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Article

Landlocked populations have small but detectable differences in ionoregulatory physiology compared to anadromous sea lamprey, *Petromyzon marinus*

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Abstract

Life histories of anadromous and landlocked sea lamprey are similar, though landlocked populations lack seawater (SW) exposure, likely experiencing relaxed selection on SW survival traits. This study investigated SW osmoregulation in juvenile sea lamprey from one anadromous and three landlocked populations from the Great Lakes and Lake Champlain. Juveniles in all populations had strongly elevated gill Na^+/K^+-ATPase activity compared to larvae, indicating all populations underwent osmoregulatory changes associated with metamorphosis. Survival in SW was high in anadromous sea lamprey (90%) and highly variable among landlocked populations (40%–100%). Plasma ions' levels were higher and hematocrit was lower after SW exposure in landlocked compared to anadromous sea lamprey. Freshwater (FW) gill ion transporter (H^+-ATPase; Na^+:Cl^- cotransporter) mRNA levels were higher in FW and remained high after SW exposure in landlocked relative to anadromous juveniles. Landlocked lamprey had 24%–33% higher gill Na^+:K^+:$2Cl^-$ cotransporter abundance after SW exposure compared to anadromous lamprey. Our results indicate ionoregulatory differences that are consistent with relaxed selection on traits for SW entry and positive selection on FW traits in landlocked populations, suggestive of a recent Great Lakes invasion.

Key words: osmoregulation, landlocked sea lamprey, anadromous sea lamprey, Na^+/K^+-ATPase, Na^+:Cl^- cotransporter, invasive species

Introduction

Sea lamprey (*Petromyzon marinus*) are an anadromous parasitic fish native to the Atlantic coasts of North America and Europe. Larvae live in the soft sediment of freshwater (FW) streams where they filter feed on detritus for 4–7 years. Once large enough, they metamorphose over a period of several months into parasitic juveniles in late summer and early fall before migrating downstream to the ocean. During metamorphosis, they undergo major morphological changes, including development of an image-forming eye, sucker-like oral disc and piston-like tongue, and isolated gill pouches used for buccal pumping while attached to hosts. Salinity tolerance, which is critical for their movement from FW to seawater (SW), occurs late in metamorphosis (Reis-Santos et al. 2008; Shaughnessy and McCormick 2020; Ferreira-Martins et al. 2021). After spending 1–2 years in the ocean feeding parasitically on other vertebrates, adults migrate upstream to spawn and die (Beamish 1980; Renaud and Cochran 2019).

Sea lamprey are generally considered to be invasive in the Laurentian Great Lakes, Lake Champlain, and Finger Lakes of New York (Aron and Smith 1971). While the invasion in the upper Great Lakes is fairly evident, with the first observations of lamprey in Lake Erie in 1921 (Dyman 1922), Lake Huron in 1936, Lake Michigan in 1937, and Lake Superior in 1946 (Applegate 1950), the origins of sea lamprey in Lake Ontario and Lake Champlain are less clear. These lakes are accessible to the Atlantic Ocean via St. Lawrence River and Richelieu River, respectively, and these populations may have originated from multiple postglacial recolonization routes, invasion by canals, marine remnants, or any combinations of these sources. Independent genetic studies (Brussard et al. 1981; Bryan et al. 2005; Waldman et al. 2006) concluded that sea lamprey populations in Lake Ontario and Lake Champlain are most likely native. However, Eshenroder (2009, 2014) has provided additional historical and ecological information, that questions the native status of lamprey in these two lakes (reviewed by Docker and Potter 2019). The first reliable observation of sea lamprey in Lake Champlain is 1929 (Greeley 1930), which correlates with the newly constructed Champlain Barge Canal which opened in 1916. The first reliable record of sea lamprey in Lake Ontario was in 1888 (Dymond et al. 1929; Eshenroder 2014; Docker and Potter 2019), which lead to the conclusion that sea lamprey were likely introduced in Lake Ontario the 1860s (Eshenroder 2014). Genetic studies of the population structure of Great Lakes sea lamprey indicate that there is little or no contemporary move-

ment of sea lamprey between the anadromous range and Lake Ontario, likely caused by the long migration distance between the Atlantic Ocean and Lake Ontario (~1200 km) and/or hydroelectric dams and navigation locks built in the mid-1900s on the St. Lawrence River indirectly preventing fish passage (Eshenroder 2014; Docker et al. 2021). Although the current prevailing hypothesis is that sea lamprey are invasive to the Great Lakes and Lake Champlain, occurring in the last 200 years, some uncertainty remains.

Sea lamprey are representative of early jawless vertebrates, having originated ~500 million years ago (Janvier 2006). Lamprey are the earliest vertebrates known to have evolved an osmoregulatory strategy in which the salt content of the blood is maintained at approximately one-third SW (Edwards and Marshall 2012). Modern teleosts have also adapted an osmoregulatory strategy and the mechanisms for regulating ions appear to be generally similar among teleosts and lampreys (Bartels and Potter 2004; Edwards and Marshall 2012; Ferreira-Martins et al. 2021; Zimmer and Perry 2022). In FW, ions are passively lost to the environment and there is an influx of water due to the higher internal osmotic concentration compared to the environment. To counteract these passive forces, monovalent ions are taken up primarily by the gill and secondarily by the intestine from food, while the kidney produces a high volume of dilute urine (Evans et al. 2005; Ferreira-Martins et al. 2021; Zimmer and Perry 2022). In SW, water is lost and ions are passively gained from the environment. To counteract these passive movements, teleosts and lamprey drink SW and the gut absorbs ions allowing water to be absorbed. The excess monovalent ions are then excreted by the gills while the kidney produces a low volume of isosmotic urine with elevated levels of divalent ions (Evans et al. 2005; Ferreira-Martins et al. 2021; Zimmer and Perry 2022). In lamprey, the gill acts as an osmoregulatory organ for ion uptake in FW and ion secretion in SW. A larva-specific FW ionocyte and an FW-type ionocyte occur in the gills during the FW stages of the sea lamprey life cycle (Bartels and Potter 2004). V-type H^+-ATPase (*atp6v1e1*) has been localized on the apical surface of the FW ionocyte in larvae and mid-metamorphic sea lamprey (Sunga et al. 2020). A gill-specific Na^+:Cl^- chloride cotransporter (NCCa/*slc12a3*) had the highest mRNA levels in the FW gill of juvenile sea lamprey relative to all other tissues, and was 16-fold greater than the levels observed in the gill of SW-acclimated juveniles (Barany et al. 2021). In contrast, Na^+/K^+-ATPase (NKA/*atp1a1*) and Na^+:K^+:2Cl^- cotransporter (NKCC1/*slc12a2*) increase during metamorphosis and after exposure to SW in the SW ionocyte as mechanisms of ion secretion (Reis-Santos et al. 2008; Shaughnessy and McCormick 2020).

