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Research paper

Corticosteroid control of Na^+/K^+-ATPase in the intestine of the sea lamprey (*Petromyzon marinus*)

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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 downstream migration and SW entry. We have previously shown that intestinal Na^+/K^+-ATPase (NKA) activity increases during metamorphosis and is critical to the osmoregulatory function of the intestine in SW. The present study investigated the role of 11-deoxycortisol (S) in controlling NKA in the anterior (AI) and posterior (PI) intestine during sea lamprey metamorphosis. In a tissue profile, *nka* mRNA and protein were most abundant in the gill, kidney, and AI. During metamorphosis, AI *nka* mRNA increased 10-fold, whereas PI *nka* mRNA did not change. Specific corticosteroid receptors were found in the AI, which had a higher binding affinity for S compared to 11-deoxycorticosterone (DOC). *In vivo* administration of S in mid-metamorphic lamprey upregulated NKA activity 3-fold in the AI and PI, whereas administration of DOC did not affect intestinal NKA activity. 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. These results indicate that intestinal osmoregulation in sea lamprey is a target for control by S during metamorphosis and the development of SW tolerance.

1. Introduction

Fossil records indicate that lampreys have remained morphologically conserved, inhabiting marine and estuarine environments from at least the late Devonian, ~360 mya (Gess et al., 2006). Modern lampreys are ion- and osmo-regulators, maintaining a constant internal osmolality at ~300 mOsm kg^{-1} H_2O (approximately one-third that of seawater (SW), which is ~1100 mOsm kg^{-1} H_2O), a strategy shared by most other vertebrate species, including the teleost fishes (Edwards and Marshall, 2013). Like many other lampreys, the sea lamprey (*Petromyzon marinus L.*) is anadromous, living as larvae in freshwater (FW) for 4–6 years before metamorphosing and migrating into SW as juveniles. The larvae-to-juvenile metamorphosis takes approximately 4 months and involves the radical transformation from substrate-dwelling, filter-feeding larvae into a free-swimming, parasitic juveniles. Juveniles spend another 2–3 years in the sea before returning to FW as reproductively mature adults to spawn and die. Larval sea lamprey cannot withstand salinity higher than one-third SW. However, SW tolerance increases dramatically during metamorphosis (Beamish et al., 1978; Reis-Santos et al., 2008; Shaughnessy and McCormick, 2020), allowing metamorphosed sea lamprey to enter SW with minimal disturbance to ion homeostasis.

The transition from FW to SW life requires a rearrangement of osmoregulatory processes. Fish living in FW counteract the passive loss of ions and gain of water by the 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. In contrast, fish living in hyperosmotic environments such as SW must counteract the passive gain of ions and water loss. To do this, fish in SW (including lamprey; Barany et al. (2020)) increase drinking and absorb water in the gut while excreting excess divalent ions *via* the gut and kidney and monovalent

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ions *via* the gills (Marshall and Grosell, 2006).

Ingested SW is consecutively processed throughout the gut. The result is a progressive desalination of the ingested SW until it is close to isosmotic with respect to the blood, allowing net water absorption to occur in the intestine (Grosell, 2006; Whittamore, 2012). As ingested SW moves through the gut, the osmolality of the lumen is reduced, allowing for simultaneous net water absorption *via* two possible paths: paracellularly and transcellularly. Both paths for water absorption from the lumen are driven by the osmotic gradient, indicating that Na^+ and Cl^- transport is essential for water uptake (Grosell et al., 2005). The 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 for Na^+ that is coupled with the movement of Cl^- through various ion transporters (Grosell, 2006; Whittamore, 2012). The excess of monovalent ions taken up by the gut in order to desalinate the imbibed SW is secreted by the gills. In teleosts, elevated NKA activity levels have been found in the gut and gill after SW acclimation (Grosell, 2006). In anadromous fish, gill and intestinal NKA increase in conjunction with increased salinity tolerance (McCormick, 2013; Veillette and Young, 2004).

Corticosteroids mediate many bodily functions in vertebrates, including metabolism, immune function, osmoregulation, and stress responses. Corticosteroids have two primary functions: i) glucocorticoid function controlling metabolism and growth; and ii) mineralocorticoid function controlling the transport of ions and water. In most tetrapods, cortisol or corticosterone carries out glucocorticoid actions by activating the glucocorticoid receptor (GR), whereas aldosterone carries out mineralocorticoid actions by activating the mineralocorticoid receptor (MR). In teleost fish, cortisol appears to act as both a glucocorticoid and mineralocorticoid (McCormick et al., 2008; Mommsen et al., 1999). Aldosterone is absent in circulation in fish due to the lack of a key enzyme involved in its synthesis in the interrenal tissue, aldosterone synthetase; likewise, stress and osmoregulatory stimuli *in vivo* or *in vitro* do not result in elevated aldosterone levels in teleost fish (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 appears to interact only with the teleost MR, not the GR (Kiilerich et al., 2011; Milla et al., 2008; 2006;; Sakamoto et al., 2011).

