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RESEARCH ARTICLE

Cortisol is an osmoregulatory and glucose-regulating hormone in Atlantic sturgeon, a basal ray-finned fish

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ABSTRACT

Our current understanding of the hormonal control of ion regulation in aquatic vertebrates comes primarily from studies on teleost fishes, with relatively little information on more basal fishes. We investigated the role of cortisol in regulating seawater tolerance and its underlying mechanisms in an anadromous chondrostean, the Atlantic sturgeon (*Acipenser oxyrinchus*). Exposure of freshwater-reared Atlantic sturgeon to seawater (25 ppt) resulted in transient (1–3 day) increases in plasma chloride, cortisol and glucose levels and long-term (6–14 day) increases in the abundance of gill $Na^+/K^+/2Cl^-$ cotransporter (NKCC), which plays a critical role in salt secretion in teleosts. The abundance of gill V-type H^+-ATPase, which is thought to play a role in ion uptake in fishes, decreased after exposure to seawater. Gill Na^+/K^+-ATPase activity did not increase in 25 ppt seawater, but did increase in fish gradually acclimated to 30 ppt. Treatment of Atlantic sturgeon in freshwater with exogenous cortisol resulted in dose-dependent increases in cortisol, glucose and gill NKCC and H^+-ATPase abundance. Our results indicate that cortisol has an important role in regulating mechanisms for ion secretion and uptake in sturgeon and provide support for the hypothesis that control of osmoregulation and glucose by corticosteroids is a basal trait of jawed vertebrates.

KEY WORDS: *Acipenser oxyrinchus*, Osmoregulation, Salinity, Ionocyte, Gill Na^+/K^+-ATPase

INTRODUCTION

Irrespective of their environmental salinity, all fish with an osmoregulatory strategy maintain a nearly constant internal osmotic concentration, roughly one-third that of seawater (SW) (Edwards and Marshall, 2013). In freshwater (FW), these fish must actively take up salts from the environment while in SW they actively secrete salts. The gill, gut and kidney are all involved in osmoregulation in both FW and SW, but the gill is the primary site of ion uptake and secretion through specialized cells termed ionocytes (also called chloride cells and mitochondrion-rich cells). The mechanism of salt secretion by the teleost gill is now well established: Na^+/K^+-ATPase (NKA) provides electric and ionic gradients that are then utilized by the $Na^+/K^+/2Cl^-$ cotransporter (NKCC) to bring Cl^- from the blood into the cell. Cl^- then leaves the cell on a favorable electrical gradient through an apical Cl^- channel which is a homolog of the cystic fibrosis transmembrane conductance regulator (CFTR) (Marshall et al., 1995). NKA also moves sodium into the paracellular space where it can move between cells on a favorable electrical gradient. NKA, NKCC and CFTR have been co-localized to ionocytes and found to be upregulated in seawater in most euryhaline teleosts (Hiroi and McCormick, 2012; McCormick et al., 2003).

There is still some uncertainty regarding the mechanisms, transport proteins and even the cell types involved in ion uptake in FW by the fish gill (Evans et al., 2005). This uncertainty may in part be because different species use different mechanisms and that more than one mechanism of ion uptake is used by the same species. Cl^- can be taken up in exchange for bicarbonate through a Cl^-/bicarbonate anion exchanger (AE) on the apical membrane of ionocytes. Similarly, Na^+ can be exchanged for hydrogen ions through a Na^+/H^+ exchanger (NHE; slc9) also located on the apical membrane of ionocytes (Kumai and Perry, 2012). An alternative pathway for absorption of Na^+ is through the epithelia Na^+ channel (ENaC) down an electrochemical gradient generated by the proton pump V-type H^+-ATPase, though, as yet there has been no molecular characterization of an ENaC in fish. Finally, an apical Na^+/Cl^- cotransporter (NCC; slc12) induced by low external ions may also be involved in ion uptake in several species including tilapia and zebrafish (Hiroi and McCormick, 2012).

Hormones play a critical role in the acclimation of fish to changes in environmental salinity (McCormick, 1995). Early studies with fish indicated that cortisol was involved in the development of mechanisms for salt secretion and it became known as the 'seawater-adapting' hormone (Foskett et al., 1983). More recent studies indicate that cortisol also has a role in promoting mechanisms of ion uptake in many teleost species (Cruz et al., 2013; Kumai et al., 2012). There is an important interaction between growth hormone and cortisol in promoting acclimation to SW (McCormick, 1995) in at least some species that includes upregulation of cortisol secretion (Young, 1988) and cortisol receptors in the gill (Shrimpton and McCormick, 1998). Prolactin also promotes osmoregulation in FW and the extent of the interaction of prolactin and cortisol is still uncertain (Breves et al., 2014).

Most of what we know about the mechanisms and hormonal control of ion regulation in aquatic vertebrates comes from studies on more advanced teleosts. Relatively little is known about more basal vertebrates, including the euryhaline and anadromous sturgeon. Sturgeon are basal Actinopterygii (ray-finned fishes) whose extant relatives include gar and paddlefish and are collectively known as chondrosteans. They hold a key evolutionary position, being the most basal vertebrates in the line that led to teleosts after the split with Sarcopterygii, a lineage that includes the tetrapods. Chondrosteans have an osmoregulatory strategy that appears to be similar to that of teleosts (though the number of studies that have examined osmoregulation in this group is limited), and thus will be a critical clade for examining the evolution of osmoregulatory mechanism and their control in vertebrates.

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As with most teleosts, the number and size of ionocytes in sturgeon increases after SW acclimation (Allen et al., 2009; Altinok et al., 1998; Carmona et al., 2004). SW acclimation results in increased gill NKA activity in some species of sturgeon (McKenzie et al., 1999; Allen et al., 2009), but apparently not in all (Wright, 2007). In Persian sturgeon (*Acipenser persicus*), gill NKCC mRNA levels are higher in brackish water than in FW (Khodabandeh et al., 2009), but there are no published reports of gill NKCC protein abundance in any sturgeon species. There has been little work to date examining ion uptake mechanisms and transporters in sturgeon. H^+-ATPase has been shown to be involved in ion uptake in several teleost species, but gill H^+-ATPase activity and abundance did not differ in sturgeon held in FW or brackish water (23–24 ppt) (McKenzie et al., 1999; Sardella and Kultz, 2009).

Our understanding of how hormones control osmoregulation in chondrosteans is even less extensive than our understanding of the mechanisms of ion regulation. As noted above, cortisol has been identified as a critical factor controlling both ion secretion and uptake in teleost fishes. Cortisol has been identified as the major corticosteroid in sturgeon (Sangalang et al., 1971; Webb et al., 2007), and has been shown to increase during physical stressors such as handling, air exposure and hypoxia (Baker et al., 2005; Webb et al., 2007). There is evidence that in some sturgeon species plasma cortisol increases in response to increased environmental salinity (Krayushkina et al., 2006), and that cortisol can affect gill NKCC abundance (Khodabandeh et al., 2009), but to our knowledge these are the only two studies that have examined any aspect of the hormonal control of osmoregulation in any chondrostean. Thus, there is a large gap in our knowledge on the endocrine control of osmoregulation in this key group for understanding the evolution of ion homeostasis in vertebrates.

