encountering barriers to upstream migration it remains to be determined whether anadromous lamprey will in fact retreat to the estuarine or coastal waters in search of suitable spawning grounds.

This is not just important from the physiological perspective of lampreys since as osmoregulators their spawning migration requires a switch from marine hypo-osmoregulation to freshwater hyper-osmoregulation (Beamish, 1980b) but also from a conservation and ecological point of view. Being a semelparous species that is vulnerable to endangered in parts of its native range due in part to loss of spawning habitat by manmade barriers such as dams, the ability of lamprey to return downstream to the ocean or estuary and search out alternative spawning river systems would be limited by seawater osmoregulatory capacity. This provides support to the hypothesis that later

during the upstream migration, seawater type ionocytes are permanently lost and energy is conducted toward the spawning process past of point of no return. Thus, these results obtained from a molecular physiology perspective, are particularly important from the conservation standpoint of this species as they provide more information about the biological limit of this species to which the efforts conservation must be made in order to obtain positive results.

5.5 Physiological challenges on the host species upon sea lamprey parasitism

As it has been stated by Alcock (2001), predator-prey interactions have an impact on the behavior, morphology, shape, size and survival of both parties. In a certain way, the results of sea lamprey parasite-host interactions (chapter three) illustrate this same trend. Of the 23 attacks registered only in seawater, 78% resulted in an open wound in the hosts skin from which lampreys could feed and from those, 17% resulted in the host's death in less than three days. Nevertheless, these numbers correspond to laboratory conditions in which water was carefully controlled to minimize infections on the debilitated fish. Thus it is expected that mortalities are greatly increased as these wounded fish are more prone to diseases and predation (Kitchell, 1990).

When sea lampreys attach and wound their host in order to feed on their body fluids a challenge on osmoregulation is expected in the host species that is influenced by the size of the host species in relation to the sea lamprey. In the worst cases, sea lampreys parasitic feeding can cause severe anemia and death.

In the study of parasite-host interactions in this thesis, the host species successfully acclimated to the higher salinity environment as it had been already shown by Hiroi and McCormick (2007). Ion secretion molecular mechanism positively responded to the salinity challenge [ex: increased NKA activity, upregulation of NKAα1b and NHE2 mRNA expression levels with the last possibly with a major role in excreting acid in seawater (Lui *et al*., 2013) and plasma ion content increased, though not statistically significant. However, when brook trout were parasitized for three consecutive days they were unable to maintain their homeostasis (general increase in plasma ions, lactate and LDH levels, loss of appetite with consequent loss of body mass which translated into a reduction of the K factor). More dramatic was the observed loss of RBC's that correlated with the increase of the LDH and lactate levels in plasma. Interestingly, in terms of osmoregulatory response to parasitism, brook trout upregulated NKAα1a mRNA

expression suggesting it plays a role in the frontline to cope with ion levels fluctuation due to loss of body fluids.

5.6 Final statements and future directions

Sea lampreys have a complex life cycle marked by profound changes in their morphology and physiology. The studies in this thesis illustrate how these mechanisms respond during these transitions, although the control mechanisms that lead to this up and down regulation of osmoregulatory mechanisms, allowing transitions between environments are not yet very clear. Particular aspects in each transitory stage occur and appear to be related with the adaptation to a new lifestyle. These changes at the molecular level seem to be related to the switch between the MRCs that characterize the gill in the sea lamprey larval, juvenile and adult stages.

In order to better understand the mechanisms that trigger these changes, future studies are needed to assess hormonal control of sea lamprey osmoregulatory mechanisms and better understand the potential role and location of carbonic anhydrases to changing demands for ion and acid-base regulation between larvae and juvenile forms. The work on the parasite-host interactions allowed the first insights into the osmoregulatory challenges and mechanisms inherent to parasitic feeding in anadromous sea lamprey populations, nevertheless more studies are needed using other hosts such as elasmobranches to deepen our knowledge of the changes of different blood and body fluid meals on the sea lamprey's osmoregulation.

Overall, this thesis contributes not only to increasing our knowledge of osmoregulatory mechanism of a representative basal vertebrate's lineage, but it can also be applied to the conservation and management of this species.

References

Alcock J (2001) Animal Behavior, 7th Edition. Sinauer Associates, Sunderland, MA.

Almeida PR, Quintella BR, Dias NM (2002a) Movement of radio-tagged anadromous sea lamprey during the spawning migration in the River Mondego (Portugal). *Hydrobiologia* 483: 1–8.

Almeida PR, Quintella BR, Dias NM, Andrade N (2002b) The anadromous sea lamprey in Portugal: Biology and Conservation Perspectives. In: *International Congress on the Biology of Fish: The Biology of Lampreys*. American Fisheries Society, Physiology Section, Bethesda, MD, USA, pp 49–58.

Andrade NO, Quintella BR, Ferreira J, Pinela S, Póvoa I, Pedro S, Almeida PR (2007) Sea lamprey (*Petromyzon marinus* L.) spawning migration in the Vouga river basin (Portugal): poaching impact, preferential resting sites and spawning grounds. *Hydrobiologia* 582: 121–132.

Anisimova M, Gil M, Dufayard J-F, Dessimoz C, Gascuel O (2011) Survey of branch support methods demonstrates accuracy, power, and robustness of fast likelihood-based approximation schemes. *Syst Biol* 60: 685–699.

Applegate VC (1950) Natural history of the sea lamprey, *Petromyzon marinus*, in Michigan. Doctoral thesis. University of Michigan, WA, USA.

Ardavin CF, Gomariz RP, Barrutia MG, Fonfria J, Zapata A (1984) The lympho-hemopoietic organs of the anadromous sea lamprey, *Petromyzon marinus*. A comparative study throughout its life span. *Acta Zool* 65: 1–15.

Arnold K, Bordoli L, Kopp J, Schwede T (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* 22: 195–201.

Bartels H, Potter I, Pirlich K, Mallatt J (1998) Categorization of the mitochondria-rich cells in the gill epithelium of the freshwater phases in the life cycle of lampreys. *Cell Tissue Res* 291: 337–349.

Bartels H, Potter IC (2004) Cellular composition and ultrastructure of the gill epithelium of larval and adult lampreys: implications for osmoregulation in fresh and seawater. *J Exp Biol* 207: 3447–3462.

Bartels H, Schmiedl A, Rosenbruch J, Potter IC (2009) Exposure of the gill epithelial cells of larval lampreys to an ion-deficient environment: A stereological study. *J Electron Microsc (Tokyo)* 58: 253–260.

Baxter EW (2009) Observations on the buccal glands of lampreys (Petromyzonidae). *Proc Zool Soc (London)* 127: 95–118.

Beamish FWH (1974) Apparent specific dynamic action of largemouth bass, *Micropterus salmoides*. *J Fish Res Board Canada* 31: 1763–1769.

Beamish FWH (1980a) Biology of the North American anadromous sea lamprey, *Petromyzon marinus*. *Can J Fish Aquat Sci* 37: 1924–1943.

Beamish FWH (1980b) Osmoregulation in juvenile and adult lampreys. *Can J Fish Aquat Sci* 37: 1739–1750.

Beamish FWH, Strachan PD, Thomas E (1978) Osmotic and ionic performance of the anadromous sea lamprey, *Petromyzon marinus*. *Comp Biochem Physiol* 60A: 435–443.