Cortisol has been shown to have a prominent role in seawater acclimation of teleosts and has important actions on intestinal function (Takahashi and Sakamoto, 2013; Utida et al., 1972). In salmon, *in vivo* implantation with cortisol upregulates gill and intestinal NKA activity, increases intestinal fluid transport, and promotes SW tolerance (Bisbal and Specker, 1991; Cornell et al., 1994; Veillette et al., 1995; McCormick et al., 2008.; Shaughnessy and McCormick, 2018). Likewise, *in vitro* experimentation using excised gill and intestinal tissue has shown that incubation with cortisol increases gill and intestinal NKA activity (McCormick and Bern, 1989; McCormick, 1995; Veillette and Young, 2004, 2005).

In lamprey, neither cortisol nor aldosterone are present in the blood, and there is increasing evidence that 11-deoxycortisol (S; a biosynthetic precursor to cortisol) is the primary corticosteroid hormone in lamprey (Bridgham et al., 2006; Close et al., 2010; Rai et al., 2015; Shaughnessy et al., 2020). Lamprey have a single 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 abundant in the gill, intestine, and testes, and *in vivo* treatment with S upregulates gill NKA in both juveniles and adults (Close et al., 2010; Shaughnessy et al., 2020). Whether osmoregulatory processes in the lamprey intestine are also controlled by S has not yet been established.

The present study aimed to determine the role of corticosteroids in mediating osmoregulatory processes in the sea lamprey intestine. Specifically, we hypothesized that S receptors would be present in the intestine of sea lamprey and that exogenous treatment with S would affect whole animal capacity for osmoregulation and the transcription, abundance, and activity of NKA in the intestine. We focused on sea lamprey metamorphosis when increases in osmoregulatory mechanisms in the gill and intestine result in the development of SW tolerance.

2. Material and methods

2.1. 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 (protocol # 2016–0009) and the US Geological Survey (protocol LB00A3O-117). Sea lamprey for the metamorphic profile were collected from 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 and Potter (1979). All other individuals were collected as stage 6 and 7 by netting from the power canal on the Connecticut River in Turners Falls, MA, adjacent to the U.S. Geological Survey, Conte Anadromous Fish Research Center, where fish were held for up to 6 months after collection. Refer to Barany et al. (2020) and Shaughnessy et al. (2020) for animal biometrics. Once in the laboratory, lamprey were held under natural photoperiod in 1.5 m diameter tanks supplied with Connecticut River water. Connecticut River sand substrate 10 cm in depth was placed in tanks to offer lamprey the opportunity to burrow. Generally, larvae and metamorphic lamprey would burrow, and post-metamorphic and migrant lamprey would remain unburrowed and attached to the tank walls. Metamorphic and post-metamorphic individuals were not offered food as they naturally cease feeding until they begin parasitic feeding in the ocean. All FW and SW experimentation was carried out in 15 °C dechlorinated tapwater (FW, <0.2 ppt) in 60 L recirculating glass aquaria equipped with aeration and mechanical, chemical, and biological filtration. Experimental SW (35 ppt) was made by dissolving artificial sea salt mix (Crystal Sea Salt, Baltimore, MD, USA) in FW.

2.2. Laboratory experiments

For the tissue profiles, lamprey were acclimated to identical FW or SW conditions for 3 weeks before sampling. We have previously shown that our *in vivo* corticosteroid treatment resulted in physiologically relevant levels of the given corticosteroid in circulation (Shaughnessy et al., 2020). For the injection experiments in the present study, mid-metamorphic lamprey (mean length and body mass, respectively: 14.6 cm and 4.6 g; early stage 7) were anesthetized in MS-222 (200 mg L^{-1} buffered with $NaHCO_3$, pH 7.0) (Argent Chemical Laboratories, Redmond, WA, USA), then injected with a slow-release implant containing either steroid (S or DOC; 5, 10, 40 or 50, and 5 or 40 μg g^{-1} body weight, respectively) or vehicle only (oil:shortening,1:1) and allowed to fully recover (~30 min) before returning to experimental tanks. This steroid administration protocol was identical to that used by Shaughnessy et al. (2020). Lamprey administered with steroid or vehicle implants were held in FW for 12 d, then were sampled in FW or after 24 h exposure to 25 ppt SW. For each experiment, uninjected lamprey in FW were sampled on the day of injections as an initial time zero (T_0) control. Our steroid treatments were carried out on mid-metamorphic lamprey (stage 7) for two reasons: (i) early in metamorphosis (stages < 6), sea lamprey have limited ability to acclimate to SW (Shaughnessy et al., 2020; Barany et al., 2020); and (ii) in the last stages of metamorphosis (late stage 7) and in fully-metamorphosed juveniles, branchial and intestinal NKA activity and transcription are at or near maximum levels, making it difficult to observe changes induced by hormone treatment.