Given their key phylogenetic position, their importance in fisheries and aquaculture, and long-standing concerns regarding their conservation status (Birstein, 1993), it is surprising how little work has been done on the control of osmoregulation in chondrosteans and sturgeon in particular. We hypothesize that, as in teleost fishes, cortisol acts as a hormone to regulate critical ion transport mechanisms during the process of SW acclimation of chondrosteans. In the present study, we used two approaches to provide evidence of a role for cortisol in ion regulation in an anadromous chondrostean, the Atlantic sturgeon (*Acipenser oxyrinchus*). First, we examined changes in plasma cortisol, osmolality and glucose during exposure to SW, as well as responses of gill ion transporters involved in both ion secretion and uptake. Second, we used three doses of *in vivo* cortisol to examine the effect of this hormone on changes in ion transporters and plasma ions in FW and after exposure to SW.

MATERIALS AND METHODS

Fish rearing

Atlantic sturgeon, *Acipenser oxyrinchus* Mitchill 1815, were obtained as fertilized eggs from the St Johns River, New Brunswick, Canada (Acadian Caviar Company, Carters Point, NB, Canada) and reared at the Conte Anadromous Fish Research Laboratory (USGS, Turners Falls, MA, USA). After hatching, fish were placed in 1 m diameter tanks and fed live *Artemia*, followed by frozen copepods and finally a commercial fish diet (Bio-Oregon, Longview, WA, USA). One month after first feeding, fish were placed in 1.5 m diameter tanks supplied with 4 l min^{-1} ambient Connecticut River water under natural photoperiod conditions and fed once daily. All rearing and experiments were carried out under US Geological Survey Institutional Animal Care and Use Committee Guidelines under protocol no. C09070.

SW exposure

On 7 October 2013, 100 one-year-old fish (25.1–45.4 cm) were transferred to four different tanks supplied with dechlorinated tap water at 2 l min^{-1} and fed *ad libitum* twice daily. Seventeen days later, two of the tanks were changed to 25 ppt; based on preliminary studies, this was the highest salinity that this size Atlantic sturgeon could be directly exposed to with no mortality. To achieve 25 ppt seawater, 50% of the water in each tank was removed and replaced with 50 ppt seawater. This approach allowed us to expose fish to elevated salinity without the added stress of transferring fish. The same water removal but with replacement by FW occurred in the two FW control tanks. Salinity was checked immediately after mixing and daily thereafter and maintained between 24.9 and 25.2 ppt throughout the experiment. After fish salinity was changed, all four tanks were maintained as recirculating systems with charcoal and biological filtration at 17–19°C with 75% water changes every other day to maintain low ammonia levels (which were checked every other day). Fish were fed *ad libitum* on days 4, 8 and 12 and 3 fish were sampled from each tank on day 0 (freshwater only) and 5 fish per tank on days 1, 2, 3, 6 and 14.

To test the effect of salinity greater than 25 ppt on gill NKA activity, NKA abundance and ionocytes, we gradually acclimated juvenile sturgeon (11.5–17.0 cm) to 30 ppt SW. These fish were part of our preliminary experiments and thus were smaller than fish in the direct transfer experiment. FW and SW tanks were maintained with charcoal and biological filtration at 15–17°C with 75% water changes made twice weekly in order to maintain low ammonia levels (which were checked every other day). Gradual acclimation occurred by increasing salinity to 15 ppt (8 days), 20 ppt (6 days), 23 ppt (9 days) and 30 ppt for 14 days after which fish were sampled on December 19 as described below. FW controls received water changes at the same time that salinity was increased in the SW group. Fish were fed *ad libitum* every other day, and food was withheld 24 h prior to terminal sampling.

Cortisol treatment

In this experiment, fish were treated with cortisol and then sampled in FW (day 14) and after 24 h in 28 ppt SW. On 22 November 2013, 80 one-year-old fish (25.5–35.8 cm) were transferred to two tanks supplied with dechlorinated tap water at 2 l min^{-1} with supplemental aeration at 14–16°C and fed *ad libitum* twice daily. On 3 December, fish were injected with three doses of cortisol and vehicle as outlined in Specker et al. (1994). The vehicle for cortisol administration was 1:1 vegetable oil:shortening heated to 30°C, which formed a semi-solid implant when injected intraperitoneally, allowing for the slow release of cortisol. Appropriate amounts of cortisol were suspended and sonicated in the vehicle to obtain 8, 40 and 200 µg g^{-1}. These doses were chosen to obtain physiological and supraphysiological levels of cortisol based on previous studies in teleost fishes (Specker et al., 1994). The 8 µg g^{-1} dose, which is lower than used in most studies on teleost fish, was chosen to match the lower levels of plasma cortisol seen in sturgeon. Fish were anesthetized with neutralized MS-222 (100 mg l^{-1}), length and mass were measured, and fish were injected with 10 µl g^{-1} body mass and given a non-toxic paint mark between the anal fin rays to identify their treatment group. In addition to the vehicle controls, 4 un-injected controls that were otherwise treated identically (including handling and paint mark) were included at each sampling. Fish were fed *ad libitum* 2 and 6 days after injection.

Food was limited particularly at the end of the study to avoid differences in feeding and growth rate that can be induced by cortisol treatment, and which may indirectly affect osmoregulation. After 12 days of treatment in FW, 8 fish were sampled from each group. One day later, all tanks (control and treated) were changed to 28 ppt by drawing down the water to 50% of its former level and adding 56 ppt water, and all fish were sampled 24 h later. The higher level of salinity relative to the direct SW exposure study (25 ppt) was chosen to provide a larger osmotic challenge to the fish so as to increase the likelihood of detecting treatment effects, similar to the seawater challenge test that has been widely used in studies on smolt development of salmonids (Clarke, 1982). Salinity was checked immediately after mixing and maintained between 27.8 and 28.2 ppt.

Fish sampling

Fish were anesthetized with neutralized MS-222 (200 mg l^{-1}) and body mass and length were measured. Blood was drawn from the caudal vessels into a 1 ml ammonium heparinized syringe then placed in a microcentrifuge tube. Blood for hematocrit measurement was collected from this pool into heparinized micro-hematocrit capillary tubes and centrifuged at 13,500 ***g*** for 5 min in a micro-hematocrit centrifuge and read on a micro-capillary reader (Damon/IEC Division, Needham, MA, USA). The remaining blood was spun at 3200 ***g*** for 5 min at 4°C, then plasma was aliquoted and stored at −80°C. Gill biopsies of four to six primary gill filaments were placed into 100 µl of ice-cold SEI buffer (250 mmol l^{-1} sucrose, 10 mmol l^{-1} EDTA, 50 mmol l^{-1} imidazole, pH 7.3) and frozen at −80°C for measurement of gill NKA activity.

Physiological assays

Plasma chloride was measured in duplicate by the silver titration method using a Buchler-Cotlove digital chloridometer (Labconoco, Kansas City, MO, USA) and external standards. Plasma glucose was measured in duplicate by enzymatic coupling with hexokinase and glucose 6-phosphate dehydrogenase (Carey and McCormick, 1998). Plasma osmolality was measured in duplicate using a Wescor 5500 vapor pressure osmometer (Logan, UT, USA).

Plasma cortisol levels were measured by a validated direct competitive enzyme immunoassay as previously outlined (Carey and McCormick, 1998). Cortisol was extracted by the addition of 10 volumes of diethylether (DEE) to 100 µl of plasma. Samples were vortexed for 30 s, centrifuged at 3000 ***g*** for 3 min, and frozen at −80°C for 10 min. The organic (liquid) supernatant was removed to a clean microcentrifuge tube and evaporated under a filtered air stream. The frozen non-organic portion of the samples were re-extracted with 10 volumes of DEE as above and the resulting supernatant was pooled with the first fraction of supernatant. Once the organic liquid containing the extracted cortisol was completely evaporated, samples were re-suspended in 100 µl of water. Average extraction efficiency as determined by extraction of known standards added to plasma samples was 69%. The standard curve ranged from 1 to 160 ng ml^{-1}. Samples that fell outside of the curve were diluted and re-assayed. Sensitivity as defined by the standard curve was determined to be 0.3 ng ml^{-1}. Using a pooled plasma sample, the average inter-assay variation (s.d./mean) was 11.52% and intra-assay variation was 3.35%.

