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PHYSIOLOGY OF A BASAL VERTEBRATE, THE SEA LAMPREY (PETROMYZON MARINUS): OSMOREGULATION AND CORTICOSTEROID ACTION

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PHYSIOLOGY OF A BASAL VERTEBRATE, THE SEA LAMPREY (*PETROMYZON MARINUS*): OSMOREGULATION AND CORTICOSTEROID ACTION

A Dissertation Presented

by

CIARAN A. S. SHAUGHNESSY

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DEDICATION

To my best friend and partner in life.

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ABSTRACT

PHYSIOLOGY OF A BASAL VERTEBRATE, THE SEA LAMPREY (*PETROMYZON MARINUS*): OSMOREGULATION AND CORTICOSTEROID ACTION

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Lamprey represent the most basal living example of a vertebrate animal which regulates its internal fluid and ion homeostasis. This phylogenetic position among vertebrates makes lamprey an important model organism for understanding the basal state, and thus the evolution, of physiological systems such as the mechanisms of osmo- and ionoregulation and the endocrine factors controlling them. Sea lamprey (*Petromyzon marinus*) are an anadromous fish, migrating from freshwater (FW) to seawater (SW) as juveniles, then returning back upstream as mature adults to spawn. Surviving this transition from a solute-poor FW environment to a solute-concentrated SW environment requires many changes to osmo- and ionoregulatory mechanisms, which are driven, in part, by signaling from corticosteroid hormones. Much of this dissertation examines the molecular mechanisms for osmo- and ionoregulation in the lamprey gill and intestine and how corticosteroids control osmoregulatory processes in these tissues. Additionally, effect of temperature on osmoregulation, and the corticosteroid response to handling and thermal stressors are also examined. I report the results of experiments that were designed

to identify molecular and organismal processes driving physiological acclimation to FW or SW residence. Special attention was given to understanding the endogenous nature of sea lamprey physiology, particularly regarding the development of the ability to survive and osmoregulate in SW that occurs during the larvae-to-juvenile metamorphosis.

In Chapter 1, I offer an introduction to basic sea lamprey biology and physiology, including background on osmoregulation and corticosteroid action in fishes.

In Chapter 2, I functionally characterize a critical ion transporter in the gills of SW-acclimated fishes responsible for Cl^- secretion, the $Na^+/K^+/2Cl^-$ cotransporter (NKCC1). I demonstrate that (i) a membrane-bound NKCC1 is upregulated in the gills of sea lamprey during metamorphosis in preparation for SW migration; (ii) the timing of gill NKCC1 upregulation corresponds to the upregulation of another critical ion transporter and biomarker of ionoregulatory activity in fish gills, Na^+/K^+-ATPase (NKA), as well as the acquisition of SW tolerance; and (iii) pharmacological inhibition of NKCC1 markedly decreases SW tolerance in juvenile sea lamprey.

In Chapter 3, I describe branchiometric changes that occur during the sea lamprey metamorphosis and SW acclimation, such as an increase in the branchiosomatic index (percentage of total body weight contributed by the gills) and a redistribution of the gill pouch-specific contribution to total gill NKA activity.

In Chapters 4 and 5, I characterize 11-deoxycortisol as the endogenous corticosteroid hormone that controls osmo- and ionoregulation in the gills and intestine of sea lamprey. I show that (i) 11-deoxycortisol and its corticosteroid receptor (CR) are upregulated during metamorphosis and after exposure to SW; (ii) that the CR present in the gill and intestine are highly specific to binding by 11-deoxycortisol; and (iii) only 11-

deoxycortisol (and not deoxycorticosterone, the other endogenous corticosteroid in lamprey blood) can upregulate osmo- and ionoregulatory mechanisms and SW tolerance in metamorphosing sea lamprey.

In Chapter 6, I describe the glucose stimulating action of 11-deoxycortisol. I demonstrate that (i) lamprey responding to acute stress upregulate circulating 11-deoxycortisol and glucose in the blood plasma; (ii) only treatment with 11-deoxycortisol (and not deoxycorticosterone) increases plasma glucose; and (iii) among other tissues, the kidney uniquely expresses the transcriptional markers of steroidogenesis.

Finally, in Chapter 7, I investigate the osmoregulatory capacity of post-metamorphic juvenile sea lamprey throughout springtime water warming and assess thermal tolerance of juvenile sea lamprey. I report that (i) elevated levels of gill NKA activity and SW tolerance persist well into the spring; (ii) survival after SW entry in late-spring is affected by temperature; and (iii) that juvenile sea lamprey can tolerate temperatures above 30 °C but begin showing signs of thermal stress, such as upregulation of heat shock proteins, at temperatures below their critical thermal maximum.

Taken together, this dissertation contributes a substantial body of work regarding mechanisms of epithelial ion transport and corticosteroid action in the most basal vertebrate group. This dissertation describes that a single endogenous corticosteroid, 11-deoxycortisol, has a critical role in promoting the development of osmo- and ionoregulatory mechanisms during metamorphosis that confer survival in SW. This work will be of interest to scientists from a range of disciplines including physiology, endocrinology, evolutionary biology, developmental biology, and ecology, as well as conservationists and managers working with sea lamprey.

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CHAPTER 1

GENERAL INTRODUCTION

Biology of the sea lamprey (*Petromyzon marinus*)

Lampreys (Petromyzontiformes) are one of two extant members (along with the hagfishes, Myxiniformes) known as 'cyclostomes' of the phylogenetically ancient group of jawless vertebrates that make up the superclass Agnatha. Genomic analysis and the fossil record indicate that Petromyzontiformes radiated more than 350–550 million years ago (Gess et al., 2006; Smith et al., 2013). Unlike hagfish, which do not regulate their internal osmotic concentration, lamprey maintain an internal osmolality of approximately one-third that of seawater (termed "osmoregulating"), which is consistent with nearly every other vertebrate species (excluding only hagfish and elasmobranchs) (Parry, 1966). Thus, lamprey represent the most basal extant order of osmoregulating vertebrates and are thus an important species in which to study the evolution of osmoregulation and its endocrine control.

Sea lamprey (*Petromyzon marinus*, Linnaeus 1758) are anadromous, making a freshwater-to-seawater migration as juveniles to access more abundant food resources in the ocean before returning upstream years later as mature adults to spawn. The general sea lamprey life history is well-described (Beamish, 1980a). As larvae (also referred to as "ammocoetes"), sea lamprey are well-adapted to live burrowed in the soft substrate of river beds, filter-feeding on suspended organic matter. After 4 to 6 years of relatively slow growth in larval life, sea lamprey undergo a true metamorphosis beginning in late Spring and concluding in early Winter, when fully-metamorphosed juveniles emerge from streambeds. Juveniles spend 1 to 3 years in the ocean exhibiting rapid growth as a

consequence of their parasitic lifestyle attaching to hosts to acquire blood meals. Once reproductively mature, adults migrate back upstream, following pheromone signals from larvae, to spawn and die in streams (Fine et al., 2004).

The larvae-to-juvenile metamorphosis is characterized by dramatic morphological changes to promote parasitism and survival in seawater (Beamish and Potter, 1972; O'Boyle and Beamish, 1977; Reis-Santos et al., 2008; Richards and Beamish, 1981; Youson, 1979; Youson, 1980). The major morphological changes during metamorphosis include a silvering in color, the appearance and development of an eye, and the restructuring of the mouth from a toothless oral hood for filter feeding to an oral disc with many teeth for attaching to and parasitizing hosts. These outward morphological changes which occur gradually over the months-long transformation are well-described and are the basis for the a widely-used classification of the metamorphosis into seven stages from larvae to juvenile (see Youson, 1979). Although the hallmark morphological changes during the lamprey metamorphosis occur primarily in the early stages (1 to 4), it appears that the most meaningful physiological changes occur in the later stages (5 to 7), including the development of salinity tolerance (Reis-Santos et al., 2008; Richards and Beamish, 1981). The physiological changes (osmoregulatory and endocrine) associated with this metamorphosis are far less represented in the literature than the morphological changes, and as such, will be a central topic in this dissertation.

Ion and water balance in fishes

Freshwater (~10 mOsm L^{-1}; <1 ppt; FW) and seawater (~1000 mOsm L^{-1}; ~35 ppt; SW) have starkly different osmotic concentrations, and so maintaining a constant internal osmolality of approximately 300 mOsm L^{-1} during migration from FW to SW

requires sea lamprey and all other anadromous fishes to modify osmoregulatory mechanisms in the gill and other organs from ion uptake in FW to ion secretion in SW.

Hyper-osmotic to their dilute environment, fishes in FW passively gain water and lose ions. As such, fishes in FW have low drinking and high glomerular filtration rates (Bath and Eddy, 1979; Jobling, 1994) and actively uptake ions (Na^+ and Cl^-) across the gill epithelium and reabsorb ions in the distal tubules of the kidney (Parry, 1966). Although cellular mechanisms of branchial uptake of Na^+ and Cl^- differ across species, the general pattern is that Na^+ crosses the gill epithelium into the blood via an apical Na^+ channel and the basolateral Na^+/K^+-ATPase (NKA), and Cl^- is absorbed via an apical Cl^-/HCO_3^- anion exchanger (AE1) and a basolateral Cl^- channel (Marshall and Grosell, 2006). NKA is present in nearly every cell type and is has a critical role in maintaining cell volume and a resting membrane potential of -30 to -70 mV (negative inside the cell) by actively transporting Na^+ out of the cell and K^+ into the cell at a ratio of 3:2 for each ATP it hydrolyses (Glynn, 2002). In tetrapods, the epithelial Na^+ channel (ENaC) is a well-described apical pathway for Na^+ retention in the kidney nephron (Rossier et al., 2015), but it is generally accepted that a non-ENaC apical Na^+ pathway, such as a Na^+/H^+-exchanger (NHE), Na^+/Cl^--cotransporter (NCC), or an acid-sensing ion channel (ASIC), is responsible for Na^+ uptake across the gill epithelium in FW fishes (Choe et al., 2005; Dymowska et al., 2014; Evans et al., 2005). Interestingly, however, recent molecular evidence suggests the ENaC may be the pathway for apical Na^+ uptake in lamprey (Ferreira-Martins et al., 2016).

As downstream-migrating fishes move from FW into SW, the passive movement of ions and water reverses—fishes in SW passively lose water and gain ions from the

solute-concentrated marine environment. In SW, fishes increase drinking rates and reduce glomerular filtration rate, releasing small volumes of isosmotic urine, and the gill epithelium transforms from a site of active ion uptake to active ion secretion (Parry, 1966). The cellular mechanism for Na^+ and Cl^- secretion is relatively well-conserved across species, and is also heavily reliant on the electrogenic gradient produced by the basolateral NKA (Silva et al., 1977). Generally, the paracellular secretion of Na^+ through 'leaky' junctions between epithelial cells is regulated by a family of proteins called tight junction proteins (Chasiotis et al., 2012; Fromter and Diamond, 1972), and the transcellular secretion of Cl^- is accomplished via the basolateral $Na^+/K^+/2Cl^-$-cotransporter member 1 (NKCC1) and an apical Cl^- channel, the cystic fibrosis transmembrane conductance regulator (CFTR) (Marshall and Grosell, 2006). The NKCC1 is responsible for the electrogenically neutral movement of Na^+, K^+, and Cl^- across the basolateral membrane with a $1Na^+:1K^+:2Cl^-$ stoichiometry, and the only evidence for a role of NKCC1 in osmoregulation in lamprey has been the transcriptional upregulation of the *slc12a2* gene (codes for NKCC1) in the gills of upstream migrating adult sea lamprey that were re-exposed to SW (Ferreira-Martins et al., 2016). The sequence of the *slc12a2* gene in the lamprey has not been described and no evidence exists on the functional importance of the NKCC1 protein in hypo-osmoregulation in sea lamprey. Thus, characterizing the role of NKCC1 in the sea lamprey will be a major chapter in my dissertation.

Corticosteroid function in sea lamprey

The terms 'mineralocorticoid' and 'glucocorticoid' have been used for many decades to describe the predominant action of various corticosteroids produced in the

mammalian adrenal gland. This distinction applies well to mammals, in which the primary mineralocorticoid, aldosterone, regulates electrolyte and water balance by controlling epithelial sodium transport, and the primary glucocorticoid, cortisol, is distinguished by its role in glucose metabolism. In mammals, these hormones function by activating mineralocorticoid (MR) and glucocorticoid (GR) receptors, respectively. In fishes, however, the distinction between 'mineralocorticoid' and 'glucocorticoid' is less applicable. In teleosts, aldosterone is present only at levels lower than are likely to be physiologically meaningful (Prunet et al., 2006). Accordingly, it is widely held that a single corticosteroid, cortisol, carries out both mineralocorticoid- and glucocorticoid-like actions in teleost fishes (Mommsen et al., 1999), mediated predominately through GRs (McCormick et al., 2008; Shrimpton and McCormick, 1999).

Evidence for the dual role of cortisol as a mineralocorticoid and glucocorticoid is abundant. Many studies in fishes have shown that cortisol increases metabolic demand and stimulates gluconeogenesis and activity of gluconeogenic enzymes (Mommsen et al., 1999). In addition to its more conserved role as a glucocorticoid, cortisol, along with growth hormone (GH) and insulin-like growth factor 1 (IGF-1), acts as a 'mineralocorticoid' in teleost and other gnathostome fishes by promoting osmoregulation in SW and the differentiation of ionocytes in the gill (McCormick, 2001).

Corticosteroid function in the most basal vertebrates, the cyclostomes, is not as well-studied. Independent sequence analyses agree that the MRs and GRs in more derived vertebrates descended from a common corticosteroid receptor (CR) found in lamprey and hagfish, but activation of the CR by its corticosteroid ligand(s) is disputed. The two reports central to this dispute are by Close et al. (2010) and Bridgham et al.

(2006). Neither study found appreciable levels of aldosterone or cortisol in lamprey serum, but report the presence of deoxycorticosterone and 11-deoxycortisol, precursors to aldosterone and cortisol, respectively. Accordingly, lamprey appear to lack the enzyme 11β-hydroxylase, which is present in the zona glomerulosa and zona fasciculata of the adrenal and interrenal glands in later evolved vertebrates and is responsible for facilitating the addition of a hydroxyl group at the 11C position of deoxycorticosterone and 11-deoxycortisol to produce aldosterone and cortisol, respectively. The central difference in the reports by Close et al. (2010) and Bridgham et al. (2006) is regarding specificity of the lamprey CR to deoxycorticosterone and 11-deoxycortisol. Using a technique of expressing a resurrected ancestral CR (AnCR) ligand binding domain in a mammalian cell line, Bridgham et al. (2006) reported a "promiscuous" CR that was transcriptionally activated by several corticosteroids, including corticosterone, deoxycorticosterone, 11-deoxycortisol, cortisol, and aldosterone, concluding that the AnCR was "structurally preadapted" for activation by cortisol and aldosterone, even when these hormones had not yet evolved. In contradiction to this finding, Close et al. (2010), using a different approach of isolating the cytosolic fraction of lamprey gill homogenate and using a competition binding assay, reported highly-specific binding of 11-deoxycortisol to the lamprey CR. To reconcile these differences, it has been suggested by Rossier et al. (2015) that phosphorylation or some other instance of post-translational modification or chaperoning of the CR or perhaps CR-chromatin interactions are different between the lamprey gill and the mammalian cell line used by Bridgham et al. (2006), leading to the differences in specificity as reported.

In teleosts and other ganathasome fishes, the role of corticosteroids and corticosteroid receptors in osmoregulation is relatively well-studied, but evidence for the physiological action of corticosteroids in agnathans is limited. In Atlantic salmon (Salmo salar) cortisol treatment increases gill NKA activity and SW tolerance (relatively lower plasma ion concentration after SW exposure) (Bisbal and Specker, 1991; McCormick et al., 2008; Specker et al., 1994; Veillette et al., 1995), and the osmoregulatory action of cortisol has been shown to be mediated through the glucocorticoid receptor (McCormick et al., 2008; Shrimpton and McCormick, 1999; Weisbart et al., 1987). Only a single study has shown an osmoregulatory role of a corticosteroid in an agnathan (Close et al., 2010), which described only an upregulation of gill NKA activity in adult lamprey administered 11-deoxycortisol-treated, but did not assess 11-deoxycortisol effects on SW tolerance and other related ionoregulatory mechanisms, nor did it address the role of the CR in mediating the osmoregulatory action of 11-deoxycortisol. Clearly, more studies are needed to address physiological questions pertaining to corticosteroid function in basal vertebrates. A central topic in my dissertation will be addressing the call that has been made (Rossier et al., 2015) for more *in vivo* studies in lamprey to provide a more complete literature on physiological action, particularly the osmoregulatory role, of corticosteroids and the CR in basal vertebrates.

CHAPTER 2

FUNCTIONAL CHARACTERIZATION AND OSMOREGULATORY ROLE OF THE $Na^+/K^+/2Cl^-$ COTRANSPORTER (NKCC1) IN THE GILL OF SEA LAMPREY (*PETROMYZON MARINUS*), A BASAL VERTEBRATE*

Authors

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Abstract

The present study provides molecular and functional characterization of $Na^+/K^+/2Cl^-$ cotransporter (NKCC1/Slc12a2) in the gills of sea lamprey (*Petromyzon marinus*), the most basal extant vertebrate with an osmoregulatory strategy. We report the full-length peptide sequence for the lamprey NKCC1, which we show to groups strongly with and occupies a basal position among other vertebrate NKCC1 sequences. In post-metamorphic juvenile lamprey, *nkcc1* mRNA was present in many tissues but was 5-fold higher in the gill than any other examined tissue and NKCC1 protein was only detected in the gill. Gill mRNA and protein abundances of NKCC1 and Na^+/K^+-ATPase (NKA/Atp1a1) were significantly upregulated (20- to 200-fold) during late

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metamorphosis in fresh water, coinciding with the development of salinity tolerance, and were upregulated an additional 2-fold after acclimation to seawater (SW). Immunohistochemistry revealed that NKCC1 in the gill is found in filamental ionocytes co-expressing NKA which develop during metamorphosis in preparation for SW entry. Lamprey treated with bumetanide, a widely used pharmacological inhibitor of NKCC1, exhibited higher plasma Cl^- and osmolality and reduced muscle water content after 24 h in SW; there were no effects of bumetanide in freshwater-acclimated lamprey. This work provides the first functional characterization of NKCC1 as a mechanism for branchial salt secretion in lampreys, providing evidence that this mode of Cl^- secretion has been present among vertebrates for ~550 million years.

Introduction

The $Na^+/K^+/2Cl^-$ cotransporter 1 (NKCC1/Slc12a2) belongs to the SLC12A family of electroneutral cation-chloride cotransporters that includes two NKCC isoforms (NKCC1 and NKCC2/Slc12a1), Na^+-Cl^- cotransporter (NCC/Slc12a2), and K^+-Cl^- cotransporters (KCC[1-3]/Slc12a[4-6]). NKCCs are responsible for the inward movements of Na^+, K^+, and Cl^- across the cell membrane with a $1Na^+$:$1K^+$:$2Cl^-$ stoichiometry and are involved in a wide variety of physiological processes including electrolyte and fluid homeostasis, regulation of blood pressure, pain perception, and neuronal signaling (Haas and Forbush III, 1998; Lambert et al., 2008; Russell, 2000). Although the two isoforms of NKCC have high sequence similarity, they are easily distinguished by their distribution throughout the body and roles in ion transport. The basolateral NKCC1 is distributed throughout the vertebrate body contributing to cell volume regulation and is highly expressed in ion-secreting epithelia and some other non-

epithelial tissues such as immature neurons (Haas and Forbush III, 1998; Lambert et al., 2008; Russell, 2000). The absorptive, apically located NKCC2 is expressed in the kidney and intestine of mammals and fishes (Cutler and Cramb, 2002; Esbaugh and Cutler, 2016; Lytle et al., 1995; Payne and Forbush, 1995; Xue et al., 2009). The importance and relevance of NKCC1 is highlighted by extensive pharmacological and genetic work targeting NKCC1 with multiple loop diuretics (primarily bumetanide and furosemide) (Shankar and Brater, 2003) and links between NKCC1 dysfunction and hypertension and neurological disorders such as epilepsy (Kahle et al., 2008). Early work that characterized NKCC in mammalian cells first described a coupled transport of Na^+ and K^+ (Wiley and Cooper, 1974) then the coordinated cotransport of Cl^- (Geck et al., 1980). A cDNA encoding NKCC1 was first cloned and its ionoregulatory role in the shark rectal gland was described (Lytle et al., 1992; Xu et al., 1994). Since these early works, NKCC1 has been shown to be a conserved basolateral pathway in epithelial Cl^- secretion, appearing within Cl^- secreting cell types of the mammalian lung and colon, shark rectal gland, reptilian/avian salt gland, and the fish gill (Haas and Forbush III, 2000).

Lampreys (Petromyzontiformes) are one of two extant members (along with the hagfishes, Myxiniformes) of the phylogenetically ancient group of jawless vertebrates known as cyclostomes that make up the superclass Agnatha. Genomic analysis indicates that Petromyzontiformes may have radiated more than 550 million years ago (Smith et al., 2013). Unlike hagfish, which do not regulate their internal osmotic concentration, lampreys maintain an internal osmolality of approximately one-third that of seawater (SW), an osmoregulatory strategy shared among nearly every other vertebrate species (Bradley, 2009). Thus, lampreys represent the most basal extant order of osmoregulating

vertebrates and are therefore an important group for understanding the evolution of osmoregulatory mechanisms.

Sea lamprey (*Petromyzon marinus*, Linnaeus 1758) are anadromous, making a freshwater (FW)-to-SW migration as juveniles to access more abundant food resources in the ocean before returning upstream years later as mature adults to spawn. The general life history of anadromous lampreys is well-described (Beamish, 1980a; Potter and Beamish, 1977; Youson, 2003), including a FW-resident, filter-feeding, larval stage and a true metamorphosis to a juvenile stage, which is characterized into seven intermediary stages between larvae and post-metamorphic juveniles primarily based on development of the eye and parasitic mouth structure (Youson, 1979; Youson, 1980). Physiological changes that accompany metamorphosis include an increase in gill Na^+/K^+-ATPase (NKA/Atp1a1) activity and protein abundance (Reis-Santos et al., 2008), an increase in abundance of ionocytes and the development of SW tolerance (Beamish, 1980b; Richards and Beamish, 1981). It is not until after metamorphosis is complete and SW tolerance has developed that juvenile anadromous lampreys migrate to the sea.

To maintain internal osmotic and ionic homeostasis, jawless and bony fishes entering SW must absorb water and secrete ions against steep gradients between their internal fluids and the more-concentrated marine environment. This is accomplished by a coordinated process of drinking SW, actively desalinating and absorbing water across gastointestinal epithelia, and secreting ions (primarily Na^+ and Cl^-) across the branchial epithelium (Marshall and Grosell, 2006). The cellular mechanism for epithelial Na^+ and Cl^- secretion in teleosts, elasmobranchs, and tetrapods is highly conserved and primarily involves three membrane-bound ion transporters: NKA, NKCC1, and a cystic fibrosis

transmembrane conductance regulator (Abcc7/CFTR) chloride channel. Activity of NKA and the presence of an inward-rectifying K^+ channel on the basolateral membrane of Cl^--secreting, mitochondrion-rich ionocytes (classically referred to as "chloride cells") are responsible for producing (i) the sodium gradient that drives Na^+-coupled transport of Cl^- from the extracellular compartment into the cell via the basolateral NKCC1, as well as (ii) the inside-negative resting membrane potential that facilitates secretion of Cl^- out of the cell through the apical CFTR. In fish, upregulation of NKA, NKCC1, and CFTR in the gill is a general response to elevated salinity. Some anadromous fishes, such as Atlantic salmon (*Salmo salar*), upregulate gill NKA, NKCC1, and CFTR in anticipation of the hypo-osmoregulatory requirements of SW before they ever experience an increase in salinity (McCormick, 2013; McCormick and Saunders, 1987).

The mechanism for branchial Cl^- section among the most basal osmoregulating fishes, sturgeons and lampreys, is not well described. It has been shown in sturgeon that gill NKCC1 and NKA may have a role in hypo-osmoregulation in SW (Sardella and Kültz, 2009; Shaughnessy et al., 2015). In sea lamprey, it has been demonstrated that a basolateral NKA is important in branchial ion transport in FW and SW (Ferreira-Martins et al., 2016; Reis-Santos et al., 2008) and that gill *nkcc1* mRNA abundance in upstream-migrating adults was observed to increase after transfer from FW back to SW (Ferreira-Martins et al., 2016). No work has been done to link the development of hypo-osmoregulatory ability to specific molecular mechanisms for Cl^- secretion (i.e., basolateral NKCC1 or apical CFTR) in the basal lampreys and no protein- or organism-level evidence exists demonstrating a functional role of NKCC1 in ion regulation or SW tolerance in lamprey. Here, we present a functional characterization of the role and

regulation of NKCC1 in the Cl^--secreting ionocytes of the sea lamprey, an extant representative of the most basal vertebrate clade.

Materials and methods

Experimentation and tissue collection

The experiments in this study were performed in accordance with protocols approved by the Institutional Animal Care and Use Committees at the University of Massachusetts (Protocol number: 2016-0009). Sea lamprey used in this study were captured from the Connecticut River watershed in Massachusetts, USA and all experiments were performed at the S.O. Conte Anadromous Fish Research Center (Leetown Science Center, U.S. Geological Survey) in Turners Falls, MA. Lamprey held in the laboratory were kept at ambient temperature under a natural photoperiod in 1.5-m diameter flow-through tanks with 10-cm deep sandy substrate for burrowing and supplied with aerated, filtered, and UV-treated Connecticut River water. All laboratory experimentation was carried out at 15 °C under a simulated natural photoperiod in 60-L recirculating aquarium tanks supplied with dechlorinated municipal water and equipped with mechanical, biological, and chemical filtration. Artificial SW (35 ‰) was made by dissolving an artificial sea salt mix (Crystal Sea Salt, Baltimore, MD) in dechlorinated municipal water.

The salinity tolerance of larvae and various stages throughout metamorphosis was tested by gradually (in the case of larvae) or directly exposing lamprey to elevated salinity and assessing survival. Tissue profile samples were collected from post-metamorphic juveniles acclimated to FW. Gill tissue for the metamorphic profile was

obtained from lamprey immediately after capture via electrofishing (larvae–Stage 7) or Fyke netting (downstream-running 'migrants') in the Sawmill River (Montague, MA) from July to November. Gill tissue for the larval FW-SW comparison was collected from larvae and post-metamorphic juveniles in FW or acclimated to SW for 3 weeks. Pharmacological inhibition experiments were conducted with post-metamorphic juveniles acclimated to FW. Lamprey were anesthetized in a non-lethal dose of MS-222 (100 mg L^{-1} buffered with $NaHCO_3$, pH 7.0) and administered via intraperitoneal injection using a 25-gauge needle a 20 μL g^{-1} volume of sterile-filtered saline (0.9% NaCl) containing one of two doses of bumetanide (1.0 or 0.1 μmol g^{-1}) or a vehicle control (VEH). Lamprey recovered within minutes after anesthetization and were allowed an additional 30–60 min recovery in FW after injection, then placed in 35‰ SW (or a FW control) and sampled for blood and tissue 24 h later.

For all field and laboratory tissue collection, lamprey were euthanized using a lethal dose of MS-222 (200 mg L^{-1}). Blood was collected into capillary tubes from the caudal vasculature and plasma was separated via centrifugation. Plasma and tissues were immediately collected, then frozen and stored at -80 °C. Plasma Cl^- was measured using a digital chloridometer (Haake Buchler Instruments Inc., Saddlebrook, NJ) and plasma osmolality was measured using a vapor pressure osmometer (Wescor, Logan, UT). For muscle water analysis, 0.1–0.2 g of white muscle was blotted dry and weighed (wet mass) then placed in a drying oven for 48 h at 60 °C until dehydrated to a stable weight (dry mass).

Sequence assembly and phylogenetic analysis of lamprey *nkcc1*

A partial sequence (one-third of the total length) of the sea lamprey nkcc1 gene containing the 3' end was identified in the sea lamprey genome assembly (Smith et al., 2018). To obtain a full-length sequence of the gene, rapid amplification of cDNA ends (RACE) was performed. Total RNA (1 μg) was isolated from the gills of three post-metamorphic juveniles and reverse transcribed using the SMARTer RACE cDNA Amplification Kit (Takara Bio USA Inc., Palo Alto, CA) following the manufacturer's protocol. Polymerase chain reaction (PCR) products between an antisense gene-specific primer (5'- CGC TCA CGA GTA GAA CGT CA-3') and a proprietary universal primer were confirmed electrophoretically on a 1% agarose gel, then extracted and purified using the UltraClean GelSpin DNA Extraction Kit (MoBio, Solana Beach, CA), then commercially sequenced (GENEWIZ, South Plainfield, NJ). A complete cDNA sequence including 5' and 3' untranslated regions was assembled by appending RACE-obtained 5' nkcc1 sequences with the partial 5' and 3' sequences available in the sea lamprey genome assembly and verified by sequencing products of long-range PCR reactions (KAPA Long Range DNA Polymerase, Kapa Biosystems, Wilmington, MA) spanning between primers at the 5' end (5'-ACA GAG GAA CGG GAG AAG CG-3') and 3' end (5'-CGC TCA CGA GTA GAA CGT CA-3').

An amino acid sequence for the sea lamprey NKCC1 gene was deduced from the obtained nkcc1 cDNA sequence and its molecular mass was predicted using the Translate and TrEMBL tools, respectively, provided by the ExPASy bioinformatics resource portal (https://www.expasy.org). Hypothetical membrane topology and potential N-linked glycosylation sites were predicted using the TMHMM and NetNGlyc tools, respectively,

provided by DTU Bioinformatics server (http://www.bioinformatics.dtu.dk). Phylogenetic analysis was carried out on a balanced selection of vertebrate NKCC1, NKCC2, and NCC (NCC1) peptides available from NCBI GenBank using a ClustalW alignment (http://www.ebi.ac.uk/clustalw) and the Neighbor-Joining method (1,000 bootstrap replicates) implemented by MEGA7 software (Kumar et al., 2016). Accession numbers for the peptides used in the phylogenetic analysis were: Ghostshark NKCC1 (XP_007890727), Ghostshark NCC (XP_007905777), Salmon NKCC1 (XP_020313370), Salmon NCC (XP_020311909), Zebrafish NKCC1 (NP_001002080), Zebrafish NKCC2 (XP_009291678), Zebrafish NCC (NP_001038545), Frog NKCC1 (XP_004910530), Frog NKCC2 (XP_012814934), Frog NCC (XP_002937217), Chicken NKCC1 (XP_003643107), Chicken NKCC2 (XP_004943856), Chicken NCC (XP_015134720), Mouse NKCC1 (NP_033220), Mouse NKCC2 (NP_001073158), Mouse NCC (NP_001192240), Human NKCC1 (NP_001243390), Human NKCC2 (NP_000329), Human NCC (NP_000330), Human KCC1 (AAH21193.1).

Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was isolated from frozen tissue using TRIzol reagent (Molecular Research Center Inc., Cincinnati, OH) following the manufacturer's protocol. Total RNA concentration and purity of each sample was determined spectrophotometrically using a Take3 micro-volume plate (BioTek Instruments, Inc., Winooski, VT) and RNA integrity was assessed using gel electrophoresis. Only high-purity ($1.9 < A260/A280 > 2.2$) and high-integrity RNA samples were used for cDNA synthesis. First-strand cDNA synthesis was accomplished using the High-Capacity Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) following the manufacturer's protocol. Quantitative real-time

PCR (qPCR) was carried out in 10 µL reactions containing 2 ng cDNA template, 200 nM forward and reverse primers, and SYBRSelect master mix (ThermoFisher, Waltham, MA). Primer pairs were identical to those already reported for nkcc1 and nka (Ferreira-Martins et al., 2016) and ef1a (Kolosov et al., 2017). Four potential reference genes were analyzed: gapdh, 18s, β-actin, and ef1α. The reference gene ef1α was determined to be stable (± 1 cycle threshold) across all metamorphic and life stages, tissues, and salinities for the analyses presented. All qPCR reactions were performed in optical 96-well reaction plates in a StepOnePlus Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA) using the following thermal profile: holding at 50 °C (2 min) then activation at 95 °C (2 min); cycling (40 cycles) from 95 °C (15 s) to 60 °C (1 min) to 72 °C (30 s). A single product was confirmed for every reaction via a dissociation step (melt curve analysis) increasing from 60 to 95 °C after the cycling protocol, and for each primer pair electrophoretically.

Immunoblotting

Gill tissue was homogenized in SEID buffer (150 mmol L^{-1} sucrose, 10 mmol L^{-1} EDTA, 50 mmol L^{-1} imidazole, and 0.1% sodium deoxycholate, pH 7.3) and centrifuged at 2,000 g for 5 min and the supernatant was used for protein abundance quantification by Western blotting. The supernatant was analyzed for protein content using the Pierce BCA Protein Assay (Thermo Scientific, Rockford, IL) and diluted in Laemmli sample buffer, denatured by heating for 15 min at 60 °C, and stored at −80 °C. For subcellular fractionation: before diluting in Laemmli buffer, the supernatant was first centrifuged again at 48,000 g for 1 h; the resulting supernatant was considered ‘cytosol’ and the pellet (resuspended in SEID) was considered ‘membrane’. Samples were run at 5 µg protein per

lane along with a Precision Plus protein standard for size reference on a 7.5% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) mini-gel (Bio-Rad, Hercules, CA) for electrophoretic separation, then transferred to Immobilon polyvinylidene fluoride (PVDF) transfer membranes (Millipore, Bedford, MA). After transfer, the PVDF membranes were dried and stored overnight at room temperature. PVDF membranes were then rehydrated in methanol then equilibrated to phosphate-buffered saline with 0.05% Triton X-100 (PBST), blocked for 1 h at 23 °C in 5% nonfat milk in PBST, then incubated overnight at 4 °C in a 1:4,000 dilution of primary antibody (Developmental Studies Hybridoma Ban, Iowa City, IA): mouse monoclonal anti-NKA α-subunit (‘α5’; RRID (Research Resource Identifier): AB_2166869), mouse monoclonal anti-NKCC/NCC (‘T4’, RRID: AB_528406), mouse monoclonal anti-NKCC (‘T9’, RRID: AB_2618107), or mouse monoclonal anti-HSP90 (‘H90’, RRID: AB_2051648). After primary incubation, membranes were washed with PBST then incubated for 2 h at 23 °C in a 1:10,000 dilution of the horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MA) in blocking buffer. After secondary antibody incubation, membranes were washed with PBST and imaged by a Syngene PXi system (SYNGENE Inc., Frederick, MD) via enhanced chemiluminescence (ECL) using a 1:1 mixture of ECL solution A (396 μmol L^{-1} coumaric acid, 2.5 mmol L^{-1} luminol, 100 mmol L^{-1} Tris-HCl, pH 8.5) and ECL solution B (0.018% H_2O_2, 100 mmol l^{-1} Tris-HCl, pH 8.5). Band intensity was measured using ImageJ (National Institutes of Health, Bethesda, MD). The use of α5 and T9 antibodies was validated for use in Western blotting as follows: (i) a specific band was present at an expected molecular weight for each protein; (ii) a linear relationship was

observed between band intensity and the amount of protein sample loaded into each well (0.1 to 20 μg); and (iii) no evidence of false positive or non-specific binding was observed in three different negative controls: (a) gill protein incubated with no primary antibody (secondary only) ([-]Ab), (b), gill protein incubated with a primary antibody produced under the same conditions ([+]T4), and (c) protein from tissues expected to have only very low NKCC1 abundance (i.e., matching mRNA and protein tissue profiles). Our lower detection limit for T9- and α5 immunoreactivity in Western blotting using a pool of SW-acclimated juveniles was approximately 0.12 and 0.11 μg lane^{-1}, which corresponds to 1/42nd and 1/47th the amount loaded in our Western blotting protocol (5 μg lane^{-1}). Undetected bands during quantification were assigned the lower limit of detection and protein abundance is expressed as relative to the lower limit of detection (i.e., undetected bands = 1). Identical pooled samples were run on each gel and used as a calibrator to correct for any inter-blot variation.