Sea lamprey in the Great Lakes have a similar life cycle as anadromous sea lamprey, where they metamorphose from FW larvae into juveniles that migrate from streams and rivers downstream into a large body water, in this case FW rather than SW. The absence of the SW life history phase should result in relaxed selection on traits associated with SW survival, including the development of salinity tolerance and associated physiological traits (Lahti et al. 2009; McCormick 2009). Loss of SW tolerance and associated traits, including reduced gill NKA activity and transcription of SW ion transporter genes, have been observed in landlocked forms that originated from anadromous populations, including Atlantic salmon (*Salmo salar*, separated for approximately 7000–12 000 years; Nilsen et al. 2007; Lemmetyinen et al. 2013; Piironen et al. 2013; McCormick et al. 2019), Arctic char (*Salvelinus alpinus*, several thousand years; Staurnes et al. 1992), and alewife (*Alosa pseudoharengus*, 300–400 years; Velotta et al. 2014, 2015). To date, there has not been simultaneous comparison of osmoregulatory physiology of anadromous and landlocked sea lamprey. In the present study, we used a direct SW exposure approach to investigate the inherent physiological capacity and capacity for acclimation in one anadromous (Connecticut River) and three landlocked (Thunder Bay, Hammond Bay, and Lake Champlain) sea lamprey populations. We measured physiological responses including survival, plasma ion levels, gill ion transporter mRNA levels, gill NKCC1 and NKA protein abundance, and gill ATPase activity prior to and after exposure to SW for 2 weeks. Given the prevailing hypothesis that sea lamprey are recent invaders of the upper Great Lakes (approximately 200 years), we predicted that there would be no or only small differences in osmoregulatory ability in SW between anadromous and landlocked populations.

Methods

Fish collection and rearing

Sea lamprey life stages are determined following Youson and Potter (1979) and Clemens (2019); briefly, larvae were considered to be immature, blind, filter-feeding individuals, transformers were considered to be metamorphosizing animals between larval and juvenile stages (outlined by Youson and Potter 1979), and juveniles were considered to be post-metamorphic animals with eyes and with the capacity to feed parasitically.

Sea lamprey were collected from four sites in May and September 2016. Transformers, which were actively undergoing metamorphosis at the time of collection, were obtained from the Connecticut River (CT) by hand during a canal drawdown (Turners Falls, MA, USA, permit No. WAV2.16CL3C), and from Lake Champlain (LC, LaPlatte River, VT, USA, permit No. 093.16LP) and Thunder Bay (TB, Neebing River, Ontario, Canada, a Lake Superior tributary, permit No. 093.16LP) by electrofishing in September 2016. A fourth population of large sea lamprey larvae (>100 mm) were collected by electrofishing from upper Great Lakes tributaries (HB, Lake Superior, Lake Michigan, and Lake Huron) in May 2016 prior to metamorphosis and were reared at the USGS Hammond Bay Biological Station (Millersburg, MI, USA) as metamorphosis commenced. Lamprey from the upper Great Lakes were reared in 200–1000 L tanks supplied with aerated, filtered, and UV-treated ambient flow-through water from Lake Huron and held under natural photoperiod from May to October 2016 (permit No. 088.16LP).

Transformers from Hammond Bay Biological Station were then transported overnight in coolers via courier, and transformers from Lake Champlain were transported in coolers via truck to the USGS EESC Conte Anadromous Fish Research Center (Turners Falls, MA, USA) in early and late October

2016. Upon arrival, oxygen was measured (>90% O_2) and rearing tanks were temperature-matched to source conditions (LC (1 October): 20 °C, TB and HB (31 October): 12.5 °C). All lamprey were reared at ambient temperature (12 °C–20 °C) under a natural photoperiod in 1 m diameter tanks (640 L) and 6–8 cm of fine sand for burrowing until experimentation commenced. Each tank was supplied with aerated, filtered, UV-treated flow-through Connecticut River water (~100–200 μS) until metamorphosis was completed at the end of November 2016. Lamprey were not fed as the absence of feeding is the natural state of sea lamprey during and immediately following metamorphosis (Youson and Potter 1979; Shaughnessy and McCormick 2023). All salinity exposure experiments occurred in tanks without substrate using dechlorinated municipal FW and were equipped with mechanical, chemical, and biological filtration. Artificial SW was prepared by dissolving sea salt (Crystal Sea Salt, Baltimore, MD, USA) in dechlorinated municipal FW. To compare the physiology of larvae and juvenile sea lamprey, larvae were collected from the Sawmill River, a tributary to the Connecticut River, in July 2016, and were reared in 1 m diameter tanks (640 L) containing 10 cm fine sand, and supplied with aerated, filtered, UV-treated flow-through ambient Connecticut River water. Larvae were fed twice a week with baker's yeast suspended in source tank water. Larval plasma chloride levels, plasma osmolality, and gill NKA activity were measured as described below as a pre-metamorphic comparison. Rearing and experiments in this study were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committees at the University of Massachusetts (protocol No. 2016-0009).

Long-term exposure to SW

Survival studies were conducted on each population of sea lamprey over 4 weeks in December 2016. Due to a limited number of landlocked sea lamprey, only eight to ten individuals were transferred directly from FW (0 ppt) into 20 L tanks containing 30 or 35 ppt SW. Experiments were performed at a constant temperature of 15 °C, and survival and water quality were assessed daily and weekly, respectively.

Time course response to SW

A 2 week SW exposure was conducted to examine inherent (day 1) and acclimation (days 5 and 14) capacity of post-metamorphic lamprey from each population in December 2016. Lamprey were acclimated to 1 m diameter tanks with recirculating FW (640 L, 175–185 μS) for 2 weeks. Lamprey ($n = 10$) were sampled as FW day 0 time point, after which the juvenile lamprey were exposed to recirculating FW (control) or 30 ppt SW within an hour. Due to limited number of lamprey from the Thunder Bay population, lamprey were only exposed to 30 ppt SW (no FW control). Lamprey ($n = 7$–10) were sampled 1, 5, and 14 days after SW or FW exposure. Experiments were performed at a temperature of 13.5 °C ± 0.4 °C, and survival and water quality were monitored daily and weekly, respectively.

Lamprey were euthanized using a lethal dose of the tricaine methanesulfonate (200 mg·L^{-1}, buffered by $NaHCO_3$, pH 7.4, MS-222; Argent Chemical Laboratories, Redmond, WA, USA) diluted in experimental tank water. Animals were measured for total length (nearest 0.1 cm) and mass (nearest 0.1 g). Tails were severed and blood was collected from the caudal vessel with ammonia-heparinized capillary tubes and centrifuged at 5000 *g* for 5 min at 23 °C. Hematocrit was measured and plasma was separated and stored at −80 °C. Gill pouches from each fish were dissected using a ventral midline incision and biopsied (limiting esophagus, cartilage, and muscle tissues), and either placed in a tube for (1) measurement of ion transporter mRNA levels, (2) measurement of ion transport protein abundance using immunoblotting, or (3) for gill NKA activity with 100 μL SEI buffer (300 mM sucrose, 20 mM EDTA, 50 mM imidazole, pH 7.5) and frozen at −80 °C.Plasma osmolality and chloride levels were measured with a vapor pressure osmometer (Wescor 5500, Logan, UT, USA) and a digital chloridometer (Labconco, Houston, TX, USA), respectively. Plasma glucose was measured using microplate Glucose (HK) reagent, and samples were determined against a four-parameter standard curve (G3293, Sigma–Aldrich, St. Louis, MO, USA).