Gill NKA (EC 3.6.1.3) activity was determined with a kinetic assay run in 96-well microplates at 25°C and read at a wavelength of 340 nm for 10 min (McCormick, 1993). Gill tissue was first homogenized in 150 µl of SEID (SEI buffer and 0.1% deoxycholic acid) and centrifuged at 5000 ***g*** for 30 s. Two sets of duplicate 10 µl samples were run, one set containing assay mixture and the other containing assay mixture and 0.5 mmol l^{-1} ouabain. The resulting ouabain-sensitive ATPase activity is expressed as µmol ADP mg^{-1} protein h^{-1}. Citrate synthase (EC 4.1.3.7) activity was run on the same homogenates using oxaloacetate as substrate and read kinetically at 412 nm (Leonard and McCormick, 2001). Protein concentrations were determined with a bicinchoninic acid (BCA) Protein Assay (Pierce, Rockford, IL, USA) using bovine serum albumin as standard. Enzyme and protein assays were run on a BioTek Synergy 2 spectrophotometer using Gen5 software (BioTek, Winooski, VT, USA).

Tissues were prepared for western blotting as described in McCormick et al. (2013). Briefly, 0.2 g of frozen gill tissue were homogenized using a handheld homogenizer in 10 volumes of SEID buffer on ice. The homogenate was centrifuged for 7 min at 3000 ***g***. The resulting supernatant was divided and one part was immediately added to an equal volume of 2× Laemmli sample buffer, and another was added to a 96-well plate in quadruplicate for BCA protein determination. Samples in Laemmli were heated to 65°C for 15 min, then stored frozen at −80°C for western blotting. Samples (10 µg) were loaded and run on 7.5% SDS-PAGE gels. Following electrophoresis, proteins were transferred to Immobilon PVDF transfer membrane (Millipore, Bedford, MA, USA) at 30 V overnight in 25 mmol l^{-1} Tris and 192 mmol l^{-1} glycine buffer, pH 8.3. PVDF membranes were blocked with 5% non-fat dried milk in PBST (PBS with 0.05% Triton X-100) for 1 h at room temperature, rinsed in PBST, and exposed to primary antibody in blocking buffer for 1 h at room temperature. Membranes were stained with 0.1% Ponceau-S in 10% acetic acid for 10 min. Samples with uneven, smeared or low levels of Ponceau-S staining lanes were rerun or a new sample from the same fish was extracted. Primary antibody to NKA (α5 concentrate, AB 2166869) and NKCC (T4 concentrate, AB 528406) were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA) were incubated at 1:5000 and 1:2000 dilution, respectively in PBST. H^+-ATPase primary antibody, a gift from Jonathan Wilson, was used at a dilution of 1:10,000 (Wilson et al., 2007). These antibodies have been widely used in studies on teleosts and basal vertebrates including lamprey, and in Atlantic sturgeon they recognize a single band of the appropriate molecular weight. Blots were washed and then exposed to goat anti-rabbit-HRP or mouse anti-rabbit-HRP antibody diluted 1:10,000 in blocking buffer for 1 h at room temperature. After rinsing in PBST, blots were incubated for 1 min in a 1:1 mixture of enhanced chemiluminescent (ECL) solution A (396 µmol l^{-1} coumaric acid, 2.5 µmol l^{-1} luminol, 100 mmol l^{-1} Tris, pH 8.5) and ECL solution B (0.018% H_2O_2, 100 mmol l^{-1} Tris, pH 8.5), then exposed to X-ray film (RPI, Mount Prospect, IL, USA). Digital photographs were taken of individual gels and band staining intensity was measured using ImageJ (National Institutes of Health, Bethesda, MD, USA); protein abundance was expressed as a cumulative 8-bit grayscale value. Dilution titration of a pooled tissue homogenate for each measured protein was completed to establish the range of linearity, and all samples fell within this range.

Statistics

For the SW exposure experiment, two-way ANOVA was used to examine the effect of salinity, time and their interaction (time 0 samples were excluded as required for symmetrical design of the two-way ANOVA). When salinity was significant ($P<0.05$) a Newman–Keuls test was used to compare FW and SW at the same time point. In cases where the assumption of homogeneity of variance was violated (plasma cortisol, gill NKCC western), non-

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parametric comparisons of FW and SW means at each time point were conducted using the Mann–Whitney *U*-test. For the cortisol injection experiment, vehicle-injected and un-injected controls did not differ significantly for any parameter. Statistical analysis with and without the un-injected controls did not differ in the overall significance of cortisol treatment for each parameter; the results of the two groups combined are reported in the text. Because SW affected the variance of most of the measured parameters (thus violating assumptions of ANOVA), the effect of cortisol was analyzed separately for FW and SW (one-way ANOVA followed by Dunnett's test). All statistical analyses were performed using Statistica version 13 (Dell Inc., Tulsa, OK, USA). Original data presented in the paper are available from the US Geological Survey database (https://doi.org/10.5066/P9KIPOF8).

RESULTS

SW exposure

Plasma osmolality and chloride remained constant in FW throughout the experiment (Fig. 1A,B). There was a significant effect of 25 ppt SW, time and their interaction on both plasma osmolality and chloride (P<0.0001). Plasma chloride and osmolality were strongly and significantly elevated after 1 and 2 days of SW exposure, followed by a decrease at day 3 to levels that remained constant (but slightly higher than FW values) through to day 14.

Plasma cortisol and glucose remained constant in FW throughout the experiment (Fig. 1C,D). Plasma cortisol and glucose were elevated after 1 day in SW and reached peak levels after 2 days in SW. Plasma glucose returned to levels similar to or slightly lower than in FW from days 3 to 14. On day 3 in SW, plasma cortisol decreased from its peak levels but remained somewhat elevated compared with FW levels until day 14. There was no SW (P=0.18) or time (P=0.067) effect on plasma glucose (P>0.067), but there was a significant interaction between them (P=0.018).

Gill NKA activity was highly variable in both FW and SW (Fig. 2A). Although there was a peak in gill NKA activity in SW at day 6 that was 2-fold higher than FW controls, there was no significant effect of SW or time on gill NKA activity (P=0.20). Gill NKA abundance was much less variable and did not change significantly with SW or time (P=0.46, Fig. 2B). Gill NKCC abundance remained constant in FW throughout the experiment (Fig. 2C). Gill NKCC increased more than 2-fold after 3 days in SW, and this was maintained through 14 days of SW exposure. Gill H^+-ATPase abundance decreased slightly in FW over the course of the study (Fig. 2D), whereas exposure to SW resulted in a 66% loss of H^+-ATPase by day 3, which remained low throughout the 14 days of SW exposure. There was a significant effect of SW on gill H^+-ATPase abundance (P<0.0001), but no effect of time (P=0.99) and no significant interaction (P=0.12). Individual values for hematocrit ranged between 22 and 31 and did not change significantly as a function of SW (P=0.98) or time (P=0.59) (data not shown).