Immunohistochemistry

Whole gill pouches were fixed in 4% paraformaldehyde in 10 mM phosphate-buffered saline (PBS) at room temperature for 2 h, then preserved in 70% ethanol. Before sectioning, gill tissue was rinsed in PBS, equilibrated to 30% (w/v) sucrose in PBS, then frozen in embedding medium (TissueTek, Sakura Finetek, Torrance, CA). Embedded gill tissue was sectioned (5 μm thick) at -20 °C, electrostatically mounted to microscope slides (Fisherbrand Colorfrost Plus, Fisher Scientific, Hampton, NH), then dried at room temperature to ensure adherence to the slide. Before incubation with primary antibody, mounted gill tissue was rehydrated in PBS, incubated for 30 min in 0.3% (w/v) Sudan Black B to reduce autofluorescence, then incubated for 30 min in a blocking solution

(10% normal goat serum in PBS). Slides were incubated overnight at 4 °C in primary antibody (T4, 1:1,000 (negative control only); T9, 1:1,000) in antibody dilution buffer (0.01% NaN_3, 0.1% BSA (bovine serum albumin), 2% normal goat serum and 0.02% keyhole limpet hemocyanin in PBS). For co-localization, slides were co-incubated with T9 and a rabbit monoclonal anti-NKA primary antibody (αRbNKA, 1:1,000) (Wilson et al., 2007). After primary incubation, slides were washed in PBS then incubated for 2 h at room temperature in fluorescently-labeled secondary antibody (goat anti-mouse, Alexa 546) diluted 1:1000 in antibody dilution buffer. After secondary incubation, slides were again washed in PBS, covered with a coverslip, and examined with a Nikon Diaphot-TMD inverted fluorescence microscope (20X) with a mercury lamp. Validation of T9 use in immunochistochemistry was similar to our validations used in Western blotting. Negative control validation included the absence of signal in (i) gill sections incubated with secondary antibody only, (ii) gill sections incubated with T4, and (iii) sections of non-target tissues incubated with T9. T9-immunoreactive cells on the primary filament or secondary lamellae were tallied from unique sections (3-5 sections per individual) from larvae, FW-acclimated, and SW-acclimated juveniles (n = 5 per group).

Calculations and statistical analyses

Muscle moisture (%) was calculated as: [(wet mass−dry mass)×100]/wet mass. Shapiro–Wilk and Levene's tests were used to test assumptions of normality and homogeneity, respectively. Significant differences between treatments were analyzed using one-way or two-way ANOVA analyses and were identified by Dunn's post hoc (all Western blot and immunohistochemistry data) or Student-Newman-Keuls post hoc analyses (all other data). An α-value of 0.05 was selected to denote statistical significance

in all analyses. Relative protein abundances of juvenile gill NKCC1 and NKA were regressed and the correlation was assessed by calculating the slope (m), coefficient of determination (r^2), and significance. Statistics and figures were completed using R statistical software Version 3.2.2 (https://www.r-project.org) and GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA).

Results

Molecular characterization and tissue profile of NKCC1

We obtained a sequence for the sea lamprey nkcc1 gene that was comprised of 3,480 bp coding for a mature NKCC1 peptide sequence of 1,160 amino acids with a predicted molecular mass of 127 kDa (NCBI GenBank accession number: MK779970). The sea lamprey NKCC1 has a 66% sequence identity with the NKCC1 of both zebrafish (Danio rerio) and human (Homo sapiens), which have a 72% sequence identity with each other (Fig. 2.1). Phylogenetic analysis of other vertebrate cation-chloride cotransporters placed the sea lamprey NKCC1 at the basal position in a clade with other vertebrate NKCC1 peptides, which was distinct from clades of vertebrate NKCC2 or NCC peptides (Fig. 2.2). In Western blotting, no banding was detected using the T4 antibody, but probing with T9 reliably detected a single, diffuse immunoreactive band centered around an approximate molecular mass of 180 kDa which appeared to be most intense in SW-acclimated juveniles (Fig. 2.3A). T9-immunoreactivity was only detected in the membrane fraction of a gill homogenate from FW- or SW-acclimated juveniles, whereas HSP90 (90 kDa) was mostly detected in the cytosolic fraction (Fig. 2.3B). In tissue profiles, mRNA abundance of nkcc1 was at least 5-fold higher in the gill of a FW-

acclimated sea lamprey than any other tissue examined (Fig. 2.3C) and T9-immunoreactivity (NKCC1 protein) was only detected in the gill (Fig. 2.3D).

Salinity tolerance

Larvae serving as a FW control all survived (Fig. 2.4A). During exposure to increasing salinity, larval mortality first occurred in 10 ‰ and all larvae died between 10 and 15 ‰, (Fig. 2.4A). Exposure to 10 ‰ for 14 d resulted in 0% survival in larvae and 30% survival in metamorphic stages 2–5 (Fig. 2.4B). Survival in 25 ‰ was 0% in larvae and early metamorphic stages 2–5, then increased dramatically to over 95% in stages 6-7 and 100% in post-metamorphic juveniles (Fig. 2.4B).

Metamorphic profile of NKCC1 and effect of SW-acclimation

In gill tissue collected from wild sea lamprey at various stages of metamorphosis, nkcc1 mRNA increased 200-fold from larvae to post-metamorphic downstream migrants ($P < 0.001$; Fig. 2.5A) and NKCC1 protein abundance increased at least 25-fold from larvae (undetected) to migrants ($P < 0.001$; Fig. 2.5B). Similarly, gill nka increased 15-fold throughout metamorphosis ($P < 0.001$; Fig. 2.5C) and gill NKA protein increased at least 30-fold from larvae (undetected) to migrants ($P < 0.001$; Fig. 2.5D). The first detection of gill NKA and NKCC1 was in stage 6 lamprey, although NKA and NKCC1 could be detected in only 2 of the 8 stage 6 lamprey in our analysis.

In our laboratory comparison between FW-acclimated larvae, and FW- and SW-acclimated post-metamorphic juveniles, gill nkcc1 was 200-fold higher in FW-acclimated juveniles than in larvae. Gill nkcc1 increased by ~70% after SW-acclimation of juveniles ($P < 0.001$; Fig. 2.6A). Gill NKCC1 protein abundance was at least 25-fold higher in

FW-acclimated juveniles than in larvae (undetected) and was ~60% higher in SW-acclimated juveniles compared to FW-acclimated juveniles ($P < 0.001$; Fig. 2.6B). Likewise, juveniles had higher abundances of gill nka mRNA ($P < 0.001$; Fig. 2.6C) and NKA protein ($P < 0.001$; Fig. 2.6D) compared to larvae, and SW-acclimation significantly increased gill nka mRNA and NKA protein. Analysis of the relationship between the relative protein abundances of gill NKA and NKCC1 in FW- and SW-acclimated juveniles revealed a significant correlation ($m = 0.76$, $r^2 = 0.66$, $F_{1,10} = 18.9$, $P < 0.005$) (Fig. 2.7A).

Immunohistochemistry of gill NKCC1

T9-immunoreactivity (NKCC1) in the gills of larval sea lamprey was very rarely observed and only ever observed in small cells on the lamellae (Fig. 2.7B). Intense NKCC1 staining was detected in the larger cells in the inter-lamellar space along the primary filament of post-metamorphic juveniles. A strong fluorescent signal was present throughout the entire body of these cells except for the nucleus, where no NKCC1 staining was present. Very rarely were NKCC1-positive cells observed on the lamellae in juveniles, and the abundance of NKCC1-positive cells on the primary filament was significantly higher in juveniles than in larvae, at approximately 140-150 immunoreactive cells per mm filament length in juveniles. There was no statistical difference in abundance of KNCC1-positive cells between FW- and SW-acclimated juveniles. Results of the two-way ANOVA in Fig. 2.7B were: $P_{life\text{-}stage} < 0.001$, $P_{location} < 0.001$, $P_{interation} < 0.001$. NKCC1 co-localized to the same filamental ionocytes as NKA (αRbNKA-immunoreactivity) (Fig. 2.7C).

Pharmacological inhibition of NKCC1

All lamprey were fully recovered (restoration of swimming activity) within 30 min of anesthetization and injection of the NKCC1 inhibitor bumetanide or saline control. In FW lamprey, bumetanide treatment had no effect on plasma Cl^- and osmolality, or muscle water (Fig. 2.8A–C). Exposure to SW resulted in slight increases in plasma Cl^-, osmolality, and a slight decrease in muscle water content in the VEH control (Fig. 2.8A–C). Lamprey injected with either 0.1 or 1.0 μmol g^{-1} bumetanide and exposed to SW had significantly higher plasma Cl^- (Fig. 2.8A) and plasma osmolality (Fig. 2.8B), and lamprey injected with 1.0 μmol g^{-1} had significantly lower muscle water (Fig. 2.8C) compared to the VEH control. Results of the two-way ANOVA were: Cl^-: $P_{treatment}$ = 0.013, $P_{salinity} < 0.001$, $P_{interaction} < 0.001$; osmolality: $P_{treatment} = 0.038$, $P_{salinity} < 0.001$, $P_{interaction} < 0.001$; muscle water: $P_{treatment} = 0.470$, $P_{salinity} < 0.001$, $P_{interaction} = 0.271$.

Discussion

Prior to this study, no protein-level evidence for the role of NKCC1 in agnathan osmoregulation was available. Here, we have presented the following lines of evidence for the importance of gill NKCC1 in lamprey hypo-osmoregulation: (i) a tissue profile showing that the gill is the primary site of NKCC1 expression (mRNA and protein abundance); (ii) a metamorphic profile showing substantial increases in gill NKA and NKCC1 expression coinciding with the development of SW tolerance; (iii) further increases in gill NKCC1 and NKA expression after SW acclimation; and (iv) reduced SW tolerance after pharmacological inhibition of NKCC1.

It has been hypothesized that the NKCC1 and NKCC2 isoforms are products of a second whole-genome duplication in the vertebrate lineage (2R), after a prior genome

duplication (1R) led to the initial divergence of NKCC from NCC (Hartmann et al., 2013), yet whether lampreys radiated after 1R or 2R is still unresolved (Holland and Ocampo Daza, 2018; Mehta et al., 2013; Smith and Keinath, 2015; Smith et al., 2013; Smith et al., 2018). In the present study, we present a full-length peptide sequence of a lamprey NKCC1-like protein. Phylogenetic analysis revealed that the sea lamprey NKCC1 groups strongly with other vertebrate NKCC1 peptides and occupies a basal position in the NKCC1 clade. The sea lamprey NKCC1 displayed a 66% sequence identity to NKCC1 in zebrafish and human with only the amino terminus displaying considerable divergence between sea lamprey and zebrafish or human. This is consistent with the defining structural characteristics of the SLC12A family of cation cotransporters, which includes a highly conserved hydrophobic region (including 12 transmembrane segments) flanked by a N-terminal region with high sequence variability and a highly conserved C-terminal region (Payne and Forbush, 1995).

In Western blotting, the sea lamprey NKCC1 appeared as a single, diffuse band detected primarily in the gill and centered on a molecular mass of approximately 180 kDa, which is larger than its predicted size of 127 kDa based on peptide sequence alone. However, glycosylation of NKCC1 often leads to higher apparent molecular weights in Western blots. Depending on the species, NKCC1 can appear in Western blotting at sizes ranging from 120-130 kDa in its deglycosylated form to 160-220 kDa when glycosylated (Lytle et al., 1995) to over 300 kDa as a homodimer (Moore-Hoon and Turner, 2000). Two of the four predicted *N*-linked glycosylation sites (N504 and N515, Fig. 2.1) on the large extracellular loop between TM7 and TM8 correspond to the two putative sites of *N*-linked glycosylation on the human NKCC1 that are important in cell surface expression,

protein turnover, and Cl^- affinity (Paredes et al., 2006). Thus, it is likely that sea lamprey NKCC1 is a glycoprotein like other vertebrate NKCC1 peptides and that glycosylation explains the higher-than-expected molecular weight of the NKCC1 immunoreactive band observed in Western blotting in the present study.

The T9 antibody used in this study was originally developed by Lytle et al. (Lytle et al., 1995) against a fusion protein of the 310 C-terminal amino acids of the human colonic NKCC1 in the same effort that produced the widely used anti-NKCC1 antibody T4. Originally, the T4 antibody was described as 'broadly specific' due to its immunoreactivity with many tissues known to possess NKCC, including NKCC1 in the human colon, shark rectal gland, and duck salt gland, and NKCC2 in the mammalian kidney. Since its development, the T4 antibody has been used extensively to detect NKCCs and NCC in a range of fishes including the teleost gill NKCC1 (Pelis et al., 2001; Wilson et al., 2000) and NCC (Hiroi et al., 2008), the teleost intestinal NKCC2 (Cutler and Cramb, 2002), and the sturgeon gill NKCC1 (Sardella and Kültz, 2009; Shaughnessy et al., 2015). Here, we show that T4 does not immunodetect any NKCC homologue in the lamprey gill. The T9 antibody was also originally produced against the human NKCC1 but described as having 'narrow specificity' as it appeared to be immunoreactive with only the mammalian NKCC (both the secretory NKCC1 in the human colon and the absorptive NKCC2 in the mammalian kidney) (Lytle et al., 1995). Since then, T9 has been sparingly used to immunodetect NKCC in published works—once to detect NKCC2 in the rat kidney (Alvarez et al., 2003) and once, along with T4, to detect an NKCC homologue in the sea urchin (D'Andrea-Winslow et al., 2001). It is not clear why T9 appears to reliably immunodetect the lamprey NKCC1 when it fails to detect NKCC1 in

the homologous and presumably less evolutionarily distant Cl^--secreting ionocytes of the shark rectal gland, teleost gill, and avian salt gland. The lamprey NKCC1 and human NKCC1 possess only a 62% sequence identity across the 310 C-terminal amino acids against which T9 was raised, however, the sequence identity between the human and lamprey NKCC1 is much higher (76%) when comparing just the 200 most C-terminal amino acids (the more likely site of antigenicity), which may explain how T9 is able to detect the lamprey NKCC1.

Early work in lampreys identified two types of ionocytes on the gill epithelium, which were presumed to be specific for either ion-uptake or ion-secretion (Bartels and Potter, 2004). After metamorphosis and migration into SW, surface exposure of the ion-secreting ionocytes in the interlamellar space increase and the intercalated cells involved in ion-uptake disappear (Peek and Youson, 1979a; Peek and Youson, 1979b). Since then, it was shown that NKA protein abundance is greater in post-metamorphic juveniles than larvae and that α5 immunoreactivity, which is a widely used marker of ionocytes, shifts from the small cells in the lamellae of larvae to large cells in the filament of juveniles (Reis-Santos et al., 2008). Taken together, these studies indicate that sea lamprey transform the branchial epithelium prior to SW entry in preparation for hypo-osmoregulation.

We expand on these previous works by resolving the timing of the increase in NKA abundance to the later stages of metamorphosis, describing for the first time the transcriptional upregulation of *nka* and *nkcc1* throughout metamorphosis and co-localizing NKCC1 and NKA to the interlamellar ionocytes along the gill filament. Larvae, which are known to have very low levels of gill NKA abundance and activity

(Reis-Santos et al., 2008) and have no detectable NKCC1 (present study), are incapable of surviving in salinities above 10‰. Much like what has been described in the Pacific lamprey (*Entosphenus tridentatus*) (Richards and Beamish, 1981), we observed the development of SW tolerance in sea lamprey to occur at metamorphic stages 6–7. The major increases in gill NKCC1 and NKA abundance between metamorphic stages 6 and 7 are both preceded by increases in *nkcc1* and *nka* mRNA at earlier stages, potentially illustrating the process of mRNA being translated into protein throughout metamorphosis—it may be that NKCC1 is being expressed in earlier metamorphic stages at levels below our detection limit. The significant and highly correlated upregulation of gill NKCC1 and NKA protein abundance and the development of SW tolerance during metamorphosis provides evidence that branchial ion secretion involves a coupling of NKCC1 and NKA. Acclimation of juvenile lamprey to SW resulted in an additional increase in NKCC1 and NKA, providing further evidence that these ion transporters are critical components of branchial ion secretion in lampreys. This coupling is further supported by the correlation of protein abundance and co-localization of NKCC1 and NKA to the large Cl- secreting ionocytes along the gill filament of post-metamorphic juveniles.

It is interesting that the sea lamprey appear to make much of the hypo-osmoregulatory adjustments before ever entering SW. The elevation of NKA and NKCC1 expression in FW prior to exposure to SW has also been shown in smolting salmonids (McCormick, 2013), which also have a strong correlation between increased levels of these ion transporters and salinity tolerance. It is likely that these proteins and their ionocytes are inactive until exposure to SW, although the mechanism(s) for

activation are currently unknown. Trafficking of ion transport proteins to the plasma membrane in response to a physiological challenge has shown to be an important regulatory process in fishes (Lingwood et al., 2005; Tresguerres et al., 2006), however, this does not appear to be the case for the lamprey NKCC1 in response to SW exposure, based on our observation that NKCC1 is found only in the membrane in the gills of both FW- and SW-acclimated juveniles (Fig. 2.3). Gill NKCC1 in juvenile sea lamprey exposed to SW may be activated by phosphorylation (Flatman, 2002), similar to what has been shown in mummichog (*Fundulus heteroclitus*) (Flemmer et al., 2010). In teleost fish, inactive ionocytes have been shown to be covered by pavement cells and then rapidly uncovered after SW exposure (Daborn et al., 2001; Van Der Heijden et al., 1997), and a similar mechanism of ionocyte activation may also be present in sea lamprey.

The hypo-osmoregulatory adjustments made during the sea lamprey metamorphosis are akin to the months-long parr-smolt transformation preceding SW entry that occurs in anadromous salmonids (Hoar, 1988; McCormick and Saunders, 1987). Smolt development and the ontogeny of SW tolerance in salmonids is driven by a coordinated endocrine program involving the stimulatory action of thyroid hormones, cortisol, and growth hormone (McCormick, 2013). Lamprey metamorphosis appears to differ from the parr-smolt transformation and most other instances of metamorphosis in vertebrates in that thyroid hormones are antagonistic to transformation, such that the onset of metamorphosis is driven by a sharp decrease in circulating thyroid hormones (Youson, 1997). Lampreys lack the steroidogenic ability to produce cortisol and so a biosynthetic precursor, 11-deoxycortisol, is presumed to be the putative corticosteroid hormone in lampreys (Close et al., 2010; Rai et al., 2015). Future work is necessary to

identify whether and how thyroid hormones, 11-deoxycortisol, or the lamprey growth hormone (Kawauchi et al., 2002) regulate the development of SW tolerance and the molecular mechanisms for osmoregulation during the lamprey metamorphosis.

We sought direct evidence for the functional importance of NKCC1 in lamprey ion secretion by pharmacologically inhibiting NKCC1 with the widely used NKCC inhibitor bumetanide and subsequently assessing multiple osmoregulatory endpoints. Here, we show that treatment with bumetanide compromises the capacity for juvenile sea lamprey to maintain ion and water homeostasis after exposure to SW. Application of "loop" diuretic compounds, such as bumetanide or furosemide, to ion-transporting epithelia has been classically performed to identify the involvement in NKCC in Cl^- transport (Haas and Forbush III, 2000). This pharmacological approach typically employs *in vitro* electrophysiological techniques such as patch clamp or Ussing chamber preparations that require uniform epithelial tissue such as the intestine, gill operculum, or culture-raised epithelia. Unfortunately, these techniques cannot be applied to the highly irregular morphology of the fish gill. Thus, our *in vivo* approach follows several studies using *in vivo* bumetanide administration in mammals (Ben-Ari et al., 1989; Dzhala et al., 2005; Dzhala et al., 2008; Javaheri and Wagner, 1993; Kahle et al., 2009; Mazarati et al., 2009; Schuchmann et al., 2006; Wang et al., 2015) as well as a previous study in fish (Prodocimo and Freire, 2006) in which a known NKCC inhibitor, furosemide, was administered by intraperitoneal injection and osmoregulatory targets such as plasma ion concentration and muscle moisture were analyzed. The obvious disadvantage of this approach is that the gill is not an isolated target of bumetanide and thus inhibition of NKCC1 or NKCC2 in other tissues could be contributing to the loss of osmoregulatory

capacity in SW. We address the potential limitations of this *in vivo* approach with the following considerations: (i) our transcriptional- and protein-level data indicate that NKCC1 abundance is far greater in the gill than any other tissue, thus any NKCC1 inhibition by bumetanide treatment is likely occurring primarily in the gill; (ii) bumetanide application to *ex vivo* intestinal preparations of SW-acclimated post-metamorphic juvenile sea lamprey does not affect ion uptake as measured by short-circuit current (A. Barany-Ruiz, Universidad de Cádiz, Cádiz, Spain; oral communication); and (iii) analysis of the Japanese and sea lamprey genomes and the hagfish genomes have failed to identify a gene encoding NKCC2. Although the inability to detect a gene in a genome must be viewed with some caution, the apparent lack of an NKCC2 in the genomes of these basal vertebrates could indicate that a duplication of NKCC occurred after the divergence of agnathans from other vertebrates or that NKCC2 has been lost in agnathans. Taken together, the evidence available to us supports that our *in vivo* approach presented here to inhibit the NKCC1 using bumetanide is likely acting in large proportion on gill NKCC1, thus providing evidence for the functional importance of branchial NKCC1 in ion and water balance of SW lamprey.

Although the role of NKCC1 in transcellular Cl^- secretion in the gills of marine fishes is widely accepted (Evans, 2008; Hwang et al., 2011) and such a role has been proposed in lampreys (Bartels and Potter, 2004), the present study offers the first protein-level and functional evidence for the role NKCC1 in lampreys. The robust upregulation of basolateral NKA and NKCC1 during metamorphosis in the anadromous sea lamprey may be a good biomarker for distinguishing anadromous sea lamprey from landlocked strains or anadromous lamprey species from their riverine species pairs. Still unresolved

is the apical pathway for Cl^- to exit the sea lamprey ionocyte, which in later-evolved fish lineages is the CFTR (Marshall and Singer, 2002). Although the gene encoding the lamprey CFTR has been cloned (Ren et al., 2015), an RNAseq approach from the same study revealed relatively low *cftr* mRNA transcript abundance in all tissues, including adult gill, with the only exception being high expression in the larval intestine. Further research is needed to establish the apical pathway of Cl^- secretion in lampreys to complete the molecular characterization of this basal vertebrate ionocyte.

In conclusion, the present study provides a molecular and functional characterization of the sea lamprey NKCC1. We show that gill NKCC1 expression (mRNA and protein abundance) increases dramatically during metamorphosis, coinciding with the development of SW tolerance, and that NKCC1 expression increases further after SW acclimation. Immunoblotting and immunohistochemical analyses co-localized NKCC1 with NKA to the plasma membrane fraction of the presumptive Cl^- secreting gill ionocytes along the filament. Finally, we demonstrated a functional role for NKCC1 in hypo-osmoregulation by showing that pharmacological inhibition of NKCC1 in post-metamorphic juveniles results in reduced SW tolerance.

Perspectives and significance

The present study provides evidence that the basolateral pathway for ion-secretion across the lamprey gill involves a coupling of NKCC1 and NKA in branchial ionocytes, moving back the most basal example of a role of NKCC1 in salt secretion in vertebrates by ~100 million years from Chondrichthyes (sharks), which first appeared as early as 450 mya, to Agnathans (lamprey) which first appeared over 550 mya. This work is a part of many recent advances in lamprey physiology (osmoregulation, endocrinology,

immunology, and neurobiology) and genetics (publication and updating of the lamprey genome), which have combined to establish lamprey as an important model system for the study of vertebrate evolution. Although our work here demonstrating that the role of a basolateral NKCC1 in a secretory epithelium is ancestral among vertebrates, we also underscore a need for further investigation into still unresolved ionoregulatory processes in lamprey and the endocrine pathways that control them.

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Author contributions

Conceptualization, methodology, and experimentation: C.A.S., S.D.M.; Data curation and formal analysis: C.A.S.; Writing and revisions: C.A.S. (original draft), S.D.M; Funding acquisition and project supervision: S.D.M.

Disclosure

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or product names is for descriptive purposes only and does not imply endorsement by the U.S. government.

Figures

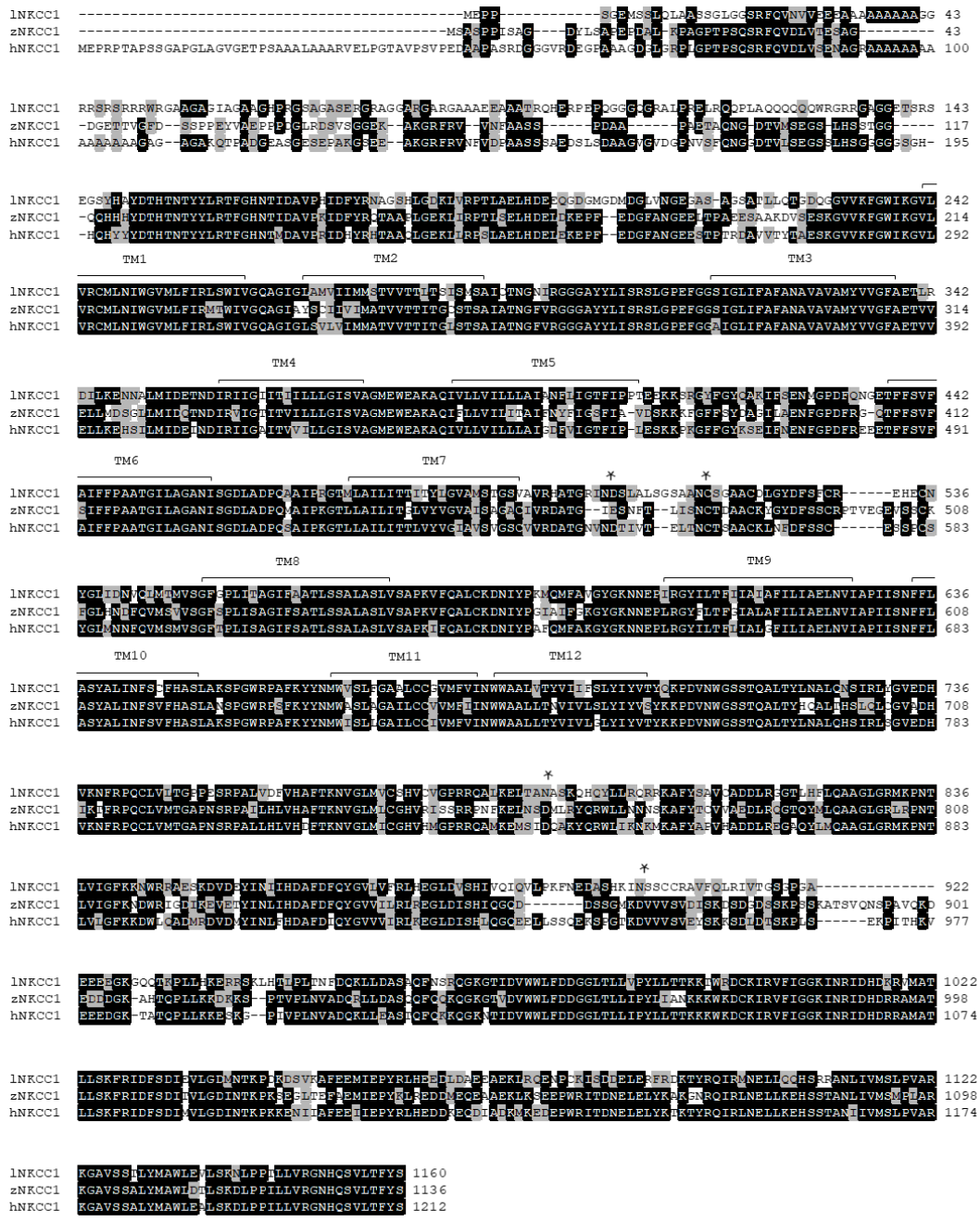

Figure 2.1: Multiple alignment of peptide sequence for the NKCC1 of zebrafish (zNKCC1) and human (hNKCC1) and the deduced amino acid sequence for sea lamprey nkcc1 (lNKCC1). Black shading indicates identical residues and grey shading indicates chemically similar residues and grey shading indicates chemically similar residues. Horizontal bars indicate the 12 predicted transmembrane segments. Asterisks indicate predicted *N*-linked glycosylation sites.

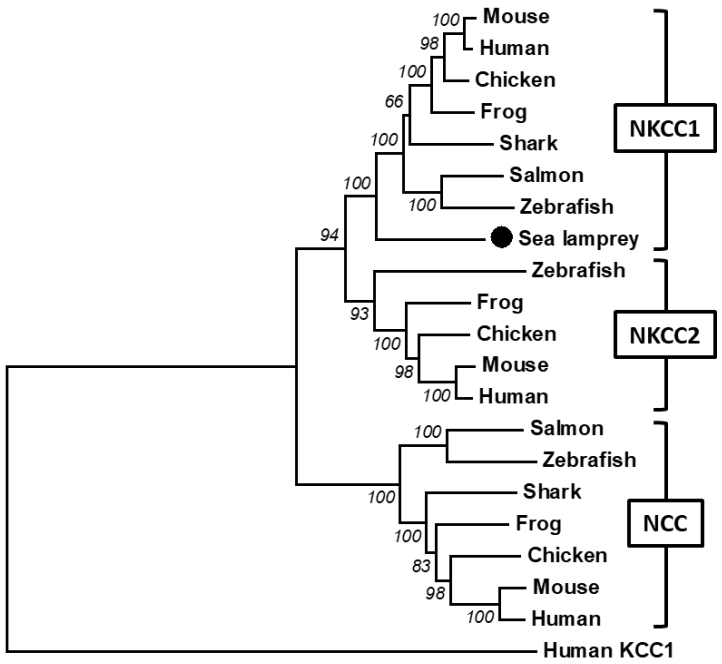

Figure 2.2: Phylogeny of the coding regions of various vertebrate cation chloride cotransporters (NKCC1, NKCC2, NCC, with a human KCC1 outgroup) constructed using the Neighbor-Joining method with 1,000 replicates. Sea lamprey NKCC1 (present study) is highlighted by a filled circle.

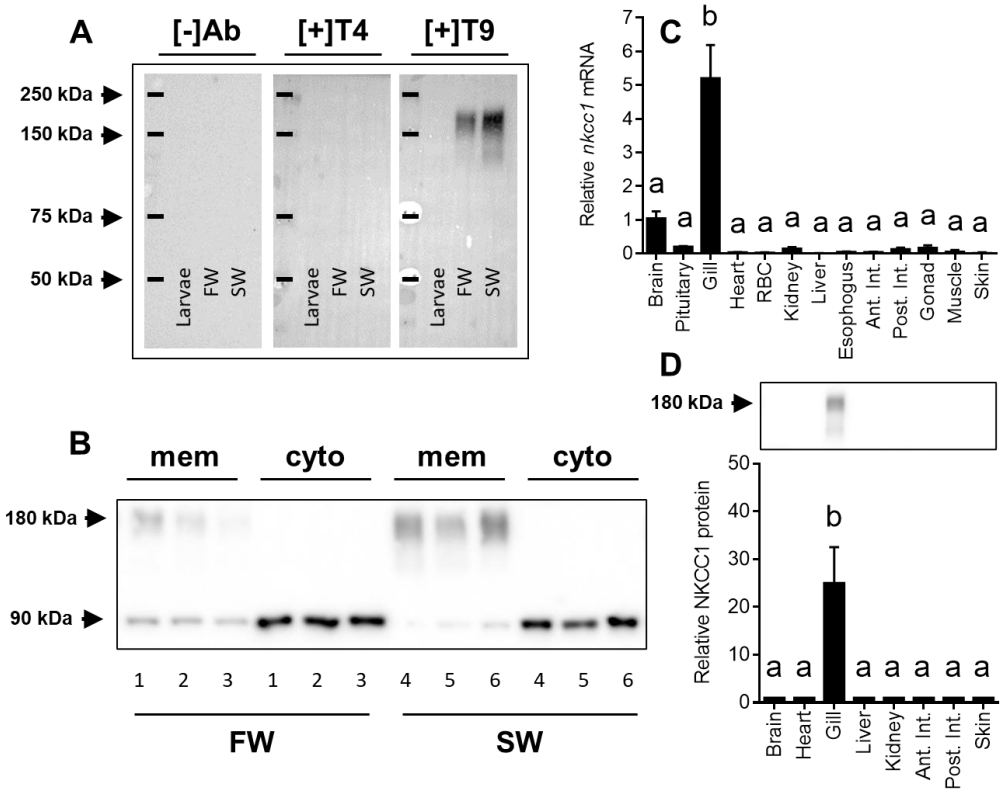

Figure 2.3: Validation blots showing T9-specific immunoreactive banding (~180 kDa) (A) and NKCC1 subcellular location (B), as well as tissue profiles for the relative abundance of *nkcc1* mRNA (C) and NKCC1 protein (D) in FW-acclimated juvenile sea lamprey. In A, lack of banding from two negative controls ([-]Ab and [+]T4) are presented on identically prepared blots alongside positive banding for NKCC1 ([+]T9). In B, banding for NKCC1 and HSP90 (~90 kDa) are shown on a single blot containing the membrane (mem) and cytosolic (cyto) fractions of gill homogenates from six individuals acclimated to either FW (1-3) or SW (35 ‰) (4-6). In D, representative NKCC1 banding is included above data. Letters depict statistically significant differences (mean ± s.e.m.; $n = 3$; one-way ANOVA; C: Student-Newman-Keuls *post hoc*; D: Dunn's *post hoc*).