Gill ion transporter mRNA levels (quantitative real-time-PCR)

Total RNA was extracted from gill tissue using TRI-reagent (Molecular Research Center Inc., Cincinnati, OH) following the manufacturer's protocol. Total RNA concentration and purity was determined spectrophotometrically using a Take3 microvolume plate (BioTek Instruments, Winooski, VT, USA) while the integrity was confirmed by gel electrophoresis. All samples with high purity ($A_{260}/A_{280} > 1.9$) and integrity (presence of only two clear bands indicative of 28s and 18s rRNA) were DNase-treated (Promega Corp., Madison, WI, USA) prior to cDNA synthesis. First-strand cDNA synthesis was accomplished using 1.6 μg of total RNA with High Capacity Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) according to manufacturer's protocol. Quantitative real-time PCR was performed using SYBR Select Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) in 10 μL reactions containing 4 ng of cDNA template and 100 nmol·L^{-1} forward and reverse primers specifically designed for sea lamprey gill *nka* (*atp1a1*), *nkcc1* (*slc12a2*), *ncca* (*slc12a3*), and h^+-*atpase* (*atp6v1e*, Table S1). Cycling profile consisted of an initial activation step at 50 °C for 2 min, a denaturation step at 95 °C for 2 min, 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 60 s, and extension at 72 °C for 30 s, with a final extension was performed at 72 °C for 2 min using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Reactions were considered satisfactory when standard curve efficiency values were 90%–105% and linear ($R^2 > 0.990$) and melt curve detected a single product. Relative gene mRNA levels were calculated using the Pfaffl (2006) method with 18s rRNA (*18 s*) as the reference gene.

Gill ion transporter abundance (Western blots)

Gill tissue samples were homogenized using a motorized pestle in 20 volumes SEI plus 0.1% sodium deoxycholate and Complete Mini Protease Inhibitor tablets (Roche, Indianapolis, IN, USA). Samples were centrifuged at 7000 *g* for 7 min

at 4 °C, and the supernatant was quantified using the Pierce BCA Protein Assay (Thermo Fisher Scientific, Rockford, IL, USA). Samples were diluted in equal volumes of 2× Laemmli buffer, denatured at 65 °C for 15 min, and stored at −80 °C. A total of 5 μg protein was loaded in 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis minigel (Bio-Rad, Hercules, CA, USA), and two lanes were reserved on each gel for Precision Plus relative molecular weight markers (Bio-Rad Laboratories, Hercules, CA, USA) and a standard consistent reference lane for interblot variation. Electrophoresis occurred in 25 mM Tris, 192 mM glycine buffer, 0.1% sodium dodecyl sulfate, pH 8.3, then proteins were transferred to Immobilon PVDF transfer membrane (Millipore, Bedford, MA, USA) at 80 V for 1 h in 25 mM Tris, 192 mM glycine buffer, pH 8.3. PVDF membranes were dried overnight at 23 °C. Immunodetection was performed by rehydrating blots in methanol, followed by deionized water, equilibration with phosphate-buffered saline with 0.05% Triton X-100 (PBST), and then blocked with 5% nonfat dry milk in PBST for 1 h at 23 °C. Blots were rinsed in PBST and exposed to a 1:2000 primary antibody in blocking buffer overnight at 4 °C: mouse monoclonal anti-NKA α-subunit ("α5"), or mouse monoclonal anti-NKCC ("T9") (Developmental Studies Hybridoma Ban, Iowa City, IA, USA). After rinsing blots in PBST three times for 15 min, blots were exposed to goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MA, USA) diluted 1:10 000 in blocking buffer for 1 h at 23 °C. After rinsing in PBST, blots were incubated for 1 min in a 1:1 mixture of enhanced chemiluminescent (ECL) solution A (396 μm coumaric acid, 2.5 μm luminol, 100 mM Tris, pH 8.5) and ECL B (0.018% H_2O_2, 100 mM Tris, pH 8.5) and imaged using a Syngene PXi system (SYNGENE, Frederick, MD, USA). Band intensity was measured using ImageJ (NIH, Bethesda, MD, USA), and protein abundance expressed as relative band intensity to the standard consistent pool.

Gill Na^+/K^+-ATPase activity

Gill NKA and residual ATPase activity were measured with a kinetic microassay (McCormick 1993) that has been validated and optimized for lamprey gill tissue (Reis-Santos et al. 2008). Samples stored in 100 μL SEI buffer were thawed on ice, sodium deoxycholate added to a final concentration of 0.1%, and the tissue was homogenized using a motorized pestle. Homogenates were centrifuged at 3200 *g* for 30 s at 4 °C and the supernatant was used for the ATPase assay. Samples of 10 μL were run in three duplicate sets at 25 °C. In one set, ouabain (0.5 mM) was added to the assay mixture to specifically inhibit NKA activity. Total protein was measured using the Pierce BCA Protein Assay. All assays were run on a THERMOmax microplate reader and SOFTmax software (Molecular Devices, Sunnyvale, CA, USA). Gill NKA activity measurements were calculated as the difference in ouabain-sensitive and -insensitive ADP production, gill residual ATPase activity was determined by the ouabain-insensitive ADP production, and both methods were expressed in μmol ADP mg^{-1} protein h^{-1}.

Statistics

Statistical differences were analyzed using SigmaPlot 14.5. A correlation of length (cm) and wet mass (g) resulted in a power regression with an exponent of 3.2 ($R^2 = 0.92$, Fig. S1); therefore, Fulton's condition factor (*K*) was calculated as (wet mass (g) × length^{-3} (cm)) × 1000. Survival was compared using the Fisher's exact test on the number of dead and alive juveniles between the anadromous and each of the landlocked populations. Normality of residuals was confirmed on all response variables using the Anderson–Darling test, and only mRNA level variables were found to be non-normal. A square root transformation was applied to all mRNA data which provided normality to the residuals. A correlation of all continuous variables was completed using Pearson correlation coefficients, with the exception of length and wet mass as they were only correlated to each other (Fig. S2). To prevent type I error inherent of multiple correlation comparisons, Bonferroni correction was conducted and *p*-values for correlation were considered significant when $p \leq 0.004$. Because there were differences in mass and condition factor among populations (Tables 1 and 2), analysis of covariance was used to test whether population, time, salinity in conjunction with mass or condition factor had significant effects on the continuous response variables tested in this experiment. No effect of mass or condition factor was found ($p > 0.05$, data not shown); therefore, three-way analysis of variance (ANOVA) tests were performed for each physiological response variable, with salinity, time, and population considered the independent categorical variables (Tables S2–S11). Because we had insufficient numbers of animals to include FW controls for TB, three-way ANOVAs were only conducted on CT, LC, and HB populations. Since we were most interested in population differences in SW and wanted to also test for differences in the TB population, we show these results graphically (along with day 0 in FW) and analyzed these results using two-way ANOVAs with population and time as independent variables for each physiological response variable in SW. FW control data and statistics (two-way ANOVA results) are included in the supplemental material (Figs. S2–S8). Tukey's multiple comparison post hoc test was used to test differences within population, time, and interaction effects. Population marginal means are represented for each physiological trait along with the statistical outcome to assess the effects of being landlocked within salinity regardless of time. Protein abundance was measured in all populations on initial FW day 0 and all SW time points (no FW controls measured), therefore only two-way ANOVAs of time and population were conducted on the SW time course. One-way ANOVA tests were conducted on all response variables in FW day 0 to compare all four populations at the start of the experiment. In all cases, results were considered significantly different when $p \leq 0.05$.