In a second experiment, juvenile Atlantic sturgeon were gradually acclimated to 30 ppt SW over 23 days and sampled after 14 days. This experiment focused on NKA because of the somewhat

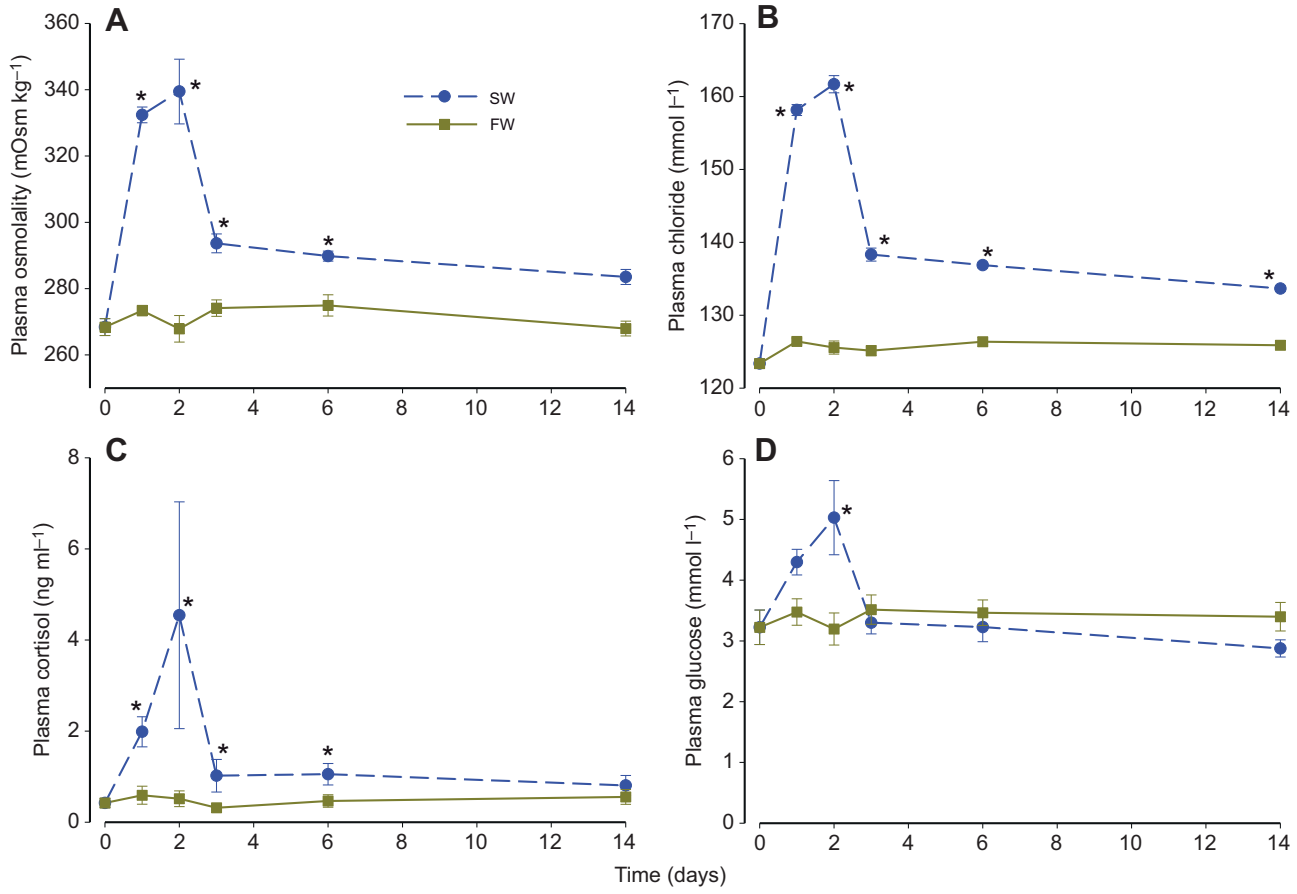

Fig. 1. Effect of seawater exposure on plasma osmolality, chloride, cortisol and glucose in juvenile Atlantic sturgeon. Plasma osmolality (A), chloride (B), cortisol (C) and glucose (D) are shown for fish exposed to 25 ppt seawater (SW; n=10) and for freshwater (FW; n=8–9) controls (means+s.e.m.). Asterisks indicate a significant difference of the SW group from the corresponding FW group at the same time point [P<0.05, Newman–Keuls test or Mann–Whitney *U*-test (cortisol)].

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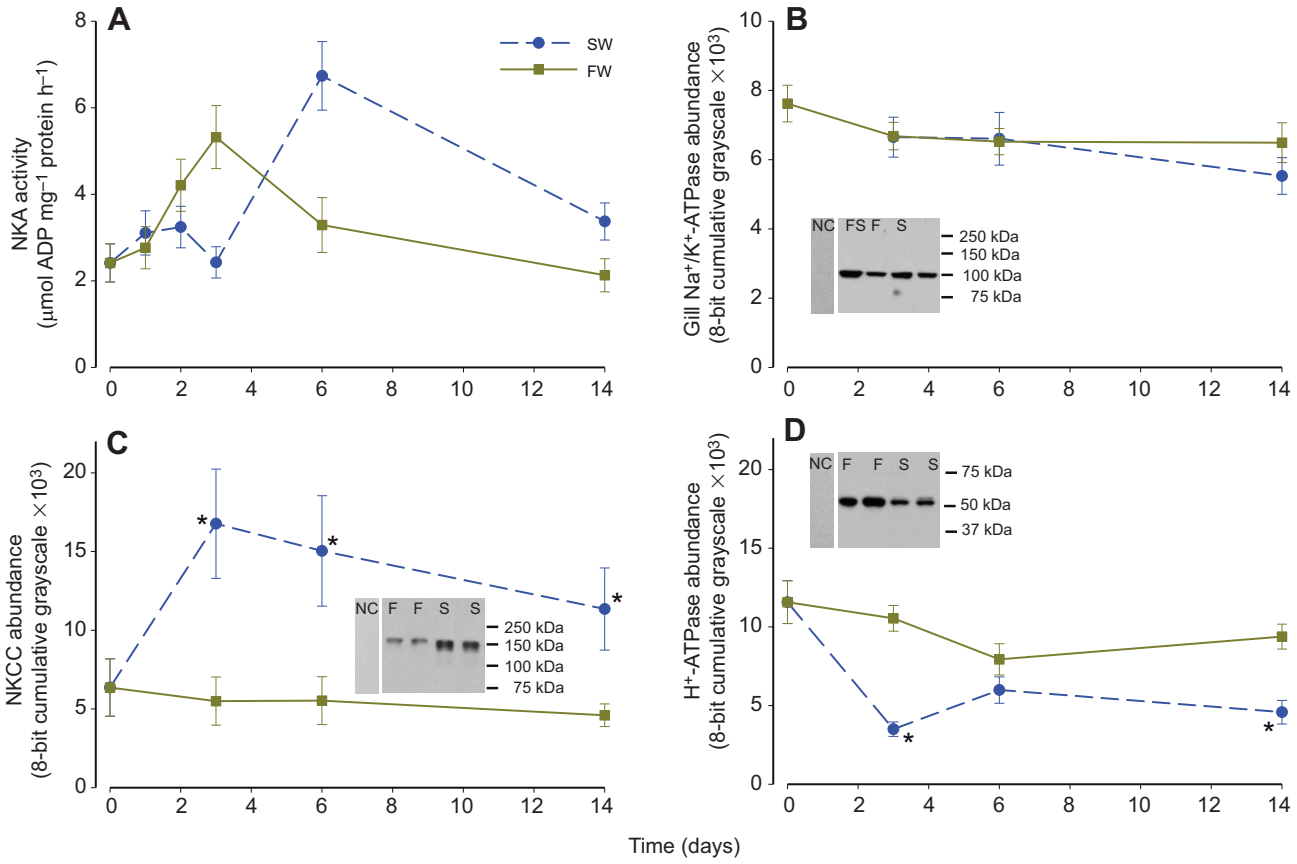