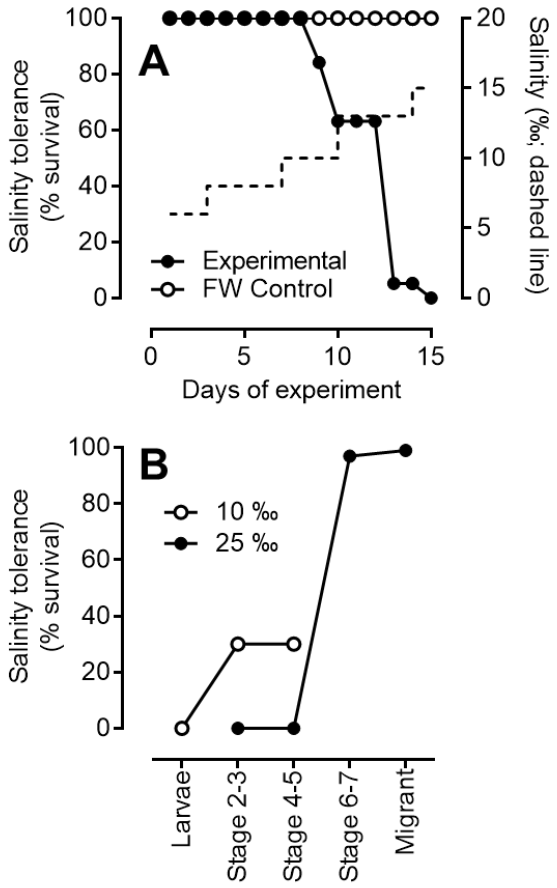

Figure 2.4: Salinity tolerance of sea lamprey larvae (n = 20) exposed to increasing salinity (A) or sea lamprey after direct exposure to elevated salinity (10 or 25 ‰) at various stages throughout metamorphosis (B). In B, survival rates were assessed 14 d after exposure to elevated salinity and calculated from various salinity trials; n = 10–20 (larvae, stage 2–3, stage 4–5) and n = 60–100 (stage 6–7, migrant).

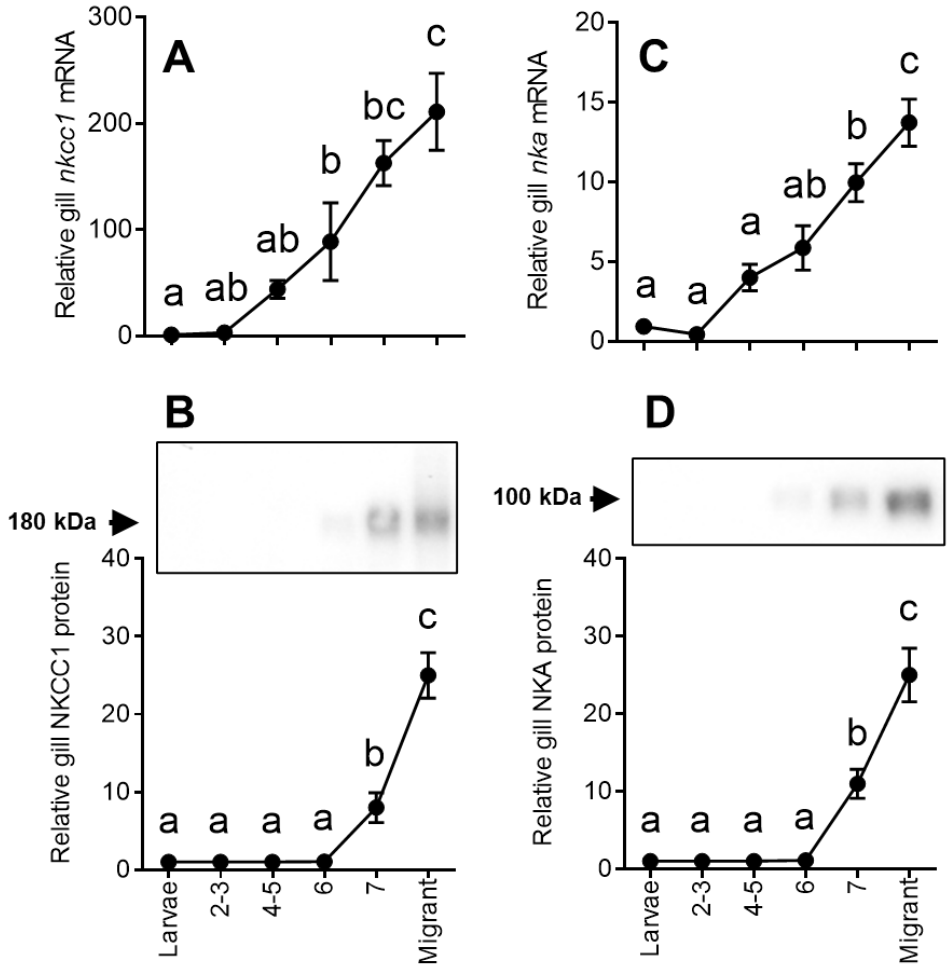

Figure 2.5: Metamorphic profile for the relative abundance of *nkcc1* (A), NKCC1 (B), *nka* (C), and NKA (D) in the sea lamprey gill. In A and C, data are presented as relative to *ef1a.* In B and D, representative T9- and α5-immunoreactive banding is included above respective data. Letters depict statistically significant differences (mean ± s.e.m.; n = 4–10; one-way ANOVA; A,C: Student-Newman-Keuls *post hoc*; B,D: Dunn's *post hoc*).

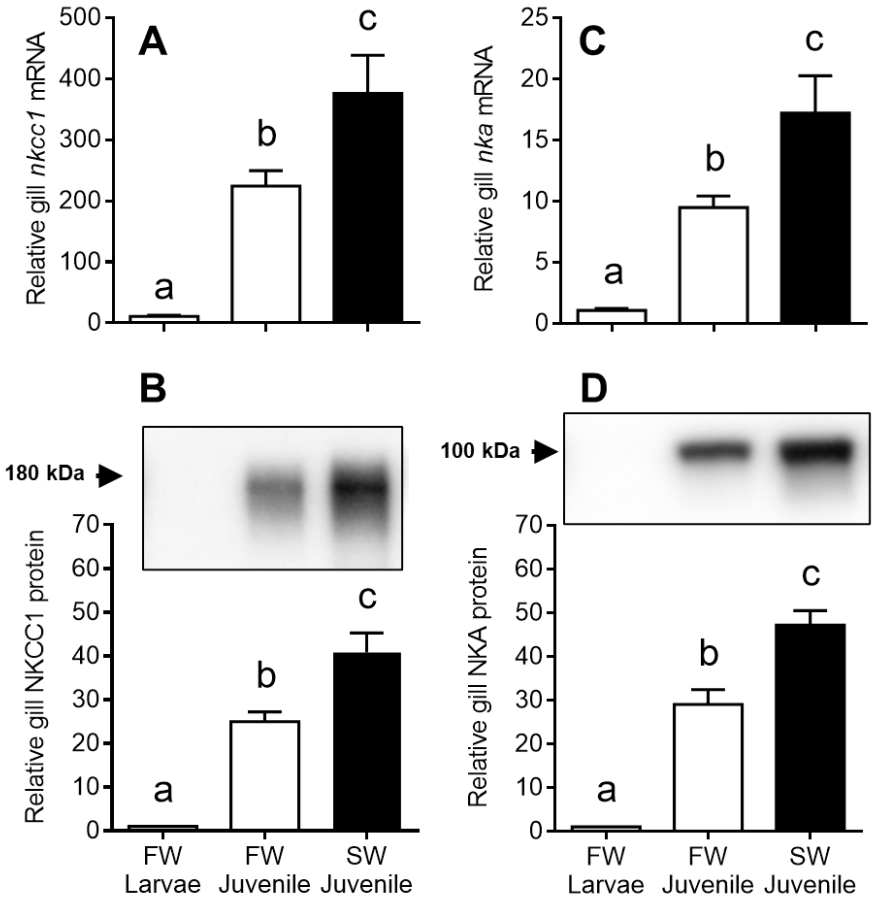

Figure 2.6: Relative abundance of *nkcc1* (A), NKCC1 (B), *nka* (C), and NKA (D) in FW-acclimated larvae, FW-acclimated juvenile, and SW-acclimated (35 ‰) juvenile sea lamprey gill. In A, the actual value for larvae (1.095 ± 0.18) has been inflated for visual clarity. In A and C, data are presented as relative to *ef1a.* In B and D, representative T9- and α5-immunoreactive banding is included above respective data. Letters depict statistically significant differences (mean ± s.e.m.; n = 8–10; one-way ANOVA; A,C: Student-Newman-Keuls *post hoc*; B,D: Dunn's *post hoc*).

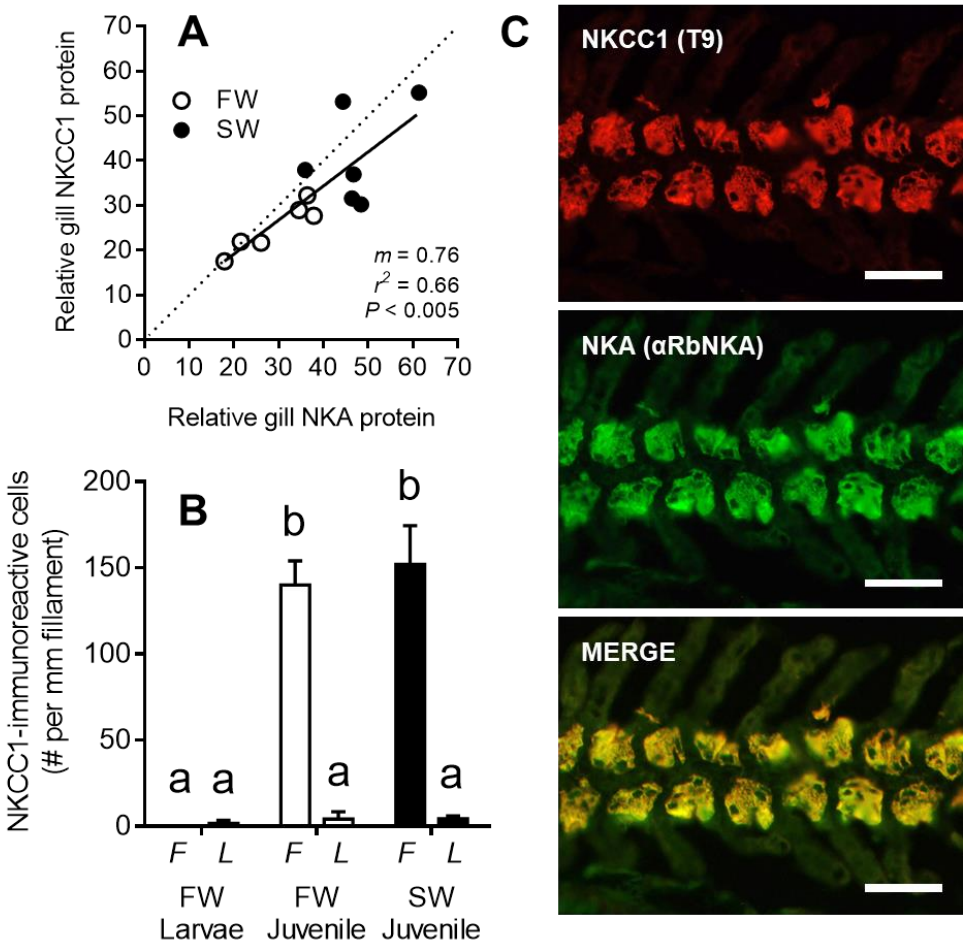

Figure 2.7: Relationship between NKA and NKCC1 protein abundance (from Western blotting) in FW- and SW-acclimated juveniles (A), abundance of T9-immunoreactive cells on the primary filament (F) and secondary lamellae (L) of larvae, FW-, and SW-acclimated juveniles (B), and representative images of anti-NKCC1 T9-immunoreactivity (red) and anti-NKA αRbNKA-immunoreactivity (green) in sagittally-sectioned (5 μm) gill tissue of SW-acclimated (35 ‰) juvenile sea lamprey (C). In C. scale bar = 50 μm. Letters depict statistically significant differences (mean ± s.e.m.; n = 5; two-way ANOVA; Dunn's *post hoc*).

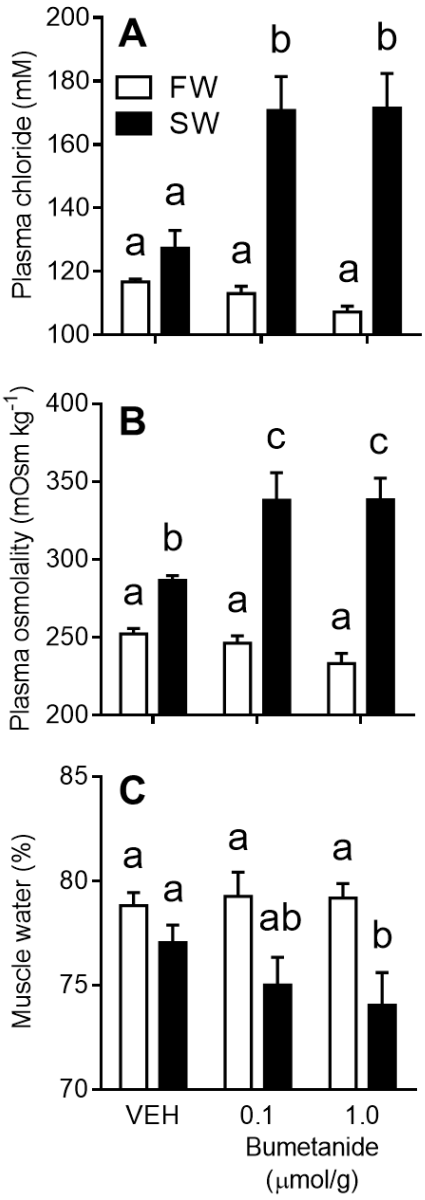

Figure 2.8: Effect of intraperitoneal injection of bumetanide (an NKCC1 inhibitor) on plasma chloride (A), plasma osmolality (B), and muscle water (C) of sea lamprey compared to a vehicle (VEH) control. After bumetanide injection, fish were exposed to SW (35 ‰; black bars) or FW (white bars) for 24 h. Letters depict statistically significant differences (mean ± s.e.m.; n = 8–10, two-way ANOVA; Student-Newman-Keuls *post hoc*).

CHAPTER 3

INTRABRANCHIAL PARTITIONING OF Na^+/K^+-ATPase ACTIVITY AND OSMOREGULATORY CONTRIBUTION IN SEA LAMPREY (*PETROMYZON MARINUS*) HAVE IMPLICATIONS FOR HOLOBRANCH EVOLUTION

Authors

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Abstract

Fish gills carry out a multitude of biochemical and physiological functions, including gas exchange, acid-base and ion regulation, and nitrogenous waste excretion. Although fish gills are comprised of several discrete units (holobranchs), the traditional view is that biochemical and physiological processes are homogenously distributed among the holobranchs of fish gills. The present study compared the branchiometrics and osmoregulatory role of each holobranch (called 'pouch') in the gills of sea lamprey (*Petromyzon marinus*). We report that gill branchiosomatic index (percent body weight that is gill tissue) increases significantly during sea lamprey metamorphosis, during which sea lamprey make physiological preparations to survive in seawater (SW). A marker for osmoregulatory capacity, gill Na^+/K^+-ATPase (NKA) activity, increased significantly during metamorphosis and to even higher levels after acclimation to SW.

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Gill NKA activity also varied significantly among gill pouches. The contribution of individual gill pouches to osmoregulation (based on gill NKA activity and relative mass) varied by 3-fold and was lowest in the anterior-most lamprey gill pouch. These findings suggest that heterogeneity in the distribution of biochemical or physiological function is present in the gills of sea lamprey. As the anterior most gill holobranch in lamprey represents a feature which evolved to form the jaw in more derived vertebrates, the reduced osmoregulatory capacity of this holobranch in lamprey may have relevance in current hypotheses for the evolution of branchial anatomy and function in vertebrates.

Introduction

The fish gill consists of discrete units called holobranchs (colloquially referred to as ‘arches’), which are arranged as several lateral pairs along the pharyngeal region. Filaments extending from the cartilaginous gill arch are the major biochemically active and physiologically relevant portion of the gill. The fish gill is a multifunctional organ, having a critical role in respiration, osmoregulation, acid-base balance, and nitrogenous waste excretion (Evans et al., 2005). As such, the gill epithelium demonstrates a site of physiological trade-off between optimal efficiency of a single function and less-than optimal efficiency of many concurrent functions. The most noticeable example of this trade-off is the ‘osmorespiratory compromise’, first introduced by Randall et al. (1972), who recognized that a large surface area on the gill epithelium is conducive for gas-exchange but counterproductive to ion homeostasis. It could be hypothesized that, in order to most efficiently perform multiple counterproductive processes, the fish gill might compartmentalize function across gill arches. However, the traditional view of the fish gill has been that biochemical and physiological function is homogeneously represented

across arches (Perry and Farrell, 1989). Within a single gill arch, heterogeneous distribution of gill ionocytes across the filament (greater density of ionocytes on leading edge of filament) have been demonstrated in a teleost fish (Christensen et al., 2012). Yet, relatively few investigations have considered possible heterogeneous partitioning of physiological function across gill arches (Duncan et al., 2011; Lyndon and Brechin, 1999).

The osmoregulatory role of the fish gill is well-described (Marshall and Grosell, 2006). In freshwater (FW), fishes are hyper-osmotic to their environment, thus passively gain water and lose ions to the dilute environment. In seawater (SW), fishes are hyper-osmotic to their environment, thus passively lose water (dehydrate) and gain ions across external surfaces, primarily the gills and skin. The role of the gill epithelium in active ion transport is considerably different between FW and SW, functioning as a site of ion uptake or ion secretion, respectively. Although major differences exist in the molecular composition of gill ionocytes between FW and SW, the basolateral Na^+/K^+-ATPase (NKA) has a central role in establishing the electrogenic gradient driving both ion uptake in FW and ion secretion in SW. For many decades, the activity of gill NKA has been commonly analyzed as a measure of osmoregulatory capacity of this tissue in determining developmental status and predicting survival in SW (McCormick, 1993; Saunders and Henderson, 1978; Zaugg, 1982). Generally, higher levels of gill NKA in SW are observed in most euryhaline species. Whether gill NKA activity varies between gill arches or distribution of gill NKA activity differs across salinities has not been widely examined.

Lamprey are extant members of the basal vertebrate superclass of jawless vertebrates, Agnatha. Unlike gnathostome fishes, which typically have five gill holobranchs along each side, lampreys have seven holobranchs with filaments arranged in a sac-like arrangement commonly referred to as gill 'pouches' (Fig. 3.1). Although the true nature of how the vertebrate jaw evolved is still being discussed, it is hypothesized that the vertebrate jaw evolved from the anterior two arches, mandibular and hyoid arches, of a 'pre-gnathastome' common ancestor to agnathans and gnathasomes (Mallatt, 1996; Mallatt, 2008; Shigetani et al., 2005).

The sea lamprey (*Petromyzon marinus*) is an anadromous lamprey, making a FW to SW migration as juveniles (Beamish, 1980a). The first 4–6 years of a sea lamprey life is spent as larvae burrowed in the benthic substrate of FW streams with no ability to tolerate SW. Before migration downstream to SW, sea lamprey undergo a larvae-to-juvenile metamorphosis during which gill NKA activity increases considerably and SW tolerance is acquired (Beamish et al., 1978; Reis-Santos et al., 2008; Shaughnessy and McCormick, 2020).

As anadromous sea lamprey represent the most basal extant osmoregulating vertebrate and exhibit an ancestral seven gill pouches which undergo dramatic osmoregulatory changes in preparation for SW, it was thought that potential pouch-by-pouch variation in osmoregulatory capacity may be present. Thus, the aims of the present study were to assess branchiometric differences across metamorphosis, salinity, and body size, as well as to evaluate the distribution of NKA activity across the seven lamprey gill pouches. The results this work may have implications at a practical level by evaluating whether the biochemistry of a single gill pouch is representative of the entire gill, and for

the evolution of gill and jaw function by shedding light on the relative contributions of each pouch to osmoregulation in a basal, jawless vertebrate.

Materials and methods

Larvae and post-metamorphic sea lamprey were held in 60 L recirculating glass aquaria equipped with mechanical, chemical, and biological filtration and held at 15 °C. Animals were acclimated under these conditions to FW or SW for 3 weeks. Artificial SW was made using a commercial marine mix (Crystal Sea Salt, Baltimore, MD) reconstituted with dechlorinated municipal FW. During sampling, animals were euthanized in neutralized MS-222 (200 mg L^{-1}), measured for mass and length. Individual gill pouches along the right side of the lamprey were carefully dissected, blotted dry and measured for mass then immediately frozen in SEI buffer (150 mmol L^{-1} sucrose, 10 mmol L^{-1} EDTA, 50 mmol L^{-1} imidazole, pH 7.4) and stored at -80 °C for later enzyme activity analysis.

Gill NKA activity was measured using a method previously described by McCormick (1993) in which the conversion of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) by NKA is enzymatically coupled to the oxidation of nicotinamide adenine dinucleotide (NADH) with a 1:1 stoichiometry. Whole gill pouches were homogenized in SEI buffer containing 0.1 % sodium deoxycholate using an electronic pestle and homogenized for 5 min at 2,000 *g*. The supernatant was incubated for with or without ouabain (0.5 mM) in a 1:3 mixture of assay buffer (4 U mL^{-1} lactate dehydrogenase, 5 U mL^{-1} pyruvate kinase, 2.8 mM phosphoenol pyruvate, 0.7 mM ATP, 0.22 mM NADH, and 50 mM imidazole, pH 7.4) and salt solution (189 mM NaCl, 10.5 mM $MgCl_2$, 21 mM KCl, and 50 mM imidazole, pH 7.4) for 10 min at 25 °C. The linear

rate of NADH oxidation in preparations with and without ouabain was measured spectrophotometrically (λ =340 nm). Additional supernatant was used to determine protein content (BCA Protein Assay Kit, Pierce, USA). Activity and protein assays were run on BioTek microplate reader (Molecular Devices, San Jose, CA, USA). The difference in ADP production between preparations with and without ouabain is considered NKA activity, which is reported at μmol ADP mg protein^{-1} h^{-1}.

Normalized gill pouch mass was calculated as the gill pouch-specific percent of body mass: *100 × [gill pouch mass ÷ body mass]*. Branchiosomatic index (BSI) was calculated as total gill percent of body mass: *100 × [total gill mass ÷ body mass]*. Values for 'contribution to gill osmoregulation' were calculated as gill pouch-specific percent of total gill NKA activity: *100 × [(gill pouch mass × gill pouch NKA activity) ÷ (total gill mass × total gill NKA activity)]*, such that the sum total of 'contribution' values for each life stage equals 100 %. Specific gill pouch positions were numbered G1–G7 (anterior–posterior) and aligned left to right in figures. All group data are presented as mean ± standard error (n = 6 individuals per life stage). Regression analysis was used to assess allometric relationships. One-way ANOVA was used to determine effect of life stage on branchiosomatic index (Tukey's *post hoc*). Two-way ANOVA was used to determine effects of life stage and gill pouch position (Dunnett's *post hoc*).

Results

Sea lamprey measured 14.0–19.1 cm body length (range) and weighed 2.9–7.5 g body mass (range). Body length was positively associated with body mass (m = 1.06 ± 0.99; R^2 = 0.878; P < 0.001) (Fig. 3.2A). Total gill mass ranged 43.0–211.0 mg (range) and was positively associated with body mass (m = 30.15 ± 3.04; R^2 = 0.861; P < 0.001)

(Fig. 3,2B). No differences between life stages were observed for the scaling of body length ($P = 0.714$) or total gill mass ($P = 0.476$) to body mass. Normalized gill pouch mass varied significantly between positions ($P_{position} < 0.001$), life stages ($P_{life\ stage} < 0.001$), and differences between gill pouch positions varied across life stages ($P_{interaction} = 0.001$) (Fig. 3.2C). Differences in normalized gill pouch mass among positions were most apparent in larvae, in which G1 was significantly smaller than all other gill pouches. BSI was greater in juveniles than larvae but not different between FW- and SW-acclimation in juveniles (Fig. 3.2D).

Gill NKA activity varied significantly between positions ($P_{position} < 0.001$), life stages ($P_{life\ stage} < 0.001$), and differences between pouch positions varied across life stages ($P_{interaction} = 0.007$) (Fig. 3.3A). Mean gill pouch NKA activity in larvae was 0.39–1.36 μmol ADP mg protein^{-1} h^{-1} (range). Juveniles had ~15-fold (FW) to ~20-fold (SW) higher gill NKA activity than larvae, and SW juveniles had significantly high gill NKA activity than FW juveniles. Significant differences in NKA activity among gill pouch positions were not detected in larvae. In juveniles, G1 had the lowest NKA activity compared to every other gill pouch. Contribution to total gill NKA activity (*i.e.*, 'contribution to gill osmoregulation') varied significantly across gill pouch position ($P_{position} < 0.001$) and the nature of positional differences varied across life stage ($P_{interaction} = 0.005$) (Fig. 3.3B). In larvae, G1 contributed the lowest NKA activity (5 %), whereas G2 contributed the highest (27 %) and G3–7 contributed 12–17 % (range) each. In juveniles, the position differences in NKA contributions were less pronounced with all gill pouches contributing 9–20 % (range) of the total NKA activity in the gills. In both FW and SW juveniles G1 had the lowest NKA contribution (9 % for both), whereas G4

had the highest NKA contribution (20 %) in FW juveniles and G5 had the highest NKA contribution (19 %) in SW juveniles.

Discussion

For the first time, we show that BSI increases during the sea lamprey metamorphosis. Along with the well documented molecular and biochemical changes to the sea lamprey gill throughout metamorphosis (*e.g.,* increases in NKA activity and abundance), the increase in BSI reported here suggests a reprioritization toward enhanced capacity for osmoregulation is being manifested at the morphological level as well. Additionally, we show that there are significant differences in relative mass and NKA activity between sea lamprey gill pouches; and that these differences change during metamorphosis and acclimation to SW. Notably, the anterior-most sea lamprey gill pouch (G1), has significantly lower NKA activity than every other pouch (G2–G7) in post-metamorphic juveniles, regardless of salinity.

Relative gill mass appears to be importantly regulated with growth and metamorphosis. All life stages of sea lamprey exhibited a similar positive correlation between body size and total gill mass. Rarely is BSI, the percent body weight of gills, reported for fishes. We were only able to find two other reports of BSI in fishes: in rainbow trout, BSI = 3.5–4.5 % (Kamunde et al., 2002); in African lungfish, BSI = 0.4–1.0 % (Okafor, 2014). Thus, the 1.5–2.5 % BSI for juvenile sea lamprey reported in the present study appears to be within the range seen for other fishes. The interesting finding regarding BSI is that it increased significantly during metamorphosis—the gills of post-metamorphic juveniles constitute a larger portion of the body than the gills of ammocoetes. The elevated BSI in post-metamorphic juveniles compared to larvae may

partly, along with the well documented increase in gill NKA activity and abundance, explain the increased ability of post-metamorphic juveniles to regulate plasma Na^+ and Cl^- in FW than larvae. In FW, larvae regulate Na^+ and Cl^- at approximately 100 and 90 mM, which is significantly lower than that of juveniles at 125 and 110 mM, respectively (Reis-Santos et al., 2008). The increased BSI in post-metamorphic juveniles in FW may also be a morphological adjustment made in preparation for hypo-osmoregulating in SW, as no adjustments in BSI were necessary (observed) after acclimation to SW.

Interesting pouch-by-pouch differences in gill NKA activity were observed in all life stages. As previously established (Beamish et al., 1978; Reis-Santos et al., 2008; Shaughnessy and McCormick, 2020), gill NKA activity increased considerably (10- to 15-fold) during metamorphosis, and increased even further in SW. In post-metamorphic juveniles in FW and SW, the lowest NKA activity was in G1 and NKA activity generally increased in more posterior gill pouches. The only other report of gill arch differences in NKA activity described a similar trend of increasing NKA activity from anterior to posterior among the gill arches of freshwater stingray (Duncan et al., 2011). This is especially interesting because freshwater stingray have to take up salts from the surrounding environments, and thus may have elevated NKA compared to most other elasmobranchs which are marine and do not excrete excess salts across the gill but do this using a specialized rectal gland (Pang et al., 1977). More studies in other species are needed to determine how universal this apparent heterogenous distribution of osmoregulatory capacity in fish gills may be. In species which do exhibit a non-homogenous distribution of osmoregulatory capacity, it should be investigated whether other biochemical and physiological transport functions of the gill, such as acid-base

balance and nitrogenous waste excretion, are distributed differently across gill arches/pouches. When accounting for relative gill pouch mass, the 'contribution to gill osmoregulation' (percent of total NKA activity) was more evenly spread across the gill pouches of juveniles than in larvae. Comparing between life stages, a posterior shift in the contribution to total gill NKA activity was apparent. The highest contribution to osmoregulation for larvae, FW juveniles, and SW juveniles came from pouches G2 (27 %), G4 (20 %), and G5 (19 %), respectively.

The relatively low osmoregulatory role of G1 is interesting with respect to the evolution of the vertebrate jaw. In the Neoclassical Hypothesis proposed by Mallatt (1996), the vertebrate jaw evolved after a rearrangement and repurposing of the mandibular and hyoid arches to form the upper and lower jaw, respectively. In this hypothesis, an anterior branchial pouch-like holobranch reduced to form the spiracle present in the pharynx of jawed vertebrates. The increasing biochemical activity from anterior to posterior pouch positions described in lamprey in the present study, particularly the relatively low osmoregulatory contribution of G1, may reflect the latent potential the anterior branchial pouches of a pre-gnathostomes ancestor to be reduced and the anterior arches be selected for some other function (*i.e.* jaws). Likewise, the ontogenetic posterior-directed shift in osmoregulatory contribution during sea lamprey metamorphosis may be a relic of a functional re-programing of branchial pouches during development of a pre-gnathostome ancestor that produces a jaw in embryonic gnathostomes (Mallatt, 2008). More functional studies on the physiological role of individual holobranchs during development in other agnathans, such as hagfish, would

help more fully define the relative physiological importance of holobranchs in early vertebrates that would help shed light on this scenario.

In conclusion, we report that NKA activity, an important marker of osmoregulatory capacity, varies between gill pouches of the sea lamprey, a basal osmoregulating vertebrate. A posterior bias in gill NKA activity and osmoregulatory contribution was observed and became more posteriorly shifted during development and after SW acclimation. These findings underscore the importance of gill position in conducting biochemical and physiological studies with gill tissue and may have meaningful significance with respect to the evolution of physiological function of branchial arches and the vertebrate jaw.

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Author contributions

Conceptualization, methodology, and experimentation: C.A.S., A.D., S.D.M.; Data curation and formal analysis: C.A.S., A.D.; Writing and revisions: C.A.S. (original draft), S.D.M; Funding acquisition and project supervision: S.D.M.

Disclosure

This work was supported by a National Science Foundation grant (IOS-1558037) to S.D.M. The authors declare no competing or financial interests. Any use of trade, firm,

or product names is for descriptive purposes only and does not imply endorsement by the U.S. government.

Figures

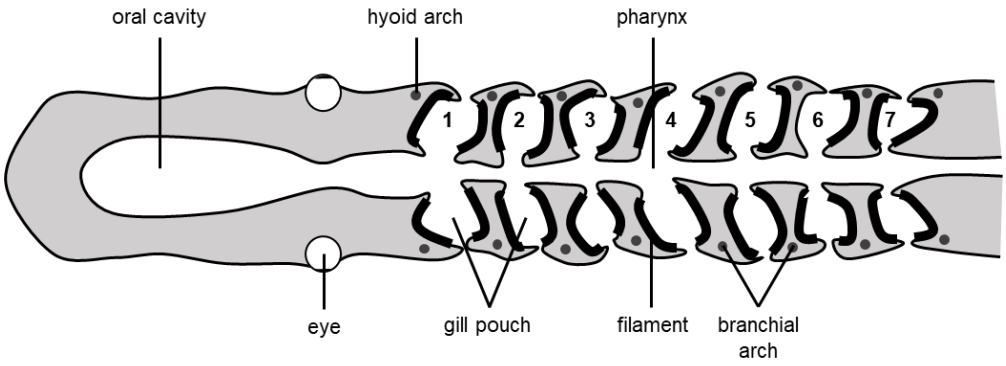

Figure 3.1: Stylized rendering of a longitudinal section along the phaynx of the sea lamprey frontal region. Gill pouches, defined by and including the filaments (thick black lines) contained within the gill pouch cavity, are labeled G1–G7 (anterior–posterior). Hyiod gill arch, which is hypothesized to have formed the gnathosome jaw, is indicated. Redrawn and modified from Wegner (2015).

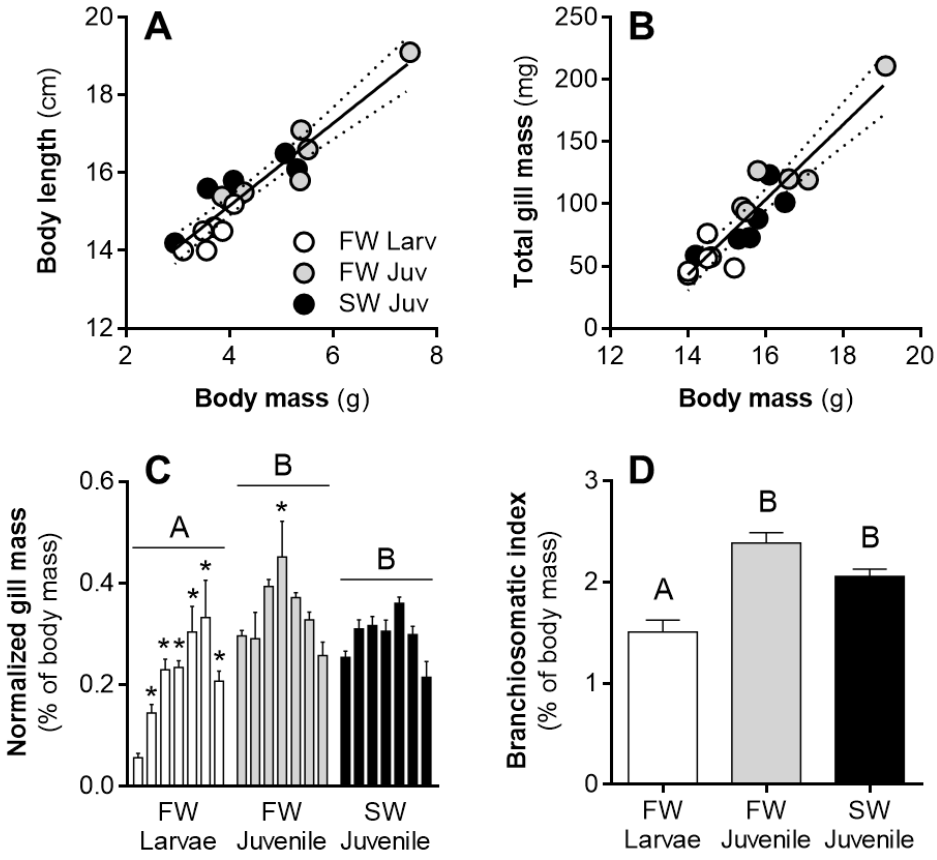

Figure 3.2: Branchiometrics for larvae or juvenile sea lamprey acclimated to freshwater (FW) or seawater (SW). A, B: allometric scaling of body length (A) and total gill mass (B) to body mass. C: mass of each gill pouch pair normalized to body mass. D: branchiosomatic index. In A and B, lines depict best fit (solid line) and 95 % confidence bands (dotted line) (n = 18). In C, gill pouches (G1–G7; anterior–posterior) are presented left-to-right for each life stage. In C and D, letters indicate mean differences between life stages and asterisks indicate difference from anterior-most gill pouch (#1) (two-way (C) or one-way (B) ANOVA; n = 6).