2.3. Sampling

Sea lamprey for the metamorphic profile were sampled immediately after capture in the field. Lamprey in the field and laboratory 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'), and then dried for two days at 60 °C. Dried muscle samples were reweighed ('dry mass'), and the difference between 'wet mass' and 'dry mass' was used to calculate values for muscle moisture (% water). Two sequential sections of intestine were collected with fine-point scissors: anterior intestine (AI), considered as the proximal intestinal section beginning at the end of the esophagus (1–1.5 cm); and posterior intestine (PI), considered as the distal intestinal section ending at the rectal sphincter (2–3 cm). Tissue samples for receptor binding, mRNA, protein analyses were immediately frozen on dry ice 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 at −80 °C.

2.4. 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. Equal loads of sample (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 alpha subunit 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 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 normalized relative to FW brain (set to a value of 1).

2.5. 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. A Take3 micro-volume plate (BioTek Instruments, Inc., Winooski, VT) was used to assess concentration and purity of each RNA sample. Only samples that 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 the 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. A pool of cDNAs from all the samples for each tissue was used for calibration plots, using five serial 10-fold dilutions from 50 ng to 5 pg, to assess the linearity and efficiency of the different primer combinations and to ensure C_T values were within the linear range for the target and reference genes. 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 (alpha subunit) mRNA was calculated using the comparative (ΔΔCT) method (Pfaffl, 2001) with elongation factor 1 (*ef1a*) or glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) used as a reference gene. Reference gene C_T values were stable across all experimental groups, life stages, and tissues. A pool of all the cDNA samples within tissues was used as a calibrator on every qPCR plate to correct for inter-assay differences. Primer sequences for *nka*, *ef1a*, and *gapdh* in the sea lamprey have been previously described in Ferreira-Martins et al. (2016) and Kolosov et al. (2017). For reactions and primer pairs across all experiments, linearities (R^2) of standard curves were 0.99–1.00 (range), and reaction efficiencies were 100 ± 4% (median ± standard deviation).

2.5.1. Receptor binding assay

Our receptor binding assay has been previously described (Shaughnessy et al., 2020). Frozen anterior intestine tissue from mid-metamorphic lamprey (early stage 7) 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 the sample was incubated with 25 µL of assay buffer (HEPES) containing [^{3}H]S, either alone (total binding; B_T) or with unlabeled 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 unlabeled 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, 150 µL of dextran-coated charcoal solution (HEPES with 0.25% w/v dextran and 2.5% w/v activated charcoal) was added to each reaction and incubated for an additional 10 min on ice. Free [^{3}H]S and unlabeled S (unbound to the receptor) were removed from the 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.

2.6. 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 the reduction of NADH to NAD^+ in a 1:1 ratio to the conversion of ATP to ADP by NKA. Supernatant samples were assayed with or without the presence of 0.5 mM ouabain, a specific inhibitor of NKA. The difference in ADP production between reactions with and without ouabain over a 10 min period 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). The protein content of supernatant samples used in the assay were determined using the BCA protein assay.

2.7. Calculations and statistics

Muscle moisture was calculated as [(wet mass − dry mass) / wet mass] × 100. All data are represented as the mean ± standard error of the mean (see figure captions for *n* values). Detection of significant differences were carried out using unpaired Students *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 were set at $P < 0.05$.