Fig. 2. Effect of SW exposure on gill Na^+/K^+-ATPase activity and abundance, $Na^+/K^+/2Cl^-$ cotransporter abundance and H^+-ATPase abundance in juvenile Atlantic sturgeon. Na^+/K^+-ATPase (NKA) activity (A), NKA abundance (B), $Na^+/K^+/2Cl^-$ cotransporter (NKCC) abundance (C) and H^+-ATPase abundance (D) are shown for fish exposed to 25 ppt SW (n=10) and for FW (n=8–9) controls (means+s.e.m.). Asterisks indicate a significant difference of the SW group from the corresponding FW group at the same time point [P<0.05, Newman–Keuls test or Mann–Whitney U-test (NKCC)]. Insets are western blot images; FW and SW samples were run on the same blot, negative control (NC) samples were run on a different blot.

surprising result that 25 ppt did not induce increases in gill NKA abundance or activity. Gill NKA activity was 2.4-fold higher in SW than in FW (P<0.00001, one-way ANOVA; Fig. 3A), whereas intestinal and renal NKA activity did not differ with environmental salinity (P>0.18; Fig. 3A). Citrate synthase activity, an index of maximum tissue aerobic capacity, was highest in gill but did not differ in gill, intestine or kidney as a function of environmental salinity (P>0.10; Fig. 3B). Plasma osmolality was 250±3 mosmol kg^{-1} in FW and 274±4 mosmol kg^{-1} in SW (P<0.0001). Plasma cortisol was 1.9±0.7 ng ml^{-1} in FW and 1.7±0.6 ng ml^{-1} in SW and did not statistically differ (P=0.67). Plasma hematocrit was 19±0.6% in FW and 20±0.6% in SW and did not statistically differ (P=0.051).

Cortisol treatment

Plasma osmolality and chloride levels (Fig. 4A,B) were not affected by 12 days of cortisol treatment in FW (P>0.47). Plasma osmolality and chloride levels after 12 days of cortisol treatment in FW followed by 24 h in 28 ppt SW were substantially higher than in FW and were especially variable in the control group (no cortisol). The lowest levels of plasma osmolality and chloride were seen in the 40 μg g^{-1} cortisol treatment group, but these differences were not significantly different (P>0.32). However, the variance in plasma osmolality in the 40 μg g^{-1} cortisol treatment group was significantly reduced compared with that of control fish (P<0.0001; Hartley F-max test).

Plasma cortisol levels in FW 2 weeks after injection were significantly elevated in the 40 and 200 μg g^{-1} groups (P<0.0001; Fig. 4C). Plasma cortisol levels 24 h after exposure to 28 ppt were elevated in the SW control group compared to the FW control group, though there was still a significant effect of cortisol treatment detected in SW (P=0.02). Plasma glucose levels in FW were significantly elevated by cortisol treatment (P<0.0001; Fig. 4D) and were 54–60% higher in the 40 and 200 μg g^{-1} groups compared with controls. There was no significant effect of cortisol treatment detectable in SW (P=0.29), likely because of the elevation of glucose in response to SW exposure in all groups (including controls), as was seen in the previous experiment.

Gill NKA activity was not significantly affected by cortisol treatment in FW or after 24 h exposure to SW (P>0.50; Fig. 5A). Gill NKA abundance in FW was significantly increased in all cortisol treatments compared with controls (P=0.002), with the largest increase (41%) occurring in the 40 μg g^{-1} group (Fig. 5B). Gill NKA abundance was higher in the control group in SW than in FW, and there was no significant effect of cortisol treatment on gill NKA abundance in SW (P=0.79).

Cortisol treatment significantly elevated gill NKCC abundance in FW (P=0.005), increasing 6-fold in the 40 μg g^{-1} group (Fig. 5C). Gill NKCC abundance was higher in the control group in SW than in FW, and there was no significant effect of cortisol treatment on gill NKCC abundance in SW (P=0.17). Gill H^+-ATPase abundance was also significantly elevated by cortisol treatment in

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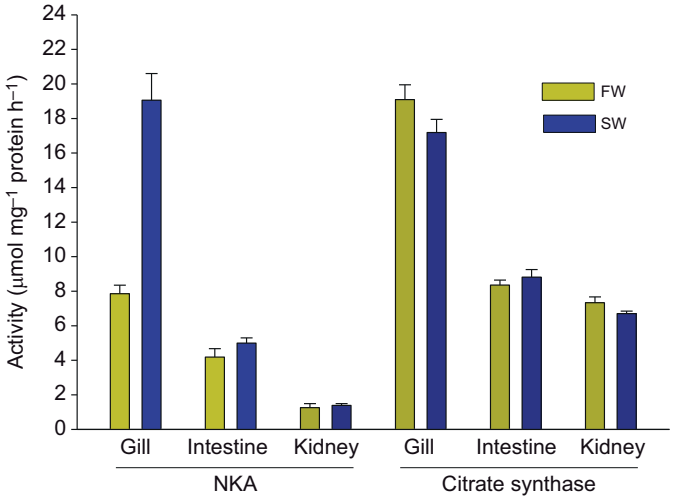

Fig. 3. Effect of 30 ppt SW exposure on gill NKA and citrate synthase activity in small juvenile Atlantic sturgeon. Gill NKA activity (A) and citrate synthase (B) activity are shown for fish gradually acclimated to 30 ppt SW and sampled after 2 weeks (n=8) and for FW controls (n=8; means+s.e.m.). Asterisks indicate a significant difference of the SW group from the corresponding FW group at the same time point (P<0.05, t-test).

FW (P=0.011) to a maximum of 30% higher than controls in the 200 µg g^{-1} group (Fig. 5D). Gill H^+-ATPase abundance was significantly lower in the control group in SW compared with FW, and there was no significant effect of cortisol treatment on gill H^+-ATPase abundance in SW (P=0.67).

DISCUSSION

Juvenile Atlantic sturgeon were able to tolerate direct transfer from FW to 25 ppt SW with no mortality. They experienced large increases in plasma osmolality and chloride in the first 2 days after SW exposure, followed by a steep decrease at day 3 and a more gradual decrease to levels that were only slightly elevated compared with initial FW levels. Similarly, peaks in plasma cortisol and glucose also occurred after 2 days of SW exposure, followed by a return to baseline levels. The coincident peaks in plasma ions, cortisol and glucose indicates that the activation of the hypothalamic–pituitary–interrenal (HPI) axis may be due to deviations in plasma ion levels. Similar concurrent changes in plasma ions and cortisol have been observed in many teleost fishes (Mommsen et al., 1999), although not all species respond similarly. Conflicting results have also been found in sturgeon species. Krayushkina et al. (2006) found that serum cortisol increased in 3 of 5 species of Acipenseridae after exposure to 12.5 ppt brackish water for 7 days, although time-matched FW controls were not included. We would also present a cautionary note that as extraction of plasma was necessary to properly detect cortisol in Atlantic sturgeon, there may be binding proteins or other interfering substances present in plasma of many chondrosteans that should be taken into consideration in future studies.