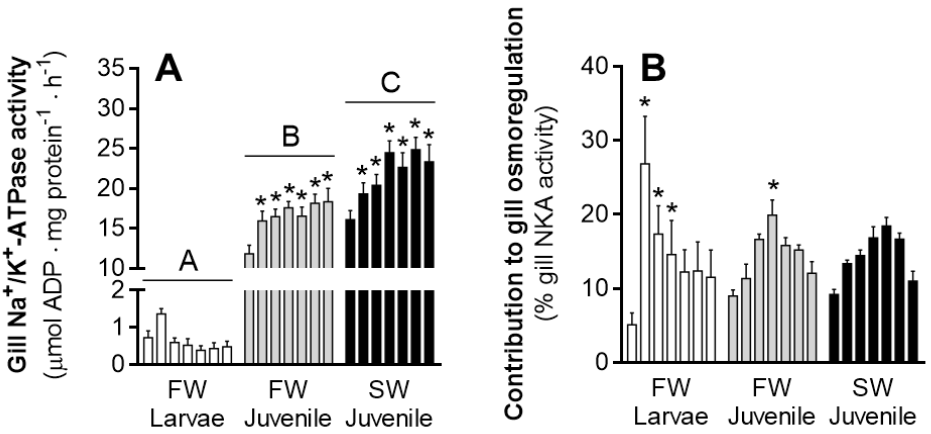

Figure 3.3: Na^+/K^+-ATPase (NKA) activity for each gill pouch (A) and percent contribution to total branchial NKA activity of each gill pouch pair (B) in larvae or juvenile sea lamprey acclimated to freshwater (FW) or seawater (SW). Gill pouches (G1–G7; anterior–posterior) are presented left-to-right for each life stage. Letters indicate mean differences between life stages and asterisks indicate difference from anterior-most gill pouch (#1) (two-way ANOVA; $n = 6$).

CHAPTER 4

11-DEOXYCORTISOL THE ENDOGENOUS CORTICOSTEROID CONTROLLING OSMOREGULATION IN SEA LAMPREY (*PETROMYZON MARINUS*)

Authors

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Abstract

Although the function of the predominant corticosteroid hormones in most vertebrate groups have been well-characterized, the physiological corticosteroids in lamprey, a member of the basal vertebrate group Agnatha, have only recently been identified. Lampreys appear to have a primitive steroid biosynthetic pathway that results in production of 11-deoxycortisol (S) and 11-deoxycorticosterone as the predominant circulating corticosteroids, but their physiological actions and specificity are uncertain. We examined the role of these endogenous corticosteroids during a critical developmental period in the sea lamprey (*Petromyzon marinus*) life cycle, the months-long metamorphosis during which sea lamprey gain seawater (SW) tolerance. We report increases in circulating [S] and gill corticosteroid receptor (CR) abundance are associated with increases in gill Na^+/K^+-ATPase (NKA) activity during metamorphosis. We show

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that *in vivo* treatment with S increases activity and abundance of gill ion transporters and improves SW tolerance. Moreover, we show that S is the only endogenous corticosteroid that has such control over osmoregulation. Together, these results demonstrate that 11-deoxycortisol is an osmoregulatory hormone in lamprey and that regulation of ion transport by corticosteroids is a basal trait among vertebrates.

Introduction

Corticosteroid signaling is central in controlling many physiological functions in vertebrates including metabolism, mood, ion homeostasis, and the stress response. Although the role of corticosteroids in controlling physiological function has been described in most vertebrate groups, the physiological role of corticosteroids and their receptors in the phylogenetically basal vertebrate group Agnatha, represented by extant lamprey and hagfish, is not well understood.

Recent investigations indicate that cortisol (F) and aldosterone (A) are lacking in lamprey serum while 11-deoxycortisol (S) and 11-deoxycorticosterone (DOC), respective steroid biosynthetic precursors to F and A in more derived vertebrates, are present at physiologically relevant levels (Bridgham et al., 2006; Close et al., 2010; Rai et al., 2015). Still it remains unclear whether the Agnathan corticosteroid receptor (CR), which is understood to be ancestral to the appearance and divergence of the mineralocorticoid (MR) and glucocorticoid (GR) receptors of later vertebrates (Eick and Thornton, 2011), has affinity for and is activated by S or DOC or both. For instance, one approach using classical *ex vivo* receptor binding studies demonstrated a lamprey CR with affinity for only S (Close et al., 2010), yet a different approach expressing the lamprey CR in mammalian cells *in vitro* demonstrated the lamprey CR can be activated by several

corticosteroids including S and DOC (Bridgham et al., 2006). Thus, there is a need to better understand the physiological role of the lamprey CR and its endogenous corticosteroid hormone(s). Recently, a call has been made for more *in vivo* studies in lamprey to address this need and provide a more complete understanding of the physiological action, particularly the osmoregulatory role, of corticosteroids and their receptors in basal vertebrates (Rossier et al., 2015).

Sea lamprey (*Petromyzon marinus*) have a complex and fascinating life history that includes a migration from freshwater (FW) to seawater (SW) as juveniles (termed 'anadromy') after a metamorphosis that allows juveniles to parasitize other aquatic vertebrates. As larvae, sea lamprey live burrowed in FW streambeds, filter feeding on suspended organic matter. After 4-6 years of larval life with no SW tolerance, sea lamprey undergo a true metamorphosis from larvae to juvenile, during which major morphological and physiological changes occur, including the development of an eye and a toothy, oral disc (Youson, 1979), proliferation of salt-secreting cells called 'ionocytes' (Bartels et al., 2011), upregulation of hypo-osmoregulatory mechanisms (Beamish et al., 1978; Reis-Santos et al., 2008; Shaughnessy and McCormick, 2020) in the gill, and acquisition of SW tolerance (Beamish et al., 1978; Reis-Santos et al., 2008; Richards and Beamish, 1981; Shaughnessy and McCormick, 2020). After metamorphosis, juvenile sea lamprey migrate downstream to the sea, where they spend 1–3 years parasitizing other aquatic vertebrates and exhibiting rapid growth before returning upstream to spawn and then die.

As in teleost fishes, lamprey have an osmoregulatory strategy in which internal osmotic concentration remains relatively constant irrespective of the salinity of the

external environment. In fishes, the transition from FW to SW includes a transformation of the gill epithelium from a site of ion uptake to a site of salt secretion, which is accomplished through the proliferation of ionocytes expressing ion transport proteins necessary for Na^+ and Cl^- secretion. The transcellular secretion of Cl^- and paracellular secretion of Na^+ are known to involve basolateral Na^+/K^+-ATPase (NKA) and $Na^+/K^+/2Cl^-$-cotransporter (NKCC1) (Marshall and Grosell, 2006). Corticosteroid control of salt-secretory mechanisms and SW tolerance in teleost fishes are well-documented (Takahashi and Sakamoto, 2013). Numerous studies in other euryhaline fishes—those which can survive in both FW and SW—have shown that F has an important role in controlling osmoregulatory processes (Takei and McCormick, 2012). In anadromous salmonids, for instance, circulating levels of F increase during the parr-smolt transformation (just before seawater entry) (McCormick, 2013), *in vivo* treatment with F increases gill Na^+/K^+-ATPase (NKA) activity and SW tolerance (Bisbal and Specker, 1991; McCormick et al., 2008; Shaughnessy and McCormick, 2018; Specker et al., 1994; Veillette et al., 1995), and the osmoregulatory action of F in salmonids is mediated mainly through the glucocorticoid receptor (GR) (McCormick et al., 2008; Shrimpton and McCormick, 1999; Weisbart et al., 1987).

In the present study, we address a conspicuous gap in the current understanding of the role of corticosteroids in the basal vertebrate group Agnatha by using *in vivo* experimentation to determine the physiological importance and specificity of corticosteroid control of osmoregulation in the sea lamprey. We focus on the critical developmental period of metamorphosis and seek to discern (i) whether circulating S and/or gill CR drive osmoregulatory changes during metamorphosis and (ii) whether only

S or both endogenous corticosteroids (S and DOC) control major osmoregulatory changes during sea lamprey metamorphosis.

Materials and methods

Refer to Appendix A for supplementary materials and methods.

Experimental subjects and animal care.

All animal care and experimentation were carried out using protocols approved by the Internal Animal Care and Use Committee at the University of Massachusetts and the U.S. Geological Survey (Protocol number: 2016-0009). Wild larval and pre-metamorphic lamprey were caught in July by electrofishing on the Sawmill River, a tributary to the Connecticut River in Western Massachusetts, USA. After capture, animals were held in 1.5 m diameter flow-through tanks supplied with Connecticut River water with 10 cm of sand. For the metamorphic profile, larval and metamorphosing lamprey were randomly selected for sampling each month from August to December. For hormone treatment experiments, mid-metamorphic lamprey were held in aquaria (80 L) containing dechlorinated, filtered, and aerated recirculating municipal fresh water kept at 15 °C. Artificial seawater was made using a commercial sea salt mix (Crystal Sea Salt, USA).

In vivo experimentation

Salinity tolerance testing and *in vivo* hormone treatments were performed in early October on mid-metamorphic (early stage 7) (Youson, 1979). In salinity tolerance tests, lamprey were exposed to varying salinities (15, 20, or 25 ‰) or a FW control and sampled for blood after 24 h. For hormone treatments, lamprey were anesthetized with

MS-222 (100 mg L^{-1} buffered by $NaHCO_3$, pH 7.4) and injected intraperitoneally with vehicle alone (Veh; 1:1, oil:shortening) or Veh containing S or DOC, then held in freshwater for 12 d prior to sampling in FW or after a 24 h exposure to 25 ‰ SW. To account for changes which may be occurring naturally during metamorphosis, uninjected lamprey were sampled on the day of injections as a time = 0 control (T_0).

Tissue sampling and analysis

Lamprey were euthanized in MS-222 (200 mg L^{-1} buffered by $NaHCO_3$, pH 7.4). Blood was collected via caudal transection into heparinized glass hematocrit tubes and plasma was separated following centrifugation. Gills were dissected and frozen at -80 °C. Gill tissue for enzyme analyses were placed in SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) before freezing. Plasma [Cl^-] was measured using a digital chloridometer (Haake Buchler Instruments Inc., USA).

Na^+/K^+-ATPase activity assay

NKA activity was determined by measuring the ouabain-sensitive ADP production of gill homogenates (McCormick, 1993). Gill tissue was thawed, homogenized in SEID buffer (SEI with 0.1 % deoxycholate) and centrifuged at 3,000 *g* for 5 min. The resulting supernatant was used in an enzyme-linked kinetic assay, which couples ADP production to NADH reduction in a 1:1 ratio to determine ATPase activity. Protein concentration was determined spectrophotometrically using a bovine serum albumin (BSA) standard curve (BCA Protein Assay, Pierce, USA) and the ouabain-sensitive ATPase activity expressed as μmol ADP mg $protein^{-1}$ h^{-1}.

Radioimmunoassay for analysis of plasma [S]

Plasma [S] was measured using a competitive radioimmunoassay (RIA) (Close et al., 2010). Plasma (10 μL) was incubated overnight with 5,000 cpm ^{3}H-labeled 11-deoxycortisol ([^{3}H]S) and a commercial antibody specific to S to achieve 50 % binding (Fig. S1). Unbound ligand was removed via centrifugation and the radioactivity of the antibody-bound fraction of each sample was compared to a standard curve to interpolate plasma [S].

Radioreceptor binding assays for analysis of gill corticosteroid receptor.

Corticosteroid receptor binding assay was modified from previous methods (1, 20). Pools of frozen gill tissue were homogenized on ice, centrifuged and the resulting supernatant was aliquoted for binding analyses and protein content determination. Samples were incubated on ice for 2 h in buffer containing [^{3}H]S (2.5–20 nM) with or without excess [cold]S. Unbound ligand was bound to charcoal and removed via centrifugation and the radioactivity of the receptor-bound fraction was measured and analyzed for total (B_T), non-specific (B_{NS}), and specific binding (B_S), binding capacity (B_{max}), dissociation constant (K_d), and ligand-specificity (Fig. S2).

Gill mRNA and protein analyses

Gill NKA and NKCC1 mRNA and protein abundance was analyzed by quantitative real-time polymerase chain reaction (qPCR) and Western blotting using previously validated molecular primers and antibodies (Shaughnessy and McCormick, 2020), respectively.

Results

Refer to Appendix A for supplementary results.

Increases in plasma [S] and gill CR abundance are associated with gill NKA activity during metamorphosis

Gill NKA activity in early- and mid-metamorphic lamprey (Aug-Sep) remained near larvae levels at ~1-2 μmol ADP mg^{-1} h^{-1} and increased to ~9 μmol ADP mg^{-1} h^{-1} by the later stages and completion (Oct-Nov) of metamorphosis (Fig. 4.1A). Plasma [S] increased above larval levels of ~1 to ~8 ng mL^{-1} in the final month of metamorphosis (Fig. 4.1B). Significant effects of 'life stage', 'month', and 'interaction' were observed for gill NKA activity and plasma [S], and a significant effect of the 'interaction' was observed for gill CR abundance (B_{max}) (Table S3). Gill CR abundance in larvae remained at ~100-150 fmol mg^{-1} throughout the time period studied, however gill CR abundance in metamorphosing lamprey increased 3-fold from ~90 in Aug to ~200 fmol mg^{-1} in Nov (Fig. 4.1C). The relationship between gill NKA activity and plasma [S] was best described as hyperbolic (y_{max} = 11.54 ± 2.34 μmol ADP mg^{-1} h^{-1}; $y_{50\%}$ = 3.66 ± 1.56 ng mL^{-1}; r^2 = 0.52; Fig. 4.2A and Table S4). The relationship between gill NKA activity and gill CR abundance was best described as linear (P = 0.003; $F_{1,10}$ = 14.74; m = 0.06 ± 0.02; r^2 = 0.60; Fig. 4.2B and Table S4). The dissociation constant of the gill CR for S (K_d = ~1-3 nM) was not different between larval and metamorphic lamprey (Fig. S5). The binding affinity hierarchy of the gill CR to corticosteroids was S > DOC >> F = A (Fig. S2C); in mid-metamorphic juveniles, the gill CR had significantly greater binding affinity (IC_{50}; half-maximal inhibition) for S than DOC, F, or A.

11-Deoxycortisol promotes SW tolerance in metamorphosing lamprey

Plasma [Cl^-] and [S] in FW control lamprey (transferred from FW to FW) remained at baseline levels of ~100 mM and ~1 ng mL^{-1}, respectively. Exposure to elevated salinities for 24 h resulted in a salinity-dependent increase in plasma [Cl^-] and plasma [S] (Fig. S6). After 24 h exposure to the highest salinity tested (25 ‰), plasma [Cl^-] was ~160 mM (Fig. S6A) and plasma [S] had risen to ~13 ng ml^{-1} (Fig. S6B). No changes in plasma [S] were detected in fully-metamorphosed lamprey exposed to SW (Fig. S6C). Similar increases plasma [Cl^-] were seen in the Veh controls transferred to SW (25 ‰) (Fig. 4.3A). After 24 h exposure to SW, plasma [Cl^-] in lamprey treated with 10 and 50 μg g^{-1} S (~140 and ~120 mM, respectively) were both maintained closer to FW control levels and significantly lower than in the Veh control (~155 mM) (Fig. 4.3A). Gill NKA activity was significantly higher in S-treated lamprey (~6-7 μmol ADP mg^{-1} h^{-1}) than the Veh control (3 μmol ADP mg^{-1} h^{-1}) (Fig. 4.3B). Lamprey treated with S had higher levels of gill *nka* (Fig. 4.3C) and *nkcc1* (Fig. 4.3D) mRNA compared to the Veh control. Lamprey treated with 50 μg g^{-1} S also had increased gill NKA (Fig. 4.3E) and NKCC1 (Fig. 4.3F) protein abundance over the Veh control. Larval lamprey treated with S showed no significant increase in gill NKA activity (Fig. S7A). After 24 h exposure to 12 ‰, plasma [Cl^-] in all larval groups were elevated and there was no significant effect of S treatment (Fig. S7B).

Only one endogenous corticosteroid, 11-deoxycortisol, stimulates lamprey osmoregulation

Intraperitoneal corticosteroid treatment resulted in a dose-dependent elevation of plasma 11-deoxycortisol concentration (Fig. 4.4B). Compared to the Veh control (~2 μmol ADP

mg^{-1} h^{-1}), gill NKA activity was significantly elevated by treatment with S to ~5 μmol ADP mg^{-1} h^{-1}, but treatment with DOC did not affect gill NKA activity (Fig. 4.4C). Similarly, gill NKA protein abundance was significantly elevated (~3-fold) by treatment with S but not DOC (Fig. 4.4D). Similarly, gill NKCC1 protein abundance was significantly elevated (~6-fold) by treatment with S but not DOC (Fig. 4.4E).

Discussion

This work provides many novel lines of evidence that S is the primary corticosteroid hormone responsible for controlling osmoregulation in sea lamprey, the basal extant osmoregulating vertebrate: (i) plasma [S] and gill CR abundance are upregulated during metamorphosis when natural increases in salinity tolerance occur, (ii) plasma [S] is upregulated during SW exposure, (iii) treatment with S upregulates osmoregulatory mechanisms and increases SW tolerance, and (iv) treatment with the only other known circulating corticosteroid, DOC, does not upregulate osmoregulatory mechanisms.

To understand how corticosteroids control osmoregulation in lamprey, it is necessary to observe the nature of endogenous osmoregulatory and endocrine changes that occur during metamorphosis and SW acclimation. In the present study, we show large increases in gill NKA activity during the later stages of metamorphosis (Oct and Nov) occurred along with increases in plasma [S] and gill CR abundance. The increase in gill NKA activity during metamorphosis corresponds with what has been observed previously with gill NKA (activity and protein abundance; (Beamish et al., 1978; Reis-Santos et al., 2008; Shaughnessy and McCormick, 2020) as well as gill NKCC1 protein abundance (Shaughnessy and McCormick, 2020). An increase in gill CR abundance was

detected earlier in metamorphosis (Oct) compared to that of plasma [S] (Nov). It makes intuitive sense that an increase in the intrinsic capacity of the lamprey gill to receive a corticosteroid signal (*i.e.*, increase in gill CR abundance) would occur prior to an elevation of the concentration of circulating corticosteroid. The opposite case—upregulating plasma [S] before increasing gill CR abundance—would less efficiently utilize the circulating hormone. A similar endocrine program is present in anadromous salmonids in which, along with circulating F, GR abundance (as measured by binding studies similar to those used in the present study) is upregulated during the parr-smolt transformation ahead of SW entry (Kiilerich et al., 2007; Kiilerich et al., 2011a; Mizuno et al., 2001; Nilsen et al., 2008; Shrimpton, 1996). In teleost fishes, the GR is known to be important in mediating osmoregulatory adaptation (Cruz et al., 2013; Shrimpton and McCormick, 1999). The hyperbolic relationship between lamprey gill NKA and plasma [S] shown in the present study suggests a saturating potential of the hormone—at some concentration of circulating S, the CRs in the gill become saturated and a further increase in plasma [S] does not further stimulate gill NKA activity. Indeed, the apparent saturating effect of [S] on gill NKA activity occurs around ~3–5 ng mL^{-1} (Fig. 4.2; NKA activity at 50 % = 3.7 ± 1.6 ng mL^{-1} = ~10.6 nM), which closely approximates the range of calculated values for K_d and IC_{50} (~2–3 and ~15 nM, respectively). Conversely, gill NKA activity was more directly related to increases in gill CR abundance, suggesting that the receptor is important in directly mediating the corticosteroid signal and affecting an osmoregulatory response in the metamorphic sea lamprey. In anadromous salmonids, increases in GR abundance during the parr-smolt transformation is regulated by growth hormone (GH) (Shrimpton et al., 1995). An

analogue to GH has been identified in sea lamprey (Kawauchi et al., 2002), but more work is needed to determine whether osmoregulation is controlled by a similar endocrine program including GH signaling in this basal anadromous fish.

Mid-metamorphic (early-Oct) lamprey exposed to elevated salinity elicit a salinity-dependent increase in both plasma [Cl^-] and [S], but fully metamorphosed lamprey do not respond to elevated salinity with increased plasma [S] (Fig. S6) or plasma osmolality (Beamish et al., 1978). These data suggest that SW exposure too early in metamorphosis (before hypo-osmoregulatory preparation is complete) is either stressful or that upregulation of circulating [S] is signaling to promote a hypo-osmoregulatory response to SW exposure, or both. Thus, S signaling seems important for lamprey to initiate osmoregulatory changes necessary to survive in SW when osmoregulatory capacity is low.

In vivo treatments with cortisol have proven a useful method for establishing its role in osmoregulatory control in other fishes, and so we used a similar approach to characterize the osmoregulatory role of S in metamorphic sea lamprey. Our hormone treatment and salinity experiments were carried out in October, which we had determined to be the best time to conduct these experiments as it was after the significant increase in gill CR abundance, but prior to the endogenous increases in plasma [S] and peak gill NKA activity. During this time frame, SW tolerance is higher than ammocoetes (which can only tolerate 12 ‰) but still lower than fully transformed juveniles. After 24 h in elevated salinity, metamorphosing lamprey in October experienced salinity-dependent loss of osmotic homeostasis, evidenced by salinity-dependent increases in plasma [Cl^-] to well above ~120 mM [Cl^-], which is the maximum ion disturbance experienced by fully

metamorphosed juveniles (Reis-Santos et al., 2008). Prior treatment with S clearly improved SW tolerance as evidenced by a greater capacity to maintain lower levels of plasma [Cl^-] after SW exposure. The effect of S on plasma [Cl^-] and ionoregulatory mechanisms is dose-dependent, which is evidence of its role as a hormone acting through a receptor. The enhanced osmoregulatory ability in lamprey treated with S is likely due to the corresponding increases in gill ion transporters (NKA and NKCC1) observed. Similar increases in gill NKA and NKCC1 have been observed in conjunction with higher levels of salinity tolerance in teleost fishes treated with F (Takei and McCormick, 2012).

In addition to characterizing the role of S and CR during metamorphosis, we sought to investigate the specificity of corticosteroid action through *in vivo* treatments with several endogenous and later-evolved corticosteroids. We confirmed that our method of intraperitoneal injection with S achieved dose-wise increases in circulating [S] within a physiological range (Fig. 4.1 and Fig. 4.4). and increased gill NKA and NKCC1. Due to the similarity in molecular structure among the various corticosteroids used in this study, it is likely that each corticosteroid treatment achieved a similar plasma concentration. In this experiment, we again observed a stimulating effect of S on NKA and NKCC1 and on hypo-osmoregulation, but observed that the other endogenous corticosteroid, DOC, did not alter these parameters critical to SW osmoregulation. The lack of effect of DOC could be explained by the relatively low binding affinity for DOC compared to S in the lamprey gill, as first reported by Close et al. (Close et al., 2010) and now replicated in the present study. Perhaps also *in situ* inactivation of DOC by a reductase (*e.g.*, 5α-reductase) or some other pathway of steroid catabolism is occurring in

the gill, much like how hydroxysteroid dehydrogenases (HSDs) are known to regulate tissue-specific physiologic actions to corticosteroids in other vertabrates (Baker, 2003).

Interestingly, we were unable to stimulate an osmoregulatory response in larvae lamprey with an identical *in vivo* approach of S treatment despite larvae having apparently adequate gill CR abundance (B_{max} ~100-150 fmol mg^{-1}) to bind the administered S. It could be that cell type-specific expression of the lamprey CR is modulating differences in osmoregulatory action of S between larvae and metamorphic lamprey—that the CR is expressed in gill ionocytes in metamorphic but not larval lamprey. Immunological studies using antibodies raised against the lamprey CR are needed to better understand the physiological role and localization of the lamprey CR. Nonetheless, the role of S on osmoregulation metamorphosing lamprey is apparent—the correspondence of plasma [S] and calculated gill CR K_d and IC_{50} values, the concordant rise in systemic S and gill CR, and the specific S effects on the gill ionoregulatory apparatus combine to suggest S is an important hormone contributing to development of SW tolerance in lamprey.

In conclusion, the present study provides much needed *in vivo* evidence and physiological context to earlier investigations and conflicting reports regarding corticosteroid function in the basal Agnathans. Our results demonstrate that a receptor-mediated signal from S, and not DOC, is a major endocrine process controlling hypo-osmoregulation in the sea lamprey. This work inspires more *in vivo* and *in vitro* research into corticosteroid function in Agnathan physiology, particularly regarding enzymatic and/or protein-protein regulatory mechanisms which may be occurring at the tissue-level to organize differential responses to corticosteroids, particularly S and DOC. That

lamprey exhibit discriminative corticosteroid control of osmoregulation (*i.e.*, stimulation by S but not DOC) indicates that such corticosteroid function appeared early in vertebrate evolution, perhaps in association with the appearance of an osmoregulatory strategy, which has been maintained in most later-evolved vertebrates.

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Author contributions

C.A.S., A.B., and S.D.M. conceived of the project and conducted all live animal experimentation. C.A.S. performed molecular, radioimmunoassay, and receptor binding analyses. C.A.S. and A.B. performed enzyme activity analyses, data curation, and statistical analyses. C.A.S., A.B., and S.D.M. wrote and revised the original draft. S.D.M. was responsible for funding acquisition and project supervision.

Disclosure

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trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. government.

Figures

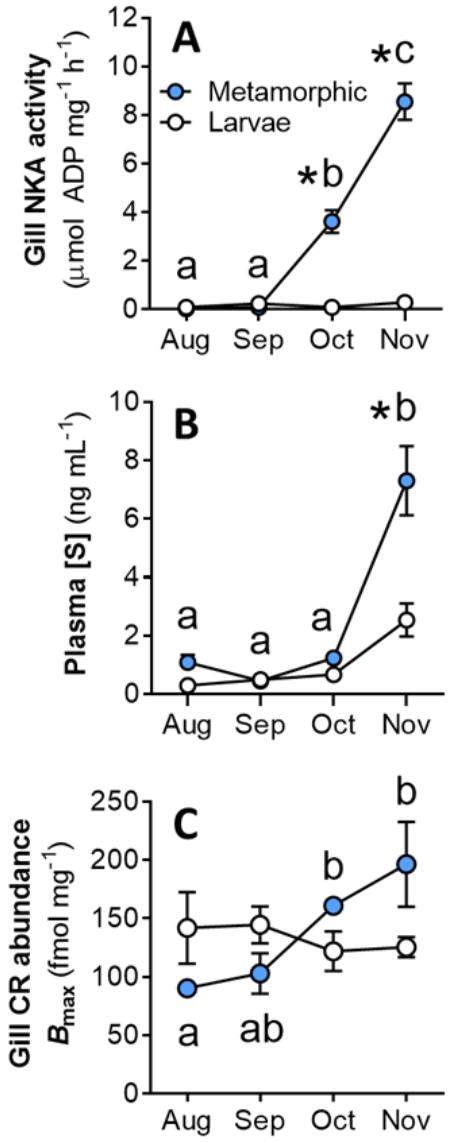

Figure 4.1: Changes in (A) gill Na^+/K^+-ATPase (NKA) activity (n = 8-12), (B) plasma 11-deoxycortisol concentration ([S]) (n = 8-12), and (C) gill corticosteroid receptor (CR) abundance (B_{max}) (n = 3) in metamorphic (blue) or larval (white) sea lamprey. Values represent mean ± s.e.m. Letters indicate differences among metamorphic group and asterisks indicate differences from time-matched larvae group (two-way ANOVA, Tukey's *post hoc*). See Supplementary Table S3 for two-way ANOVA results.

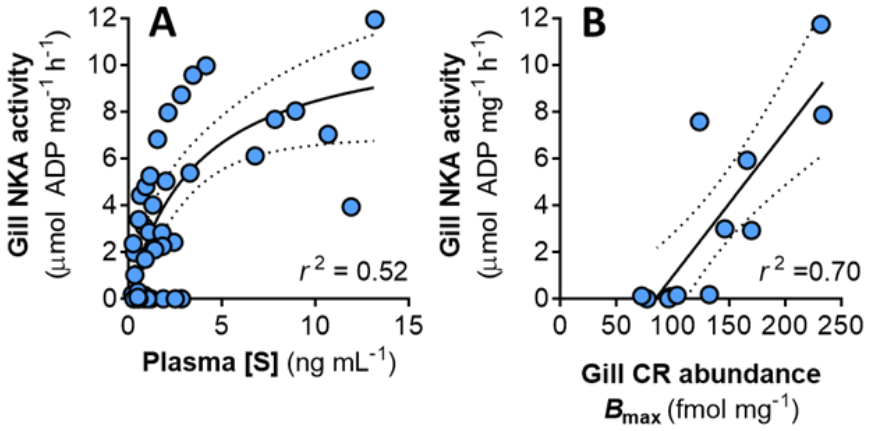

Figure 4.2: Relationships between gill Na^+/K^+-ATPase (NKA) activity and (A) plasma 11-deoxycortisol concentration ([S]) or (B) gill corticosteroid receptor (CR) abundance (B_{max}) in metamorphic sea lamprey. Data from metamorphic lamprey are fit by (A) hyperbolic or (B) linear ($P < 0.001$) regression (black line); dotted lines represent 95 % confidence bands. See Supplementary Table S4 for curve-fitting results.

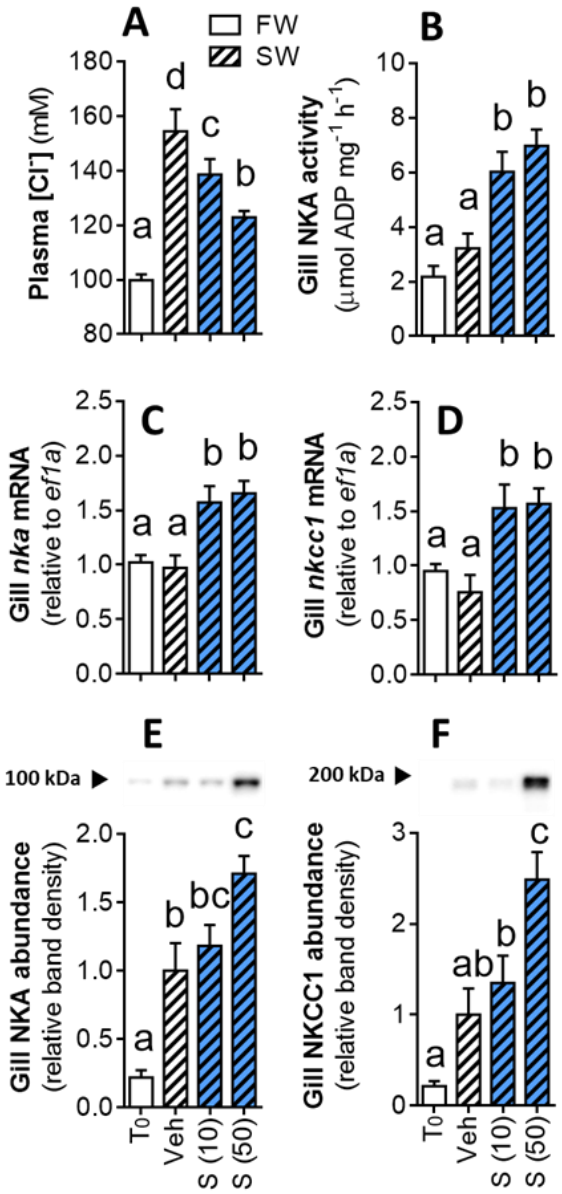

Figure 4.3: Changes in (A) plasma chloride concentration ([Cl^-]), (B) gill Na^+/K^+-ATPase (NKA) activity, (C-D) relative gill mRNA abundance of *nka* and *nkcc1*, and (E-F) representative bands and quantification of gill NKA and NKCC1 protein abundance in mid-metamorphic sea lamprey administered 11-deoxycortisol (S) treatment and then exposed to 25 ‰ SW for 24 h. Dose of S is stated in parenthesis (μg g^{-1} body weight). Values represent mean ± s.e.m and letters indicate differences (n = 8-10, one-way ANOVA (A-F: $P < 0.001$), Tukey's *post hoc*).

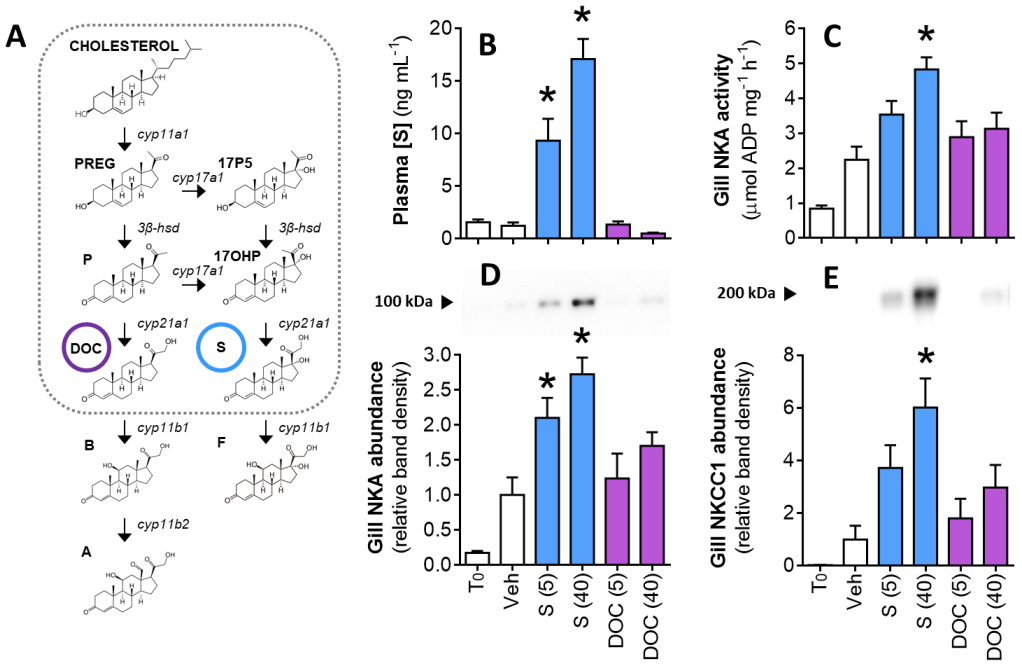

Figure 4.4: (A) Corticosteroid biosynthetic pathway; dotted box represents the pathway present in sea lamprey, which lack the gene *cyp11b1*. Changes in (B) plasma 11-deoxycortisol concentration ([S]), (C) gill Na^+/K^+-ATPase (NKA) activity, and (D-E) representative bands and quantification of gill NKA and NKCC1 protein abundance in mid-metamorphic sea lamprey administered corticosteroids in fresh water. Dose of S and DOC is stated in parenthesis (μg g^{-1} body weight). Values represent mean ± s.e.m. and asterisks indicate difference from Veh (n = 8-12, one-way ANOVA (B-E: $P < 0.001$), Dunnett's *post hoc*).