Results

Long-term survival in SW

Mortality occurred in the first 2 days after transfer to 30 or 35 ppt SW (Fig. 1). In 30 ppt, survival was 90% for the anadro-

Table 1. Sample sizes and biometric data of Connecticut River (CT), Lake Champlain (LC), Hammond Bay (HB), and Thunder Bay (TB) juvenile sea lamprey (*Petromyzon marinus*) for long-term survival in 30 and 35 ppt.

	30 ppt				35 ppt			
	CT	LC	HB	TB	CT	LC	HB	TB
Sample size	10	10	10	8	10	10	10	8
Length, cm	16.8 ± 0.2^{a}	15.1 ± 0.4^{b}	13.9 ± 0.1^{b}	17.6 ± 0.4^{a}	15.5 ± 0.3^{ab}	16.6 ± 0.5^{bc}	14.3 ± 0.4^{a}	17.0 ± 0.5^{c}
Mass, g	6.1 ± 0.2^{a}	4.6 ± 0.4^{b}	3.1 ± 0.2^{c}	7.1 ± 0.5^{a}	4.7 ± 0.3^{a}	5.7 ± 0.5^{ab}	3.1 ± 0.3^{a}	5.9 ± 0.6^{ab}
Condition factor, *K*	1.3 ± 0.01^{ab}	1.3 ± 0.03^{a}	1.1 ± 0.07^{b}	1.3 ± 0.02^{ab}	1.3 ± 0.02^{a}	1.2 ± 0.03^{a}	1.0 ± 0.03^{b}	1.2 ± 0.06^{ab}

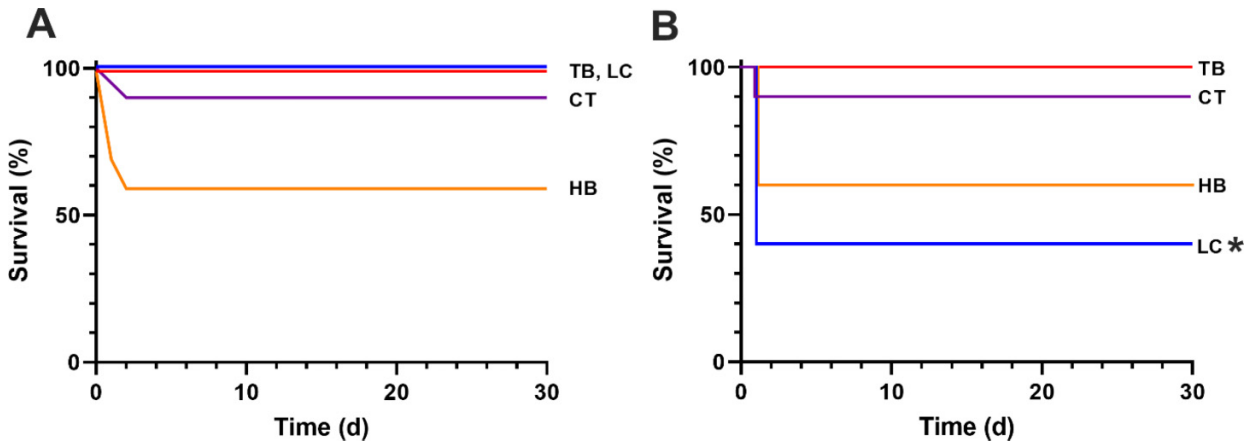
Note: Values represent means (±SEM) of mass, total length, and condition factor. Different letters indicate significant differences among populations ($p \leq 0.05$, one-way ANOVA, Tukey's post hoc).

Table 2. Sample sizes, biometric data, and survival of Connecticut River (CT), Lake Champlain (LC), Hammond Bay (HB), and Thunder Bay (TB) juvenile sea lamprey (*Petromyzon marinus*) for 2 week exposure to 30 ppt.

	CT	LC	HB	TB
Sample size	60	64	57	40
Length, cm	14.9 ± 0.1^{a}	16.3 ± 0.1^{b}	13.7 ± 0.1^{c}	17.2 ± 0.2^{d}
Mass, g	4.5 ± 0.1^{a}	5.8 ± 0.1^{b}	3.1 ± 0.1^{c}	6.6 ± 0.2^{d}
Condition factor, *K*	1.4 ± 0.10^{a}	1.4 ± 0.12^{a}	1.2 ± 0.13^{b}	1.3 ± 0.09^{c}
Survival	97.5%	100%	97.5%	100%

Note: Values represent means (±SEM) of mass, total length, and condition factor. Different letters indicate significant differences among populations ($P \leq 0.05$, one-way ANOVA, Tukey's post hoc).

Fig. 1. Survival of Connecticut River (CT, purple), Lake Champlain (LC, blue), Hammond Bay (HB, orange), and Thunder Bay (TB, red) juvenile sea lamprey (*Petromyzon marinus*) over 30 days in (A) 30 ppt and (B) 35 ppt. An asterisk indicates significant differences in survival compared to CT sea lamprey ($p \leq 0.05$, χ^2).