While increases in cortisol have been widely examined after SW exposure of teleost fishes, other aspects of the HPI axis in teleosts have not been widely examined. Increases in circulating adrenocorticotropic hormone (ACTH) and corticotropin-releasing factor (CRF) mRNA levels in the hypothalamus and preopotic area have been found after SW exposure in rainbow trout, indicating stimulation of the HPI axis originates in the brain (Craig et al., 2005). ACTH release by the pituitary appears to be insensitive to changes in plasma osmolality in Mozambique tilapia (Seale et al., 2002), lending further support to the importance of CRF signaling during salinity stress, though the mechanism of CRF stimulation remains to be established. Our finding of a clear stimulation of cortisol in Atlantic sturgeon provides inroads for further studies of salinity regulation of the HPI axis of chondrosteans.

Both the basal levels of circulating cortisol and the response to increased salinity observed in this study and other studies on sturgeon (Krayushkina et al., 2006) are lower than those observed in most studies on teleosts (Takei and McCormick, 2013). This lower magnitude of the cortisol response also includes handling stresses, which can rise to between 50 and 1500 ng ml^{-1} in teleosts (Carey and McCormick, 1998; Shrimpton et al., 2001), whereas in sturgeon the response to handling stress is usually in the range of 10 to 50 ng ml^{-1}, but may rise to as high as 400 ng ml^{-1} (Webb et al., 2007). This does not necessarily mean that the action of cortisol in response to salinity and other stressors is less physiologically relevant in sturgeon, as cortisol receptors and their affinity for cortisol have not been fully examined in this group. The Amur sturgeon mineralocorticoid receptor (MR) expressed in a human embryonic kidney cell line has a 4-fold higher affinity for cortisol than the zebrafish MR (Sugimoto et al., 2016). Unfortunately, the glucocorticoid receptor (GR), which in teleosts appears to be the most important corticosteroid receptor for ion and water homeostasis (Takei and McCormick, 2013), has not yet been characterized in sturgeon. Information on the binding affinity, localization and abundance of GR and MR, as well as possible binding proteins and tissue-specific degradation pathways for cortisol will be critical to understanding the full complexity of the HPI axis in this important clade of fishes.

We found no significant change in gill NKA abundance or activity after exposure of Atlantic sturgeon to 25 ppt over a 2 week period (Fig. 2A). However, gradual acclimation of Atlantic sturgeon to 30 ppt resulted in 2.2-fold higher levels of gill NKA activity (Fig. 3), while there were no changes in intestinal or renal NKA activity. It should also be noted that the fish exposed to 30 ppt were smaller than those used for direct exposure to 25 ppt, and thus a size-dependent response to salinity cannot be ruled out, especially as size-dependent salinity tolerance has been observed in Atlantic and green sturgeon (Niklitschek and Secor, 2009; Allen et al., 2009). Citrate synthase is a rate-limiting enzyme in the TCA cycle and is widely used as an index of maximum tissue aerobic capacity. Citrate synthase activity did not differ with salinity in any of the osmoregulatory tissues (gill, intestine and kidney; Fig. 3) that were examined in this study. These results indicate that maximum aerobic capacity of osmoregulatory tissue does not change with salinity in Atlantic sturgeon and that the metabolic costs of osmoregulation between FW and 30 ppt SW may not be large for this species.

The response of gill NKA activity to salinity appears to be highly variable among sturgeon species. Gill NKA activity was shown to be significantly higher after exposure of Siberian sturgeon (*Acipenser baerii*) (Rodríguez et al., 2002), Adriatic sturgeon (*Acipenser naccarii*) (McKenzie et al., 1999) and Persian sturgeon (*Acipenser persicus*) (Shirangi et al., 2016) to hyperosmotic conditions (11–24 ppt). However, no significant differences in gill NKA activity were found in green sturgeon (*Acipenser medirostris*) between FW and 24 or 34 ppt (Allen et al., 2009; Sardella and Kultz, 2009). As noted within our own studies, there is a great difference in size of sturgeon used among these studies, and size-dependent responses may vary among species. Gill NKA has been found to be abundant in gill ionocytes of Persian sturgeon (Shirangi et al., 2017), and the abundance of NKA in

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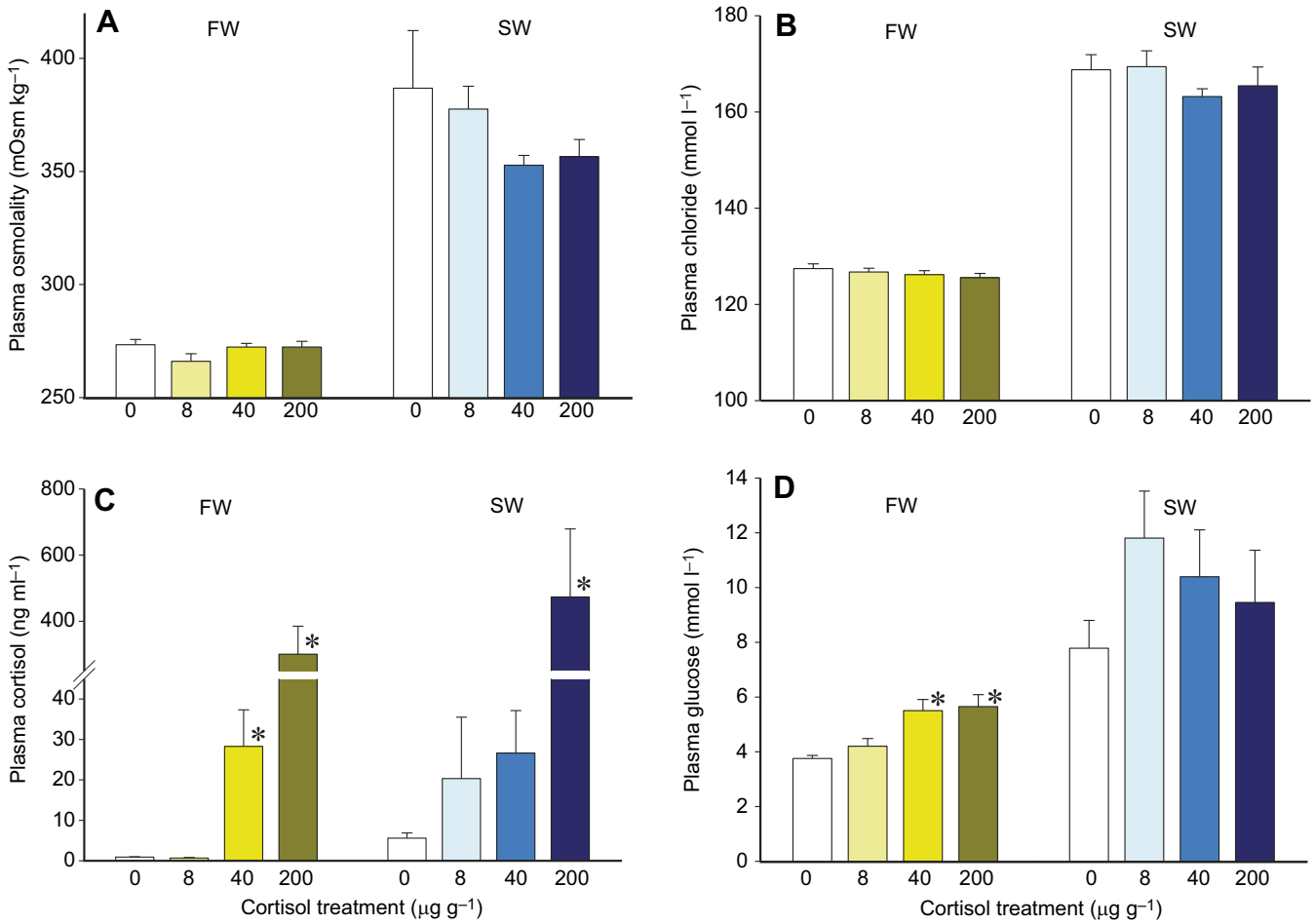

Fig. 4. Effect of cortisol treatment on plasma osmolality, chloride, cortisol and glucose in juvenile Atlantic sturgeon. Plasma osmolality (A), chloride (B), cortisol (C) and glucose (D) are shown for fish treated with 0 (control), 8, 40 and 200 μg g^{-1} cortisol and sampled after 12 days in FW (n=8–10; left) or after 12 days in FW followed by exposure to 28 ppt SW for 24 h (n=8–10; right; means±s.e.m.). Asterisks indicate a significant difference of the SW group from the corresponding FW group at the same time point (P<0.05, Newman–Keuls test).

individual ionocytes has been estimated to increase following SW acclimation of green sturgeon (Sardella and Kultz, 2009).