Beamish RJ, Youson JH (1987) Life history and abundance of young adult *Lampetra ayresi* in the Fraser River and their possible impact on salmon and herring stocks in the Strait of Georgia. *Can J Fish Aquat Sci* 44: 525–537.

Behrens T, Zhu A-X, Schmidt K, Scholten T (2010) Multi-scale digital terrain analysis and feature selection for digital soil mapping. *Geoderma* 155: 175–185.

Bergstedt RA, Seelye JG (1995) Evidence for lack of homing by sea lampreys. *Trans Am Fish Soc* 124: 235–239.

Betts, Matthew J. RRB (2003) Amino acid properties and consequences of substitutions. In: Gray IC, Barnes MR, eds. *Bioinformatics for Geneticists*. John Wiley & Sons, Ltd, pp 289–304.

Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, Kiefer F, Cassarino TG, Bertoni M, Bordoli L, *et al.* (2014) SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res* 42: W252–W258.

Bigelow H, Schroeder W (1953) Fishes of the Western North Atlantic. Part 2.(sawfishes, guitarfishes, skates, rays and chimaeroides). In: *Fishes of the Western North Atlantic. Memoirs of the Sears Foundation for Marine Research.* Yale University, CT, USA 1

(2): pp 588.

Bjellqvist B, Basse B, Olsen E, Celis JE (1994) Reference points for comparisons of two-dimensional maps of proteins from different human cell types defined in a pH scale where isoelectric points correlate with polypeptide compositions. *Electrophoresis* 15: 529–539.

Bjellqvist B, Hughes GJ, Pasquali C, Paquet N, Ravier F, Sanchez J-C, Frutiger S, Hochstrasser D (1993) The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences. *Electrophoresis* 14: 1023–1031.

Blom N, Sicheritz-Pontén T, Gupta R, Gammeltoft S, Brunak S (2004) Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. *Proteomics* 4: 1633–1649.

Bordoli L, Kiefer F, Arnold K, Benkert P, Battey J, Schwede T (2009) Protein structure homology modeling using SWISS-MODEL workspace. *Nat Protoc* 4: 1–13.

Boutet I, Long Ky CL, Bonhomme F (2006) A transcriptomic approach of salinity response in the euryhaline teleost, *Dicentrarchus labrax*. *Gene* 379: 40–50.

Boutilier RG, Ferguson RA, Henry RP, Tufts BL (1993) Exhaustive exercise in the sea lamprey (*Petromyzon marinus*): relationship between anaerobic metabolism and intracellular acid-base balance. *J Exp Biol* 178: 71–88.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254.

Brett CL (2005) Evolutionary origins of eukaryotic sodium/proton exchangers. *AJP Cell Physiol* 288: C223–C239.

Bury NR, Grosell M, Wood CM, Hogstrand C, Wilson RW, Rankin JC, Busk M, Lecklin T, Jensen FB (2001) Intestinal iron uptake in the European flounder (*Platichthys flesus*). *J Exp Biol* 204: 3779–3787.

Cameron MacEachen D, Russell RW, Michael Whittle D (2000) Spatial distribution of mercury and organochlorine contaminants in Great Lakes sea lamprey (*Petromyzon marinus*). *J Great Lakes Res* 26: 112–119.

Campos A, Puerto M, Prieto A, Cameán A, Almeida AM, Coelho A V, Vasconcelos V

(2013) Protein extraction and two-dimensional gel electrophoresis of proteins in the marine mussel *Mytilus galloprovincialis*: an important tool for protein expression studies, food quality and safety assessment. *J Sci Food Agric* 93: 1779–1787.

Carrie D, Gilmour KM (2016) Phosphorylation increases the catalytic activity of rainbow trout gill cytosolic carbonic anhydrase. *J Comp Physiol B* 186: 111–122.

Casillas E, Sundquist J, Ames WE (1982) Optimization of assay conditions for, and the selected tissue distribution of, alanine aminotransferase and aspartate aminotransferase of English sole, *Parophrys vetulus* Girard. *J Fish Biol* 21: 197–204.

Choe KP, O'Brien S, Evans DH, Toop T, Edwards SL (2004) Immunolocalization of Na^+/K^+-ATPase, carbonic anhydrase II, and vacuolar H^+-ATPase in the gills of freshwater adult lampreys,*Geotria australis*. *J Exp Zool* 301A: 654–665.

Cholette C, Gagnon A, Germain P (1970) Isosmotic adaptation in *Myxine glutinosa* L. I. Variations of some parameters and role of the amino acid pool of the muscle cells. *Comp Biochem Physiol* 33: 333–346.

Clemens BJ, Binder TR, Docker MF, Moser ML, Sower SA (2010) Similarities, differences, and unknowns in biology and management of three parasitic lampreys of North America. *Fisheries* 35: 580–594.

Close DA, Fitzpatrick MS, Li HW (2002) The ecological and cultural importance of a species at risk of extinction, Pacific lamprey. *Fisheries* 27: 19–25.

Close DA, Yun S-S, McCormick SD, Wildbill AJ, Li W (2010) 11-Deoxycortisol is a corticosteroid hormone in the lamprey. *Proc Natl Acad Sci* 107: 13942–13947.

Cochran PA (1984) The Foraging Behavior of Parasitic Lampreys. Doctoral thesis. University of Wisconsin, WI, USA.

Cochran PA, Lyons J (2010) Attachments by parasitic lampreys within the branchial cavities of their hosts. *Environ Biol Fishes* 88: 343–348.

Conley DM, Mallatt J (1988) ATPase and carbonic anhydrase activity in gills of 17 fish species. *Can J Zool* 66: 2398–2405.

Consoli L, Damerval C (2001) Quantification of individual zein isoforms resolved by two-dimensional electrophoresis: Genetic variability in 45 maize inbred lines. *Electrophoresis* 22: 2983–2989.

Cutler CP, Cramb G (2001) Molecular physiology of osmoregulation in eels and other teleosts: the role of transporter isoforms and gene duplication. *Comp Biochem Physiol A Mol Integr Physiol* 130: 551–564.

Cutler CP, Sanders IL, Luke G, Hazon N, Cramb G (1996) Ion transport in teleosts: identification and expression of ion transporting proteins in branchial and intestinal epithelia of the European eel (*Anguilla anguilla*). In: Ennion SJ, Goldspink G, eds. *Seminar Series-Society For Experimental Biology*. Cambridge University Press, Cambrige University, pp 43–74.

Davis R (1967) Parasitism by newly-transformed anadromous sea lampreys on landlocked salmon and other fishes in a coastal Maine lake. *Trans Am Fish Soc* 96: 11–16.

Dawson J (1905) The breathing and feeding mechanism of the lampreys. I. *Biol. Bull.* 9: 91–111.

Delarbre C, Gallut C, Barriel V, Janvier P, Gachelin G (2002) Complete mitochondrial DNA of the hagfish, *Eptatretus burgeri*: the comparative analysis of mitochondrial DNA sequences strongly supports the cyclostome monophyly. *Mol Phylogenet Evol* 22: 184–192.

Dimberg K, Höglund LB (1987) Carbonic anhydrase activity in the blood and the gills of rainbow trout during long-term hypercapnia in hard, bicarbonate-rich freshwater. *J Comp Physiol B* 157: 405–412.

Docker MF (2015) Lampreys: biology, conservation and control, Springer. Springer Netherlands, Dordrecht.