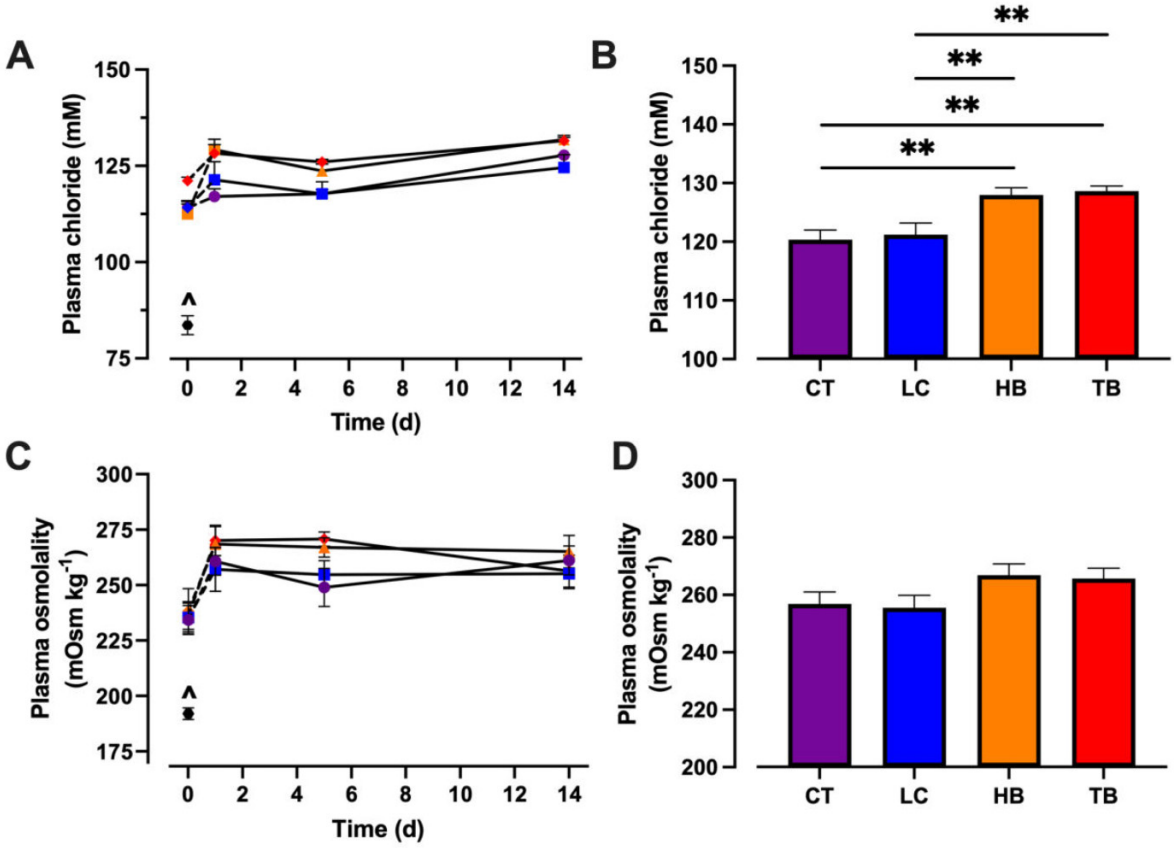
mous (CT) population, 100% for TB and LC, and 60% for HB, with no statistical difference among populations ($p \geq 0.15$). In 35 ppt SW, survival was 90% for CT, 100% for TB, 60% for HB, and 40% for LC sea lamprey. LC sea lamprey had significantly lower survival compared to CT sea lamprey ($p = 0.03$).

Time course response in SW

Plasma chloride concentration

Plasma chloride levels were affected by population, salinity, and population × salinity interaction effect based on the three-way ANOVA (Table S2). All FW juveniles had higher plasma chloride levels on day 0 compared to larvae, and among populations, TB was lower than HB (one-way ANOVA, $p = 0.034$, Fig. 2A). All populations had an increase in plasma chloride levels from initial FW values to day 1 after SW exposure (Fig. 2A, CT: 2.5%, LC and TB: 6%, and HB: 15%). Within the SW time course, plasma chlorides were affected by time ($p < 0.01$) and population ($p < 0.01$) but not population × time interaction ($p = 0.70$). HB and TB populations had elevated plasma chloride levels compared to CT lamprey throughout the time course. Within SW marginal means, HB and TB lamprey had higher plasma chloride levels from CT (6% and 7%, respectively, $p < 0.01$; Fig. 2B). Plasma chloride levels were positively correlated to plasma osmo-

Fig. 2. Plasma chloride and osmolality levels of juvenile sea lamprey transferred from freshwater to seawater. (A) Time course changes in plasma chloride levels in CT (Connecticut River, purple circles), LC (Lake Champlain, blue squares), HB (Hammond Bay, orange triangles), and TB (Thunder Bay, red diamonds) sea lamprey (*Petromyzon marinus*) exposed to 30 ppt seawater (SW). Larval sea lamprey plasma chloride levels in freshwater (FW) are shown in black hexagon. (B) Plasma chloride levels from SW population marginal mean (data from all individuals exposed to SW) of two-way ANOVA. (C) Time course changes in plasma osmolality in CT, LC, HB, and TB sea lamprey exposed to 30 ppt SW. Larval sea lamprey plasma osmolality levels are shown in black. (D) Plasma osmolality levels of SW population marginal mean from two-way ANOVA results. Values are means (±SEM, $n = 5$–10). Dashed line in time course figures indicates that fish previously in freshwater (Time 0) have been exposed to SW, which occurred within one hour after day 0 sampling. Asterisks indicate effect of population ($^{**}p < 0.01$) as determined by a two-way ANOVA followed by Tukey's HSD post hoc test. A caret indicates significant differences between larvae and all populations of juvenile sea lamprey ($p < 0.01$).



lality ($p < 0.001$) and gill NKA activity ($p < 0.001$, Fig. S2) in SW.