CHAPTER 5

CORTICOSTEROID CONTROL OF Na^+/K^+-ATPASE ACTIVITY IN THE INTESTINE OF SEA LAMPREY (*PETROMYZON MARINUS*)

Authors

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Abstract

Anadromous sea lamprey (*Petromyzon marinus*) larvae undergo a months-long true metamorphosis during which they develop seawater (SW) tolerance prior to SW entry. The present study investigated the role of 11-deoxycortisol (S) in controlling osmoregulatory mechanisms, namely Na^+/K^+-ATPase (NKA), in the sea lamprey anterior (AI) and posterior (PI) intestine during metamorphosis. In a tissue profile, *nka* mRNA was most highly expressed in the gill, kidney, and AI. During metamorphosis, *nka* mRNA increased 10-fold from larvae to juvenile in the AI. The sea lamprey AI had a high binding affinity for S compared to 11-deoxycorticosterone (DOC), and *in vivo* administration of S in mid-metamorphic lamprey upregulated NKA activity 3-fold in the AI and PI, whereas administration of DOC had no effect on intestine NKA activity.

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During a 24 h SW challenge test, dehydration of white muscle moisture was rescued by prior treatment with S, which was associated with increased intestinal *nka* mRNA and NKA activity. *In vivo* administration with corticosteroid receptor antagonists did not block the endogenous increase in intestinal NKA activity; spironolactone and eplerenone increased NKA activity in AI during metamorphosis. Together, these results indicate that intestinal osmoregulation in sea lamprey is a target for control by S during metamorphosis and the development of SW tolerance.

Introduction

Fossil records indicate that lampreys have inhabiting marine and estuarine environments and remained relatively morphologically unchanged since at least the late Devonian, ~360 mya (Gess et al., 2006). Modern lampreys are iono- and osmoregulators, maintaining a constant internal osmolality at ~300 mOsm kg^{-1} (approximately one-third that of seawater; SW), a strategy shared by most other vertebrate species including the teleost fishes (Hughes et al., 2018). Sea lamprey (*Petromyzon marinus* L.), which are a basal anadromous fish, live as larvae in freshwater (FW) for 4-6 years before metamorphosing and migrating into SW as juveniles. The larvae-to-juvenile metamorphosis takes many months and involves a radical transformation from a substrate-dwelling, filter-feeding larvae into a free-swimming, parasitic juvenile. Juveniles spend another 2-3 years in the sea before returning to FW as reproductively mature adults to spawn and die. The capacity for osmoregulation in SW is very low in larval sea lamprey but increases dramatically during metamorphosis (Barany et al., 2020; Beamish et al., 1978; Reis-Santos et al., 2008; Shaughnessy and McCormick, 2020).

Transition from FW to SW life requires a rearrangement of osmoregulatory processes. Fishes living in FW counteract the passive loss of ions and absorption of water by a process of active, ATP-dependent uptake of ions (primarily Na^+ and Cl^-) across the gill epithelium and removal of excess water via the production of dilute urine. Fishes living in hyperosmotic environments such as SW (~1050 mOsm kg^{-1}) must counteract the passive gain of ions and loss of water. To do this, fishes in SW increase drinking and absorb water in the gut while excreting excess divalent ions (primarily Ca^{2+} and Mg^{2+}) across epithelia in the intestine and kidney and secrete monovalent ions across the gill epithelium (Marshall and Grosell, 2006).

Ingested SW is consecutively processed throughout the gut. Ingested SW is progressively desalinated as it moves through the gut until approximately isosmotic with respect to the blood plasma (Grosell and Taylor, 2007; Skadhauge, 1969), thus allowing for simultaneous net water absorption in the intestine via two possible paths: transcellularly, in which aquaporins are involved (Cerdà and Finn, 2010; Whittamore, 2012; Wilson et al., 2002), and paracellularly (Preston et al., 1992). Both paths for water absorption from the lumen are driven by the osmotic gradient, suggesting Na^+ and Cl^- transport is an important driver of the reduction of gut osmolality (up to ~50 %) (Grosell et al., 2005; Musch et al., 1982). Transcellular movement of Na^+ is driven by basolateral Na^+/K^+-ATPase (NKA), which exchanges extracellular K^+ for intracellular Na^+ with a 3:2 stoichiometry, thus providing an electrical gradient that favors movement of Cl^- into the blood through voltage-gated ion channels in intestinal epithelial cells. The excess of monovalent ions taken up by the gut in order to desalinate the imbibed SW is secreted by the gills (Grosell, 2006; Hirano and Mayer-Gostan, 1976); other SW-derived ions such as

Ca^{2+} are incorporated into biological functions, including bone growth and muscle function. In teleosts, elevated levels of NKA activity have been found in the gut and gill after SW acclimation (Grosell, 2006; Grosell et al., 2005; Tresguerres et al., 2010).

Corticosteroids mediate many bodily functions in vertebrates, including osmoregulation in fishes. In vertebrates, corticosteroids are responsible for controlling, among other functions, metabolism, immunology, osmoregulation, and stress responses. This is accomplished through two major corticosteroid signaling pathways, glucocorticoid function controlling metabolism and growth and mineralocorticoid function controlling transport of ions and water. In non-avian tetrapods, two distinct hormones, cortisol (F) and aldosterone (A), serve these respective functions by activating their respective receptors. The glucocorticoid receptor (GR) is activated by cortisol and the mineralocorticoid receptor (MR) is activated by aldosterone. In teleost fishes, it is generally accepted that cortisol acts as both a glucocorticoid and mineralocorticoid (McCormick et al., 2008; Mommsen et al., 1999), whereas aldosterone circulates in blood only in extremely low levels and is not responsive to stress and osmoregulatory stimuli (Bury et al., 2003; Prunet et al., 2006). Like cortisol, 11-deoxycorticosterone (DOC) is also present in the blood of teleosts in significant concentrations, but it interacts only with the teleost MR, not the GR (Kiilerich et al., 2011b; Milla et al., 2006; Milla et al., 2008; Sakamoto et al., 2011). As a result, DOC has been proposed as a teleost mineralocorticoid (Bury and Sturm, 2007; Prunet et al., 2006; Stolte et al., 2008; Sturm et al., 2005). Molecular studies of the fish MR and GR indicate that cortisol and DOC both activate the MR, whereas the fish GR is only activated by cortisol at physiological concentrations (Bury and Sturm, 2007; Stolte et al., 2008; Sturm et al., 2005). Several

studies in teleosts have shown cortisol to control osmoregulatory function in many tissues in fishes, including the intestine (Takahashi and Sakamoto, 2013; Utida et al., 1972).

In lamprey, neither cortisol nor aldosterone are present in the blood, and 11-deoxycortisol (S; a biosynthetic precursor to cortisol) has been established as a putative corticosteroid hormone in lamprey (Close et al., 2010; Rai et al., 2015). Lamprey also have a corticosteroid receptor (CR) that is ancestral to the diverged GR and MR of later-evolved vertebrates (Eick and Thornton, 2011). In sea lamprey, the CR is most highly abundant in the gill, intestine, and testes and treatment with S *in vivo* upregulated gill NKA activity (Close et al., 2010). Whether osmoregulatory processes in the lamprey intestine are also controlled by S has not been established.

The present study aimed to establish the role of endogenous lamprey corticosteroids in mediating osmoregulatory processes in the sea lamprey intestine. We focused on the sea lamprey metamorphosis, when increases in osmoregulatory mechanisms in the gill and intestine lead to the development of SW tolerance. Specifically, we examined whether endogenous corticosteroids were able to upregulate NKA activity in intestinal regions during metamorphosis.

Materials and methods

Sea lamprey collection and care

All animal care and use procedures were approved by the Internal Animal Care and Use Committee at the University of Massachusetts and U.S. Geological Survey. Sea lamprey for the metamorphic profile were collected in July to November from a tributary of the Connecticut River (Massachusetts, USA) by electrofishing (larvae to stage 7) or

Fyke net capture (downstream migrants). The metamorphic stages were determined according to Youson (1979). All other individuals were collected as stage 6 and 7 by netting from the Connecticut River adjacent to the laboratory, where all fish were held after collection. Once in the laboratory, mid- and post-metamorphic individuals were not offered food as the naturally cease feeding until the enter the begin parasitic feeding in the ocean. Rearing and all FW and SW experimentation were carried out in 15 °C dechlorinated FW in 60 L recirculating glass aquaria equipped with aeration and mechanical, chemical, and biological filtration. Experimental SW was made by dissolving artificial sea salt mix (Crystal Sea Salt, Baltimore, MD, USA) in FW.

Laboratory experiments

For the tissue profiles, lamprey were acclimated to identical FW or SW conditions for 3 weeks before sampling. For the injection experiments, mid-metamorphic lamprey acclimated to FW were anesthetized in MS-222 (200 mg L^{-1} buffered with $NaHCO_3$, pH 7.0) (Argent Chemical Laboratories, Redmond, WA, USA) then administered a slow-release implant containing either steroid, receptor blocker, or vehicle only (1:1, oil/shortening; Veh) and allowed to fully recover (~30 min) before returning to experimental tanks. Lamprey administered hormones (S or DOC) were held in FW for 12 d, then either sampled in FW, or sampled after 24 h exposure to SW. Lamprey administered receptor blockers (spironolactone, SPIRO; mifepristone, RU486; or eplerenone, EPL) were maintained for 3 weeks then sampled in FW. In each experiment, uninjected lamprey in FW were sampled on the day of injections as a time = 0 control (T_0).

Sampling

Sea lamprey for the metamorphic profile were sampled immediately after capture in the field. Lamprey were euthanized in MS-222 (400 mg L^{-1} buffered with $NaHCO_3$, pH 7.4) then sampled for various tissues. Muscle (~0.2 g) was taken from a mediolateral location, blotted dry, weighed ('wet mass'), then dried for two days at 60 °C. Dried muscle samples were reweighed ('dry mass') and the difference between 'wet mass' and 'dry mass' (presumably from evaporation of water) was used to calculate values for muscle moisture (% water). Two sections of intestine were collected with fine-point scissors: anterior intestine (AI), considered as the intestinal section beginning at the end of the esophagus (1–1.5 cm); and posterior intestine (PI), considered as a section of distal intestine delimited by the posterior/rectal sphincter (2–3 cm). Samples for receptor binding, mRNA, protein analyses were immediately frozen and stored at -80 °C. Samples for NKA activity analysis were placed in SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.4) before immediate freezing and storage.

Western blotting

Tissues (~30 mg) were homogenized in 150 µL SEID (0.1 % sodium deoxycholate in SEI buffer, pH 7.4) and centrifuged at 2,000 g for 5 min at 4 °C. The resulting supernatant was aliquoted for either protein determination (BCA protein assay, Thermo Scientific, Rockford, Il, USA) or diluted in 2X Laemmli buffer and heated for 15 min at 60 °C and stored at -80 °C for later use in the Western blotting protocol. Samples (10 µg) were electrophoretically separated on a 7.5% SDS-PAGE gel (Bio-Rad, Hercules, CA, USA) and transferred onto Immobilon PVDF transfer membranes (Millipore, Bedford, MA, USA) in transfer buffer (25 mmol l^{-1} Tris, 192 mmol l^{-1}

glycine, pH 8.3), which were stored dry overnight. Identical pooled samples were run on each gel and subsequent membrane to account for any potential inter-blot variation. Membranes were rehydrated in methanol, equilibrated in PBST (phosphate-buffered saline with 0.05% Triton X-100), and blocked in blocking buffer (PBST with 5 % nonfat milk) for 1 h at room temperature. Membranes were then probed with 1:4,000 dilution of a mouse monoclonal anti-NKA primary antibody ('α5'; RRID: AB_2166869; Developmental Studies Hybridoma Bank, Iowa City, IA) overnight at 4 °C, washed in PBST, then probed with a 1:10,000 dilution of a horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MA) for 2 h at room temperature. After a final round of washing in PBST, membranes were imaged via enhanced chemiluminescence (ECL) using a 1:1 mixture of homemade ECL solutions (solution A: 396 μmol L^{-1} coumaric acid, 2.5 mmol L^{-1} luminol, 100 mmol L^{-1} Tris-HCl, pH 8.5; solution B: 0.018% H_2O_2, 100 mmol l^{-1} Tris-HCl, pH 8.5) using Syngene PXi system (SYNGENE Inc., Frederick, MD). Densitometric band intensity was analyzed using ImageJ (National Institutes of Health, Bethesda, MD) and relative band intensity for all tissues was calculated relative to FW brain (set to a value of one).

RNA isolation and polymerase chain reaction

Following the manufacturers' protocol, TRIzol reagent (Molecular Research Center Inc., Cincinnati, OH) was used to isolate total RNA from frozen intestinal tissue, and Take3 micro-volume plate (BioTek Instruments, Inc., Winooski, VT) was used to assess concentration and purity of each RNA sample. Only samples which were determined to have high-purity (1.9 < A260/A280 > 2.2) were used for cDNA synthesis

and real-time quantitative polymerase chain reaction (qPCR). A High-Capacity Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) was used to synthesize first-strand cDNA used in qPCR reactions using SYBRSelect master mix (ThermoFisher, Waltham, MA). Reactions (10 µL) contained 2 ng cDNA, 150 nM forward and reverse primers, and 1X master mix. Reactions were analyzed using a StepOnePlus Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA) with the following thermal profile: holding (2 min at 50 °C), activation (2 min at 95 °C); 40 cycles: 15 s at 95 °C , 1 min at 60 °C, 30 s at 72 °C. A dissociation step (melt curve analysis, 60 to 95 °C) was used to confirm a single product in each reaction. Relative nka mRNA was calculated using the comparative ($\Delta\Delta C_T$) method (Pfaffl, 2001) with elongation factor 1 (*ef1a*) or glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) used as a reference gene. Primer pairs for *nka*, *ef1a*, and *gapdh* in sea lamprey have been previously reported (Ferreira-Martins et al., 2016; Kolosov et al., 2017).

Receptor binding assay

Our receptor binding assay was modified from the protocol reported by Close et al. (2010). Frozen anterior intestine tissue from mid-metamorphic lamprey were homogenized using a ground glass manual homogenizer in HEPES assay buffer (300 µL: 25 mM HEPES. 10 mM NaCl, 1 mM monothioglycerol, pH 7.4) and kept on ice. Homogenized intestine samples were centrifuged at 2,000 g for 10 min at 4 °C. An aliquot of the resulting supernatant was reserved to determine protein concentration (BCA Protein Assay), and the rest of the supernatant sample was kept on ice for receptor binding analysis. In a non-binding 96-well microplate, 25 µL of sample was incubated with 25 µL of assay buffer (HEPES) containing [^{3}H]S, either alone (total binding; B_T) or

with a [cold]S (non-specific binding; B_{NS}). Each reaction (50 µL) contained intestine sample and 2.5, 5, 10, or 20 nM [^{3}H]S with or without 500-fold excess [cold]S. The intestine protein content in each assay was 4-6 mg mL^{-1}. Each reaction was incubated on ice for 2 h. After incubation, each reaction was incubated for 10 min on ice with dextran-coated charcoal solution (150 µL: HEPES with 0.25 % w/v dextran and 2.5 % w/v activated charcoal). Free [^{3}H]S and [cold]S (unbound to receptor) were removed from solution by centrifugation at 2,000 **g** for 10 min at 4 °C. The final supernatant (100 µL) was mixed with scintillation fluid (2 mL: ECONO-SAFE, Research Products International Corp., USA) and counted in a scintillation counter (LS 6000IC, Beckman Instruments Inc., USA). Specific binding (B_S) was calculated ($B_S = B_T - B_{NS}$) and receptor binding capacity (B_{max}) and equilibrium dissociation constant (K_d) were determined by hyperbolic regression analysis. Binding specificity was analyzed following an identical protocol as above, with the exception that 1 nM [^{3}H]S was incubated in competition with 1, 10, 100, 1000 nM of unlabeled S, DOC, F, and A.

Measurement of Na^+/K^+-ATPase activity

NKA activity was analyzed according to McCormick (1993). Frozen intestinal samples were homogenized in SEID on ice then centrifuged at 2,000 g for 5 min at 4 °C. NKA activity of the supernatant was determined using an enzyme-linked kinetic assay run at 25 °C in a 96-well plate, which links in a 1:1 ratio the reduction of NADH to NAD^+ to the conversion of ATP to ADP by NKA. Supernatant samples were assayed with or without the presence of 1 mM ouabain, a specific inhibitor of NKA at this concentration. The difference in ADP production between reactions with and without ouabain over a 10 min was considered the activity of NKA and reported as µmol ADP

mg^{-1} protein h^{-1}. The depletion of NADH was measured spectrophotometrically at 340 nm by a BioTek microplate reader using SOFTmax software (Molecular Devices, Menlo Park, CA, USA). Protein content of supernatant samples used in the assay were determined using the BCA protein assay.

Calculations and statistics

Muscle moisture was calculated as [(wet mass − dry mass) x 100] / wet mass. All data are represented as the mean ± standard error (n values reported in figure captions). Detection of significant differences were carried out by using unpaired Student´s t-test, one-way ANOVA, or two-way ANOVA, followed by a Tukey's or Sidak's post hoc analysis. All statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). Significance for all tests was set at $P < 0.05$.

Results

Tissue and metamorphic profiles

In a tissue profile of post-metamorphic sea lamprey, NKA protein abundance was highest in brain and kidney in FW-acclimated lamprey and highest in brain, gill, kidney, and AI in SW-acclimated lamprey (Fig. 5.1A). Abundance of nka mRNA abundance was similar between FW- and SW-acclimated lamprey and highest in brain, gill, kidney, and AI (Fig. 5.1B). In a metamorphic profile, nka mRNA did not change throughout metamorphosis in PI, but increased nearly 10-fold from larvae to migrant in AI (Fig. 5.1C).

Receptor binding assay

Specific binding of S was observed in the lamprey AI (B_{max} = 329.4 ± 68.7 fmol mg^{-1}; K_d = 3.5 ± 2.4 nM) (Fig. 5.2A,B). Binding affinity of the lamprey AI was significantly greater for S (IC_{50} = 157 ± 49 nM) than DOC (IC_{50} = 1352 ± 330 nM) (t-test: P = 0.023) (Fig. 5.2C). Binding by F and A were not detected.

Steroid hormone experiments

Prior to S or DOC administration (T_0), intestinal NKA activity in the AI and PI was 2.97 ± 1.33 and 0.91 ± 0.22 μmol ADP mg $protein^{-1}$ h^{-1}, respectively (Fig. 5.3). After 12 d, a dose-wise increase in AI and PI NKA activity was observed. In lamprey administered 40 μg g^{-1} S, AI and PI activity had increased to 11.57 ± 4.10 and 2.50 ± 0.61 μmol ADP mg $protein^{-1}$ h^{-1}, respectively, significantly above the vehicle control. No significant increase in AI or PI NKA activity above the vehicle control was observed in lamprey administered DOC.

Initial muscle moisture in uninjected lamprey (T_0) was 68.0 ± 1.1 % water (Fig. 5.4A). After 24 h SW exposure (12 d post-injection) muscle moisture in the vehicle control significantly decreased to 62.9 ± 3.0 % water. Lamprey administered S did not exhibit reduced muscle moisture after SW exposure; these lamprey had muscle moisture that was not significantly different from the T_0 FW control, but that was significantly higher than the vehicle control in SW. Initial (T_0) AI and PI NKA activity was 8.47 ± 1.90 and 2.86 ± 0.35 μmol ADP mg $protein^{-1}$ h^{-1}, respectively (Fig. 5.4B,C). After 12 d with an S implant, NKA activity in both AI and PI had increased dose-wise and significantly above the vehicle control. In both AI and PI, nka mRNA was not upregulated after 24 h in SW in the vehicle control compared to T_0. In AI nka mRNA was

upregulated in lamprey treated with either dose of S after 24 h in SW compared to the vehicle control (Fig. 5.4D). In PI, only lamprey treated with the 50 µg g-1 S exhibited elevated nka mRNA after 24 h SW exposure (Fig. 5.4E).

Receptor blocker experiment

Prior to administration with receptor blockers (T_0), AI and PI NKA activity was 3.43 ± 1.62 and 1.08 ± 0.54 µmol ADP mg protein^{-1} h^{-1}, respectively (Fig. 5.5). After 3 wk, AI and PI activity in the vehicle control had increased to 9.05 ± 3.53 and 2.39 ± 2.17 µmol ADP mg protein^{-1} h^{-1}, respectively. In AI, SPIRO and EPL treatment increased NKA activity to ~17 µmol ADP mg protein^{-1} h^{-1}, significantly above the vehicle control, but RU486 had no effect on NKA activity. In PI, NKA activity was not affected by treatment with any receptor blocker.

Discussion

In the present study, we demonstrated that the intestine is a target for the endogenous corticosteroid S in sea lamprey and that the intestine has high affinity to S compared to the other endogenous corticosteroid in lamprey, DOC. We further show that S and a mineralocorticoid receptor-like CR may have a role in controlling the endogenous increases of *nka* mRNA and NKA activity in the intestine during the sea lamprey metamorphosis leading to the development of SW tolerance prior to SW entry.

The sea lamprey intestine exhibits regionalized osmoregulatory function and is abundant with NKA. In the present study we show that acclimation to SW increases NKA mRNA and protein expression in osmoregulatory tissues such as the gill, kidney, and intestine. This finding is consistent with other recent studies in sea lamprey (Barany

et al., 2020; Beamish et al., 1978; Ferreira-Martins et al., 2016; Reis-Santos et al., 2008; Shaughnessy and McCormick, 2020). The generally higher NKA activity in AI compared to PI, as described in the present study and previously (Barany et al., 2020), indicates that regionalization of osmoregulatory function is occurring in the sea lamprey intestine. It should be noted that the levels of NKA activity observed in the AI of mid- and post-metamorphic sea lamprey in FW (present study; Barany et al., 2020) are much higher than NKA activity of the AI in adult sea lamprey in FW (Ferreira-Martins et al., 2016). Increases in NKA mRNA (present study) and activity (Barany et al., 2020) in the sea lamprey intestine during metamorphosis, prior to SW entry, indicates that sea lamprey physiologically prepare for marine life before ever reaching the ocean. This phenomenon is akin to the osmoregulatory adjustments made during the parr-to-smolt transformation in anadromous salmonids (McCormick, 2013). Earlier work in salmonids described increases in intestinal fluid transport capacity during smolting and after SW exposure (Collie and Bern, 1982; Veillette et al., 1995) and more recent molecular investigations have demonstrated that claudins, NKCC2, and specific NKA isoforms may be involved in fluid transport in the salmonid intestine in SW (Sundh et al., 2014; Tipsmark et al., 2010). In larvae lamprey (which have no SW tolerance), tight-junction proteins have been identified, but their role in ion and water transport remains unclear (Kolosov et al., 2017). In adult FW lamprey re-exposed to SW, NKA mRNA, protein, and activity were relatively high in the anterior intestine compared to other tissues, and moderately upregulated in SW (Ferreira-Martins et al., 2016). Further studies of the sea lamprey intestine are needed to establish molecular mechanisms of water transport in addition to the role of NKA.

A corticosteroid receptor is abundant in the lamprey intestine and is specific to S. It has been shown previously that the lamprey CR is abundant in the gill and intestine and that the lamprey gill specifically binds S (Close et al., 2010). In the present study, we show for the first time that the lamprey intestine also specifically binds S with significantly higher affinity than the other endogenous corticosteroid, DOC. The receptor-ligand interactions of the lamprey CR are not resolved. *Ex vivo* receptor binding assays (such as the method used in the present study) describe a highly specific receptor for S (Close et al., 2010), whereas *in vitro* transactivation studies describe a receptor that is promiscuous to many corticosteroids including S, DOC, and the later-evolved corticosteroids, corticosterone, cortisol, and aldosterone (Bridgham et al., 2006). In the present study, we report a dissociation constant (~3 mM) of the CR in AI that is similar to what was previously reported using a similar method to study the gill CR (Close et al., 2010). Interestingly, the CR abundance (B_{max}) we report for the AI of metamorphosing sea lamprey (~330 fmol mg^{-1}) is much higher than what has been reported for the intestine of adult lamprey (~60 fmol mg^{-1}; Close et al., 2010). Life-stage differences may account for such differences in intestine CR abundance. Indeed, elevated CR abundance in the AI reported in the present study could indicate the importance of the CR in mediating the very high levels of NKA activity observed in the AI mid- and post-metamorphic sea lamprey metamorphosis compared to adults. It is also unclear which intestinal region was used by (Close et al., 2010) (perhaps it was a transection of the whole intestine) and so regional tissue differences may account for the higher CR abundance observed in the present study—the AI is known to have a higher NKA activity and capacity for osmoregulation than other regions of the lamprey gut (present study;

Barany et al., 2020), which may be due, in part, to higher CR abundance in the AI than other regions of the gut or the gut as a whole. Thus, future investigations are warranted into potential intestinal regionalization of CR expression and regionalized osmoregulatory response to S in regional intestine preparations *ex vivo*.

In vivo treatment with S upregulated intestinal NKA activity. In the 12 d between injection (T_0) and sampling, intestinal NKA did not change post injection in metamorphosing sea lamprey treated with the vehicle only (Veh), which ensures no changes due to metamorphosis complicated the observed responses to the S injection. Although no significant effects on NKA activity were observed after treatment with DOC, the putative physiological ligand for MR in teleost fishes (Prunet et al., 2006). Treatment with S showed a dose-dependant upregulating effect on NKA activity that was similar in magnitude in both the AI and PI. The greater potency of S compared to DOC in upregulating NKA activity is likely due in part to the greater binding affinity of the lamprey intestine CR to S compared to DOC. The *in vivo* results of the present study further indicate that S has a greater osmoregulatory role compared to DOC in lamprey, which is much like the greater osmoregulatory role of cortisol compared to DOC in euryhaline teleosts (Takei and McCormick, 2012).

Previous studies in our lab on lamprey have shown that NKA, among other osmoregulatory mechanisms, are upregulated in main osmoregulatory tissues after acclimation to SW (Barany et al., 2020; Reis-Santos et al., 2008; Shaughnessy and McCormick, 2020). Additionally, an extensive literature primarily of teleost fishes demonstrates that treatment with corticosteroids (*i.e.*, cortisol) improves hyper-osmoregulatory capacity and SW tolerance (Takei and McCormick, 2012). Thus, the

upregulation of intestinal NKA and improved SW tolerance we observed in the sea lamprey after S treatment reflects a role of S that is similar to the role of cortisol in teleost fishes. Treatment with S in in the present study resulted in improved maintenance of hydration after SW exposure. This is likely due to processes related to desalination of imbibed SW and increased intestinal water absorption, such as has been observed in lamprey and teleosts (Barany et al., 2020; Fuentes et al., 1996; Rankin, 2002; Veillette et al., 1995). There is an apparent similarity regarding the role of corticosteroids in controlling the osmoregulatory adjustments between the salmonid parr-smolt transformation (McCormick, 2013) and the lamprey metamorphosis (present study). For instance, in Atlantic salmon, increased intestinal water absorption has been shown to be driven by elevated circulating levels of cortisol during the parr-smolt transformation, even prior to experiencing exposure to SW (Veillette et al., 1995). The corticosteroid-driven increase in hypo-osmoregulatory capacity in both the salmon parr-smolt transformation and the lamprey metamorphosis in preparation for SW entry underscores the importance (and likely fitness-related benefits) of this endocrine program in reducing potential negative impacts of a direct transfer from FW to SW (Barton et al., 1985; Sundell et al., 2003). Improved SW tolerance requires coordinated physiological adjustments (including elevated NKA activity in gills and intestine and increased intestinal water absorption) and ethological adjustments (such as increased drinking to facilitate desalination of imbibed fluid to counteract the overall body surface dehydration) (Fuentes et al., 1996; McCormick et al., 2008; Takahashi and Sakamoto, 2013). Such coordination of physiological and behavioral adjustments to SW exposure appear to be present in lamprey (Barany et al., 2020). Interestingly, even elasmobranchs,

which are osmoconformers and do not drink in SW, upregulate drinking when exposed to artificially higher salinities (Anderson et al., 2002), demonstrating the physiological importance and potentially evolutionarily basal trait of desalination processes related to drinking.

We sought to obtain further direct evidence for the roles of S and the CR in mediating osmoregulatory adjustments during metamorphosis by administering known MR and GR antagonists *in vivo*. In mammalian clinical research, antagonists of MR or GR (such as spironolactone and eplerenone (both derivatives of progesterone) or RU486, respectively) have been used for more than half a century, due to their anti-aldosterone, -cortisol, and -progesterone activity in affecting hypertension, obesity, and pregnancy (Cadepond et al., 1997; Funder, 2013). In fishes, MR and GR antagonists has been used to examine the osmoregulatory roles of the MR- and GR-mediated action of corticosteroids, namely cortisol, with mixed results (Cruz et al., 2013; Kiilerich et al., 2007; Kiilerich et al., 2011b; Marshall et al., 2005; McCormick et al., 2008; Pippal et al., 2011; Scott et al., 2005; Sloman et al., 2001). We predicted that *in vivo* treatment with corticosteroid receptor antagonists early in metamorphosis would block the endogenous rise in intestinal NKA activity during metamorphosis, thus implicating the role of the S and the CR in lamprey osmoregulation. Theoretically, this may have been an adequate approach but, experimentally, it was flawed by the lack of information about whether known MR and GR antagonists are effective in blocking the ancestral lamprey CR. We observed an expected rise in NKA activity in the AI and PI between T_0 and the Veh control three weeks later, but none of the receptor antagonists blocked the endogenous increase in NKA activity. Interestingly, in the AI, spironolactone and eplerenone actually

promoted NKA activity much like the results of our experiments with S. Indeed, spironolactone apparently serves as an agonist, not an antagonist, to the MR in older lineages of fishes (Katsu et al., 2019; Sugimoto et al., 2016). It has recently been discovered that a substitution of leucine by threonine in helix 8 of the MR of terrestrial vertebrates confers the switch of progesterone and its derivatives, spironolactone and eplerenone, from MR agonists to antagonists, and it has been suggested that this molecular switch may be causally related to the appearance of aldosterone synthesis and mineralocorticoid function of aldosterone in terrestrial vertebrates (Fuller et al., 2019). In light of these recent works, our present findings of stimulatory action of spironolactone and eplerenone, but not RU486, treatment *in vivo* on NKA activity in sea lamprey should be expected, and suggest an MR-like functional quality of the lamprey CR, which has been suggested by molecular and structural analyses of the lamprey CR (Baker and Katsu, 2019; Baker et al., 2007; Baker et al., 2013; Bridgham et al., 2006). To our knowledge, our *in vivo* results demonstrating the stimulatory action of progesterone derivatives on osmoregulation are the only such *in vivo* evidence in any vertebrate lineage predating the actinopterygian-sarcopterygian split.

In conclusion, the results in the present study demonstrate that changes in NKA activity in intestinal regions and the acquisition of SW tolerance during the sea lamprey metamorphosis is controlled by only one endogenous corticosteroid, S. Our results regarding the corticosteroid receptor demonstrate that the CR in the lamprey AI has specific binding affinity for S compared to the other known endogenous corticosteroid, DOC, and that treatment with MR agonists produce a similar physiological action to that

of treatment with S. This work provides much needed *in vivo* physiological context to many previous *in vitro* studies on ancestral corticosteroid action.

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Author contributions

Conceptualization, methodology, and experimentation: C.A.S., A.B., S.D.M.; Data curation and formal analysis: C.A.S., A.B.; Writing and revisions: C.A.S., A.B. (original draft), S.D.M; Funding acquisition and project supervision: S.D.M.

Disclosure

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Figures

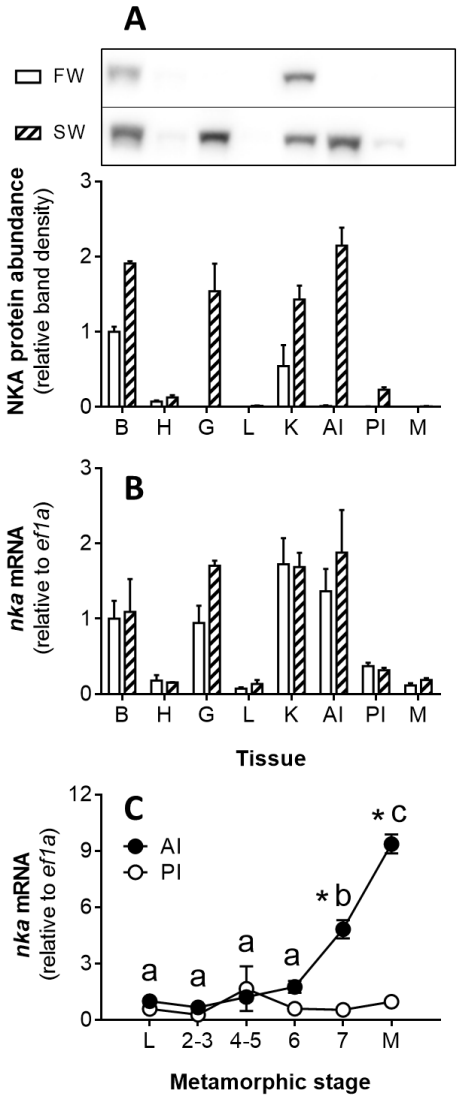

Figure 5.1: Na^+/K^+-ATPase (NKA) protein and mRNA abundance in sea lamprey. Relative abundance of (A) NKA protein (bands appear at ~100 kDa) and (B) *nka* mRNA across a tissue profile. (C) relative abundance of *nka* mRNA throughout metamorphosis. In A and B, data are shown for FW- and SW- (35 ppt) acclimated juveniles. Tissue abbreviations: B, brain (FW set to 1); P, pituitary; G, gill; H, heart; L, liver; AI, anterior intestine; PI, posterior intestine; K, kidney; M; muscle. Life-stage abbreviations: L, larvae; M, downstream migrant. Data are presented as mean ± standard error (A, B: n = 3; C: n = 4-12, two-way ANOVA). Letters indicate difference within intestine region; asterisks indicate difference from larvae.