Dolinsky TJ, Nielsen JE, McCammon JA, Baker NA (2004) PDB2PQR: an automated pipeline for the setup of Poisson-Boltzmann electrostatics calculations. *Nucleic Acids Res* 32: W665–W667.

Dymowska AK, Schultz AG, Blair SD, Chamot D, Goss GG (2014) Acid-sensing ion channels are involved in epithelial Na^+ uptake in the rainbow trout *Oncorhynchus mykiss*. *Am J Physiol Cell Physiol* 307: C255–C265.

Edsall CC, Swink WD (2001) Effects of nonlethal sea lamprey attack on the blood chemistry of lake trout. *J Aquat Anim Health* 13: 51–55.

Edwards SL, Wall BP, Morrison-Shetlar A, Sligh S, Weakley JC, Claiborne JB (2005) The

effect of environmental hypercapnia and salinity on the expression of NHE-like isoforms in the gills of a euryhaline fish (*Fundulus heteroclitus*). *J Exp Zool Part A Comp Exp Biol* 303: 464–475.

Ehrenfeld J, Klein U (1997) The key role of the H^+ V-ATPase in acid-base balance and Na^+ transport processes in frog skin. *J Exp Biol* 200: 247–256.

Epstein F, Katz A, Pickford G (1967) Sodium-and potassium-activated adenosine triphosphatase of gills: role in adaptation of teleosts to salt water. *Science* 156: 1245–1247.

Ernst SA, Dodson WC, Karnaky KJ (1980) Structural diversity of occluding junctions in the low-resistance chloride-secreting opercular epithelium of seawater-adapted killifish (*Fundulus heteroclitus*). *J Cell Biol* 87: 488–497.

Esbaugh AJ (2005) Cytoplasmic carbonic anhydrase isozymes in rainbow trout *Oncorhynchus mykiss*: comparative physiology and molecular evolution. *J Exp Biol* 208: 1951–1961.

Esbaugh AJ, Gilmour KM, Perry SF (2009) Membrane-associated carbonic anhydrase in the respiratory system of the Pacific hagfish (*Eptatretus stoui*). *Respir Physiol Neurobiol* 166: 107–116.

Esbaugh AJ, Lund SG, Tufts BL (2004) Comparative physiology and molecular analysis of carbonic anhydrase from the red blood cells of teleost fish. *J Comp Physiol B* 174: 429–438.

Esbaugh AJ, Tufts BL (2006) Tribute to R. G. Boutilier: evidence of a high activity carbonic anhydrase isozyme in the red blood cells of an ancient vertebrate, the sea lamprey *Petromyzon marinus*. *J Exp Biol* 209: 1169–1178.

Evans DH (2008) Teleost fish osmoregulation: what have we learned since August Krogh, Homer Smith, and Ancel Keys. *AJP Regul Integr Comp Physiol* 295: R704–R713.

Evans DH, Piermarini PM, Choe KP (2005) The multifunctional fish gill : dominant site of gas exchange , osmoregulation , acid-base regulation , and excretion of nitrogenous waste. *Physiol Rev* 85: 97–177.

Evans DH, Piermarini PM, Potts WTW (1999) Ionic transport in the fish gill epithelium. *J Exp Zool* 283: 641–652.

Farmer GJ (1980) Biology and physiology of feeding in adult lampreys. *Can J Fish Aquat*

Sci 37: 1751–1761.

Farmer GJ, Beamish FWH (1973) Sea lamprey (*Petromyzon marinus*) predation on freshwater teleosts. *J Fish Res Board Canada* 30: 601–605.

Farmer GJ, Beamish FWH, Lett PF (1977) Influence of water temperature on the growth rate of the landlocked sea lamprey (*Petromyzon marinus*) and the associated rate of host mortality. *J Fish Res Board Canada* 34: 1373–1378.

Farmer GJ, Beamish FWH, Robinson GA (1975) Food consumption of the adult landlocked sea lamprey, *Petromyzon marinus*, L. *Comp Biochem Physiol Part A Physiol* 50: 753–757.

Faux MC, Scott JD (1996) More on target with proteinphosphorylation: conferring specificity by location. *Trends Biochem Sci* 21: 312–315.

Ferreira-Martins D, Coimbra J, Antunes C, Wilson JM (2016) Effects of salinity on upstream-migrating, spawning sea lamprey, *Petromyzon marinus*. *Conserv Physiol* 4: cov064.

Forey P, Janvier P (1993) Agnathans and the origin of jawed vertebrates. *Nature* 361: 129–134.

Foskett J, Scheffey C (1982) The chloride cell: definitive identification as the salt-secretory cell in teleosts. *Science* 215: 164–166.

Foskett JK, Machen TE (1985) Vibrating probe analysis of teleost opercular epithelium: Correlation between active transport and leak pathways of individual chloride cells. *J Membr Biol* 85: 25–35.

Fuentes J, Soengas JL, Rey P, Rebolledo E (1997) Progressive transfer to seawater enhances intestinal and branchial Na^+-K^+-ATPase activity in non-anadromous rainbow trout. *Aquac Int* 5: 217–227.

Fujikawa-Adachi K, Nishimori I, Taguchi T, Onishi S (1999) Human mitochondrial carbonic anhydrase VB: cDNA cloning, mRNA expression, subcallular localization, and mapping to chromosome X. *J Biol Chem* 274: 21228–21233.

Fulton T (1902) Rate of growth of seas fishes. 20th Annual Report of the Fishery Board of Scotland , Part 3, pp 326–446.

Gabriel U, Akinrotimi O, Ariweriokuma V (2012) Changes in metabolic enzymes activities

in selected organs and tissue of *Clarias cariepinus* exposed to cypermethrin. *J Environ Eng Technol* 1: 13–19.

Gage SH (1928) The lampreys of New York State: Life history and economics. In: *Biological Survey of the Oswego River System*. New York Conserv. Dept. Suppl. 17th Ann. Rep. 1927. pp 158–191.

Gaim K, Gebru G, Abba S (2015) The effect of arsenic on liver tissue of experimental animals (fishes and mice) - A Review article. *Int J Sci Res Publ* 5: 1–9.

Galloway T (1933) The osmotic pressure and saline content of the blood of *Petromyzon fluviatilis*. *J Exp Biol* 10: 313–316.

Garcia-Romeu F, Ehrenfeld J (1975) In vivo Na^+ and Cl minus independent transport across the skin of *Rana esculenta*. *Am J Physiol* 228: 839–844.

Gasteiger E., Hoogland C., Gattiker A., Duvaud S., Wilkins M.R., Appel R.D., Bairoch A (2005) Protein identification and analysis tools on the ExPASy server. In: Walker J, ed. *The Proteomics Protocols Handbook*. Humana Press, pp 571–607.

Georgalis T (2006) The role of branchial carbonic anhydrase in acid-base regulation in rainbow trout (*Oncorhynchus mykiss*). *J Exp Biol* 209: 518–530.

Georgopoulos C, Welch WJ (1993) Role of the major heat shock proteins as molecular chaperones. *Annu Rev Cell Biol* 9: 601–634.

Gilmour KM (2010) Perspectives on carbonic anhydrase. *Comp Biochem Physiol A Mol Integr Physiol* 157: 193–197.