In the present study, a robust response of gill NKCC abundance to transfer from FW to 25 ppt was observed, with values increasing within 3 days and maintained 2-fold higher throughout the exposure period. This is similar in magnitude to the response that has been observed in several teleost species (Flemmer et al., 2002; Pelis et al., 2001). NKCC has also been co-localized with NKA to gill ionocytes of Persian sturgeon (Shirangi et al., 2017). NKCC immunofluorescence of individual ionocytes has also been estimated to increase following transfer of green sturgeon to 24 ppt SW (Sardella and Kultz, 2009). Together, these data provide evidence that NKCC is upregulated in ionocytes following SW exposure of sturgeon, where it likely acts to transport Cl^- from the blood into ionocytes, which would then allow Cl^- to leave through an apical transporter. To date, the apical transporter for Cl, which is known to be CFTR in teleosts (Marshall et al., 1995; McCormick et al., 2003), has not been identified in sturgeon. Studies in teleost fish have also shown increasing phosphorylation (and presumptive activation) of NKCC following SW exposure (Flemmer et al., 2002), and it would be of interest to determine whether a similar phenomenon occurs in sturgeon.

There is strong evidence in many teleost fish that H^+-ATPase is involved in ion uptake in FW and works by acidifying the external apical membrane, providing an electrical gradient for inward movement of Na^+ through a sodium channel (Hwang, 2009). A specific inhibitor of H^+-ATPase, bafilomycin, has been shown to inhibit Na^+ uptake in several species (Fenwick et al., 1999). H^+-ATPase activity, abundance and number of H^+-ATPase-rich ionocytes have been shown to be higher in FW than in SW (Bystriansky and Schulte, 2011; Huang et al., 2010), and increase further after exposure to low ion content water or acidic conditions when demand for ion uptake is further increased (Horng et al., 2009; Huang et al., 2010). In green and Adriatic sturgeon, acclimation to 23–24 ppt SW did not result in significant changes in H^+-ATPase activity (McKenzie et al., 1999; Sardella and Kultz, 2009). In the present study, we measured H^+-ATPase abundance by western blots in sturgeon for the first time and found decreased abundance (−50%) following exposure to 25 ppt SW, suggesting that this enzyme plays a role in ion uptake in sturgeon as it does in many teleost species. Given this evidence, it will be valuable to conduct further studies to localize H^+-ATPase in the gill and determine the characteristics of the Na^+ channel that likely works in concert with H^+-ATPase to promote ion uptake.

We treated juvenile Atlantic sturgeon with cortisol to determine its potential role in controlling the transport proteins involved in ion regulation. Plasma cortisol levels at the end of the 2 week treatment period were significantly elevated by the 40 and 200 μg g^{-1}

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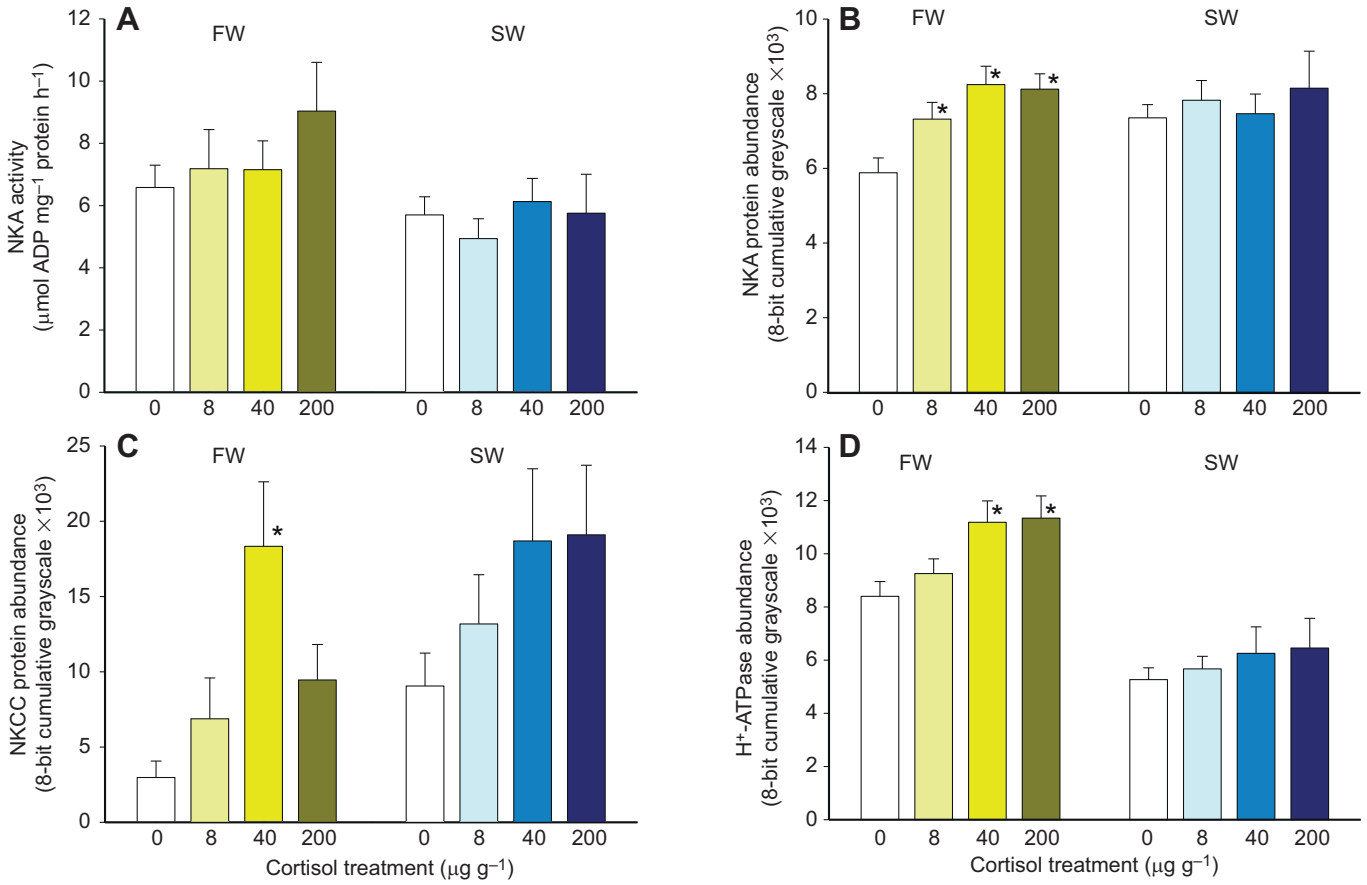