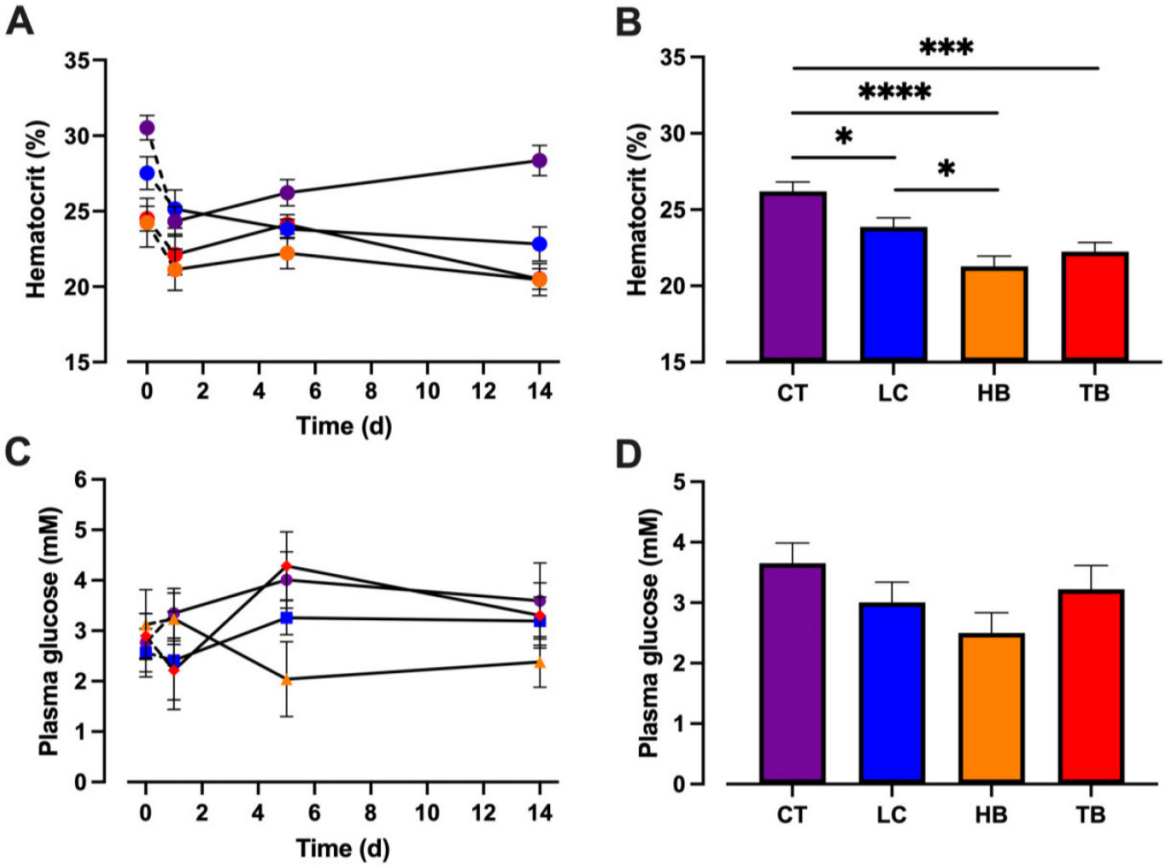
Plasma osmolality

Plasma osmolality was affected by salinity based on the three-way ANOVA (Table S3). Plasma osmolality levels in all FW juveniles on day 0 were 23%–24% higher than those in larvae and showed no differences among populations (one-way ANOVA, $p = 0.99$, Fig. 2C). After SW exposure, all populations increased plasma osmolality from 9% to 14% relative to initial FW values (Fig. 2C). However, plasma osmolality had no differences in time ($p = 0.61$), population ($p = 0.11$), or population × time interaction ($p = 0.73$) over the 2 week SW exposure. Plasma osmolality remained elevated in all populations compared to FW day 0. Populations were not different among the SW marginal mean (Fig. 2D). Plasma osmolality was positively correlated to plasma chloride levels ($p < 0.001$) and negatively correlated to hematocrit ($p < 0.001$) in SW (Fig. S2).

Hematocrit

Hematocrit was affected by salinity and population (three-way ANOVA, Table S4). On FW day 0, CT was higher than HB and TB ($p < 0.002$ and $p < 0.004$, respectively), but not LC ($p = 0.226$). After SW exposure, hematocrit was affected by population ($p < 0.01$) and population × time interaction ($p = 0.03$) but not by time ($p = 0.29$, Fig. 3A). CT lamprey experienced a transient decrease in hematocrit on day 1, with values returning to initial FW levels by day 14. Landlocked populations had a reduction of hematocrit from initial FW levels, which remained low throughout the SW time course. On day 14, landlocked lamprey had 25%–40% lower hema-

Fig. 3. Hematocrit and plasma glucose levels of juvenile sea lamprey transferred from freshwater to seawater. (A) Time course changes in hematocrit in CT (Connecticut River, purple circles), LC (Lake Champlain, blue squares), HB (Hammond Bay, orange triangles), and TB (Thunder Bay, red diamonds) sea lamprey (*Petromyzon marinus*) exposed to 30 ppt seawater (SW). (B) Hematocrit levels from SW population marginal mean (data from all individuals exposed to SW) of two-way ANOVA. (C) Time course changes in plasma glucose in CT, LC, HB, and TB sea lamprey exposed to 30 ppt SW. (D) Plasma glucose levels of SW population marginal mean from two-way ANOVA results. Values are means (±SEM, $n = 5$–10). Dashed line in time course figures indicates that fish previously in freshwater (Time 0) have been exposed to SW, which occurred within 1 h after day 0 sampling. Asterisks indicate effect of population ($^{*}p < 0.02$, $^{***}p < 0.001$, $^{****}p < 0.0001$) as determined by a two-way ANOVA followed by Tukey's HSD post hoc test.



tocrit than CT lamprey (all populations: $p < 0.01$). Within SW marginal mean, hematocrit was different among populations; all landlocked populations had 10%–23% lower hematocrit levels compared to CT lamprey (LC: $p = 0.03$, HB and TB: $p < 0.01$; Fig. 3B). Hematocrit was negatively correlated with gill *nka* mRNA levels ($p = 0.003$, Fig. S2) in SW. In the FW control, hematocrit did not change over time ($p = 0.62$) or show a population × time effect ($p = 0.12$), but there were population differences ($p < 0.01$) in the FW marginal means (Figs. S4A and S4B). HB lamprey had 21% lower hematocrit in FW compared to CT lamprey ($p < 0.01$, Fig. S4B).