Gilmour KM, Bayaa M, Kenney L, McNeill B, Perry SF (2007) Type IV carbonic anhydrase is present in the gills of spiny dogfish (*Squalus acanthias*). *Am J Physiol Regul Integr Comp Physiol* 292: R556–R567.

Gilmour KM, Perry SF (2009) Carbonic anhydrase and acid-base regulation in fish. *J Exp Biol* 212: 1647–1661.

Gschwind A, Fischer OM, Ullrich A (2004) Timeline: The discovery of receptor tyrosine kinases: targets for cancer therapy. *Nat Rev Cancer* 4: 361–370.

Halligan BD (2009) Phospho-Proteomics. Humana Press, Totowa, NJ, USA.

Halligan BD, Ruotti V, Jin W, Laffoon S, Twigger SN, Dratz EA (2004) ProMoST (protein modification screening tool): a web-based tool for mapping protein modifications on

two-dimensional gels. *Nucleic Acids Res* 32: W638–W644.

Hardisty MW (1979) Biology of the Cyclostomes. Chapman & Hall, London, pp 272–292.

Hardisty MW (1982) Lampreys and hagfishes: analysis of cyclostome relationships. In: Hardisty MWH and Potter IC, ed. *The Biology of Lampreys*. Academic Press, London, pp 165–259.

Hardisty MW (2006) Lampreys: Life without Jaws. Forrest Text. Ceredigion, pp. 272.

Hardisty MW, Potter IC (1971a) The Biology of Lampreys: Vol. 1. Academic Press, London.

Hardisty MW, Potter IC (1971b) The behaviour, ecology and growth of larval lampreys. In: *The Biology of Lampreys*. Academic Press, London, pp 85–125.

Hardisty MW, Potter IC (1971c) The general biology of adult lampreys. In: T*he Biology of Lampreys*. Academic Press, London, pp 127–206.

Hardisty MW, Potter IC, Hilliarda RW (1989) Physiological adaptations of the living agnathans. *Trans R Soc Edinb Earth Sci* 80: 241–254.

Hartl FU (1996) Molecular chaperones in cellular protein folding. *Nature* 381: 571–580.

Hartl FU (2002) Molecular Chaperones in the cytosol: from nascent chain to folded protein. *Science* 295: 1852–1858.

Harvey BJ (1988) Intracellular pH controls cell membrane Na^+ and K^+ conductances and transport in frog skin epithelium. *J Gen Physiol* 92: 767–791.

Harvey BJ, Ehrendeld J (1986) Regulation of intracellular sodium and pH by the electrogenic H^+ pump in frog skin. *Pflügers Arch Eur J Physiol* 406: 362–366.

Henry R, Tufts B, Boutilier R (1993) The distribution of carbonic anhydrase type I and II isozymes in lamprey and trout: possible co-evolution with erythrocyte chloride/bicarbonate exchange. *J Comp Physiol B* 163: 380–388.

Henry RP (1996) Multiple roles of carbonic anhydrase in cellular transport and metabolism. *Annu Rev Physiol* 58: 523–538.

Hewett-Emmett D (2000) Evolution and distribution of the carbonic anhydrase gene families. In: Chegwidden WR, Carter ND, Edwards YH, eds. *The Carbonic Anhydrases*. Birkhäuser Basel, Basel, pp 29–76.

Hewett-Emmett D, Tashian RE (1996) Functional diversity, conservation, and convergence in the evolution of the α-, β-, and γ-carbonic anhydrase gene families. *Mol Phylogenet Evol* 5: 50–77.

Hiroi J, McCormick SD (2007) Variation in salinity tolerance, gill Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter and mitochondria-rich cell distribution in three salmonids *Salvelinus namaycush*, *Salvelinus fontinalis* and *Salmo salar*. *J Exp Biol* 210: 1015–1024.

Hoar W (1988) The physiology of smolting salmonids. In: *Fish Physiology*, Vol. XIB. pp 275–343.

Holbrook CM (2015) Dynamics of sea lamprey, *Petromyzon marinus*, spawning migrations in large rivers, with application to population assessment and control in the Great Lakes. Doctoral thesis. Michigan State University, MI, USA.

Holmes WN, Donaldson EM (1969) The body compartments and distribution of electrolytes. In: Hoar WS, Randall DJ, eds. *Fish Physiology* Vol. I. Academic Press., New York, pp 1–89.

Huang C (2008) Molecular origin and variability of the Rh gene family: an overview of evolution, genetics and function. *Haematol Educ Educ Program Annu Congr Eur Hematol Assoc* 2: 149–157.

Huang C, Peng J (2005) Evolutionary conservation and diversification of Rh family genes and proteins. *Proc Natl Acad Sci* 102: 15512–15517.

Hunter T (1995) Protein kinases and phosphatases: The Yin and Yang of protein phosphorylation and signaling. *Cell* 80: 225–236.

Hwang P-P, Lee T-H, Lin L-Y (2011) Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. *AJP Regul Integr Comp Physiol* 301: R28–R47.

Ivanis G, Esbaugh AJ, Perry SF (2008) Branchial expression and localization of SLC9A2 and SLC9A3 sodium/hydrogen exchangers and their possible role in acid-base regulation in freshwater rainbow trout (*Oncorhynchus mykiss*). *J Exp Biol* 211: 2467–2477.

Janvier P (1999) Catching the first fish. *Nature* 402: 21–22.

Jorgensen JC, Kitchell JF (2005) Growth potential and host mortality of the parasitic phase of the sea lamprey (*Petromyzon marinus*) in Lake Superior. *Can J Fish Aquat*

Sci 62: 2343–2353.

Karnaky K, Degnan K, Zadunaisky J (1977) Chloride transport across isolated opercular epithelium of killifish: a membrane rich in chloride cells. *Science* 195: 203–205.

Karnaky KJ (1980) Ion-secreting epithelia: chloride cells in the head region of *Fundulus heteroclitus*. *Am J Physiol* 238: R185–R198.

Karnaky KJ (1986) Structure and function of the chloride cell of *Fundulus heteroclitus* and other teleosts. *Integr Comp Biol* 26: 209–224.

Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* 30: 772–780.

Kelso JRM, Gardner WM (2000) Emigration, upstream movement, and habitat use by sterile and fertile sea lampreys in three Lake Superior tributaries. *North Am J Fish Manag* 20: 144–153.

Keys A, Willmer EN (1932) “Chloride secreting cells” in the gills of fishes, with special reference to the common eel. *J Physiol* 76: 368–378.

Kiilerich P, Kristiansen K, Madsen SS (2007) Cortisol regulation of ion transporter mRNA in Atlantic salmon gill and the effect of salinity on the signaling pathway. *J Endocrinol* 194: 417–427.

Kitchell JF (1990) The scope for mortality caused by sea lamprey. *Trans Am Fish Soc* 119: 642–648.

Kott E (1971) Characteristics of pre-spawning American brook lampreys from Big Creek, Ontario. *Can F Nat* 85: 235–240.

Krogh A (1939) Osmotic regulation in aquatic animals. Cambridge University Press, London.

Kültz D, Bastrop R, Jürss K, Siebers D (1992) Mitochondria-rich (MR) cells and the activities of the and carbonic anhydrase in the gill and opercular epithelium of *Oreochromis mossambicus* adapted to various salinities. *Comp Biochem Physiol Part B Comp Biochem* 102: 293–301.