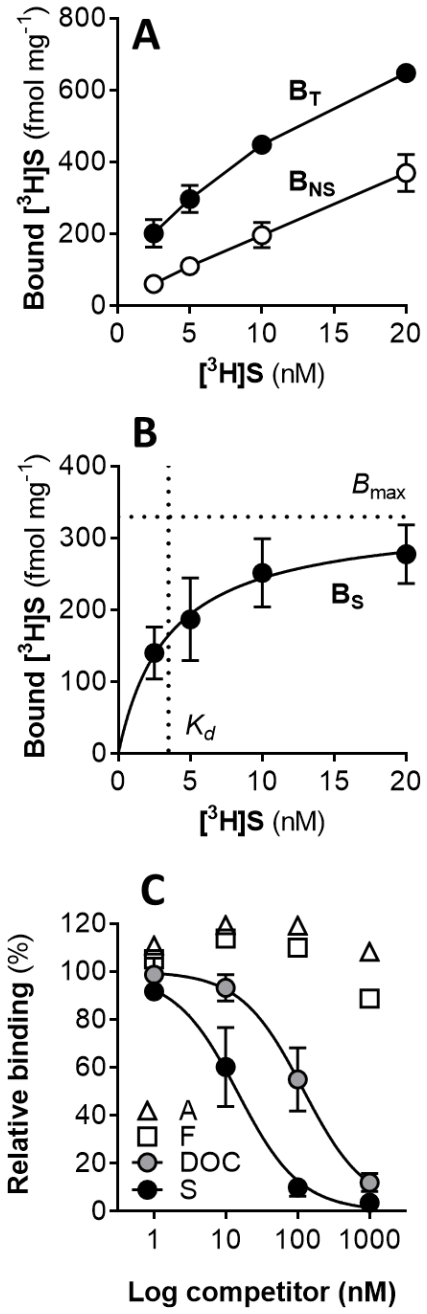

Figure 5.2: Characterization of corticosteroid receptor binding in the anterior intestine of metamorphosing sea lamprey. (A) Receptor binding assay results showing total (B_T) and nonspecific (B_{NS}) binding of [^{3}H]11-deoxycortisol ([^{3}H]S). (B) Specific, saturating binding (B_S) plotted as hyperbolic regression showing calculated B_{max} and K_d. (C) Specificity of corticosteroid binding: S, 11-deoxycortisol; DOC, 11-deoxycorticosterone; F, cortisol; A, aldosterone. Data presented as mean ± standard error (n = 3).

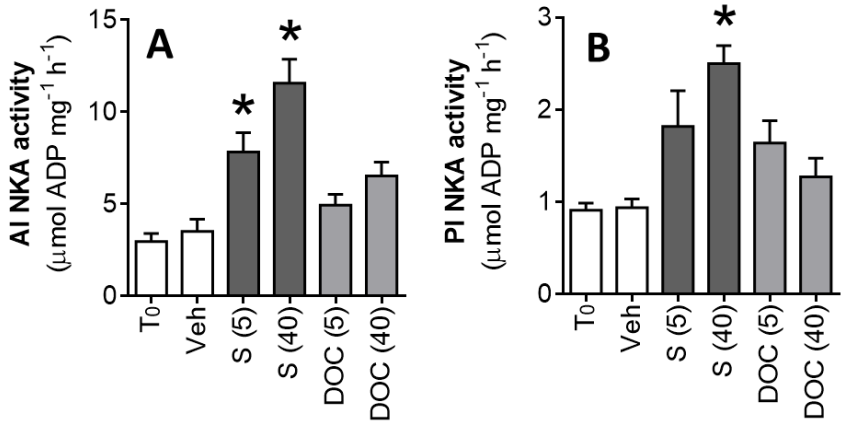

Figure 5.3: Effect of endogenous corticosteroids on Na^+/K^+-ATPase (NKA) activity in metamorphosing sea lamprey. Tissues: anterior (AI; A) and posterior (PI; B) intestine. Parentheses indicate injected dose (μg g^{-1}): S, 11-deoxycortisol; DOC, 11-deoxycorticosterone. Data presented as mean ± standard error (*n* = 8-10). Asterisk denotes difference from vehicle control.

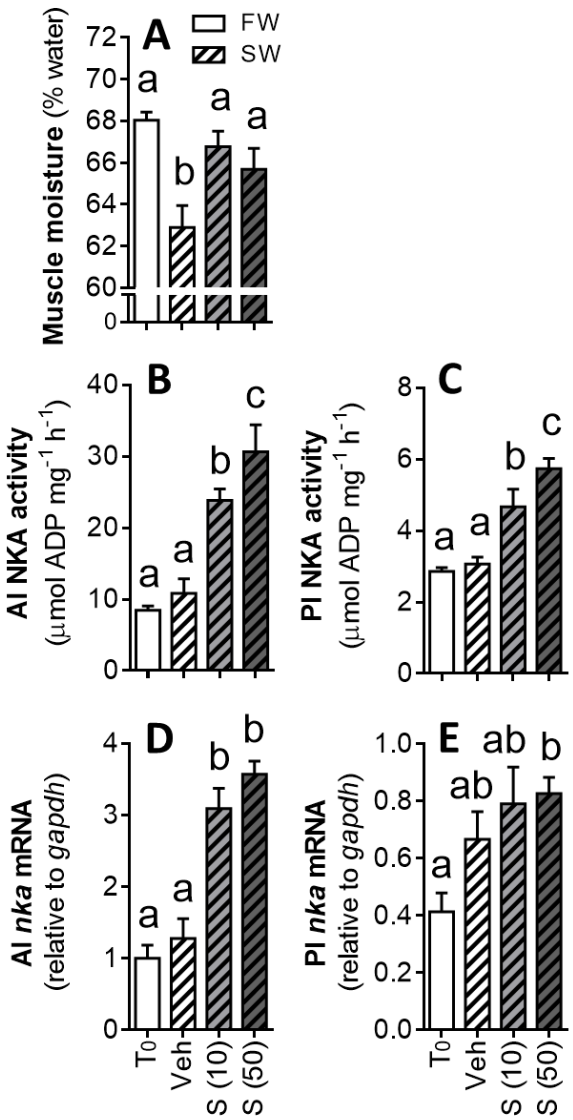

Figure 5.4: Effect of 11-deoxycortisol (S) on SW osmoregulation in metamorphosing sea lamprey. (A) muscle moisture. (B,C) Na^+/K^+-ATPase (NKA) activity. (D,E) relative *nka* mRNA abundance. Tissues: anterior (AI) and posterior (PI) intestine. Parentheses indicate injected dose (μg g^{-1}). Data presented as mean ± standard error (n = 8-10). Letters denote significant differences.

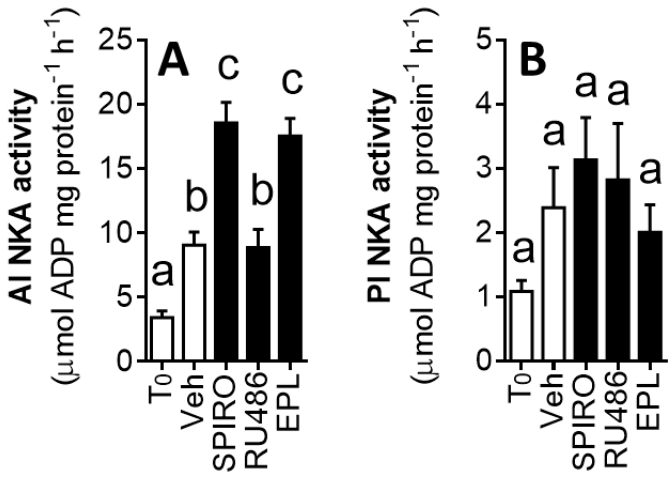

Figure 5.5: Effect of receptor antagonists on Na^+/K^+-ATPase (NKA) activity in metamorphosing sea lamprey. Tissues: anterior (AI) and posterior (PI) intestine. T_0, uninjected time = 0 control. Black bars represent lamprey sampled 3 weeks post-injection of (400 μg g^{-1}) spironolactone (SPIRO), mifepristone (RU486), and eplerenone (EPL). Data presented as mean ± standard error (*n* = 10-12). Letters denote significant differences.

CHAPTER 6

GLUCOSE STIMULATING ACTION OF 11-DEOXYCORTISOL DURING THE STRESS RESPONSE OF SEA LAMPREY (*PETROMYZON MARINUS*)

Authors

Ciaran A. Shaughnessy[1‡] and Stephen D. McCormick[2,3]

Abstract

Although corticosteroid-mediated hepatic gluconeogenic activity in response to stress has been extensively studied in fishes and other vertebrates, there is little information on the endocrine stress response in basal vertebrates. The present study examined the response of 11-deoxycortisol (the major corticosteroid in lamprey) to a physical stressor and the gluconeogenic actions of 11-deoxycortisol in sea lamprey (*Petromyzon marinus*), a member of the most basal extant vertebrate group (Agnatha). After exposure to an acute handling stress, larval and juvenile sea lamprey plasma 11-deoxycortisol increased 15- and 6-fold, respectively within 6 h post-stress, and plasma glucose increased 3- and 4-fold, respectively. Intraperitoneal administration of the two prominent endogenous corticosteroids in lamprey (11-deoxycortisol and deoxycorticosterone) demonstrated that only 11-deoxycortisol resulted in upregulation of plasma glucose. Radioreceptor binding studies revealed that a corticosteroid receptor

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(CR) is present in the liver at lower abundance than other tissues (gill, anterior intestine) and that binding affinity of the liver CR was similar between 11-deoycortisol and deoxycorticosterone. Transcriptional tissue profiles for CR and steroidogenic acute regulatory protein (StAR) indicate a wide distribution of CR expression and kidney-specific expression of StAR. Together, these results demonstrate the potential utility of gene-specific markers to identify components of steroidogenesis in lamprey and provide direct evidence for the gluconeogenic activity of 11-deoxycortisol, indicating corticosteroid regulation of plasma glucose is a basal trait among vertebrates.

Introduction

Lampreys (Petromyzontiformes) are a basal order of vertebrates within the superclass of jawless vertebrates, Agnatha, containing the extant representatives, hagfishes and lampreys. The sea lamprey (*Petromyzon marinus*) is an anadromous fish (exhibiting freshwater and seawater life stages) with a native geographic range broadly distributed along eastern and western coasts of the Atlantic Ocean, including established populations in invaded territory such as the Great Lakes of North America. As an extant representative of the basal vertebrates, sea lamprey are an excellent model organism to gain insight into the evolution of physiological and endocrine traits in vertebrates. The present study aimed to investigate aspects of corticosteroid regulation and actions during stress in sea lamprey.

Release of catecholamines and corticosteroids in response to stress is a fundamental and classically described component of a ‘fight or flight’ response to stress in vertebrates (Cannon, 1929; Romero and Butler, 2007). Catecholamines such as epinephrine and norepinephrine are produced in the adrenal medulla and released rapidly

after the detection of a ‘stressor’, a change in or stimuli from outside of the body of the animal that promotes a stress response. The pathway for steroid biosynthesis and release in the adrenal cortex (steroidogenesis) involves the serial enzymatic conversion of cholesterol beginning with the rate-limiting transfer of cholesterol within the mitochondria by the steroidogenic acute regulatory protein (StAR) (Stocco, 2001). The terms ‘mineralocorticoid’ and ‘glucocorticoid’ have been used to describe the predominant action of corticosteroids produced in the adrenal cortex of mammals. The distinction between mineralocorticoid and glucocorticoid applies fairly well to mammals, in which the predominant mineralocorticoid, aldosterone, regulates electrolyte and water balance by controlling epithelial sodium transport, and the predominant glucocorticoids, cortisol and corticosterone, is distinguished by its role in glucose metabolism. In mammals, these hormones function by activating mineralocorticoid (MR) and glucocorticoid (GR) receptors, respectively. In fishes, however, the distinction between mineralocorticoid and glucocorticoid is less applicable. In teleosts, aldosterone is present only at levels lower than are likely to be physiologically meaningful (Prunet et al., 2006). Accordingly, it is widely held that a single corticosteroid, cortisol, carries out both mineralocorticoid- and glucocorticoid-like actions in teleost fishes, mediated predominately through GRs (Mommsen et al., 1999; Takahashi and Sakamoto, 2013). The role of corticosteroid signaling in response to stress in the mobilization of glucose from the liver in fishes is well-described (Faught and Vijayan, 2016; Suarez and Mommsen, 1986). Glucose can be produced in the liver either by the breakdown of glycogen stores (glycogenolysis) or the synthesis of glucose from lactate, amino acids, and glycerol (gluconeogenesis).

Both lamprey and hagfish appear to have a reduced steroid biosynthesis pathway resulting in terminal corticosteroids that only serve as biosynthetic precursors in more derived vertebrates. In lamprey, the putative stress-responsive, terminal corticosteroid is 11-deoxycortisol acting through a single corticosteroid receptor (CR) (Bridgham et al., 2006; Close et al., 2010; Rai et al., 2015; Roberts et al., 2014). Although it has been shown that lampreys subjected to stress respond with increases in circulating 11-deoxycortisol (Close et al., 2010; Rai et al., 2015) and glucose (Larsen, 1976; Wilkie et al., 2007), evidence for the direct action of 11-deoxycortisol in stimulating gluconeogenesis is lacking. Thus, the objectives of the present study were (i) to demonstrate synchrony in corticosteroid and glucose responses to an acute stressor, and (ii) provide direct evidence for the role of corticosteroids in gluconeogenesis in lamprey.

Materials and methods

Study animals

Animal care and use followed procedures previously approved by the Internal Animal Care and Use Committee at the University of Massachusetts and U.S. Geological Survey. Sea lamprey were collected from the Connecticut River and brought to the Conte Anadromous Fish Research Center in Turners Falls, Massachusetts. Prior to experimentation, lamprey were maintained in 1.5 m diameter tanks supplied with flow-through Connecticut River water (ambient temperature) and equipped with mechanical, chemical, and biological filtration. Acute stress, steroid administration, and tissue profile experiments were carried out in larvae, metamorphic (early stage 7; Youson 1979), or post-metamorphic juvenile lamprey after one week acclimation to 60 L glass aquarium

tanks supplied with recirculating, dechlorinated, and aerated municipal fresh water at 15 °C equipped with mechanical, chemical, and biological filtration**.**

Experimentation and sampling

Prior to acute stress treatment, larvae and post-metamorphic juvenile sea lamprey were sampled as a pre-stress control. The protocol for acute stress treatment consisted of 1 min netting followed by 10 min exposure to a low water level (depth of body). Following this, water was restored to the tank and fish were left in recovery for sampling 3 and 6 h post-stress. For steroid administration, larvae and metamorphic lamprey were anaesthetized with MS-222 (100 mg L^{-1} buffered with $NaHCO_3$, pH 7.4). Steroids were suspended in a molten 1:1 mixture of oil:shortening (Veh) by sonication and administered by intraperitoneal injection. Metamorphic lamprey were administered either 11-deoxycortisol or deoxycorticosterone at 5 or 40 μg g^{-1} doses**.** Lamprey were sampled 12 d after injection.

Prior to sampling, lamprey were euthanized in a lethal dose of MS-222 (200 mg L^{-1} buffered with $NaHCO_3$, pH 7.4) and measured for body length and mass. Blood was collected from the caudal vasculature into heparinized capillary tubes and plasma was separated from blood after centrifugation at 2,000 g for 5 min. Tissue (brain, pituitary, gill, heart, liver, kidney, intestine, muscle) was collected, flash frozen, then stored at -80 °C.

Plasma glucose and 11-deoxycortisol determination

Plasma glucose was determined against a standard curve in an assay utilizing the enzymatic coupling of hexokinase and glucose-6-phosphate dehydrogenase (Stein, 1963).

Plasma 11-deoxycortisol was determined using a radioimmunoassy modified from those reported by Close et al. (2010), using a commercial antibody (Ab; CET-M8, Absolute Antibodies Inc.) and a commercially-labeled 11-deoxycrotisol (([^{3}H]S; American Radiolabeled Chemicals, Inc.). In glass culture tubes, 10 μL plasma was combined with 100 μL PBS assay buffer (50 mM NaH_2PO_4, 137 mM NaCl, 0.4 mM EDTA, BSA 0.2 % w/v, pH 7.4, 5,000 cpm [^{3}H]S) and 50 μL of CET-M8 antibody (diluted 1:5,000). The tubes were treated at 37 °C for 1 h then incubated overnight at 4 °C to allow the reaction to reach equilibrium. The next day, to remove any unbound 11-deoxycortisol, 500 μL of ice-cold dextran-coated charcoal (PBS, 0.25 % w/v dextran, 2.5 % w/v activated charcoal) was added to each tube and incubated on ice for 15 min, then centrifuged at 2,000 g for 15 min. The supernatant was added to 10 volumes of scintillation fluid (ECONO-SAFE, Research Products International Corp.) and counted on a liquid scintillation counter (LS-6500, Beckman Coulter). Plasma 11-deoxycortisol concentration was determined against a standard curve.

Receptor binding

Receptor binding was characterized using a binding assay modified from the protocols described by Shrimpton and McCormick (1999) and Close et al. (2010). Frozen liver was homogenized on ice in HEPES assay buffer (25 mM HEPES. 10 mM NaCl, 1 mM monothioglycerol, pH 7.4) using a ground glass homogenizer, then centrifuged at 2,000 g for 10 min. The supernatant was determined for protein content using the BCA protein assay (Pierce) and used in the binding assay at 4-6 mg mL^{-1}. The receptor binding assay was carried out on a non-binding 96-well microplate. For each reaction, 25 μL of supernatant was incubated for 2 h on ice with 25 μL of assay buffer containing either

[^{3}H]S alone (total binding) or [^{3}H]S with 500-fold excess of unlabeled S (non-specific binding). Each 50 μL reaction contained 2.5, 5, 10, or 20 nM [^{3}H]S. After incubation, any unbound steroids were removed by adding 150 μL of ice-cold dextran-coated charcoal in (0.25 % w/v dextran and 2.5 % w/v activated charcoal in assay buffer), incubating on ice for 10 min, then centrifuging for 10 min at 2,000 g. The supernatant (containing receptor-bound steroids) was combined with 10 volumes of scintillation fluid and counted using a liquid scintillation counter. Specificity of the receptor to 11-deoxycortisol or deoxycorticosterone was determined using an assay identical to that described above, except that 1 nM [^{3}H]S was incubated with 1, 10, 100, or 1,000 nM of unlabeled S or unlabeled DOC.

Gene expression analysis

Total RNA was isolated from frozen tissue using the TRI-zol method following manufacturer's protocol (Molecular Research Center Inc.) and quantified and analyzed for purity using a Take3 micro-volume plate reader (BioTek Instruments, Inc.). Only high-purity samples (1.9 < A260/A280) were used for cDNA synthesis and real-time PCR analyses. First-strand cDNA was synthesized using a high-capacity reverse transcription kit following the manufacturer's protocol (Applied Biosystems Inc.). Real-time quantitative PCR was carried out in 10 μL reactions containing 2 ng cDNA, 150 nM forward and reverse primers, and 1X SYBRselect master mix, following the manufacturer's protocol (ThermoFisher Inc.). The thermal profile of the reactions was first 2 min at 50 °C then 2 min at 95 °C (holding and activation), then 40 cycles of 15 s at 95 °C , 1 min at 60 °C, 30 s at 72 °C (cycling), then finally, a ramp from 60 to 95 °C (melt curve analysis) was used to confirm a single product in each reaction. Relative

mRNA abundance of *cr* and *star* was calculated using the comparative method ($\Delta\Delta C_T$; Pfaffl 2001) using elongation factor 1 (*ef1a*) as a reference gene. Primer pairs for *cr* and *ef1a* in sea lamprey have been previously described (Ferreira-Martins et al., 2016; Kolosov et al., 2017). Primers for *star* were as follows: (forward) 5'-GTGAATCTCCGCCACTCGAT-3', T_m = 59.9 °C; (reverse) 5'-AAGTCGAGCTGCATTCGTGA-3', T_m = 60.0; product size = 101 bp.

Calculations and statistics

Specific receptor binding was calculated as: *total binding - non-specific binding*. Receptor abundance (B_{max}) and dissociation constant (K_d) were determined using GraphPad Prism 6.0 (GraphPad Software Inc.). One-way and two-way ANOVA and *post hoc* analyses (denoted in figure captions and/or text) were performed using GraphPad Prism 6.0 and significance for all statistical tests was set at $P < 0.05$.

Results

Acute stress

Pre-stress levels of plasma 11-deoxycortisol were 2.0 ± 0.8 ng mL^{-1} in larvae and 1.7 ± 1.0 ng mL^{-1} in juveniles (Fig. 6.1A). In response to acute stress, plasma 11-deoxycortisol increased similarly in larvae and juveniles ($P_{time} < 0.001$; $P_{interaction} = 0.098$; two-way ANOVA). Life stage differences were observed ($P_{life\ stage} < 0.001$), represented by an overall greater-in-magnitude effect of stress in larvae. At 6 h post-stress, plasma 11-deoxycortisol had increased to 16.7 ± 4.5 and 6.4 ± 1.7 ng mL^{-1} in larvae and juvenile, respectively.

Pre-stress levels of plasma glucose were 1.0 ± 0.1 mM in larvae and 1.8 ± 0.1 mM in juveniles (Fig. 6.1B). In response to acute stress, plasma glucose significantly increased in both larvae and juveniles ($P_{time} < 0.001$) to greater than 4 mM, and life stage differences in response to stress were observed ($P_{life\ stage} < 0.001$; $P_{interaction} = 0.002$), represented by an earlier upregulation in plasma glucose in larvae (at 3 h) than juveniles.

Corticosteroid administration

Plasma glucose in lamprey administered the vehicle only was 2.2 ± 0.2 mM (Fig 6.2). An 11-deoxycortisol dose-dependent increase in plasma glucose was observed ($P = 0.003$). Lamprey receiving a 40 μg g^{-1} dose of 11-deoxycortisol had significantly elevated plasma glucose to 3.2 ± 0.3 mM.

Receptor binding

Receptor binding of 11-deoxycortisol was observed in the liver (Fig. 6.3). Liver CR abundance (B_{max}) was 91.6 ± 3.1 fmol mg^{-1} and dissociation constant (K_d) was 7.5 ± 5.6 mM (Fig 6.3A). CR B_{max} varied among tissues ($P = 0.023$; one-way ANOVA), with CR of the anterior intestine exhibiting the highest B_{max} at 344.5 ± 81.9 fmol mg^{-1} (Fig. 6.3B). No difference was detected in binding affinity of the liver to CR 11-deoxycortisol (15.3 ± 15.6 mM) and deoxycorticosterone (26.3 ± 28.1) (Fig. 6.3C).

Gene expression

Transcript abundance of *cr* varied among tissues, and was relatively high in brain, gill, heart, kidney, and anterior intestine, and relatively low in pituitary, liver, posterior

intestine, and muscle (Fig. 6.4A). Transcript abundance of *star* was much higher in the kidney (12–1,600-fold) than any other tissue (Fig. 6.4A).

Discussion

The present study establishes a role of the corticosteroid 11-deoxycortisol in regulating plasma glucose levels during the stress response of sea lamprey. This work is the first in lamprey, or any agnathan, to describe concurrent increases in plasma 11-deoxycortisol and glucose in response to acute stress. Furthermore, this work provides the first direct evidence for glucose stimulating action of 11-deoxycortisol by showing that administration of 11-deoxycortisol *in vivo* results in increased plasma glucose. We follow up these novel findings with a characterization of 11-deoxycortisol binding in the liver, as well as evidence that mRNA transcribed from genes which code for enzymes involved in the production of corticosteroids exist in high abundance in the lamprey kidney. These findings, taken together, confirm previous hypotheses and advance current understanding of lamprey stress physiology and endocrinology.

Increases in circulating 11-deoxycortisol and glucose have been observed in response to stress in lamprey before, but not until the present study had concurrent increases in both physiological markers of stress been observed in the same study. We report that lamprey at both larvae and juvenile life stages exhibit an increase in plasma 11-deoxycortisol and glucose within 6 h after acute stress. Changes in plasma 11-deoxycortisol were more immediate and greater in larvae than juveniles, whereas changes in plasma glucose were more immediate and greater in juveniles compared to larvae. This may suggest that the glucose stimulating potential of 11-deoxycortisol is generally lower in larvae than juveniles—that, in larvae, a greater corticosteroid signal is needed to

achieve a glucose response. Gluconeogenic action of stress-responsive catecholamines may also be contributing to the differences in stress responses between larvae and juveniles. It has been shown that in prespawning adult sea lamprey, administration of epinephrine significantly increased plasma glucose levels (Dashow and Epple, 1983), but the role of catecholamines in stimulating plasma glucose in larvae and juvenile lamprey life stages has not been reported.

The levels of plasma 11-deoxycortisol to an acute stressor reported in the present study in larvae and juvenile sea lamprey (6 and 16 ng mL^{-1}, respectively) is greater than what has been observed in adult lamprey. In one previous study, adult sea lamprey subjected to netting, air exposure, and salt exposure acute stressors exhibited a significant rise in plasma 11-deoxycortisol from 0.75 to 1.75 ng mL^{-1} within 4 h (Close et al., 2010). In a separate study, adult Pacific lamprey (*Entosphenus tridentatus*) subjected to netting and air exposure acute stressors exhibited a significant elevation in plasma 11-deoxycortisol from 0.6 to 1.2 ng mL^{-1} within 30 min (Rai et al., 2015). These reports describe an approximate 2-fold increase in plasma 11-deoxycortisol in adult lamprey following an acute stressor. Although pre-stress levels of plasma 11-deoxycortisol in larvae and juvenile sea lamprey in the present study were similar to those previously reported for adults (~1–2 ng mL^{-1}), post-stress levels in larvae and juvenile lamprey were higher in absolute value (~17 and ~6 ng mL^{-1}, repsectively) and magnitude (3- and 8-fold, respectively). As experimental subjects, adult sea lamprey are in sharp contrast to larvae and juvenile sea lamprey—adults are several orders of magnitude larger than larvae and juveniles. Despite the large differences in size, the methods of applying an acute stressor used in the present study for larvae and juveniles was similar to the

methods used by Close et al. (2010) and Rai et al. (2015) for adults. These previous studies netted adult lamprey subjects from holding tanks and placed them into a dry bucket for 5 min before returning subjects to recovery tanks. In the present study, we netted larval and juvenile subjects from holding and placed them in shallow water (body depth) for 10 min, before returning to recovery tank conditions. Considering the relative similarity in methods used between the present study and previous studies, life stage differences (and not experimental differences) likely explain the larger response to acute stress of plasma 11-deoxycortisol in the larval and juvenile sea lamprey used in the present study compared to the adults used in previous studies.

Plasma glucose levels of pre-stress sea lamprey in the present study (~1–2 mM) is similar to those reported in previous studies for larval and adult lamprey (Larsen, 1976; Wilkie et al., 2007). These baseline levels of circulating glucose represent a generally lower homeostatic set point compared to that of later-derived fishes and tetrapods (Polakof et al., 2011; Polakof et al., 2012). In larval sea lamprey, exposure to a chemical toxic lampricide (3-trifluoromethyl-4-nitrophenol) increased plasma glucose 2-fold from 1 to 2 mM (Wilkie et al., 2007). In adult river lamprey (*Lampetra fluviatilis*), prolonged exposure to anesthesia (from 5 to 70 min) resulted in a time-dependent increase in blood glucose from 1 to 5 mM (Larsen, 1976). In the same study by Larsen (1976), intraperitoneal injection with a saline solution resulted in an increase in blood glucose from 2 to 7 mM within 2 h before returning nearer to pre-injection levels within 24 h (Larsen, 1976). In this context, we report a similar magnitude of change (3-fold) in plasma glucose in larvae (1.8 to 5.6 mM) and juvenile (1.0 to 3.3 mM) lamprey following an acute stressor.

The present study is the first to report direct evidence for the glucose stimulating potential of 11-deoxycortisol. It has been established that, in lieu of the steroidogenic enzymes needed to produce cortisol, corticosterone, or aldosterone, there are likely only two terminal corticosteroids circulating at physiological levels in lamprey blood, 11-deoxycortisol and deoxycorticosterone (Bridgham et al., 2006; Close et al., 2010; Rai et al., 2015). Although it has been observed that both endogenous corticosteroids increase following an acute stressor (11-deoxycortisol much more so than deoxycorticosterone) (Close et al., 2010), no evidence for the role of either of these corticosteroids as a glucocorticoid hormone has been presented before the present study. By administering these hormones by intraperitoneal injection, we demonstrated that only treatment with 11-deoxycortisol resulted in an increase in plasma glucose above the vehicle control 12 d after initial treatment. In teleosts, as well as mammals, cortisol treatment generally increases plasma glucose levels within days not hours (Mommsen et al., 1999), whereas more rapid changes (~hours) in carbohydrate metabolism are attributed to the action of catecholamines such as epinephrine and norepinephrine (Fabbri et al., 1998). Such is the case with sea lamprey, in which treatment with exogenous epinephrine has been shown to increase circulating glucose within 1 h (Bentley and Follett, 1965; Dashow and Epple, 1983).

The role of the lamprey liver in glucose production and release is unclear. We confirm the findings of a previous report in adult sea lamprey (Close et al., 2010) that the abundance of CR (B_{max}) in the liver is lower than that of other tissues such as the gill and intestine. The gills and intestine of juvenile and adult lamprey are prominent osmoregulatory tissues (Barany et al., 2020; Ferreira-Martins et al., 2016; Reis-Santos et

al., 2008; Shaughnessy and McCormick, 2020). As 11-deoxycortisol is presumed to serve both glucocorticoid- and mineralocorticoid-like function acting through a single CR, the relatively high abundance of CR in the juvenile and adult sea lamprey gills and intestine is likely due to the osmoregulatory action of 11-deoxycortisol in these tissues. In addition to our binding comparison, we show that mRNA transcript abundance of *cr* is also relatively low in the lamprey liver. Previous work has demonstrated that, compared to other tissues in lamprey such as the brain, heart, kidney, and muscle, the lamprey liver has relatively low stores of glycogen and glycerol and relatively low activity of enzymes which participate in gluconeogenesis such as glucose-6-phosphatase and glycerol-3-phosphate dehydrogenase (Murat et al., 1979; Savina and Wojtczak, 1977). This is striking contrast to the physiology of more derived fishes in which the liver is the predominant tissue for processes related to gluconeogenesis (Knox et al., 1980). Curiously, whereas the lamprey gill and intestine both exhibit specificity for binding of 11-deoxycortisol over deoxycorticosterone (Close et al., 2010), the lamprey liver appears to bind both corticosteroids equally (present study). It is known that post-translational modifications to corticosteroid receptors can alter ligand specificity, translocation of the receptor or receptor-ligand complex, or transcriptional activity of the receptor-ligand complex in binding DNA (Faus and Haendler, 2006). Additionally, reductases and hydroxysteroid dehydrogenases are known to regulate tissue-specific responses to circulating corticosteroids in lieu of later-evolved specific receptor-ligand interactions (Baker, 2003). Whether similar modes of regulation are present leading to the different binding properties in the lamprey liver compared to other tissue remains unanswered and warrants further investigation. *Ex vivo* studies using isolated lamprey liver tissue to

evaluate the gluconeogenic effects of corticosteroids and catecholamines would greatly advance the understanding of the how glucose production in the lamprey liver is regulated.

Characterizing the site of steroid production and its neuroendocrine regulation has been a topic of investigation for over half a century. Prior to the identification of the putative corticosteroids in lamprey, early histochemical work identified "presumptive interrenal tissue" associated with the kidney, but experimental approaches to identify steroidogenic enzyme activity or morphological responses to acute stressor or adrenocorticotropin hormone (ACTH) stimuli were largely unsuccessful (Hardisty, 1972; Youson, 1973a; Youson, 1973b). More recent *in vivo* and *ex vivo* work has identified the kidney as the site of 11-deoxycortisol production, yet still ACTH appears to lack steroidogenic potential (Rai et al., 2015; Roberts et al., 2014). Interestingly, in these more recent works, treatment with the hypothalamus-derived corticotropin releasing factor (CRF) appears to result in the upregulation of plasma 11-deoxycortisol. Thus, it is still unresolved whether 11-deoxycortisol synthesis is regulated by a complete hypothalamus-pituitary-interrenal (HPI) axis, as is well described in more derived fishes. StAR has been used as a molecular marker for steroidogenic tissue in sturgeon (Kusakabe et al., 2009) and elasmobranchs (Evans and Nunez, 2010), and thus *star* and other gene-specific markers could help elucidate the molecular components involved in steroidogenesis in lamprey. Our initial work here showing kidney-specific expression of *star* mRNA is thus a demonstration of the potential utility of gene-specific markers for resolving the various components of steroidogenesis and its neuroendocrine control, whether by a complete HPI axis or a more primitive ACTH-independent endocrine program.

In summary, this work has provided the first example in lamprey that the response to an acute stressor includes simultaneous increases in circulating 11-deoxycortisol and glucose, and has provided direct evidence that 11-deoxycortisol increases circulating plasma glucose *in vivo*. Lastly this work has provided an example of the utility of examining gene-specific markers in a tissue profile to help resolve the components involved in steroidogenesis in lamprey. A similar approach using gene-specific markers in a tissue profile should be employed to identify markers of HPI regulation and gluconeogenesis, particularly using tissue from *in vivo* and *ex vivo* studies using acute stressor experimentation and corticosteroid administration. Such work will shed light on the evolution of the essential endocrine programs of HPI regulation of steroidogenesis and steroidogenic control of gluconeogenesis in vertebrates.

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Author contributions

Conceptualization, methodology, and experimentation: C.A.S., S.D.M.; Data curation and formal analysis: C.A.S.; Writing and revisions: C.A.S. (original draft), S.D.M; Funding acquisition and project supervision: S.D.M.