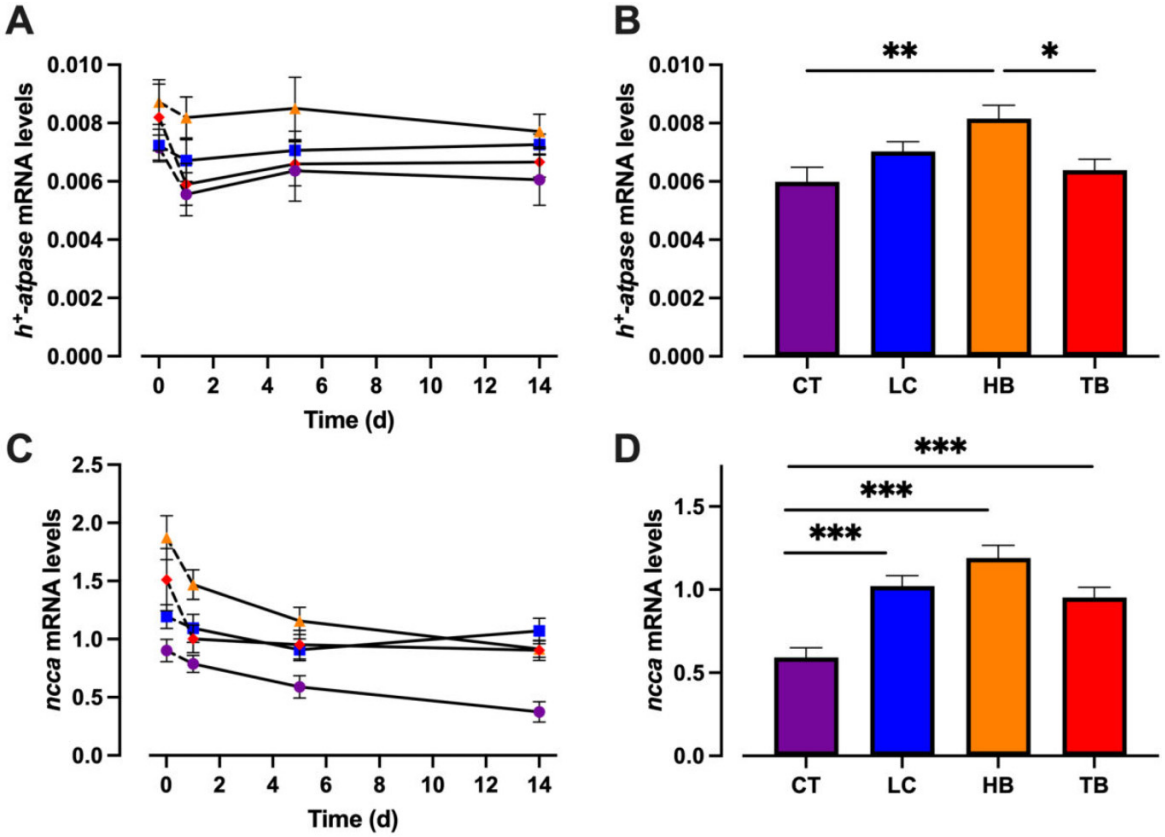
Plasma glucose

Plasma glucose levels were not different among any variables (three-way ANOVA, Table S5). On FW day 0, plasma glucose was not affected by population (one-way ANOVA; $p = 0.912$). After SW exposure, there were no effects of time ($p = 0.40$), population ($p = 0.16$), or population × time effects ($p = 0.34$) on plasma glucose (Figs. 3C and 3D).

Gill ion transporter mRNA levels

Gill h^{+}-*atpase* mRNA levels were different among populations but not between salinities or over time (three-way ANOVA, Table S6). On FW day 0, all populations had similar h^{+}-*atpase* levels (one-way ANOVA, $p = 0.37$, Fig. 4A). After SW exposure, gill h^{+}-*atpase* did not change from initial FW levels (Fig. 4A). Gill h^{+}-*atpase* was different among populations ($p < 0.01$) but not over time ($p = 0.59$) or in the population × time interaction ($p = 0.99$) after SW exposure. HB lamprey gill h^{+}-*atpase* levels were 100% and 66% greater than CT lamprey on days 1 and 5, respectively. Within the SW marginal mean, HB had 71% higher gill h^{+}-*atpase* mRNA levels compared to CT lamprey ($p < 0.01$, Fig. 4B). Gill h^{+}-*atpase* did not change in FW over time ($p = 0.07$) but population mRNA levels were different ($p < 0.01$, Fig. S4A). HB lamprey had 85%

Fig. 4. Freshwater (FW) ion transporter mRNA levels of juvenile sea lamprey transferred from freshwater to seawater. (A) Time course changes in gill *h*$^+$*-atpase* (*atp6v1e1*/V-type H$^+$-ATPase gene) mRNA levels in CT (purple circles), LC (blue squares), HB (orange triangles), and TB (red diamonds) sea lamprey (*Petromyzon marinus*) exposed to 30 ppt SW. (B) SW population marginal mean (data from all individuals exposed to SW) of gill *h*$^+$*-atpase* mRNA levels from two-way ANOVA results. (C) Time course changes in gill *ncca* (*slc12a3*/gill NCC gene) mRNA levels in CT, LC, HB, and TB sea lamprey exposed to 30 ppt SW. (D) SW population marginal mean of gill *ncca* mRNA levels from two-way ANOVA results. Values are means (±SEM, $n = 5$–10). Dashed line in time course figures indicates that fish previously in freshwater (Time 0) have been exposed to SW, which occurred within 1 h after day 0 sampling. Asterisks indicate effect of population ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) as determined by a two-way ANOVA followed by Tukey's HSD post hoc test. All mRNA levels are relative to *18 s* mRNA levels.



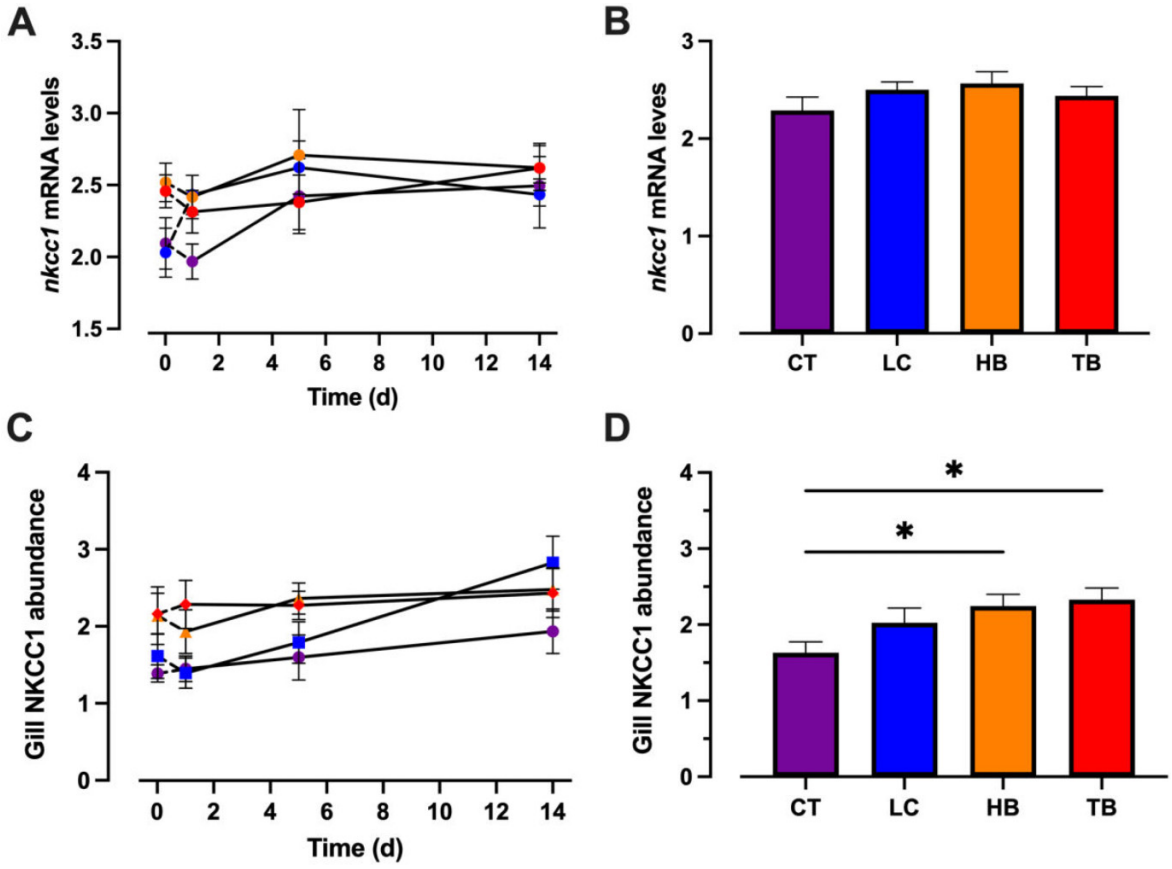
higher *h*$^+$*-atpase* mRNA FW marginal mean compared to CT lamprey ($p < 0.01$, Fig. S5B).