Kumari K, Ranjan N, Sinha RC (2011) Multiple biomarker response in the fish , *Labeo rohita* due to hexavalent chromium. In: *2011 2nd International Conference on Biotechnology and Food Science IPCBEE*. IACSIT Press, Singapore, pp 155–158.

Kuraku S, Hoshiyama D, Katoh K, Suga H, Miyata T (1999) Monophyly of lampreys and hagfishes supported by nuclear DNA–coded genes. *J Mol Evol* 49: 729–735.

Kustu S, Inwood W (2006) Biological gas channels for NH_3 and CO_2: evidence that Rh (Rhesus) proteins are CO_2 channels. *Transfus Clin Biol* 13: 103–110.

Laemmli UK (1970) Cleavage of Structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.

Lanfranchi G, Pallavicini A, Laveder P, Valle G (1994) Ancestral hemoglobin switching in lampreys. *Dev Biol* 164: 402–408.

Larinier M (2000) Dams and fish migration. World Commission on dams. *FAO Fish Tech Pap* 419: 45–89 Toulouse.

Larsen EH (1991) Chloride transport by high-resistance heterocellular epithelia. *Physiol Rev* 71: 235–283.

Larsen LO (1980) Physiology of adult lampreys, with special regard to natural starvation, reproduction, and death after spawning. *Can J Fish Aquat Sci* 37: 1762–1779.

Lehtonen J, Shen B, Vihinen M, Casini A, Scozzafava A, Supuran CT, Parkkila AK, Saarnio J, Kivel AJ, Waheed A, *et al.* (2004) Characterization of CA XIII, a novel member of the carbonic anhydrase isozyme family. *J Biol Chem* 279: 2719–2727.

Lennon RE (1954) Feeding mechanism of the sea lamprey and its effect on host fishes. *Fish Bull US Fish Wildl Serv* 56: 247-293.

Lewis JH, Ferguson EE (1966) Osmotic fragility of premammalian erythrocytes. *Comp Biochem Physiol* 18: 589–595.

Li X, Jayachandran S, Nguyen H-HT, Chan MK (2007) Structure of the *Nitrosomonas europaea* Rh protein. *Proc Natl Acad Sci* 104: 19279–19284.

Li Z, Lui EY, Wilson JM, Ip YK, Lin Q, Lam TJ, Lam SH (2014) Expression of key ion transporters in the gill and esophageal-gastrointestinal tract of euryhaline mozambique tilapia *Oreochromis mossambicus* acclimated to fresh water, seawater and hypersaline water. *PLoS One* 9: e87591.

Lin CH, Tsai RS, Lee TH (2004) Expression and distribution of Na, K-ATPase in gill and kidney of the spotted green pufferfish, *Tetraodon nigroviridis*, in response to salinity challenge. *Comp Biochem Physiol A Mol Integr Physiol* 138: 287–295.

Lin T-Y, Liao B-K, Horng J-L, Yan J-J, Hsiao C-D, Hwang P-P (2008) Carbonic anhydrase 2-like a and 15a are involved in acid-base regulation and Na^+ uptake in zebrafish H^+-ATPase-rich cells. *Am J Physiol Cell Physiol* 294: C1250–C1260.

Lindquist S, Craig EA (1988) The heat-shock proteins. *Annu Rev Genet* 22: 631–677.

Lionetto MG, Schettino T (2006) The Na^+-K^+-$2Cl^-$ cotransporter and the osmotic stress response in a model salt transport epithelium. *Acta Physiol* 187: 115–124.

Liu S-T, Tsung L, Horng J-L, Lin L-Y (2013) Proton-facilitated ammonia excretion by ionocytes of medaka (*Oryzias latipes*) acclimated to seawater. *AJP Regul Integr Comp Physiol* 305: R242–R251.

Loretz CA (1995) Electrophysiology of ion transport in teleost intestinal cells. In: Shuttleworth CMW and TJ, ed. *Cellular and Molecular Approaches to Fish Ionic Regulation*. Academic Press., Toronto, pp 25–56.

Lucas M, Bubb D, JANG M (2009) Availability of and access to critical habitats in regulated rivers: effects of low-head barriers on threatened lampreys. *Freshw Biol* 54: 621-634.

Lund SG, Dyment P, Gervais MR, Moyes CD, Tufts BL (2002) Characterization of erythrocyte carbonic anhydrase in an ancient fish, the longnose gar (*Lepisosteus osseus*). *J Comp Physiol B* 172: 467–476.

Lutz P (1975) Adaptive and evolutionary aspects of the ionic content of fishes. *Copeia* 2: 369-373.

Mallatt J, Sullivan J (1998) 28S and 18S rDNA sequences support the monophyly of lampreys and hagfishes. *Mol Biol Evol* 15: 1706–1718.

Malsure S, Wang Q, Charles R-P, Sergi C, Perrier R, Christensen BM, Maillard M, Rossier BC, Hummler E (2014) Colon-specific deletion of epithelial sodium channel causes sodium loss and aldosterone resistance. *J Am Soc Nephrol* 25: 1453–1464.

Maren TH, Friedlandl BR, Rittmaster RS (1980) Kinetic properties of primitive vertebrate carbonic anhydrases. *Comp Biochem Physiol Part B Comp Biochem* 67: 69–74.

Marino S, Hayakawa K, Hatada K, Benfatto M, Rizzello A, Maffia M, Bubacco L (2007) Structural features that govern enzymatic activity in carbonic anhydrase from a low-temperature adapted fish, *Chionodraco hamatus*. *Biophys J* 93: 2781–2790.

Marmulla G (2001) Dams, fish and fisheries opportunities, challenges and conflict resolution. *FAO Fish Tech Pap* 419: 1–166.

Marshall WS (2002) Na^+, Cl^-, Ca^{2+} and Zn^{2+} transport by fish gills: Retrospective review and prospective synthesis. *J Exp Zool* 293: 264–283.

Marshall WS, Bryson SE, Darling P, Whitten C, Patrick M, Wilkie M, Wood CM, Buckland-Nicks J (1997) NaCl transport and ultrastructure of opercular epithelium from a freshwater-adapted euryhaline teleost, *Fundulus heteroclitus*. *J Exp Zool* 277: 23–37.

Marshall WS, Grosell M (2005) Ion Osmoregulation , and acid – base balance. In: *The Physiology of Fishes*. pp 177–230.

Marshall WS, Grosell M (2006) Ion transport, osmoregulation, and acid-base balance. *Physiol fishes* 3: 177–230.

Marshall WS, Howard JA, Cozzi RRF, Lynch EM (2002a) NaCl and fluid secretion by the intestine of the teleost *Fundulus heteroclitus*: involvement of CFTR. *J Exp Biol* 205: 745–758.

Marshall WS, Lynch EM, Cozzi RRF (2002b) Redistribution of immunofluorescence of CFTR anion channel and NKCC cotransporter in chloride cells during adaptation of the killifish *Fundulus heteroclitus* to sea water. *J Exp Biol* 205: 1265–1273.

Mateus CS, Rodríguez-Muñoz R, Quintella BR, Alves MJ, Almeida PR (2012) Lampreys of the Iberian Peninsula: distribution, population status and conservation. *Endanger Species Res* 16: 183–198.

McCormick S (1995) Hormonal control of gill Na^+, K^+-ATPase and chloride cell function. In: Wood CM, Shuttleworth TJ, eds. *Fish Physiology*. Academic Press, San Diego, pp 285–315.