3. Results

3.1. Tissue and metamorphic profiles

NKA protein abundance was highest in the brain and kidney of larvae (Fig. 1**A**). NKA protein was significantly higher in the brain, gill, kidney, and AI in juvenile lamprey compared to larval lamprey (Fig. 1**A**). The abundance of *nka* mRNA was generally similar between FW- and SW-acclimated juvenile lamprey and highest in brain, gill, kidney, and AI (Fig. 1**B**). In the gills, *nka* mRNA was significanly higher in SW- compared to FW-acclimated juveniles. In the metamorphic profile, *nka* mRNA did not change throughout metamorphosis in PI, but increased nearly 10-fold from larvae to migrant in AI (Fig. 1**C**). The results of the two-way ANOVA results for Fig. 1 were as follows: (A) $P_{stage} < 0.001$, $P_{tissue} < 0.001$, $P_{interaction} < 0.001$; (B) $P_{salinity} = 0.150$, $P_{tissue} < 0.001$, $P_{interaction} = 0.581$; (C) $P_{stage} < 0.001$, $P_{tissue} < 0.001$, $P_{interaction} < 0.001$.

3.2. Receptor binding assay

Specific binding of S was observed in the lamprey AI ($B_{max} = 329.4 \pm 68.7$ fmol mg^{-1}; $K_d = 3.5 \pm 2.4$ nM) (Fig. 2**A, B**). Binding affinity of the lamprey AI was significantly greater for S ($IC_{50} = 157 \pm 49$ nM) than DOC ($IC_{50} = 1352 \pm 330$ nM) (*t*-test: $P = 0.023$) (Fig. 2**C**). Specific binding of cortisol and aldosterone were not detected.

3.3. Steroid hormone experiments

Prior to S or DOC administration (T_0), intestinal NKA activity in the AI and PI was 3.0 ± 1.3 and 0.9 ± 0.2 μmol ADP mg protein^{-1}h^{-1}, respectively (Fig. 3). After 12 d, a dose-dependent increase in AI and PI NKA activity in response to S was observed. In lamprey administered 40 μg g^{-1} body weight S, AI and PI activity had increased to 11.6 ± 4.1 and 2.5 ± 0.6 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 with DOC.

Initial muscle moisture in uninjected lamprey (T_0) was 68.0 ± 1.1% water (Fig. 4**A**). 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 had significantly higher muscle moisture after SW exposure compared to the vehicle control and were not significantly different from the T_0 group. Initial (T_0) AI and PI NKA activity were 8.5 ± 1.9 and 2.9 ± 0.4 μmol ADP mg protein^{-1}h^{-1}, respectively (Fig. 4**B, C**). After 12 d with an S implant, NKA activity in both AI and PI had increased in a dose-dependent manner and were significantly greater than the vehicle controls. In both AI and PI, *nka* mRNA in vehicle controls was not upregulated after 24 h in SW. In AI, *nka* mRNA was higher in lamprey treated with either S dose (10, and 50 μg g^{-1} body weight) after 24 h in SW compared to the vehicle control (Fig. 4**D**). In PI, only lamprey treated with the 50 μg g^{-1} body weight S had elevated *nka* mRNA after 24 h SW exposure (Fig. 4**E**).

4. Discussion

In the present study, we demonstrate that the intestine of sea lamprey contains highly specific corticosteroid receptors that have a higher affinity for S compared to the other endogenously produced corticosteroid in lamprey, DOC. We further show that exogenous S can upregulate NKA mRNA and activity during the sea lamprey metamorphosis, leading to the development of SW tolerance. These results indicate that S is a physiological regulator of the osmoregulatory function of the sea lamprey intestine and whole animal capacity for ion regulation in SW.

Metamorphosis and acclimation to SW increased NKA expression in osmoregulatory tissues including the gill, kidney, and AI (Reis-Santos et al., 2008; Barany et al., 2020). The higher NKA activity in AI compared to PI, as described in the present study, indicates that