Disclosure

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Figures

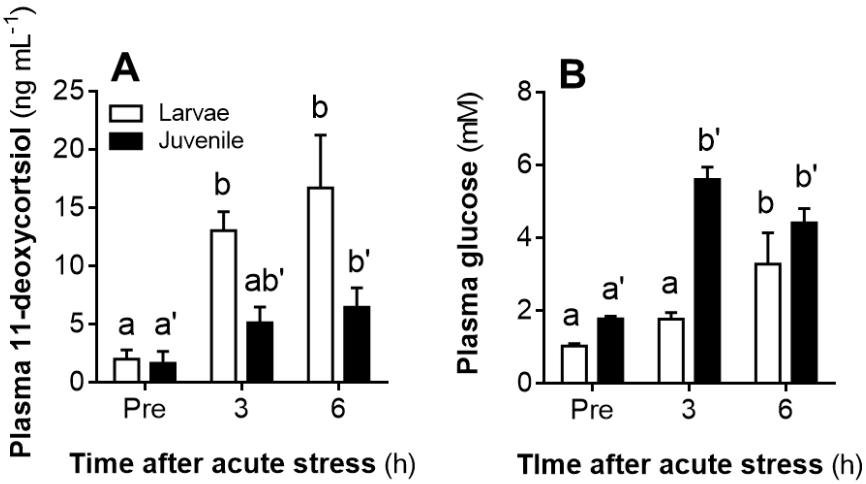

Figure 6.1: Acute stress response. Plasma concentrations of (A) 11-deoxycortisol (S) and (B) glucose before (Pre) and at 3 and 6 h after acute netting stress in larval (white) and juvenile (black) sea lamprey. Data represent mean ± SEM. Letters indicate differences (two-way ANOVA; Tukey's *post hoc*).

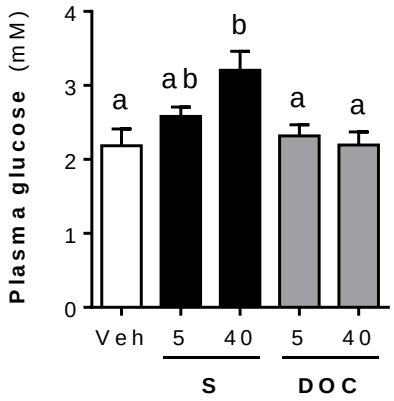

Figure 6.2: Glucose stimulating action of corticosteroids. Plasma glucose concentration in metamorphic sea lamprey sampled 12 d after intraperitoneal administration of 11-deoxycortisol (S), deoxycorticosterone (DOC), or a vehicle (Veh) control. Numbers on x-axis indicate dose (μg/g). Data represent mean ± SEM. Letters indicate differences (one-way ANOVA; Tukey's *post doc*).

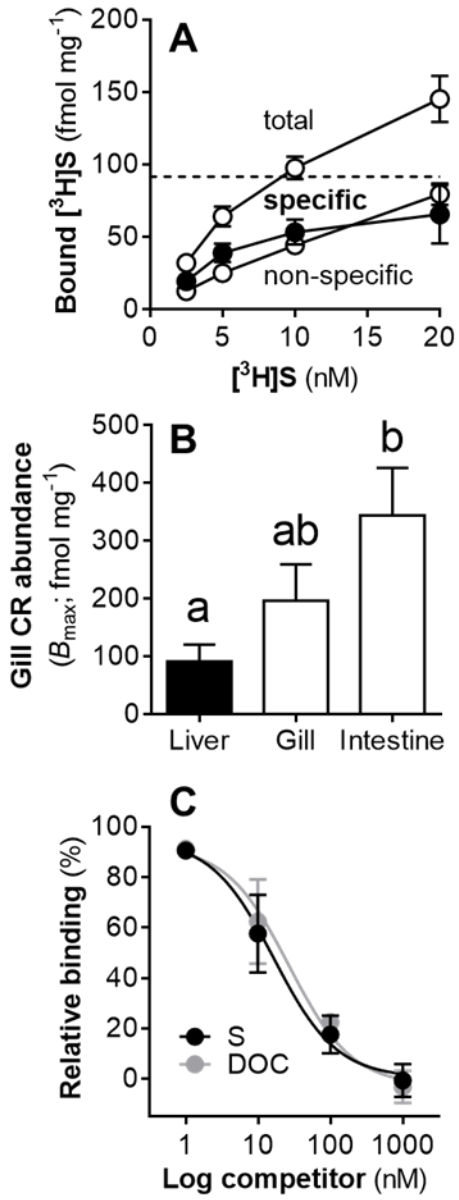

Figure 6.3: Liver corticosteroid receptor binding. (A) Specific binding of liver corticosteroid receptor (CR) to 11-deoxycortisol (S); total and non-specific binding (white) of radiolabeled S are used to calculate specific binding (black) and B_{max} (dashed line). (B) Tissue comparison of CR abundance (B_{max}) in metamorphic sea lamprey. (C) Relative binding affinity for 11-deoxycortisol (S) and deoxycorticosterone (DOC) in the liver. Data points represent mean ± SEM (B: one-way ANOVA, Tukey's *post hoc*).

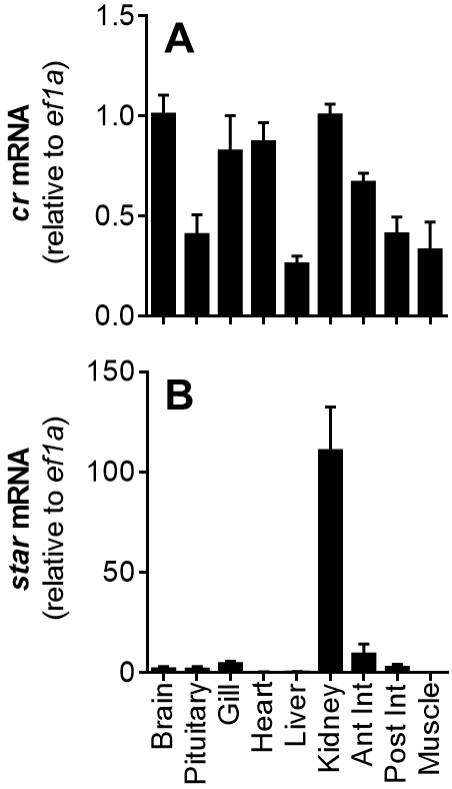

Figure 6.4: CR and StAR gene expression. Tissue profile for mRNA abundance of (A) corticosteroid receptor (*cr*) and (B) steroidogenic acute regulatory protein (*star*) in juvenile lamprey. Relative mRNA calculated by the comparative method ($\Delta\Delta C_T$) using elongation factor 1 (*ef1a*) as a reference gene. Data represent mean ± SEM.

CHAPTER 7

JUVENILE SEA LAMPREY (*PETROMYZON MARINUS*) HAVE A WIDE WINDOW OF ELEVATED SALINITY TOLERANCE THAT IS LIMITED BY SPRINGTIME WARMING

Authors

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Abstract

Sea lamprey (*Petromyzon marinus*) are an important ecological component of many river systems on Atlantic Ocean coasts, serving as a food resource to fishes and birds on their seaward migration and returning with marine-derived nutrients on their upstream spawning migration. The timing of the juvenile sea lamprey seaward migration is largely unknown, but likely varies widely from early-winter to late-spring, and thus, may be susceptible to springtime warming. The present study examined (i) the osmoregulatory capacity and seawater (SW) tolerance of juvenile sea lamprey throughout late fall, winter and spring river warming, and (ii) the thermal tolerance performance and physiology in May, corresponding to the initial rise in river and estuary temperatures. Body length, mass, and condition factor were maintained until April, when significant

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declines in mass and condition factor were observed to correspond with increasing temperature. Nearly 100 % survival in SW at 5 ^{o}C was maintained from December April. In May, after temperatures in FW had increased, significant mortality in SW (up to 50 %) was observed at both 5 and 12 ^{o}C. Plasma chloride elevated to a new set point after SW acclimation and was not affected by springtime warming. Gill Na^{+}/K^{+}-ATPase (NKA) activity was maintained throughout the spring and increased further after SW acclimation. The critical thermal maximum (CT_{max}) of sea lamprey in May was 33–35 °C (range). Plasma 11-deoxycortisol was elevated in sea lamprey at 29 °C and at CT_{max}. Plasma glucose was only elevated in sea lamprey reaching CT_{max}. Expression (mRNA) of gill heat shock proteins *hsp70* and *hsp90* increased with increasing temperature and was elevated in sea lamprey reaching CT_{max}. Together, these results provide a first characterization of the osmoregulatory and thermal performance of juvenile sea lamprey in springtime and show that the window of increased hypo-osmoregulatory performance for SW entry lasts for several months, but will be limited by increases in river water temperatures in late spring.

Introduction

Sea lamprey (*Petromyzon marinus*, Linnaeus 1758) are anadromous fishes of North America and Europe. Like all anadromous fishes, sea lamprey make a freshwater (FW)-to-seawater (SW) migration as juveniles to access the greater food resources and potential for growth in the ocean, then return upstream to spawn. As larvae (also referred to as ‘ammocoetes’), sea lamprey live burrowed in the soft substrate of river beds, filter-feeding on suspended organic matter. After 4 to 6 years of relatively slow growth in larval life, sea lamprey undergo a months-long, larvae-to-juvenile metamorphosis lasting

from mid-summer to late-Autumn, characterized by dramatic morphological and physiological changes to allow parasitism and survival in SW (Barany et al., 2020; Beamish and Potter, 1972; Beamish et al., 1978; O'Boyle and Beamish, 1977; Reis-Santos et al., 2008; Richards and Beamish, 1981; Shaughnessy and McCormick, 2020; Youson, 1979; Youson, 1980). Although the hallmark morphological changes such as the appearance of eyes and teeth occur early in the metamorphosis of anadromous lamprey (stages 1 to 4) (Youson, 1979), the critical development of SW tolerance occurs in the later stages (stages 5 to 7) (Barany et al., 2020; Richards and Beamish, 1981; Shaughnessy and McCormick, 2020).

Lamprey at each life-stage have an important ecological role and some populations are of conservation concern. Filter-feeding larval lamprey make up a large portion of the biomass in streams where they are present and are important in nutrient processing and cycling (Moore and Mallatt, 1980). Importantly, larval lamprey release pheromone signals to guide upstream-migrating, pre-spawning adults (Fine et al., 2004). Downstream migrating juveniles are an important prey item for many freshwater and estuarine fishes and birds (Kelly and King, 2001). Upstream-migrating adults in the spring bring back with them an abundance of marine-derived nutrients at an important time of the year when riverine habitats tend to be nutrient-poor (Nislow and Kynard, 2009; Weaver et al., 2016). Although the sea lamprey is considered a species of 'least concern' (IUCN, 2019), there are many vulnerable native populations of anadromous sea lamprey, particularly along the southern coast of Europe (Maitland et al., 2015). For instance, sea lamprey populations on the Iberian Peninsula, Italy, and the eastern Adriatic

Sea are expected to disappear under predicted climate change scenarios (Lassalle et al., 2008).

For anadromous fishes to survive seawater entry they must be able to maintain ion and water homeostasis despite transitioning between environments with drastically different salinities. The transformation of the gill epithelium from a site of active ion uptake to active ion secretion is critical to survive SW entry (Marshall and Grosell, 2006), and can only be carried out by a minority of fish species (~4%) that are amphihaline (Schultz and McCormick, 2013). The cellular mechanism by which lamprey accomplish branchial salt secretion appears to be similar to that of bony fishes, involving the paracellular transport of Na^+ and the transcellular transport of Cl^- facilitated by electrogenic gradients produced by the activity of Na^+/K^+-ATPase (NKA) in the gill ionocytes. Much like the parr-to-smolt transformation in salmonids (McCormick, 2013), increases in NKA activity and abundance during the lamprey metamorphosis correspond to increases in SW tolerance (Beamish et al., 1978; Reis-Santos et al., 2008; Shaughnessy and McCormick, 2020).

The timing of the downstream migration made by post-metamorphic juvenile sea lamprey appears to be bimodal. Studies in North American and European sea lamprey populations typically report migration occurring in either late-Autumn/early-winter (November-December) or early-spring (March-April) and is thought to correspond to periods of increased discharge (Kelly and King, 2001; Potter, 1980). Although several studies have examined the timing of emigration from natal tributaries, the timing of SW entry of juvenile sea lamprey is largely unknown. Whether sea lamprey out-migrate in Autumn or overwinter and out-migrate the following spring may have physiological

consequences as lamprey stop filter-feeding after the onset of metamorphosis and only resume feeding as newly parasitic juveniles once at sea. This dramatic change in feeding behavior can result in a prolonged period of fasting lasting many months (Potter, 1980; Youson, 1980).

Physiologists examining the effects of temperature on fishes often assess both organismal and cellular responses to thermal stress. At the organismal level, critical thermal maximum (CT_{max}) is a widely used metric for estimating the upper limit of thermal tolerance in a species (Becker and Genoway, 1979; Beitinger et al., 2000; Lutterschmidt and Hutchison, 1997). Experimentally, CT_{max} is most often defined as the temperature at which an individual exhibits a loss of equilibrium. Analysis of heat shock proteins (HSPs), molecular chaperones that help maintain structure and function of proteins, is a widely used metric for determining cellular stress in response to thermal stress (Feder and Hoffman, 1999). Among a large family of HSPs, two stress-induced members (HSP70 and HSP90) and one constitutively-expressed members (HSC70) are commonly assessed in fishes (Basu et al., 2002; Deane and Woo, 2011; Iwama et al., 1999).

Although, it has been shown several times that post-metamorphic lamprey have high SW tolerance in late-Autumn/early-winter, seawater tolerance after this period has not been examined. Similarly, the upper thermal tolerance for juvenile sea lamprey has not been described, nor has the thermal onset of induction of HSPs in lamprey been investigated by gene-specific molecular analyses. Thus, the aims of the present research were to (i) determine whether SW tolerance in post-metamorphic juvenile sea lamprey was maintained throughout springtime warming, and (ii) characterize the thermal

tolerance and physiological response in post-metamorphic juvenile sea lamprey to increasing temperatures.

Materials and methods

Animal Collection and Care

For all experimentation, mid-metamorphic sea lamprey were collected in September by hand and netting during an annual draw-down of a hydroelectric canal near the laboratory on the Connecticut River (Conte Anadromous Fish Research Center, U.S. Geological Survey, Massachusetts, USA) where they were brought back to and reared for future experimentation and sampling. Animals were reared in 1.5 m diameter flow-through tanks in filtered and UV-treated river water (freshwater) under ambient river temperatures and natural photoperiod.

Series 1: Biometric monitoring

Individual sea lamprey were given a color tag so that we could follow changes in weight and length of individuals over time. Sea lamprey were anesthetized using a non-lethal dose of MS-222 (100 mg L-1 buffered by NaHCO3, pH 7.4) and individually color-coded using acrylic paint along the caudal fin. For the duration of growth monitoring, these individually marked sea lamprey were held in duplicate 1.5 m diameter recirculating tanks equipped with chemical and biological filtration and maintained in ambient Connecticut River water. Approximately monthly, each individual was anesthetized and measured for body length and mass. Length, mass, and condition factor was monitored from December to June. As ambient river temperature fluctuated naturally

during this time, the relationship between change in length, mass, or condition factor and the cumulative degree-days from the initial December biometric sampling was assessed.

Series 2: Seawater acclimation trials

Artificial SW (35 ‰) was made using a commercial sea salt formula (Crystal Sea Salt, Baltimore, MD, USA) in dechlorinated municipal water. For each SW trial, lamprey were transferred from holding in ambient river water to SW at 5 °C or 12 °C (May only) and held in SW for 30 d; lamprey were sampled at Day 0 (FW), 2 (SW) and 30 (SW). A total of five SW trials were conducted throughout the winter and spring; the dates and ambient river temperature at the start of trial were: 9 December, 5.2 °C; 20 January, 1.6 °C; 22 March, 5.3 °C; 3 May, 11.5 °C (Fig. 7.1). Since a major goal of our study was to examine developmental changes over time, we chose a constant SW temperature to control for possible temperature-related changes. The SW temperature of 5 °C that we chose represented an average winter and early-spring temperature of the estuary; the 12 °C SW trial was added in May to capture the spring increase in temperature and provide an isothermal SW transfer in early May.

Series 3: Thermal tolerance testing

On 17 May, sea lamprey were transferred from rearing conditions to 1.5 m diameter flow through tanks supplied with dechlorinated municipal water maintained at 20 °C. Sea lamprey were acclimated to these experimental conditions for 24 h before thermal tolerance testing began. Water temperature was controlled as previously reported (Chadwick and McCormick, 2017; Shaughnessy and McCormick, 2018) using a feedback-based system in which water from a heated header tank was added through

solenoid valves (Granzow, Inc., Charlotte, NC, USA) controlled by digital temperature controllers (Omega cn7500, Omega Engineering, Inc., Stamford, CT, USA). Every tank was provided supplemental aeration to ensure dissolved oxygen remained at saturating levels. During 'heat shock' testing, water temperature was increased at a rate of 3 °C h^{-1} until the target temperature for each tank (i.e., 20 (control), 23, 26, 29 °C) was reached. The temperature of each tank was then maintained for 18 h until sampling. For CT_{max} testing, the temperature was increased at a rate of 2 °C h^{-1}. Each sea lamprey was removed from CT_{max} testing and immediately sampled once it had exhibited an 'endpoint' behavior, which was characterized as a loss of equilibrium (LOE) and lack of response to stimuli.

Sampling

Sea lamprey were euthanized in a lethal dose of MS-222 (200 mg L-1 buffered by NaHCO3, pH 7.4) and measured for length and weight. Blood was collected via caudal severance in to heparinized capillary tubes and plasma was obtained by centrifugation. Gill tissue (filaments only, no cartilaginous arch) excised and flash frozen. For enzyme activity analysis, gill tissue was frozen and stored in SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3). Gill and blood plasma samples were immediately flash frozen and stored at -80 °C.

Gill Na^+/K^+-ATPase activity

Gill Na^+/K^+-ATPase activity was measured as previously described (McCormick, 1993). Gill tissue was homogenized in SEID buffer (SEI + 0.1 % deoxycholate) using an electronic handheld microcentrifuge tube homogenizer (Fisherbrand, FisherScientific,

USA). The homogenized samples were centrifuged at 2,000 g for 5 min and the supernatant was used in the assay. The ATPase activity specific to NKA was measured by exploiting the ouabain-inhibition of NKA activity in an enzyme-linked assay which connected in a 1:1 ratio the production of ADP to the reduction of NADH to NAD^+. Gill homogenates were run in duplicate without and with 0.5 mM ouabain. Absorbance of NADH (340 nm) was measured every 20 s throughout a 10 min kinetic assay, and the difference between the rate of NADH reduction (i.e., ADP production) under control and ouabain-inhibited conditions was considered the rate of ADP production by NKA. Protein concentration of each sample was determined spectrophotometrically (BCA Protein Assay Kit, Pierce, USA) activity reported as μmol ADP mg $protein^{-1}$ h^{-1}. Activity and protein assays were run on a ThermoMax microplate reader using SoftMax software (Molecular Devices, San Jose, CA, USA).

Hematological analyses

Plasma chloride concentration was measured using a standard chloidometer (Haake Buchler Instruments Inc., USA). Plasma glucose concentration was measured using an enzymatic coupling of hexokinase and glucose-6-phosphate dehydrogenase (Glucose Assay Kit, Sigma Aldrich, St. Louis, USA).

Plasma 11-deoxycortisol concentration was measured using a competitive radioimmunoassay using a commercial antibody (CET-M8, Absolute Antibodies Inc., UK) and a commercial ^{3}H-labeled 11-deoxycortisol ([^{3}H]S; American Radiolabeled Chemicals, Inc., St. Louis, MO). In glass culture tubes, 10 μL of plasma was incubated overnight on ice with 100 μL of PBS assay buffer (50 mM NaH_2PO_4, 137 mM NaCl, 0.4 mM EDTA, BSA 0.2 % w/v, pH 7.4; containing 5,000 cpm [^{3}H]S) and 50 μL of CET-M8

antibody (diluted 1:5,000 for ~50 % binding). After overnight incubation, 500 μL of a dextran-coated charcoal solution (PBS assay buffer containing 0.25 % w/v dextran and 2.5 % w/v activated charcoal) was added. The mixture was incubated on ice for 15 min then centrifuged at 2,000 **g** for 15 min to pellet any unbound 11-deoxycortisol. The supernatant was added to 10 volumes of scintillation fluid (ECONO-SAFE, Research Products International Corp., USA), then analyzed by a liquid scintillation counter (LS-6500, Beckman Coulter). Final determination of plasma 11-deoxycortisol concentration was made by comparison to a serial dilution standard curve of unlabeled 11-deoxycortisol (Riechstien's Substance S, Sigma-Aldrich, USA) ranging from 32 to 0.25 ng mL^{-1} run in triplicate.

RNA extraction and quantitative polymerase chain reaction

Total RNA was extracted from frozen gill tissue using the TRIzol method (TRI reagent, Molecular Research Center Inc., Cincinnati, OH). Total RNA was tested for purity and integrity and all samples fell within an optimal purity range of 2.2 > A260/A280 >1.8 (Take3, BioTek Instruments, Inc., USA). Synthesis of cDNA was performed using High Capacity Reverse Transcription Kit (Applied Biosystems, USA). Real-time, quantitative PCR was performed using SYBRSelect master mix (ThermoFisher, Waltham, MA) in 10 μL reactions containing 5 ng cDNA template, 150 nM forward and reverse primers. All real-time PCR reactions were carried out in 96-well plates using StepOnePlus Real-Time PCR System (Applied Biosystems, Inc., USA) using the following protocol: activation at 95 °C (2 min); cycling (40 cycles) from 95 °C (15 s) to 60 °C (1 min) to 72 °C (30 s); melt-curve analysis (to confirm single product). Primers for ef1α were identical to those previously reported (Kolosov et al., 2017). Primers for

hsp90, hsp70, and hsc70 (Table 7.1) were designed from sequences discovered using the sea lamprey genome portal (https://genomes.stowers.org; accessed 20 May 2019; Smith et al., 2018)

Calculations and statistics

Condition factor was calculated as: (weight ÷ length^3) × 1000. Degree-days was calculated as the sum total of average daily temperatures since the initial December biometric sampling. Changes in (Δ) biometric data and degree-days were fit by linear regression. All group data are presented as mean ± standard error (SE). Normality and homogeneity of variance assumptions were tested using Shapiro–Wilk and Levene's tests. Statistical analyses (ANOVA, Kruskal-Wallis, t-test), post hoc analyses, and *P*-values are presented in figures and figure captions. An α-value of 0.05 was used to demarcate significance in all statistical analyses.

Results

The ambient temperature of the river changed seasonally reaching a low of ~2 °C in January and February and reaching ~16 °C by the end of all experimentation in mid-June (Fig. 7.1). Lamprey behavior was nearly always observed to be attached via suction to the wall of the tank in FW and SW. The only consistent divergence from this behavior was during CT_{max} testing at temperatures above 30 °C, when some lamprey were observed detaching from the wall and swimming freely. All lamprey eventually detached from the wall as they approached CT_{max}.

Series 1: Biometric monitoring

Initial body length of sea lamprey in December was 15.7 ± 0.3 cm and did not change significantly over time, although a slight shortening trend was observed (Fig. 7.2A). Initial body mass was 4.6 ± 0.3 g and remained stable until April (4.4 ± 0.4), but by June had decreased significantly to 3.2 ± 0.4 g (Fig. 7.2B). Initial condition factor (1.18 ± 0.02) was also maintained through April (1.2 ± 0.3) until decreasing to 1.02 ± 0.04 in June (Fig. 7.2C). The cumulative degree-days between biometric sampling times and initial biometric sampling in December were: January, 210; March, 490; April, 825; June, 1213 (Fig. 7.2D-E). Increasing degree-days had a significant effect on changes in (Δ) body length, body mass, and condition factor. By sampling in June body length, body mass, and condition factor had declined by 6, 27, and 16 %, respectively. Relationships between Δ_{length}, Δ_{mass}, and $\Delta_{condition}$ and $\Delta_{degree\text{-}days}$ were fit by linear regression: $\Delta_{length} = -7.69\text{x}10^{-3}(\Delta_{degree\text{-}days}) + 0.48$; $\Delta_{mass} = -3.68\text{x}10^{-2}(\Delta_{degree\text{-}days}) + 3.38$; $\Delta_{condition} = -2.43\text{x}10^{-2}(\Delta_{degree\text{-}days}) + 3.85$.

Series 2: Seawater acclimation trials

Tank temperature of each 30 d SW acclimation trials was maintained within ± 1 °C at either target temperature of 5 or 12 °C (Fig. 7.1A). Body length, mass, and condition of FW controls (time = 0) of each respective salinity trial declined from December to May (Table 7.2). Survival was nearly 100 % for the first three SW trials through April, but significant mortality was observed in both May trials (Fig. 7.3A). In May, transfer from ambient (~12 °C) to 5 °C SW caused over 50 % mortality within the first three days, then no further mortality was observed among the surviving lamprey. When lamprey were transferred from ambient to 12 °C SW, less initial mortality was

observed (~10 %), but significant mortality (50 %) occurred after two weeks of SW exposure. Plasma chloride in freshwater remained consistent ~115 mM throughout spring and exposure to SW resulted in the elevation of plasma chloride to ~130–140 mM (Fig. 7.3B). Only an effect of salinity on plasma chloride was detected; there was no effect of the moth of salinity trial (P_{date} = 0.782) or an interaction effect ($P_{interaction}$ = 0.701) (two-way ANOVA, Dunnet's *post hoc*). Elevated gill NKA activity in FW was maintained at ~15–20 μmol ADP mg^{-1} h^{-1} throughout the spring (Fig. 7.3C). In the January, March, and the 12 °C May trials, gill NKA activity increased after 30 d acclimation to SW, but gill NKA activity did not increase after SW acclimation in the December or 5 °C May trials. An effect of salinity on gill NKA activity was detected, but no effect of date (P_{date} = 0.097) or the interaction ($P_{interaction}$ = 0.231) was detected (two-way ANOVA, Dunnet's *post hoc*).

Series 3: Thermal tolerance testing

Maintenance of water temperature in experimental tanks was controlled within 0.25 °C (standard deviation) of desired temperature throughout experimentation (Fig. 7.4A). LOE occurred between 33–35 °C, which is considered the range of CT_{max} (Fig. 7.4B). Plasma 11-deoxycortisol was significantly affected by heat shock temperature, increasing from 1.4 ± 0.5 ng mL^{-1} in the 20 °C control to 3.9 ± 1.1 ng mL^{-1} in lamprey exposed to 29 °C (Fig. 7.5A). Lamprey sampled after reaching CT_{max} also had elevated plasma 11-deoxycortisol levels at 3.0 ± 0.4 ng mL^{-1}. Plasma glucose in the 20 °C control was 2.9 ± 0.4 mM (Fig. 7.5B). Lamprey reaching CT_{max} had significantly higher plasma glucose levels (4.5 ± 0.1 mM) than the 20 ° C control. There was no significant effect on plasma chloride by heat shock temperature or reaching CT_{max} (Fig. 7.5C).

Heat shock temperature had significant effects on gill *hsp70* and *hsp90* mRNA levels but no significant effect on gill *hsc70* mRNA levels. Gill *hsp70* mRNA levels increased with increasing heat shock temperature and was ~1.5-fold higher at 29 °C than the 20 °C control (Fig. 7.6A). Gill *hsp90* mRNA levels also increased with increasing heat shock temperature and were ~1.75-fold higher at 29 °C than 20 °C (Fig. 7.6B). Gill *hsc70* did not change with respect to heat shock temperature but was significantly lower (~43 %) in lamprey reaching CT_{max} compared to the 20 °C control (Fig. 7.6C).

Discussion

Post-metamorphic sea lamprey appear to exhibit a large window of hypo-osmoregulatory capacity and SW tolerance. It has been observed that the increase in gill NKA activity and protein abundance during sea lamprey metamorphosis corresponds to the increase in SW tolerance (Beamish et al., 1978; Reis-Santos et al., 2008; Shaughnessy and McCormick, 2020). As expected, post-metamorphic juvenile sea lamprey in the present study exhibited gill NKA activity that was 10–15-fold higher than what has been reported for pre-metamorphic larvae (Reis-Santos et al., 2008), and acclimation to SW tended to further increase gill NKA. This concurrent increase in gill NKA activity and SW tolerance during the sea lamprey larvae-to-juvenile metamorphosis is akin to similar physiological adjustments made during the parr-smolt transformation of anadromous salmonids. In anadromous salmonids such as the Atlantic salmon (*Salmo salar*), peak gill NKA activity and SW tolerance are maintained for a relatively short period, beginning in March or April, typically reaching a maximum in May before rapidly declining to parr levels by June (McCormick, 2013). The width of the smolt window is decreased by elevated temperatures, which have only a moderate effect on the onset of smolting but

causes a rapid decrease in gill NKA activity and salinity tolerance in 'post-smolts' (Mccormick et al., 1999). The rapid onset of a post-smolt decline in osmoregulatory performance likely limits the optimal window for SW entry in Atlantic salmon to only ~2–4 weeks, corresponding to the observed downstream migratory behavior for this species (McCormick et al., 1998; McCormick et al., 2014; Zydlewski et al., 2014). Whether a similarly transient window of osmoregulatory performance existed for sea lamprey juveniles had not been investigated before the present study. Unlike what has been observed in anadromous salmonids, we show here that the window of peak osmoregulatory capacity and SW tolerance in sea lamprey juveniles is much broader than in salmonids, lasting for 5–6 months from November to April.

Unlike the highly synchronized timing of lamprey metamorphosis (Youson, 2003), the timing of the downstream migration made by post-metamorphic juvenile lamprey within a population can vary considerably. An important distinction must be made between when juvenile lamprey emigrate from natal streams and when they subsequently enter SW. Many studies have reported that the downstream migration of lamprey populations from natal streams corresponds to periods of high discharge and occurs either in late-winter, early-spring, or is bimodally distributed between those two windows, but it is largely unknown whether they immediately enter SW (see review by Dawson *et al.*, 2015). Understanding that newly metamorphosed sea lamprey exhibit rapid growth after initiation of feeding behavior begins in estuary (Silva et al., 2013) and that remaining upstream means many more months of fasting, it might be presumed that late-winter out-migrants have the advantage in adult growth, and thus reproductive potential. However, in a comparison between early- and late- out-migrants, no difference

in adult growth was detected (Swink and Johnson, 2014). Therefore, it could be that the prolonged maintenance of hypo-osmoregulatory capacity and SW tolerance observed in the present study is an adaptive trait in anadromous sea lamprey—a larger window for out-migration allows for more options to select for conditions favorable to migration.

Despite having an apparently large osmoregulatory window to out-migrate, sea lamprey body condition and SW survival is eventually negatively impacted by increases in river temperatures in late-spring. A low metabolic rate determined by low activity and cold water temperatures between December and April (< 6 °C) is a likely explanation for the apparent maintenance of body mass and condition over the first several months of fasting observed in the present study. Likewise, an increasing metabolic rate during the ~8 °C increase in river temperatures between April and June could explain the significant decline in body mass and condition that occurred during this time. In larval sea lamprey metabolism has been shown to decline beginning in September and remain at very low levels over winter until March, when dramatic springtime increases in metabolism were observed (O'Boyle and Beamish, 1977). By regressing the change in temperature between samplings, it was clear that changes in temperatures significantly negatively correlated to changes in body length, mass, and condition. Our late-season SW acclimation trials in May were carried out after large increases in river temperatures had occurred corresponding with declining body mass and condition. Considering that gill NKA activity, and indicator of hypo-osmoregulatory capacity, remained high during this time, the temperature-induced decline in body mass and condition likely contributed in large-part to the reduction of SW tolerance in May.

Along with the likely effect of reduced body mass and condition on SW tolerance, temperature also had a discernable effect on the nature of the SW tolerance observed in May. Mortality among lamprey transferred from ambient (12 °C) FW to 5 °C SW was immediate (~days), whereas mortality among lamprey transferred from ambient to 12 °C SW occurred much more gradually (~weeks). A similar finding of more rapid mortality occurring in low temperature SW transfer has been observed in Atlantic salmon smolts (Handeland et al., 2000; Sigholt and Finstad, 1990). These differences in survival based on SW temperature (5 versus 12 °C) are likely not explained by metabolic rate—that critical cellular and metabolic adjustments that are immediately required upon SW entry are slowed at lower temperatures, resulting in mortality that is more immediate in nature. In the present study, little to no immediate mortality was observed in the three preceding SW trials, which were all carried out at an equally low temperature (5 °C). Perhaps the reduced body condition in late-spring exacerbated the effect of temperature on metabolism or cellular processing during SW entry. It should be noted that the net temperature change during transfer from ambient river water to SW was much greater in the 5 °C SW trial in May (~7 °C) than that of the preceding SW trials (~0–3 °C)—experiencing a simultaneous thermal and salinity transfer certainly could have also contributed to the immediate mortality in the 5 °C SW trial in May. When we transferred lamprey to 12 °C SW in May, which both removed any simultaneous thermal stress and was more ecologically relevant considering the ~12 °C temperature of the estuary in May, mortality occurred at the end of the 30 d SW trial rather than in the beginning, as occurred in the 5 °C SW. This finding suggests that salinity tolerance was initially high in the 12 °C SW fish, and we hypothesize that the mortality that occurred later in this group

was due to the impact of elevated temperature on energetic reserves. In either event, it appears that SW entry as late in spring as May (after temperatures have risen to ~10 °C) is beyond the window for optimal osmoregulatory performance, which is likely related to prolonged fasting and increased metabolism.

The only reports of sea lamprey thermal tolerance before the present study were on embryo and larvae life stages (Potter and Beamish, 1975; Rodríguez-Muñoz et al., 2001), thus we report here a measure of upper thermal tolerance in juvenile lamprey for the first time. Sea lamprey larvae acclimated to 15 °C had an incipient lethal temperature of ~30 °C (Potter and Beamish, 1975). A particularly interesting finding of this previous study was that larval lamprey acclimated to either 5, 15, or 25 °C only differed in their upper lethal temperature by 1.5 °C (29.5–31 °C). This demonstrates a relatively low acclimation effect on upper thermal tolerance compared to teleosts, which generally exhibit a 1 °C increase in upper thermal tolerance for every 3 °C increase in acclimation temperature (Fry, 1971; Gunderson and Stillman, 2015). Grossly comparing the 33–35 °C (range) CT_{max} of sea lamprey in the present study acclimated to 12 °C to other diadromous fishes of the North Atlantic (Beitinger et al., 2000), it appears sea lamprey thermal tolerance falls on the higher end of the range. For example, salmonids acclimated to 15 °C have a CT_{max} of ~27–33 °C (range) (Elliott and Elliott, 1995) and clupeids acclimated to 15 °C have a CT_{max} of ~26–35 °C (range) (Monirian et al., 2010; Otto et al., 1976). In the European eel (*Anguilla anguilla*), a fish with a similar body shape to sea lamprey, acclimation to 18 °C corresponded to a CT_{max} of 31.5 °C (mean) (Claësson et al., 2016).