McCormick S, Saunders R (1987) Preparatory physiological adaptations for marine life of salmonids: osmoregulation, growth, and metabolism. In: *Am Fish Soc Symp*. pp 211–229.

McCormick SD (1993) Methods for nonlethal gill biopsy and measurement of Na^+, K^+ -ATPase activity. *Can J Fish Aquat Sci* 50: 656–658.

McCormick SD, Farrel AP, Brauner CJ, eds (2013) Fish Physiology: Euryhaline Fishes, Vol 32. Academic Press.

McCormick SD, Regish AM, Christensen AK (2009) Distinct freshwater and seawater isoforms of Na^+/K^+-ATPase in gill chloride cells of Atlantic salmon. *J Exp Biol* 212: 3994–4001.

McFarland WN, Munz FW (1965) Regulation of body weight and serum composition by hagfish in various media. *Comp Biochem Physiol* 14: 383–398.

Mehta TK, Ravi V, Yamasaki S, Lee AP, Lian MM, Tay B-H, Tohari S, Yanai S, Tay A, Brenner S, *et al.* (2013) Evidence for at least six Hox clusters in the Japanese lamprey (*Lethenteron japonicum*). *Proc Natl Acad Sci U S A* 110: 16044–16049.

Morimoto R, Tissières A, Georgopoulos C (1990) Stress proteins in biology and medicine. cold spring harbor laboratory. *Cold Spring Harbor Monograph Archive* 19: 1-36.

Morris R (1956) The osmoregulatory ability of the lampern (*Lampetra fluviatilis* L.) in sea water during the course of its spawning migration. *J Exp Biol* 33: 235–248.

Morris R (1957) Some aspects of the structure and cytology of the gills of *Lampetra fluviatilis*. *Q J Microsc Sci* 44: 473–485.

Morris R (1958) The mechanism of marine osmoregulation in the lampern (*Lampetra fluviatilis* L.) and the causes of its breakdown during the spawning migration. *J Exp Biol* 35: 649–665.

Morris R (1972) Osmoregulation. In: Potter IC, Hardisty WM, eds. *The Biology of Lampreys*, Vol. 2. Academic Press, London, pp 192–239.

Morris R (1980) Blood composition and osmoregulation in ammocoete larvae. *Can J Fish Aquat Sci* 37: 1665–1679.

Morris R, Pickering A (1976) Changes in the ultrastructure of the gills of the river lamprey, *Lampetra fluviatilis* (L.), during the anadromous spawning migration. *Cell Tissue Res* 173: 271–277.

Moser ML, Almeida PR, Kemp PS, Sorensen PW (2015) Lamprey spawning migration. In: Docker MF, ed. *Lampreys: Biology, Conservation and Control* vol.1. Springer Netherlands., pp 215–263.

Murray RL (1984) Aspartate aminotransferase. In: Kaplan LA, Pesce AJ, eds. *Clinical Chemistry. Theory, Analysis and Correlation*. CV Mosby Company, St. Louis, Toronto, Princeton, pp 1141–1145.

Nagel W, Dörge A (1996) The role of mitochondria-rich cells in sodium transport across amphibian skin. *Pflügers Arch Eur J Physiol* 433: 146–152.

Nah GSS, Tay B-H, Brenner S, Osato M, Venkatesh B (2014) Characterization of the Runx gene family in a jawless vertebrate, the Japanese lamprey (*Lethenteron japonicum*). *PLoS One* 9: e113445.

Nawata CM, Hung CCY, Tsui TKN, Wilson JM, Wright PA, Wood CM (2007) Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H^+-ATPase involvement. *Physiol Genomics* 31: 463–474.

Nawata CM, Wood CM (2008) The effects of CO_2 and external buffering on ammonia excretion and Rhesus glycoprotein mRNA expression in rainbow trout. *J Exp Biol* 211: 3226–3236.

Neuhoff V, Arold N, Taube D, Ehrhardt W (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* 9: 255–262.

Nichols OC, Tscherter UT (2011) Feeding of sea lampreys *Petromyzon marinus* on minke whales *Balaenoptera acutorostrata* in the St Lawrence Estuary, Canada. *J Fish Biol* 78: 338–343.

Noyes CJ, Caudill CC, Clabough TS, Joosten DC, Keefer ML (2013) Adult Pacific lamprey migration behavior and escapement in the Bonneville and Lower Columbia River monitored using the juvenile salmonid acoustic telemetry system (JSATS), 2012. Idaho.

Olsen Y, Falk K, Reite O (1992) Cortisol and lactate levels in atlantic salmon *Salmo salar* developing infectious anemia (isa). *Dis Aquat Organ* 14: 99–104.

Pakhira C, Nagesh TS, Abraham TJ, Dash G, Behera S (2015) Stress responses in rohu, *Labeo rohita* transported at different densities. *Aquac Reports* 2: 39–45.

Pancer Z, Amemiya CT, Ehrhardt GRA, Ceitlin J, Gartland GL, Cooper MD (2004) Somatic diversification of variable lymphocyte receptors in the agnathan sea lamprey. *Nature* 430: 174–180.

Pandey A, Mann M (2000) Proteomics to study genes and genomes. *Nature* 405: 837–846.

Parker PS, Lennon RE (1956) Biology of the sea lamprey in its parasitic phase. *US Fish Wildl Serv Kes Rep* 44: 32.

Peek WD, Youson JH (1979a) Transformation of the interlamellar epithelium of the gills of the anadromous sea lamprey, *Petromyzon marinus* L., during metamorphosis. *Can J Zool* 57: 1318–1332.

Peek WD, Youson JH (1979b) Ultrastructure of chloride cells in young adults of the anadromous sea lamprey, *Petromyzon marinus* L., in fresh water and during adaptation to sea water. *J Morphol* 160: 143–164.

Pelham HRB (1982) A regulatory upstream promoter element in the Drosophila Hsp 70 heat-shock gene. *Cell* 30: 517–528.

Pelis RM, Zydlewski J, McCormick SD (2001) Gill Na(+)-K(+)-2Cl(-) cotransporter abundance and location in Atlantic salmon: effects of seawater and smolting. *Am J Physiol Regul Integr Comp Physiol* 280: R1844–R1852.

Penczak T (1964) Sea lamprey, *Petromyzon marinus* L. caught in river Pilica, the left tributary of middle Vistula. *Przegl Zool* 8: 264–265.

Peng J, Huang CH (2006) Rh proteins vs Amt proteins: an organismal and phylogenetic perspective on CO_2 and NH_3 gas channels. *Transfus Clin Biol* 13: 85–94.

Perry SF (1997) the chloride cell:structure and function in the gills of freshwater fishes. *Annu Rev Physiol* 59: 325–347.

Perry SF, Gilmour KM (2006) Acid-base balance and CO_2 excretion in fish: unanswered questions and emerging models. *Respir Physiol Neurobiol* 154: 199–215.

Pickering AD, Morris R (1973) Localization of ion-transport in the intestine of the migrating river lamprey, *Lampetra fluviatilis* L. *J Exp Biol* 58: 165–176.

Pickering ADA, Morris R (1970) Osmoregulation of *Lampetra fluviatilis* L. and *Petromyzon marinus* (Cyclostomata) in hyperosmotic solutions. *J Exp Biol* 53: 231–243.