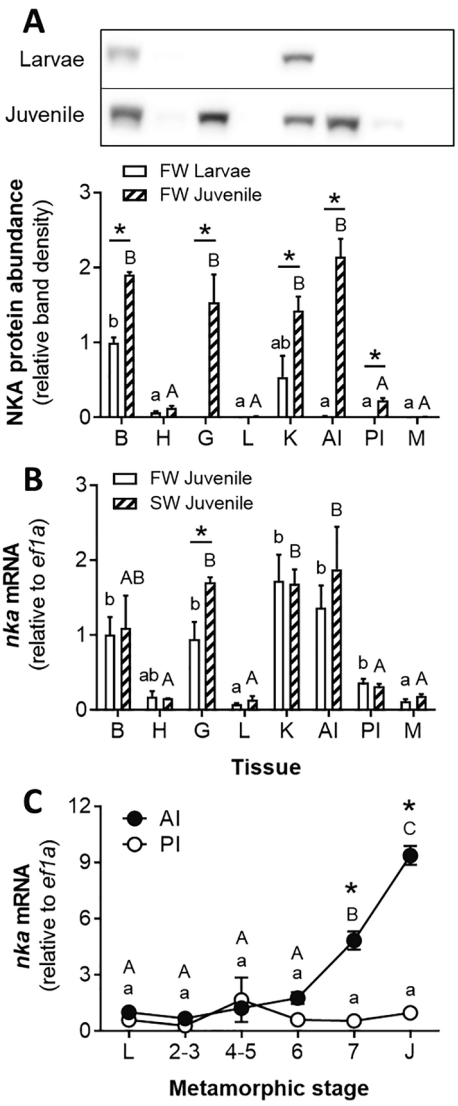

Fig. 1. Na^+/K^+-ATPase (NKA) protein and mRNA abundance in sea lamprey. The relative abundance of NKA protein (Western blot bands appear at ~100 kDa) (A) and *nka* mRNA across a tissue profile (B) and in AI and PI throughout metamorphosis (C). In A and B, data are shown for FW- and/or SW- (35 ppt) acclimated larvae (FW only) and juveniles. Tissue abbreviations: B, brain (FW set to 1); H, heart; G, gill; L, liver; K, kidney; AI, anterior intestine; PI, posterior intestine; M; muscle. Life-stage abbreviations: L, larvae; J, juvenile. Data are presented as mean ± standard error of the mean (A, B: n = 3; C: n = 4–12, two-way ANOVA). Asterisks indicate a significant difference between FW and SW (in A and B) or tissues (in C). In A and B, case-matched letters indicate differences between tissues for respective life stage (in A) or salinity (in B). In C, case-matched letters indicate significant differences between life stages for respective tissue.

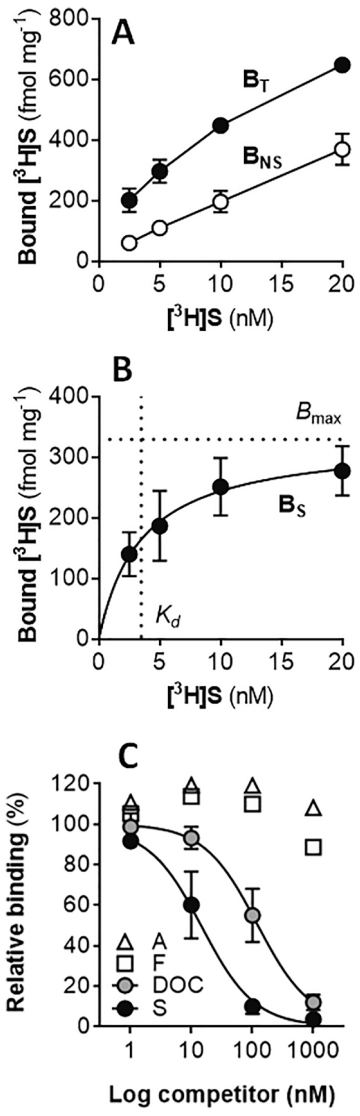

Fig. 2. Characterization of corticosteroid receptor binding in the anterior intestine of metamorphosing (early stage 7) sea lamprey. (A) Receptor binding assay results showing total (B_T) and non-specific (B_{NS}) binding of [^{3}H]11-deoxycortisol ([^{3}H]S). (B) Specific, saturating binding (B_S) plotted 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 of the mean ($n = 3$).

regionalization of osmoregulatory function occurs in the sea lamprey intestine. Although data for sea lamprey intestinal NKA activity are sparse, our findings are consistent with other studies of sea lamprey. Morphologically, the AI is much shorter and more elastic than the PI, which allows this region to easily increase its volume after drinking is initiated in SW (Barany et al., 2020). Functionally, the AI exhibits much greater NKA activity and higher rates of water and Cl^- absorption (Ferreira-Martins et al., 2016; Barany et al., 2020). Upstream migrating adult sea lamprey that were re-exposed to SW exhibited significantly higher NKA activity and mRNA expression in the anterior region of the intestine than the middle and posterior regions (Ferreira-Martins et al., 2016). In the study by Ferreira-Martins et al. (2016), upstream-migrating adult sea lamprey that were acclimated to FW for only a short time (one week) were still capable of upregulating AI NKA activity to high levels (~30 μmol ADP $mg^{-1}h^{-1}$) upon re-introduction to SW, whereas adults that were acclimated to FW for a long time (two months) were unable to upregulate AI NKA activity when re-introduced to SW. Together with the results of the present study, it seems that increased intestinal NKA expression and activity in juvenile sea lamprey in preparation for SW entry may be maintained through adulthood while at sea. Although difficult to conduct, analyses of intestinal osmoregulation in adult sea lamprey caught at sea prior to upstream migration are needed to complete our understanding of intestinal osmoregulatory function throughout the sea lamprey life cycle.