Fig. 5. Effect of cortisol treatment on gill NKA activity and abundance, NKCC abundance and H^+-ATPase abundance in juvenile Atlantic sturgeon. NKA activity (A), NKA abundance (B), NKCC abundance (C) and H^+-ATPase abundance (D) are shown for fish treated with 0, 8, 40 and 200 μg g^{-1} cortisol and sampled after 12 days in FW (*n*=8–10; left) or after 12 days in FW followed by exposure to 28 ppt SW for 24 h (*n*=8–10; right; means±s.e.m.). Asterisks indicate a significant difference of the SW group from the corresponding FW group at the same time point (*P*<0.05, Newman–Keuls test).

treatment levels, but not by the lowest dose of 8 μg g^{-1} (Fig. 4C). The levels achieved by the 40 μg g^{-1} dose overlapped with the peak levels seen during SW exposure, whereas the levels of the 200 μg g^{-1} group were substantially higher and possibly supraphysiological. It should be noted that while the method of cortisol treatment is known to give a prolonged release of cortisol, plasma cortisol levels are higher in the first several days of treatment compared with those after 2–3 weeks of treatment (Specker et al., 1994). Thus, there may have been significant elevations of plasma cortisol in the early phases of the 8 μg g^{-1} treatment, which would explain the significant effect this dose had on gill NKA abundance. It should also be noted that while the levels of plasma cortisol in the cortisol-treated fish were similar in FW and SW, we cannot rule out the possibility that prior cortisol treatment altered the pattern of endogenous cortisol release. Such an effect may be the reason why highly variable levels of plasma cortisol were seen in the 8 μg g^{-1} group after SW exposure. A different experimental approach using cortisol agonists would be necessary to accurately measure such an effect.

Cortisol treatment of juvenile Atlantic sturgeon in FW resulted in significant increases in gill NKA and NKCC abundance, with peak levels occurring at 40 μg g^{-1} and intermediate levels at 8 μg g^{-1}, although statistical significance occurred only with gill NKA. The maximum observed effect on gill NKA abundance was moderate (+41%) but much larger for NKCC (6-fold), suggesting that the latter is more sensitive to cortisol and potentially its upregulation is more important for salt secretion. This is consistent with the higher levels of NKCC after exposure to 25 ppt SW, whereas gill NKA abundance was not altered at this salinity. Although the levels of gill NKA and NKCC were similar in each dose of cortisol in FW and after SW treatment, there was no significant increase detected in SW, likely due to the higher levels in control fish after 24 h of SW exposure. Clear increases in gill NKCC abundance were seen within 3 days of exposure to 25 ppt (Fig. 2C). Increased gill *nkcc* mRNA and NKA activity were found in Persian sturgeon after 24 h of aqueous cortisol treatment (Khodabandeh et al., 2009). These results indicate that responses to cortisol, and by extension SW itself, can be relatively rapid in sturgeon. Cortisol has been shown to upregulate gill NKA and NKCC abundance and NKA activity in many euryhaline teleosts (McCormick, 1995; Pelis and McCormick, 2001; Pickford et al., 1970). The results of the present study indicate that cortisol upregulates the abundance of ion transport proteins that are involved in salt secretion in chondrosteans as it does in teleosts.

Cortisol treatment had no statistically significant effect on plasma osmolality or chloride levels in FW (Fig. 4A,B). These results are consistent with studies in teleosts that have shown that steady-state levels of plasma ions in FW are not altered by cortisol (McCormick, 1995). In contrast, a large number of studies have shown that cortisol treatment of euryhaline teleosts with cortisol in FW improves salinity tolerance as judged by lower levels of plasma ions and osmolality after exposure to SW (McCormick, 1995). In the present study, we

did not observe a significant effect of cortisol on plasma levels of osmolality or chloride after exposure to SW. While there was a clear trend for plasma osmolality to be lower with increasing doses of cortisol, the statistical significance of this was likely masked by the high variability within the control group (Fig. 4A). This variability was not explained by size differences as there was no significant correlation between size of fish and plasma osmolality or chloride (P>0.14) in the control group and may simply be reflective of high levels of individual variability in salinity tolerance among juvenile Atlantic sturgeon. The variance in plasma osmolality after 24 h in SW was significantly lower in the 40 µg g^{-1} relative to controls, indicating that cortisol has the capacity to improve the salinity tolerance of juvenile Atlantic sturgeon with poor salinity tolerance.

We found that cortisol upregulated gill H^+-ATPase in FW, providing the first evidence that cortisol is involved in regulating mechanisms of ion uptake in chondrosteans. Cortisol has been shown to upregulate H^+-ATPase mRNA abundance, activity and number of H^+-ATPase-rich ionocytes in zebrafish (Cruz et al., 2013), whereas cortisol does not appear to impact H^+-ATPase mRNA levels in Atlantic salmon (*Salmo salar*) (Kiilerich et al., 2007). This difference in the impact of cortisol may relate to species-specific differences in the importance of H^+-ATPase to ion uptake among teleosts. It is of interest to note that there was a rapid loss of gill H^+-ATPase abundance following exposure of Atlantic sturgeon to 28 ppt for 24 h (Fig. 5D), and that the effect of cortisol on gill H^+-ATPase abundance that was apparent in FW was no longer present. These observations indicate that there is signaling, potentially endocrine in nature, that rapidly downregulates the abundance of gill H^+-ATPase after SW exposure, including reversing the upregulation induced by cortisol.

It is now well established that cortisol is involved in regulating glucose levels in teleosts following exposure to environmental stressors (Mommsen et al., 1999). In the classic stress response, adrenaline induces a rapid release of glucose by promoting glycogenolysis, whereas glucose plays a role in its long-term elevation by increasing key hepatic gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK) (Faught and Vijayan, 2016). Increases in plasma glucose after SW exposure have been observed in studies on teleost fishes, often in conjunction with elevated plasma cortisol (Mommsen et al., 1999). Glucose is the preferred oxidative substrate for gill metabolism in teleost fishes (Mommsen, 1984), and may be important in supplying energy for synthesis of ion transport proteins and other alterations in protein synthesis that are required by the gill (and other osmoregulatory tissues) to carry out integrated water conservation and salt secretion during the transition from FW to SW (Hiroi and McCormick, 2012). Increased plasma glucose after 1–3 days of exposure to increased salinity has also been observed in green sturgeon, with higher levels seen with increasing ration size (Haller et al., 2015). We have shown for the first time in a chondrostean that exogenous cortisol results in dose-dependent increases in plasma glucose that are similar in magnitude and time course to those seen in teleost fishes. The temporal correspondence between plasma cortisol and glucose after exposure to SW provides further, albeit indirect, evidence for cortisol regulation of circulating glucose in sturgeon. An important next step will be to determine the targets (e.g. gluconeogenic enzymes) and other aspects of intermediary metabolism that are regulated by cortisol in sturgeon.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: S.D.M.; Methodology: S.D.M., M.L.T., A.M.R.; Validation: M.L.T., A.M.R.; Formal analysis: S.D.M.; Investigation: S.D.M., M.L.T., A.M.R.; Resources: S.D.M.; Data curation: M.L.T., A.M.R.; Writing - original draft: S.D.M.; Writing - review & editing: S.D.M., A.M.R.; Supervision: S.D.M.; Project administration: S.D.M.; Funding acquisition: S.D.M.

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Data availability
Data are available from the US Geological Survey database: https://doi.org/10.5066/P9KIPOF8

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