Potter IC (1980) The Petromyzoniformes with particular reference to paired species. *Can J Fish Aquat Sci* 37: 1595–1615.

Potter IC, Beamish FWH (2010) The freshwater biology of adult anadromous sea lampreys *Petromyzon marinus*. *J Zool* 181: 113–130.

Potter IC, Gill HS, Renaud CB, Haoucher D (2015) The taxonomy, phylogeny, and

distribution of lampreys. In: Docker MF, ed. *Lampreys: Biology, Conservation and Control*. Springer Netherlands, pp 35–74.

Putnam NH, Butts T, Ferrier DEK, Furlong RF, Hellsten U, Kawashima T, Robinson-Rechavi M, Shoguchi E, Terry A, Yu J-K, *et al.* (2008) The amphioxus genome and the evolution of the chordate karyotype. *Nature* 453: 1064–1071.

Qiu Y (2000) Lamprey Hemoglobin. Structural basis of the BOHR effect. *J Biol Chem* 275: 13517–13528.

Quintella BR (2006) Biology and conservation of the sea lamprey (*Petromyzon marinus* L.) in Portugal. Doctoral thesis. University of Lisbon, Portugal.

Randall D (2014) Hughes and Shelton: the fathers of fish respiration. *J Exp Biol* 217: 3191–3192.

Rankin CJ (2002) Drinking in hagfishes and lampreys. In: Hazon N, Flik G, eds. *Osmoregulation and Drinking in Vertebrates*. BIOS Scientific Publishers Ltd, pp 1–18.

Reinhardt UG, Binder T, McDonald DG (2009) Ability of adult sea lamprey to climb inclined surfaces. In: *Am Fish Soc Symp*. pp 000–000.

Reis-Santos P, McCormick SD, Wilson JM (2008) Ionoregulatory changes during metamorphosis and salinity exposure of juvenile sea lamprey (*Petromyzon marinus* L.). *J Exp Biol* 211: 978–988.

Renaud C (2011) Lampreys of the world. An annotated and illustrated catalogue of lamprey species known to date. FAO Species Catalogue for Fisheries Purposes No. 5. *FAO Fish Tech Paper*.

Renaud CB (1997) Conservation status of northern hemisphere lampreys (Petromyzontidae). *J Appl Ichthyol* 13: 143–148.

Reynolds TE (1931) Hydrostatics of the suctorial mouth of the lamprey. *University of California Publications in Zoology* 37:15–34.

Richards JG (2003) Na^+/K^+-ATPase -isoform switching in gills of rainbow trout (*Oncorhynchus mykiss*) during salinity transfer. *J Exp Biol* 206: 4475–4486.

Ricker WE (1975) Computation and interpretation of biological statistics of fish populations. *Bull Fish Res Board Can* 191: 382.

Righetti PG, Bossi A (1997) Isoelctric focusing in immobilized pH gradients: recent

analytical and preparative developments. *Anal Biochem* 247: 1–10.

Robertson JD (1957) The habitat of the early vertebrates. *Biol Rev* 32: 156–187.

Robertson JD (1974) Osmotic and ionic regulation in cyclostomes.. *Chem Zool 8:* 149–193.

Rohlfing K, Stuhlmann F, Docker MF, Burmester T (2016) Convergent evolution of hemoglobin switching in jawed and jawless vertebrates. *BMC Evol Biol* 16: 1–9.

Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. In: Misener S, Krawetz SA, eds. *Bioinformatics Methods and Protocols*. Humana Press, Totowa New Jersey, pp 365–386.

Rzhetsky A, Nei M (1992) A simple method for estimating and testing minumum-evolution trees. *Mol Biol Evol* 9: 945–967.

Santovito G, Marino SM, Sattin G, Cappellini R, Bubacco L, Beltramini M (2012) Cloning and characterization of cytoplasmic carbonic anhydrase from gills of four Antarctic fish: insights into the evolution of fish carbonic anhydrase and cold adaptation. *Polar Biol* 35: 1587–1600.

Sardet C, Pisam M, Maetz J (1979) The surface epithelium of teleostean fish gills. Cellular and junctional adaptations of the chloride cell in relation to salt adaptation. *J Cell Biol* 80: 96–117.

Schindler JE, Krabbenhoft DP (1998) The hyporheic zone as a source of dissolved organic carbon and carbon gases to a temperate forested stream. *Biogeochemistry* 43: 157–174.

Scott GR, Baker DW, Schulte PM, Wood CM (2008) Physiological and molecular mechanisms of osmoregulatory plasticity in killifish after seawater transfer. *J Exp Biol* 211: 2450–2459.

Scott WB, Crossman EJ (2012) Freshwater fishes of Canada. *Monogr Soc Res Child Dev* 77: 81–86.

Seidelin M, Madsen SS, Blenstrup H, Tipsmark CK (2000) Time-course changes in the expression of Na^+, K^+-ATPase in gills and pyloric caeca of brown trout (*Salmo trutta*) during acclimation to seawater. *Physiol Biochem Zool* 73: 446–453.

Shi R, Chiang VL (2005) Facile means for quantifying microRNA expression by real-time

PCR. *Biotechniques* 39: 519–525.

Shifman MI, Yumul RE, Laramore C, Selzer ME (2009) Expression of the repulsive guidance molecule RGM and its receptor neogenin after spinal cord injury in sea lamprey. *Exp Neurol* 217: 242–251.

Shu D, Luo H, Conway Morris S, Zhang X, Hu S, Chen L-Z, Han J, Zhu M, Li Y, Chen L-Z (1999) Lower cambrian vertebrates from South China. *Nature* 402: 42–46.

Shuttleworth TJ, Thompson J, Munger RS, Wood CM (2006) A critical analysis of carbonic anhydrase function, respiratory gas exchange, and the acid-base control of secretion in the rectal gland of *Squalus acanthias*. *J Exp Biol* 209: 4701–16.

Sidon EW, Youson JH (1983) Morphological changes in the liver of the sea lamprey, *Petromyzon marinus* L., during metamorphosis: I. atresia of the bile ducts. *J Morphol* 177: 109–124.

Silva S, Araújo MJ, Bao M, Mucientes G, Cobo F (2014) The haematophagous feeding stage of anadromous populations of sea lamprey *Petromyzon marinus*: low host selectivity and wide range of habitats. *Hydrobiologia* 734: 187–199.

Singer TD, Tucker SJ, Marshall WS, Higgins CF (1998) A divergent CFTR homologue: highly regulated salt transport in the euryhaline teleost *F. heteroclitus*. *Am J Physiol - Cell Physiol* 274: C715–C723.

SLIS (1980) Proceedings of the 1979 Sea lamprey international symposium (SLIS). In: *Can J Fish Aqua Sci*. pp 1585–2214.

Smith HW (1930) The absorption and excretion of water and salts by marine teleosts. *Am J Physiol Content* 93: 480–505.

Smith HW (1932) Water regulation and its evolution in the fishes. *Q Rev Biol* 7: 1–26.

Smith JJ, Keinath MC (2015) The sea lamprey meiotic map improves resolution of ancient vertebrate genome duplications. *Genome Res* 25: 1081–1090.

Smith JJ, Kuraku S, Holt C, Sauka-Spengler T, Jiang N, Campbell MS, Yandell MD, Manousaki T, Meyer A, Bloom OE, *et al.* (2013) Sequencing of the sea lamprey (*Petromyzon marinus*) genome provides insights into vertebrate evolution. *Nat Genet* 45: 415–421.