Increases in NKA mRNA expression and activity in the sea lamprey intestine during metamorphosis and prior to SW entry are an indication that the sea lamprey physiologically prepares for marine life before 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 larval lamprey (which has limited SW tolerance), tight-junction proteins have been identified, but their role in ion and water transport remains unclear (Kolosov et al., 2017). Ouabain, a specific inhibitor of NKA, has been used to demonstrate the NKA dependence of water absorption in SW-acclimated teleosts (Ando, 1981) and lamprey (Barany et al., 2020), indicating a critical role of NKA in intestinal function in SW. Further studies of the SW-acclimated sea lamprey intestine are needed to elucidate mechanisms of ion and water transport in addition to the role of NKA, such as the possible participation of Na^+ and Cl^- absorptive molecular pathways (Shaughnessy and Breves, 2021).

It has been shown previously that the lamprey CR is abundant in the gill and that the gill CR specifically binds S (Close et al., 2010; Shaughnessy et al., 2020). In the present study, we show for the first time that the lamprey intestine specifically binds S with significantly higher affinity than the other endogenously produced corticosteroid, DOC. However, the receptor-ligand interactions of the lamprey CR are still unresolved. Studies using an *ex vivo* receptor binding assay have described a highly specific receptor for S (Close et al., 2010; Shaughnessy et al., 2020), whereas *in vitro* transactivation studies have described a lamprey CR that is promiscuous to many corticosteroids, including S, DOC, and the later-evolved corticosteroids, corticosterone, cortisol, and aldosterone (Bridgham et al., 2006). We report the level of specificity to S and a dissociation constant (~3 nM) of the CR in the AI that is similar to what was previously reported using gill *ex vivo* binding assays (Close et al., 2010; Shaughnessy et al., 2020).

Interestingly, the CR abundance (B_{max}) we report for the AI of metamorphosing sea lamprey (early stage 7, ~330 fmol mg^{-1}) is much higher than what has been previously reported for the intestine of adult lamprey (~60 fmol mg^{-1}; Close et al., 2010). A possible explanation for these differences might be the life-stage of the animals used. Indeed, the elevated CR abundance in the AI reported here could indicate the importance of the CR in mediating the very high levels of NKA activity observed in the AI of mid- and post-metamorphic sea lamprey (Barany et al., 2020). We have recently described such a relationship between CR abundance and NKA activity in the lamprey gill; increases in gill CR likely have a large role in regulating the increases in gill NKA during the lamprey metamorphosis (Shaughnessy et al., 2020). Likewise, higher CR abundance in the AI compared to other regions of the gut or the gut as a whole may account for the higher NKA activity and capacity for ion uptake observed in the AI (Barany et al., 2020). Thus, future investigations using intestinal preparations *ex vivo* on the potential regionalization of CR expression and osmoregulatory response to S are warranted.

The remarkable similarity in how corticosteroids control the osmoregulatory adjustments during the parr-smolt transformation in salmonids and the lamprey metamorphosis underscores the importance of this endocrine program on fitness by reducing potential negative impacts of a direct transfer from FW to SW without prior physiological preparation