In addition to testing for CT_{max}, we exposed lamprey to three elevated 'heat shock' temperatures for 18 h to examine the onset of a physiological or cellular response to temperature. It has been shown before in fishes that acclimation to salinity impairs thermal tolerance and that ion homeostasis in fishes is impaired at warmer temperatures (Metzger et al., 2016; Shaughnessy and McCormick, 2018; Vargas-Chacoff et al., 2018). Although lamprey at 29 °C and CT_{max} had the lowest mean values for plasma chloride, the expected result if warm temperatures impaired ionoregulation in FW, these trends were not statistically significant. We did observe indicators of physiological and cellular stress several degrees Celsius prior to the loss of equilibrium. 11-Deoxycortisol is the putative corticosteroid involved in the general stress response in lamprey (Close et al., 2010; Rai et al., 2015). In the present study, we show plasma 11-deoxycortisol concentration increases significantly with increasing heat shock temperature and is elevated in lamprey reaching CT_{max}. The mobilization of glucose as indicated by an elevation of plasma glucose concentration is an expected result of stress and has been previously observed in lamprey (Close, 2001). Here we show a detectable rise in plasma glucose concentration in lamprey reaching CT_{max}. Together, these data are the first description of a corticosteroid or gluconeogenic stress response to elevated temperature in lamprey.

HSPs are important to combatting the negative effects of thermal stress and are a commonly used biomarker to detect thermal stress in fishes (Deane and Woo, 2011). In the gills of sea lamprey acclimated to 13 °C, newly synthesized protein labeled with [^{35}S]methionine of molecular weights of 70 and 90 kDa were detected in lamprey exposed to 29 °C, but not 25, 21, or 17 °C (Wood et al., 1998). The same study was

unable to confirm a temperature-dependent induction of HSP70 by Western blotting, and the authors suggested that the antibody used was cross-reacting with both the inducible and constitutive forms of HSP70 (Wood et al., 1998). In the present study, we use a gene-specific analysis to examine regulation of *hsp70*, *hsp90*, and *hsc70* in lamprey for the first time. Both *hsp70* and *hsp90* are known to be induced by thermal stress in teleost fishes (Basu et al., 2002), and were induced by exposure to elevated temperatures in sea lamprey gill in the present study. Induction of HSP70 has traditionally been thought to be more sensitive and have a response to thermal stress that is greater in magnitude compared to HSP90 in teleost fishes (Basu et al., 2002), but a more recent thorough transcriptomic analysis revealed HSP70 and HSP90 are equivalent biomarkers of thermal stress in a salmonid (Houde et al., 2019). In the present study, induction of gill *hsp70* was only observed in sea lamprey exposed to the 29 °C heat shock temperature and those reaching CT_{max}, whereas induction of gill *hsp90* appeared more sensitive to increasing heat shock temperature, induced by all temperatures above 20 °C. It's worth noting that slight, 1.5–2-fold upregulation of *hsp70* and *hsp90* observed in lamprey in the present study in response to temperature is on the low end of the range of magnitudes of transcriptional induction observed in other fishes, which are ~2–10-fold and can be as high as 100-fold (Deane and Woo, 2011). As expected, the constitutive heat shock protein *hsc70* was not induced by exposure to increasing heat shock temperatures. In fact, gill *hsc70* mRNA abundance was significantly reduced in lamprey reaching CT_{max}, potentially indicating cellular dysfunction or reprioritization away from constitutive processes at the brink of cellular and metabolic collapse.

The present study provides important insight into the critical downstream migratory life stage of anadromous sea lamprey. We show that rising springtime temperature reduces survival in SW and may determine the end of the window of opportunity for SW entry in late-season out-migrating juvenile sea lamprey. For the first time, we describe endocrine and gene-specific biomarkers of thermal stress in juvenile sea lamprey. Although it is unlikely that northerly populations of sea lamprey would experience mean spring temperatures as high as 29 °C, coastal watersheds with mean temperatures as high as 24 °C are present throughout the North American range of sea lamprey (Letcher, 2016), and hydroelectric facilities are known to cause large fluctuations (up to 7 °C) to downstream river habitats (Cushman, 1985). In the southerly European range, sea lamprey populations are found in rivers with early-spring temperatures that reach over 10 °C (Kamarianakis et al., 2016) and are considered vulnerable to warming trends posed by climate change (Mateus et al., 2012). In closing, the present study highlights meaningful sensitivities to warming temperatures exhibited by juvenile sea lamprey, particularly with regard to survival upon SW entry, which should be considered in management and conservation efforts throughout the North American and European range for this species where warming river or estuarine temperatures is of concern.

Acknowledgments

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Author contributions

Conceptualization, methodology, and experimentation: C.A.S., S.D.M.; Data curation and formal analysis: C.A.S.; Writing and revisions: C.A.S. (original draft), S.D.M; Funding acquisition and project supervision: S.D.M.

Disclosure

This work was supported by a National Science Foundation grant (IOS-1558037) to S.D.M. The authors declare no competing or financial interests. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. government.

Tables

Gene	Primer	Primer sequence (5' to 3')	T_m (°C)	Amplicon (bp)	Gene ID
hsp70	Forward	AAGCAGTTCATGAGGGAGGC	60.03	138	PMZ_0041511
	Reverse	GAGCTCCGTGAGTTGGTTGA	59.97		
hsp90	Forward	GAGTTTGACGGCAAAACGCT	59.97	273	PMZ_0016150
	Reverse	CATGGTGGACGAGTCACGAA	60.04		
hsc70	Forward	GTGGCCTTCAATGACTCGGA	60.04	208	PMZ_0044616
	Reverse	TTTCGCCCTTGTACTCGACC	60.04		

Table 7.1: Information for primers used in gene expression analyses of HSP70, HSP90, and HSC70 in sea lamprey. Tm, annealing temperature. Gene ID refers to the unique sequence identifier in the gene assembly (https://genomes.stowers.org/).

Date	**Body length** (cm)	**Body mass** (g)	**Condition factor**
9 Dec	15.41 (0.37)	4.48 (0.3)	1.21 (0.04)
20 Jan	15.23 (0.22)	3.5 (0.2)	0.98 (0.03)
22 Mar	14.69 (0.35)	3.9 (0.26)	1.22 (0.02)
3 May	14.49 (0.19)	3.46 (0.2)	1.13 (0.04)

Table 7.2: Biometrics for seawater performance trials. Values are presented as mean (SE) of FW controls (time = 0) for respective salinity trials.

Figures

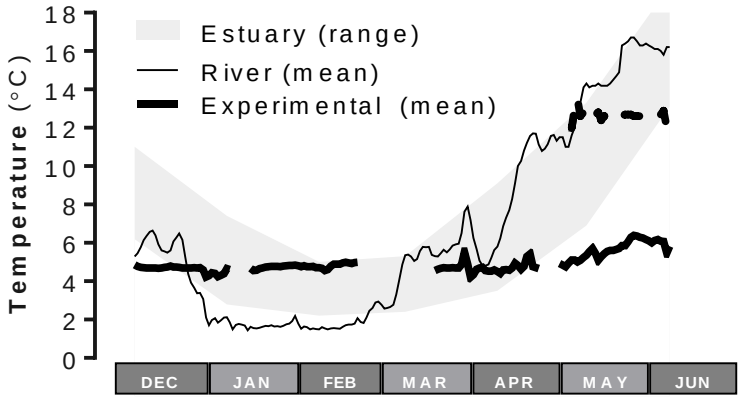

Figure 7.1: Water temperatures and experimental design. Temperature traces of ambient Connecticut river estuary (grey area), ambient Connecticut river rearing water (thin line), and 30 d experimental seawater (SW) exposures (thick lines). SW exposures were carried out at 5 °C (solid line) or 12 °C (May only; dashed line). Estuary temperature data were obtained from NOAA Center for Operational Oceanographic Products and Services (https://tidesandcurrents.noaa.gov).

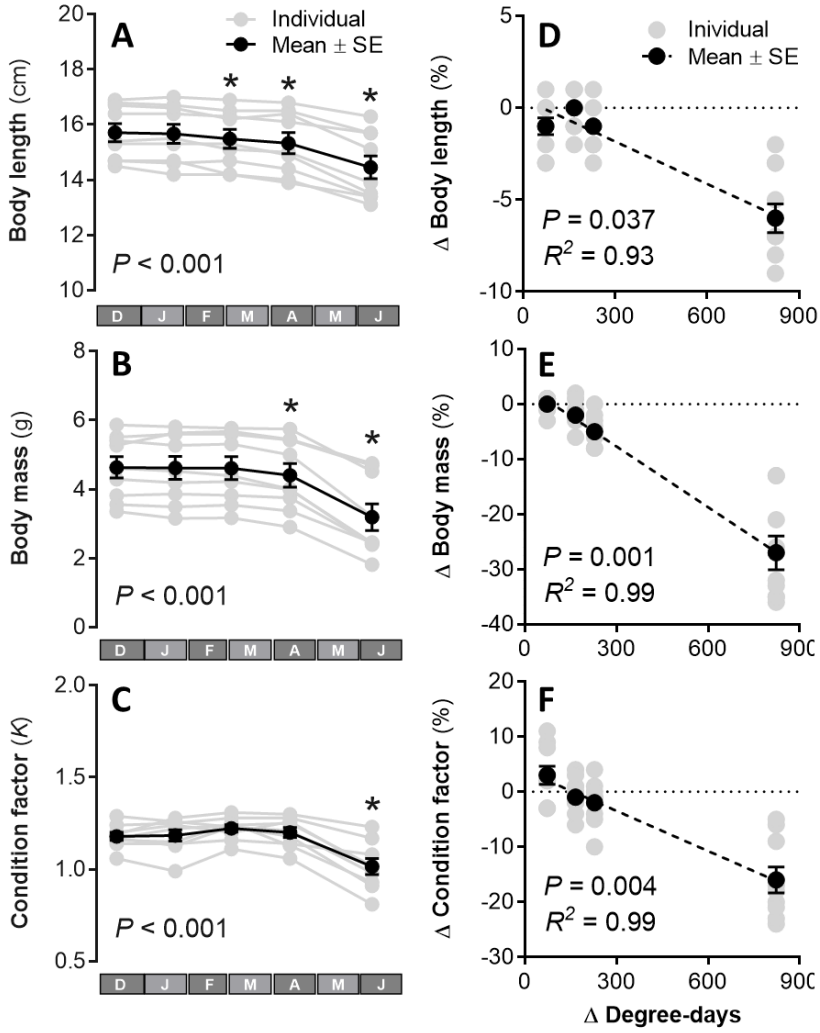

Figure 7.2: Seasonal changes in biometric data for tagged sea lamprey. (A, D) Body length. (B, E) Body mass. (C, F) Fulton's condition factor, *K*. In all panels, data are presented as individuals (grey) and mean ± SE (black; n = 8-10). In A-C: asterisk indicates difference from December value (ANOVA, Student-Newman-Keuls). In D-F: percent change in (Δ) respective biometric data since the previous sampling (y-axis) is plotted against the respective Δ degree-days over that time (x-axis); dashed line represents linear fit.

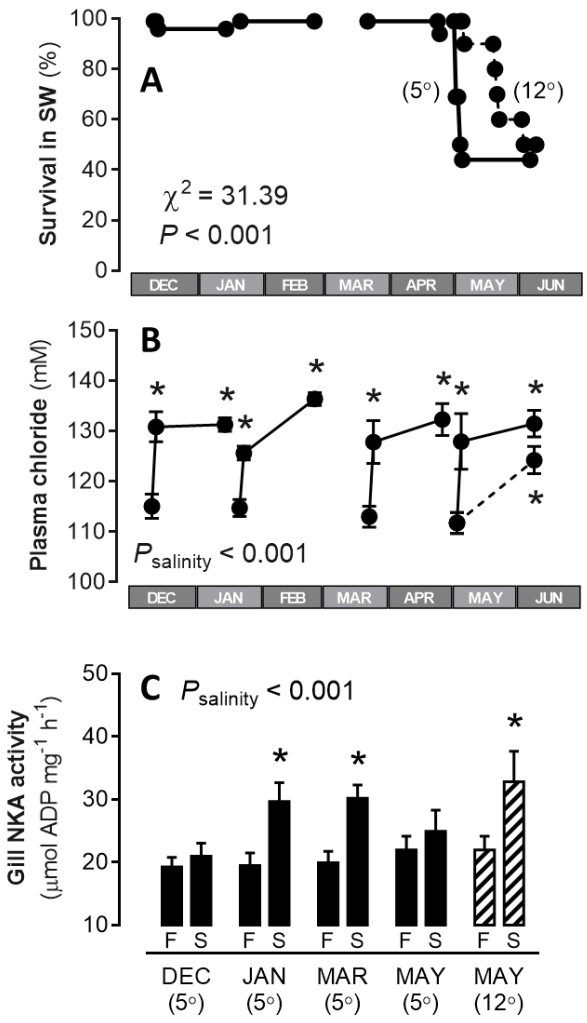

Figure 7.3: Seasonal changes in seawater performance. Seawater (SW) trials were carried out for 30 d at either 5 °C (solid lines) or 12 °C (May only; dashed line). (A) Survival during SW trials (Log-rank test). (B) Plasma chloride concentration at day 0 (freshwater; FW), 2, and 30 of SW trials. (C) Gill NKA activity at day 0 (F) and 30 (S) of SW trials 5 °C (solid) or 12 °C (May only; dashed). Data in B and C are presented as mean ± SE (n = 8-10). Asterisks indicate difference from FW of respective SW trial (B: two-way ANOVA, Dunnet's *post hoc*; C: *t*-test).

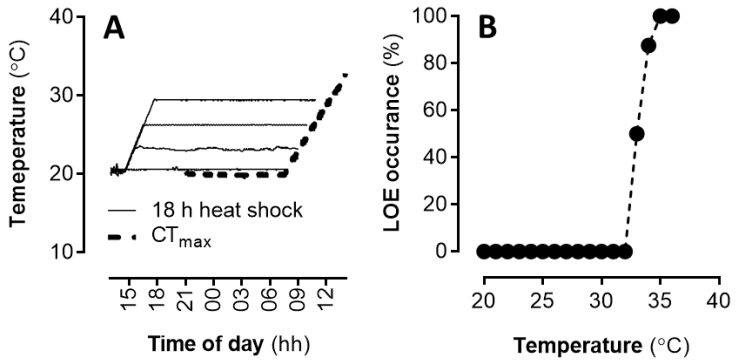

Figure 7.4: Thermal tolerance testing. (A) Temperature traces of 18 h heat shock (solid lines) and CT_{max} (dashed line) trials. (B) Percent individuals exhibiting loss of equilibrium (LOE) during exposure to increasing temperature (rate: 2 °C h^{-1}). Temperature at LOE is considered critical thermal maximum (CT_{max}). (C) Plasma 11-deoxycortisol. (D) Plasma glucose. Data in C and D are presented as mean ± SE (*n* = 6-8). Asterisks indicate difference from 20 °C control (heat shock: (C) Kustal-Wallis, Dunn's; (C) ANOVA, Student-Newman-Keuls; CT_{max}: *t*-test).

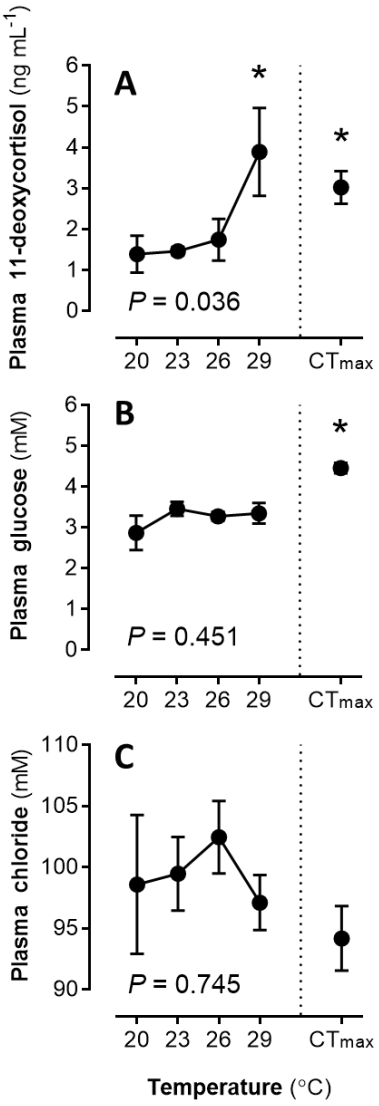

Figure 7.5: Physiological responses of sea lamprey during thermal tolerance testing. Plasma concentrations for (A) 11-deoxycortisol, (B) glucose, and (C) chloride in sea lamprey after 18 h heat shock or reaching CT_{max}. Data in are presented as mean ± SE (n = 6-8). Asterisks indicate difference from 20 °C control (heat shock: (C) Kustal-Wallis, Dunn's; (D, E) ANOVA, Student-Newman-Keuls; CT_{max}: t-test).

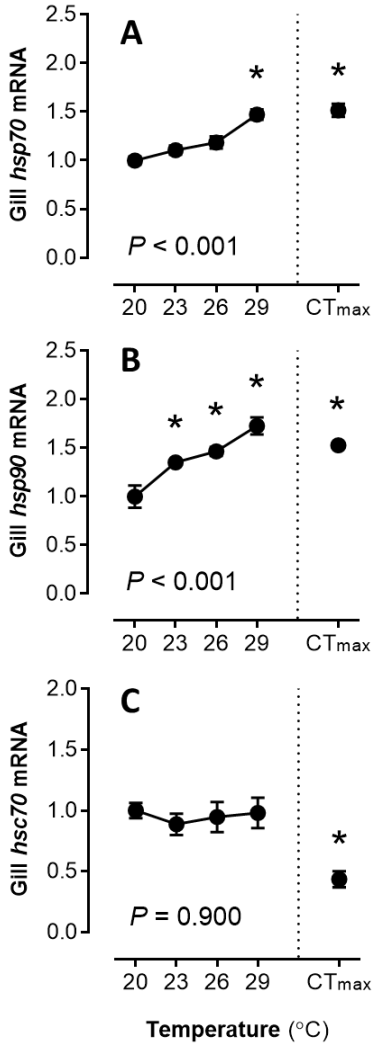

Figure 7.6: Gill heat shock protein expression during thermal tolerance testing. Relative mRNA abundance of (A) heat shock protein 70 (*hsp70*), (B) *hsp90*, or (C) heat shock cognate 70 (*hsc70*) in sea lamprey after 18 h heat shock or reaching CT_{max}. Data are presented as fold-change relative to the housekeeping gene *ef1α* (comparative $\Delta\Delta C_T$ method). Data are presented as mean ± SE (n = 6-8). Asterisk indicates difference from 20 °C control (heat shock: ANOVA, Student-Newman-Keuls; CT_{max}: t-test).

APPENDIX A

SUPPLEMENTARY MATERIAL FOR CHAPTER 4

Supplementary materials and methods

Radioimmunoassay for analysis of plasma [S]

The RIA was carried out in glass culture tubes (10 x 75 mm) using a commercial antibody (Ab; CET-M8, Absolute Antibodies Inc.) and commercial ^{3}H-labeled 11-deoxycortisol ([^{3}H]S; American Radiolabeled Chemicals, Inc., St. Louis, MO). Each reaction consisted of 10 μL of plasma or standard sample, 100 μL of PBS assay buffer (50 mM NaH_2PO_4, 137 mM NaCl, 0.4 mM EDTA, BSA 0.2 % w/v, pH 7.4) containing 5,000 cpm [^{3}H]S, and 50 μL of Ab diluted 1:5,000 in assay buffer (determined to be appropriate for 50 % [^{3}H]S binding; Fig. S1A). The reactions were prepared on ice, incubated for 1 h at 37 °C, then incubated at 4 °C overnight. After overnight incubation, 500 μL of dextran-coated charcoal (PBS, 0.25 % w/v dextran, 2.5 % w/v activated charcoal) was added to each reaction and incubated on ice for 15 min. Unbound 11-deoxycortisol that associates with charcoal was pulled out of solution by centrifugation at 2,000 ***g*** for 15 min. A 325 μL aliquot of the supernatant containing S and [^{3}H]S bound to Ab was added to 2.5 mL scintillation fluid, then analyzed by a liquid scintillation counter (LS-6500, Beckman Coulter). Final determination of plasma [S] was made by interpolation using a serial dilution standard curve of unlabeled S (Riechstien's Substance S, Sigma-Aldrich, USA) ranging from 32 to 0.25 ng mL^{-1} run in triplicate (Fig. S1B). The CET-M8 Ab used in our assay was determined to have high specificity for S, compared to DOC, F, and A (Fig. S1C).

Radioreceptor binding assays for analysis of gill corticosteroid receptor

Frozen gill tissue pooled from two individuals (~8-10 gill pouches) were placed in 300 μL of ice-cold HEPES assay buffer (25 mM HEPES. 10 mM NaCl, 1 mM monothioglycerol, pH 7.4) and homogenized on ice using a ground glass homogenizer. Homogenates were centrifuged for 10 min at 2,000 *g* at 4 °C and the resulting supernatant (crude cytosolic preparation) was placed on ice for use in receptor binding assay which was carried out on ice in a 96-well plate. An aliquot of supernatant was reserved to determine protein concentration (BCA Protein Assay, Pierce, USA). For each sample, total (B_T) and non-specific (B_{NS}) binding curves were obtained by incubating 25 μL of gill supernatant with 25 μL of assay buffer (HEPES) containing various concentrations of [^{3}H]S, either alone (B_T) or with a 500-fold excess of [cold]S (B_{NS}). The final assay in each well was 50 μL and contained 4-6 mg mL^{-1} gill protein and 2.5, 5, 10, or 20 nM [^{3}H]S and was incubated on ice for 2 h to allow the reaction to reach equilibrium. After incubation, 150 μL of an ice-cold dextran-coated charcoal solution (HEPES, 0.25 % w/v dextran, 2.5 % w/v activated charcoal) was added to each well and incubated for 10 min on ice, then centrifuged for 10 min at 2,000 *g* at 4 °C to remove any unbound [^{3}H]S. Finally, 100 μL of supernatant was transferred to scintillation vial containing 2 mL of scintillation fluid (ECONO-SAFE, Research Products International Corp., USA) and counted in a liquid scintillation counter (LS 6000IC, Beckman Instruments Inc., USA). Specific binding (B_S) was calculated ($B_S = B_T - B_{NS}$; Fig. S2A) and the corticosteroid receptor binding capacity (B_{max}) and equilibrium dissociation constant (K_d) were determined by hyperbolic regression analysis (Fig. S2B). Binding

specificity was analyzed identically as above but using 1 nM [^{3}H]S in competition with various concentrations (1, 10, 100, 1000 nM) of unlabeled S, DOC, F, or A (Fig. S2C).

Gill mRNA transcription analysis

Total RNA isolated from gill (TRIzol reagent, Molecular Research Center Inc., Cincinnati, OH) was analyzed for concentration and purity (Take3, BioTek Instruments, Inc., USA) as well as integrity (gel electrophoresis). Only high-purity (A260/A280 > 1.9) and high-integrity RNA samples were used for cDNA synthesis. Synthesized cDNA (High-Capacity Reverse Transcription Kit, Applied Biosystems, USA) was used for quantitative real-time polymerase chain reaction (qPCR), which was carried out in 10 µL reactions containing 2 ng cDNA template, 200 nM forward and reverse primers, and SYBRSelect master mix (ThermoFisher, Waltham, MA). All qPCR reactions were performed using previously published primers for *nka*, *nkcc1*, and *ef1a* in optical 96-well reaction plates (StepOnePlus™ Real-Time PCR System, Applied Biosystems, Inc., USA) using the following thermal profile: holding at 50 °C (2 min) then activation at 95 °C (2 min); cycling (40 cycles) from 95 °C (15 s) to 60 °C (1 min) to 72 °C (30 s). A single product was confirmed by melt curve analysis (every reaction) and electrophoresis (each primer pair).

Immunoblotting

Gill samples prepared by crude homogenization in SEID buffer plus 0.1 % protease inhibitors (complete mini EDTA-free; Roche, USA) and stored in Laemmli sample buffer were run at 5 µg protein per lane for electrophoretic separation then transferred to Immobilon PVDF transfer membranes (Millipore, USA). The PVDF

membranes were equilibrated to phosphate-buffered saline with 0.05% Triton X-100 (PBS-T), blocked for 1 h at 23°C in 5% nonfat milk in PBS-T, then incubated for 2 h in Ponceau stain (stains all proteins in all lanes) to assess equal protein loading across all lanes. Ponceau stain was then washed off in PBS-T and the membrane was incubated overnight at 4 °C in blocking buffer containing a 1:4,000 dilution of primary antibodies: mouse monoclonal anti-NKA α-subunit ('α5'; RRID: AB_2166869) or mouse monoclonal anti-NKCC1 ('T9'; RRID: AB_2618107) (Developmental Studies Hybridoma Ban, Iowa City, IA, USA). After primary incubation, membranes were washed with PBS-T, incubated for 2 h at 23 °C in blocking buffer containing a 1:10,000 dilution of a goat anti-mouse secondary antibody (WVR), then washed again. Images were acquired using a Syngene PXi system (SYNGENE Inc., USA) via enhanced chemiluminescence. Band intensity was measured using ImageJ (National Institutes of Health, USA).

Calculations and statistics

To understand the nature of the relationships between hormone or receptor and the developmentally regulated rise in gill NKA activity, we compared three theoretical curve-fitting analyses (linear, hyperbolic, and quadratic) to determine the simplest model for the relationship (most degrees of freedom) that best fit (highest r^2) the data. Normality and homogeneity of variance assumptions were tested using Shapiro–Wilk and Levene's tests, respectively. All other statistics used in analyses and *P*-values are indicated in figure captions. An α-value of 0.05 was selected to denote statistical significance in all analyses. All regression and statistical analyses and figures were completed using GraphPad Prism 6.0 (GraphPad Software, USA).

Supplementary results

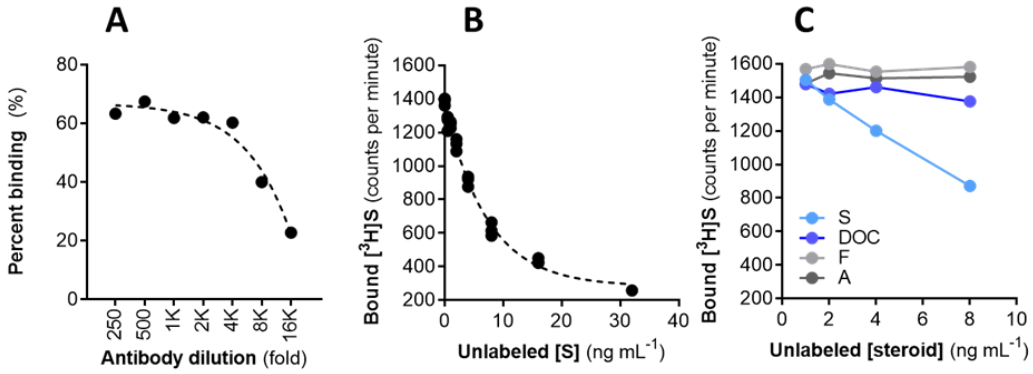

Figure S1: Description of radioimmunoassay for measurement of plasma [S]. (A) Percent binding of radiolabeled 11-deoxycortisol ([^{3}H]S; 5,000 cpm total in reaction) to CET-M8 antibody at various dilutions. (B) Representative competitive displacement standard curve from which unknown plasma [S] values were interpolated. (C) Specificity analysis of CET-M8 antibody to competition by unlabeled corticosteroids; S, 11-deoxycortisol, DOC, 11-deoxycorticosterone; F, cortisol; A, aldosterone.

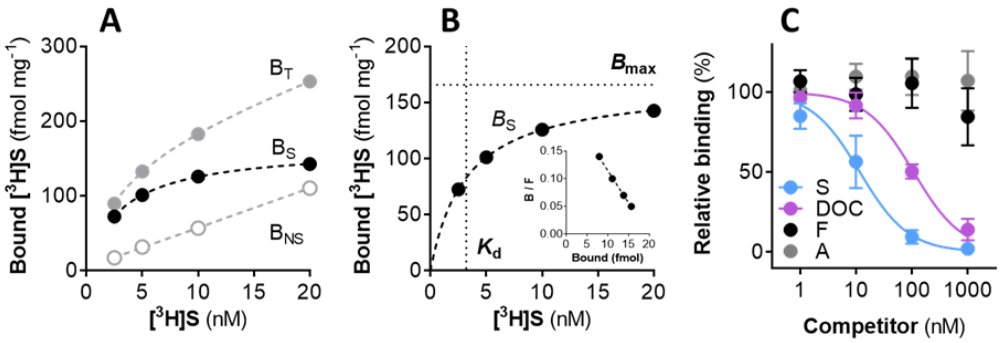

Figure S2: Description of radioreceptor binding assay gill corticosteroid receptor analysis. (A) Representative receptor binding curves; B_T = Total binding; B_{NS} = Nonspecific binding; B_s = Specific binding (calculated; B_T - B_{NS}). (B) Representative receptor binding saturation curve showing calculated values for corticosteroid receptor abundance (B_{max}) and equilibrium dissociation constant (K_d); insert: Rosenthal plot of presented curve. (C) Ligand binding affinity analysis (n = 3; values represent mean ± s.e.m.); IC_{50} (half-maximal inhibition): IC_{50}(S) = 15.7 nM; IC_{50}(DOC) = 89.1 nM (unpaired t test: $P < 0.001$; n = 3).

Table S3: Endocrine and osmoregulatory changes throughout sea lamprey metamorphosis. Results of two-way ANOVA analyses for gill Na^+/K^+-ATPase (NKA) activity, plasma 11-deoxycortisol concentration ([S]), and gill corticosteroid receptor (CR) abundance (B_{max}) in larvae and mid-metamorphic sea lamprey during August-November.

	Life Stage		**Month**		**Interaction**	
	F (DFn, Dfd)	*P*	*F* (DFn, Dfd)	*P*	*F* (DFn, Dfd)	*P*
Gill NKA Activity	120.0 (1, 93)	< 0.001	57.75 (3, 93)	< 0.001	54.56 (3, 93)	< 0.001
Plasma [S]	12.81 (1, 72)	0.001	27.86 (3, 72)	< 0.001	7.48 (3, 72)	< 0.001
Gill CR Abundance, B_{max}	0.15 (1, 16)	0.702	2.75 (3, 16)	0.077	6.64 (3, 16)	0.004

Table S4: Relationships between endocrine and osmoregulatory changes during sea lamprey metamorphosis. Relationships between gill Na^+/K^+-ATPase (NKA) activity and plasma 11-deoxycortisol concentration ([S]) or gill corticosteroid receptor (CR) abundance (B_{max}) in metamorphic sea lamprey fit by either linear, hyperbolic, or quadratic regression.

Relationship (vs. gill NKA activity)	Plasma [S]			Gill CR abundance (B_{max})		
	n	df	r^2	*n*	df	r^2
Linear	51	49	0.41	12	10	0.70
Hyperbolic	51	49	0.52	12	10	0.47
Quadratic	51	48	0.51	12	9	0.70

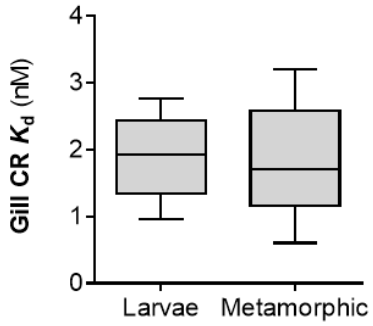

Figure S5: Dissociations constants (K_d) from all larvae and metamorphic individuals (unpaired *t* test: $P = 0.817$; $n = 12$).

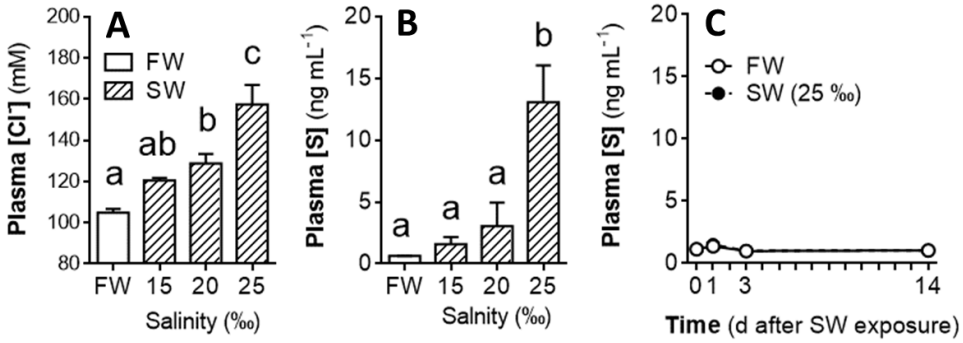

Figure S6: Changes in concentrations of (A) plasma chloride ([Cl^-]) ($P < 0.001$) and (B) plasma 11-deoxycortisol ([S]) ($P = 0.007$) in mid-metamorphic (early-Oct) sea lamprey after 24 h exposure to freshwater (FW) or various dilutions of seawater (SW; dashed bars). Changes in (C) plasma [S] in fully metamorphosed (late-Nov–early-Dec) sea lamprey during SW acclimation ($P_{salinity} = 0.770$, $P_{time} = 0.049$, $P_{interaction} = 0.978$). Values represent mean ± s.e.m and letters indicate differences (A, B: n = 4-6, one-way ANOVA, Tukey's *post hoc*; C: n = 10-12, two-way ANOVA, Tukey's *post hoc*).

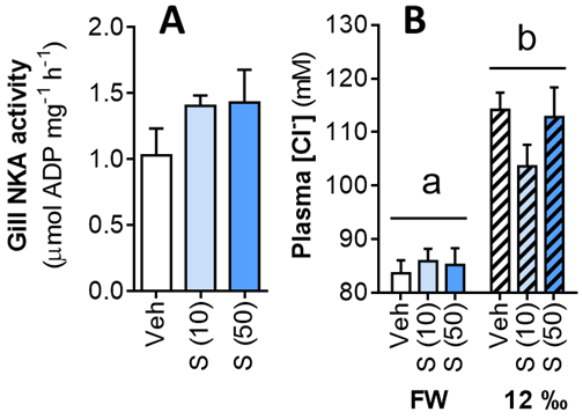

Figure S7: Effect of 11-deoxycortisol (S) treatment in larval sea lamprey on (A) gill Na^+/K^+-ATPase (NKA) activity (P = 0.297; one-way ANOVA, Tukey's *post hoc*) and (B) plasma chloride concentration ($[Cl^-]$) in larval lamprey in freshwater (FW; open bars) or after exposure to seawater (SW; 12 ‰; dashed bars) 12 d after injection with an oil implant alone (Veh) or containing 11-deoxycortisol (blue bars). Dose of S is stated in parenthesis (μg g^{-1} body weight). In (B), only the effect of salinity was significant (P < 0.001); neither effects of S treatment (P = 0.262) nor the interaction (P = 0.476) were significant (two-way ANOVA). Values represent mean ± s.e.m (n = 6-8).

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