Stock D, Whitt G (1992) Evidence from 18S ribosomal RNA sequences that lampreys and

hagfishes form a natural group. *Science* 257: 787–789.

Sullivan G, Fryer J, Perry S (1995) Immunolocalization of proton pumps (H^+-ATPase) in pavement cells of rainbow trout gill. *J Exp Biol* 198: 2619–2629.

Summerfelt R, Smith L, Schreck C, Moyle P (1990) Anesthesia, surgery, and related techniques. In: Schreck CB, Moyle PB, eds, *Methods for fish biology*. *Am Fish Soc*, Bethesda, MD, pp 213–272

Sutton TM, Bowen SH (1994) Significance of organic detritus in the diet of larval lampreys in the Great Lakes basin. *Can J Fish Aquat Sci* 51: 2380–2387.

Swink WD (1993) Effect of water temperature on sea lamprey growth and lake trout survival. *Trans Am Fish Soc* 122: 1161–1166.

Swink WD (1995) Growth and survival of newly parasitic sea lampreys at representative winter temperatures. *Trans Am Fish Soc* 124: 380–386.

Swink WD (2003) Host selection and lethality of attacks by sea lampreys (*Petromyzon marinus*) in laboratory studies. *J Great Lakes Res* 29: 307–319.

Takeyasu K, Tamkun M, Renaud K, Fambrough D (1988) Ouabain-sensitive (Na^++K^+)-ATPase activity expressed in mouse L cells by transfection with DNA encoding the alpha-subunit of an avian sodium pump. *J Biol Chem* 263: 4347–4354.

Tashian RE (1992) Genetics of the mammalian carbonic anhydrases. *Adv Genet* 30: 321–356.

Taylor JR, Grosell M (2009) The intestinal response to feeding in seawater gulf toadfish, *Opsanus beta*, includes elevated base secretion and increased epithelial oxygen consumption. *J Exp Biol* 212: 3873–3881.

The Telegraph (2012) Traditional lamprey pie for the Queen has to come from Canada for the first time in centuries. *Telegr*.

Tipsmark CK, Madsen SS, Seidelin M, Christensen AS, Cutler CP, Cramb G (2002) Dynamics of Na(+),K(+),2Cl(-) cotransporter and Na(+),K(+)-ATPase expression in the branchial epithelium of brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*). *J Exp Zool* 293: 106–118.

Tufts BL, Boutilier RG (1989) The absence of rapid chloride/bicarbonate exchange in lamprey erythrocytes: implications for CO_2 transport and ion distributions between

plasma and erythrocytes in the blood of *Petromyzon marinus*. *J Exp Biol* 144: 565–576.

Tufts BL, Esbaugh A, Lund SG (2003) Comparative physiology and molecular evolution of carbonic anhydrase in the erythrocytes of early vertebrates. *Comp Biochem Physiol Part A Mol Integr Physiol* 136: 259–269.

Vedel NE, Korsgaard B, Jensen FB (1998) Isolated and combined exposure to ammonia and nitrite in rainbow trout (*Oncorhynchus mykiss*): effects on electrolyte status, blood respiratory properties and brain glutamine/glutamate concentrations. *Aquat Toxicol* 41: 325–342.

Villeval JL, Testa U, Vinci G, Tonthat H, Bettaieb A, Titeux M, Cramer P, Edelman L, Rochant H, Breton-Gorius J (1985) Carbonic anhydrase I is an early specific marker of normal human erythroid differentiation. *Blood* 66: 1162–1170.

Wang AM, Doyle M V, Mark DF (1989) Quantitation of mRNA by the polymerase chain reaction. *Proc Natl Acad Sci U S A* 86: 9717–21.

Weiller GF, Caraux G, Sylvester N (2004) The modal distribution of protein isoelectric points reflects amino acid properties rather than sequence evolution. *Proteomics* 4: 943–949.

Wilson JM (2011) Role of the gills | Morphology of branchial ionocytes. In: Farrell AP, ed. *Encyclopedia of Fish Physiology, First Edition*. Academic Press, pp 1381–1388.

Wilson JM, Laurent P (2002) Fish gill morphology: inside out. *J Exp Zool* 293: 192–213.

Wilson JM, Laurent P, Tufts BL, Benos DJ, Donowitz M, Vogl AW, Randall DJ (2000) NaCl uptake by the branchial epithelium in freshwater teleost fish: an immunological approach to ion-transport protein localization. *J Exp Biol* 203: 2279–2296.

Wilson JM, Leitão A, Gonçalves AF, Ferreira C, Reis-Santos P, Fonseca A-V, Silva JM, Antunes JC, Pereira-Wilson C, Coimbra J (2007a) Modulation of branchial ion transport protein expression by salinity in glass eels (*Anguilla anguilla* L.). *Mar Biol* 151: 1633–1645.

Wilson JM, Randall DJ, Donowitz M, Vogl AW, Ip AK (2000) Immunolocalization of ion-transport proteins to branchial epithelium mitochondria-rich cells in the mudskipper (*Periophthalmodon schlosseri*). *J Exp Biol* 203: 2297–2310.

Wilson JM, Reis-Santos P, Fonseca A-V, Antunes JC, Bouça PD, Coimbra J (2007b)

Seasonal changes in ionoregulatory variables of the glass eel *Anguilla anguilla* following estuarine entry: comparison with resident elvers. *J Fish Biol* 70: 1239–1253.

Wright PA, Randall DJ, Perry SF (1989) Fish gill water boundary layer: a site of linkage between carbon dioxide and ammonia excretion. *J Comp Physiol B* 158: 627–635.

Wright PA, Wood CM (2009) A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. *J Exp Biol* 212: 2303–2312.

Wu C (1995) Heat shock transcription factors: structure and regulation. *Annu Rev Cell Dev Biol* 11: 441–469.

Yamashita M, Yabu T, Ojima N (2010) Stress protein HSP70 in fish. *Aqua-BioScience Monogr* 3: 111–141.

Yap MR, Bowen SH (2003) Feeding by northern brook lamprey (*Ichthyomyzon fossor*) on sestonic biofilm fragments: habitat selection results in ingestion of a higher quality diet. *J Great Lakes Res* 29: 15–25.

Youson JH, Potter IC (1979) A description of the stages in the metamorphosis of the anadromous sea lamprey, *Petromyzon marinus* L. *Can J Zool* 57: 1808–1817.

Zadunaisky JA (1984) The chloride cell: the active transport of chloride and the paracellular pathways. In: Randall WSH and DJ, ed. *Fish Physiology*, Vol. 10B. Academic Press, Orlando, pp 130–176.

Zhou L, Cryan E V, D'Andrea MR, Belkowski S, Conway BR, Demarest KT (2003) Human cardiomyocytes express high level of Na^+/glucose cotransporter 1 (SGLT1). *J Cell Biochem* 90: 339–346.

Zhu K, Zhao J, Lubman DM, Miller FR, Barder TJ (2005) Protein pI shifts due to posttranslational modifications in the separation and characterization of proteins. *Anal Chem* 77: 2745–2755.

Zydlewski J, Mccormick SD (1997) The ontogeny of salinity tolerance in the American shad, *Alosa sapidissima. Can J Fish Aquat Sci* 54: 182–189.