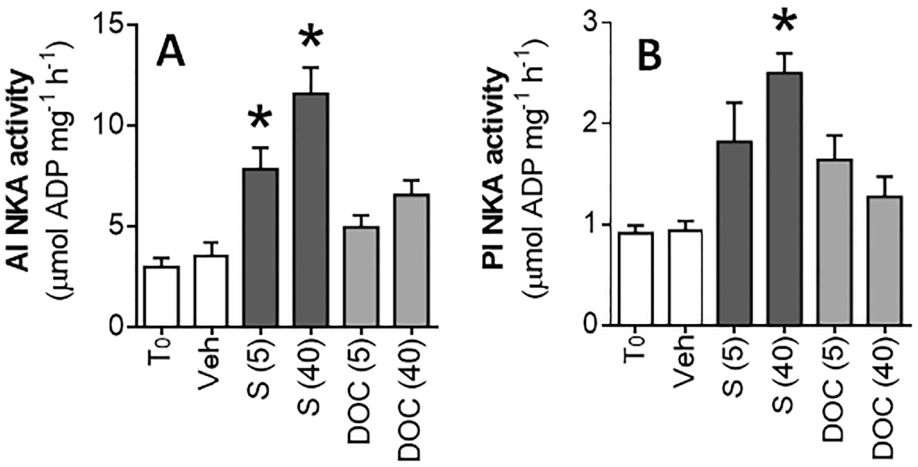

Fig. 3. Effect of endogenous corticosteroids on Na^+/K^+-ATPase (NKA) activity in metamorphosing (early stage 7) sea lamprey in FW. Tissues: anterior (AI; A) and posterior (PI; B) intestine. Parentheses indicate injected dose (μg g^{-1} body weight): S, 11-deoxycortisol; DOC, 11-deoxycorticosterone. Data presented as mean ± standard error (n = 8–10). Asterisks indicate significant differences from vehicle control (oil:shortening, 1:1).

(Barton et al., 1985; Sundell et al., 2003). Previous studies on Atlantic salmon (*Salmo salar*) have shown that increased intestinal water absorption is related to elevated circulating cortisol levels in this species during the parr-smolt transformation, even before experiencing a hypersaline environment (Veillette et al., 1995). Additionally, the transfer of cortisol-implanted salmon presmolts to SW resulted in a more immediate and fuller drinking response (Fuentes et al., 1996). Furthermore, gill GR abundance (B_{max}) increases during the parr-smolt transformation (Shimpton et al., 1996), and gill NKA activity is correlated with GR abundance (Shrimpton and McCormick, 1999).

We have previously shown that increases in gill NKA activity during sea lamprey metamorphosis is strongly correlated with increases in circulating levels of S and gill CR abundance (Shaughnessy et al., 2020). The upregulation of intestinal NKA induced by S treatment that we observed in the present study mirrors our previous findings in the lamprey gill. As with the sea lamprey gill, it seems likely that intestinal CR abundance also increases during metamorphosis and that increased interaction between S and intestinal CR drives the upregulation in intestinal NKA that occurs during the lamprey metamorphosis (Barany et al., 2020).

We have already shown in previous studies that active, NKA-dependent osmoregulatory mechanisms in the intestine are upregulated during sea lamprey metamorphosis (Barany et al., 2020). In the present study, we sought to determine whether S and/or DOC were factors controlling these osmoregulatory changes. In the Veh treated lamprey, NKA activity was not significantly upregulated compared to the T_0 control (Fig. 3), suggesting that the 12 d of metamorphic progression between injection and sampling was not responsible for the increase in intestinal NKA activity observed in the lamprey treated with S. We therefore conclude that S, but not DOC, is a hormone controlling active osmoregulatory mechanisms in both AI and PI. We surmise that the increase in intestinal NKA activity after S treatment is due to an increase in NKA transcription and protein abundance since an increase in gill NKA mRNA, protein abundance, and NKA activity increase in the sea lamprey gill after S treatment (Shaughnessy et al., 2020). The higher potency of S compared to DOC in upregulating NKA activity is likely due to the greater binding affinity of the lamprey intestine CR to S compared to DOC (Fig. 2). The *in vivo* results of the present study further indicate that S has a greater osmoregulatory role than DOC in lamprey, which is likely analogous to a greater osmoregulatory role of cortisol compared to DOC in euryhaline teleosts (Takei and McCormick, 2013).

Lastly, we aimed to relate the osmoregulatory action of S in upregulating intestinal NKA to the overall impacts of this hormone on the whole animal capacity for osmoregulation in SW. Previous studies on lamprey in our laboratory have shown that NKA and other ion transport mechanisms are upregulated in the gill, gut, and kidney after acclimation to hyperosmotic salinities (Reis-Santos et al., 2008; Barany et al., 2020; Shaughnessy and McCormick, 2020; Shaughnessy et al., 2020). Furthermore, numerous studies in teleost fishes have demonstrated that treatment with corticosteroids (*i.e.,* cortisol) prior to exposure to hyperosmotic salinities enhances osmoregulatory acclimation (Takei and McCormick, 2013). In the present study, the muscle dehydration exhibited in the Veh lamprey indicates that exposure to 25 ppt for 24 h (Fig. 4) was a substantial osmoregulatory challenge. Moreover, that the Veh group did not exhibit elevated NKA activity or mRNA compared to the T_0 control indicates that the upregulation of NKA in lamprey administered S was due to the corticosteroid treatment alone. Ultimately, prior treatment with S led to improved fluid homeostasis after SW exposure, which was likely due, at least in part, to the increase in intestinal NKA activity. Future investigations should examine whether S and/or DOC control the ethological adjustments during SW acclimation, such as increased drinking or the emergence from the substrate, and the subsequent physiological responses such as desalination and water absorption of imbibed SW (Barany et al., 2020; Fuentes et al., 1996; Rankin, 2002; Veillette et al., 1995). It is interesting to note that sharks, which are osmoconformers and do not substantially drink while in SW, upregulate drinking when exposed to elevated salinities (Anderson et al., 2002). The present and previous work in lamprey (Barany et al., 2020; Rankin, 2002) provide evolutionary context to this curious drinking behavior of sharks, suggesting that drinking and active osmoregulatory mechanisms in the intestine may be a basal trait among vertebrates and shared by a common osmoregulating ancestor.

In conclusion, the results in the present study demonstrate that changes in NKA activity in intestinal regions and the development of SW tolerance during the sea lamprey metamorphosis is controlled by the 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. This work provides important information regarding corticosteroid action in controlling ion and water transport processes in the vertebrate intestine. Future research should seek to further characterize the regulation of the hypothalamic-pituitary-interrenal axis in lamprey during metamorphosis and salinity acclimation. There is also a need to identify the molecular mechanisms for Na^+ and Cl^- absorption present in the lamprey intestine and how these might be regulated by 11-deoxycortisol.

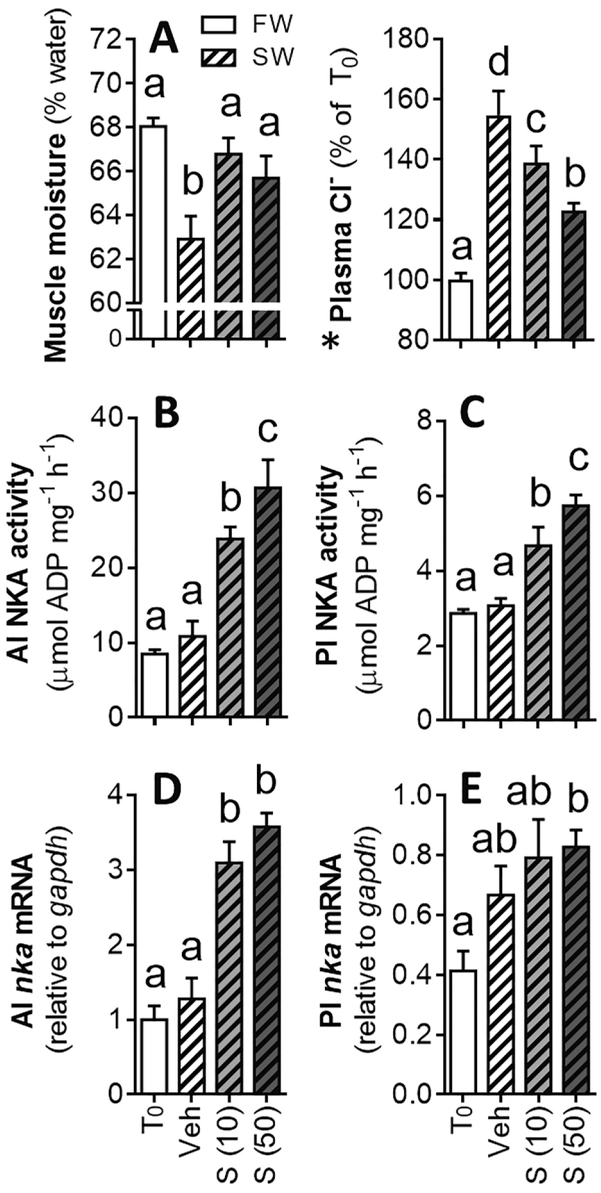

Fig. 4. Effect of 11-deoxycortisol (S) on SW osmoregulation in metamorphosing (early stage 7) sea lamprey. (A) Muscle moisture. (B, C) Na^+/K^+-ATPase (NKA) activity. (D, E) relative *nka* mRNA abundance. Tissues: anterior (AI) and posterior (PI) intestine. T_0 lamprey were sampled in FW, whereas the other groups were treated with Veh or S for 12 d and then transferred to SW for 24 h. Parentheses indicate injected dose (µg g^{-1} body weight). Data presented as mean ± standard error of the mean (n = 8–10). Letters indicate significant differences. *Original plasma Cl^- data can be found in Shaughnessy et al. (2020).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

A.B., C.A.S., and S.D.M. conceived and designed research; A.B. and C. A.S. conducted all live animal experimentation; C.A.S. performed radioimmunoassay, receptor binding and western blot analyses; A.B., and C.A.S. performed enzyme activity and molecular 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.

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