Gill *ncca* mRNA levels were different across populations, salinities, and time (three-way ANOVA, Table S7). On FW day 0, CT lamprey had lower *ncca* mRNA levels compared to HB lamprey ($p < 0.01$), but not to LC or TB lamprey (one-way ANOVA, $p = 0.64$ and $p = 0.10$, respectively, Fig. 4C). After SW exposure, gill *ncca* mRNA levels decreased in all populations from initial FW levels (Fig. 4C). HB lamprey gill *ncca* mRNA levels were approximately 2.5-fold greater than CT *ncca* on day 1 ($p < 0.01$). Landlocked lamprey gill *ncca* were 3.5- to 5.4-fold greater than CT *ncca* on day 14 (LC14: $p < 0.001$, HB14: $p = 0.03$, TB14: $p = 0.04$). All landlocked populations had 1.3- to 2.6-fold higher gill *ncca* SW marginal mean mRNA levels compared to CT lamprey gill *ncca* mRNA levels (all populations: $p < 0.01$, Fig. 4D). Gill *ncca* mRNA levels did not change over time in FW ($p = 0.39$), but there was a population effect ($p < 0.01$, Fig. S4C). Landlocked lamprey had 2.2- to 3.9-fold higher gill *ncca* levels than CT lamprey among FW marginal means (both populations: $p < 0.01$, Fig. S5D).

Gill *nkcc1* mRNA levels were affected by salinity, but not time or population (three-way ANOVA, Table S8). Populations were not different on day 0 in FW (one-way ANOVA, $p = 0.07$, Fig. 5A). After SW exposure, gill *nkcc1* were not affected by time ($p = 0.09$), population ($p = 0.32$), or population × time interaction ($p = 0.80$) in SW time course (Figs. 5A and 5B).

Gill *nka* mRNA levels were different between salinities and among populations (three-way ANOVA, Table S9). There were no differences in gill *nka* on FW day 0 among populations (one-way ANOVA, $p = 0.290$, Fig. 6A). After SW exposure, gill *nka* was not affected time ($p = 0.16$), population ($p = 0.09$), or population × time effects ($p = 0.46$) in SW time course (Figs. 6A and 6B).Gill *nka* showed time ($p = 0.02$) and population ($p = 0.02$) effects but no population × time effect ($p = 0.87$) in FW (Fig. S7A). HB lamprey had 97% higher gill *nka* FW marginal mean compared to CT lamprey ($p = 0.03$, Fig. S7B).

Fig. 5. NKCC1 mRNA levels and relative protein abundance of juvenile sea lamprey transferred from freshwater to seawater. (A) Time course changes in gill *nkcc1* (*slc12a2*/NKCC1 gene) mRNA levels in CT (purple), LC (blue), HB (orange), and TB (red) sea lamprey (*Petromyzon marinus*) exposed to 30 ppt SW. (B) SW population marginal mean (data from all individuals exposed to SW) of gill *nkcc1* mRNA levels from two-way ANOVA results. (C) Time course changes in gill NKCC1 protein abundance in CT, LC, HB, and TB sea lamprey exposed to 30 ppt SW. (D) SW population marginal mean of gill NKCC1 protein abundance from two-way ANOVA results. Values are means (±SEM, $n = 5$–10). Dashed line in time course figures indicates that fish previously in freshwater (Time 0) have been exposed to SW, which occurred within 1 h after day 0 sampling. Asterisks indicate effect of population ($^*p < 0.05$) as determined by a two-way ANOVA followed by Tukey's HSD post hoc test.



Gill ion transporter protein abundance

Gill NKCC1 protein abundance was different over time ($p < 0.01$) and among population ($p = 0.02$) but did not have a population × time effect ($p = 0.28$). Populations were not different on FW day 0 (one-way ANOVA, $p = 0.15$, Fig. 5C). Gill NKCC1 abundance was upregulated in LC lamprey, doubling in abundance from day 1 to 14 ($p < 0.01$, Fig. 5C), while all other populations had stable protein abundance throughout the time course. HB and TB lamprey had higher NKCC1 abundance in the SW marginal mean compared to CT lamprey (HB: 54%, $p = 0.04$; TB: 55%, $p = 0.02$; Fig. 5D). Gill NKCC1 abundance was positively correlated to gill NKA protein abundance ($p < 0.004$, Fig. S2) in SW.

Gill NKA protein abundance was different over time ($p = 0.03$) but not among populations ($p = 0.60$) or in population × time interaction in the SW time course ($p = 0.46$, Fig. 6C). Populations were not different on FW day 0 (one-way ANOVA, $p = 0.56$, Fig. 6C). Gill NKA abundance was 22% and 30% higher in HB and TB lamprey, respectively, on day 1 compared to CT lamprey. Population SW marginal means of gill NKA abundance were not different (Fig. 6D).

Gill ATPase activity

Gill NKA activity was affected by population, time, salinity, and time × salinity (three-way ANOVA, Table S10). Gill NKA activity levels in all FW juveniles were 8.5- to 10-fold greater than those in larvae, but there were no population differences on FW day 0 (one-way ANOVA, $p = 0.86$, Fig. 7A). After SW exposure, LC, HB, and TB lamprey had 55%–70% increase in gill NKA activity over time. CT lamprey gill NKA levels peaked on day 5 and remained elevated on day 14, increasing a total of 34% from day 1. Populations were not different among SW marginal means (Fig. 7B). Gill NKA activity was positively correlated to gill residual ATPase activity ($p < 0.004$, Fig. S2) in SW.

Residual gill ATPase activity was different among populations, over time, and in time × salinity (three-way ANOVA,

Fig. 6. NKA mRNA levels and relative protein abundance of juvenile sea lamprey transferred from freshwater to seawater. (A) Time course changes in gill *nka* (*atp1a1*/NKA α subunit gene) mRNA levels in Connecticut River (CT, purple circles), Lake Champlain (LC, blue squares), Hammond Bay (HB, orange triangles), and Thunder Bay (TB, red diamonds) sea lamprey (*Petromyzon marinus*) exposed to 30 ppt seawater (SW). (B) SW population marginal mean (data from all individuals exposed to SW) of *nka* mRNA levels from two-way ANOVA results. (C) Time course changes in gill NKA protein abundance in CT, LC, HB, and TB sea lamprey exposed to 30 ppt SW. (D) SW population marginal mean of gill NKA protein abundance from two-way ANOVA results. Values are means (±SEM, n = 5–10). Dashed line in time course figures indicates that fish previously in freshwater (Time 0) have been exposed to SW, which occurred within 1 h after day 0 sampling. Asterisks indicate effect of population ($^*p < 0.05$) as determined by a two-way ANOVA followed by Tukey's HSD post hoc test.

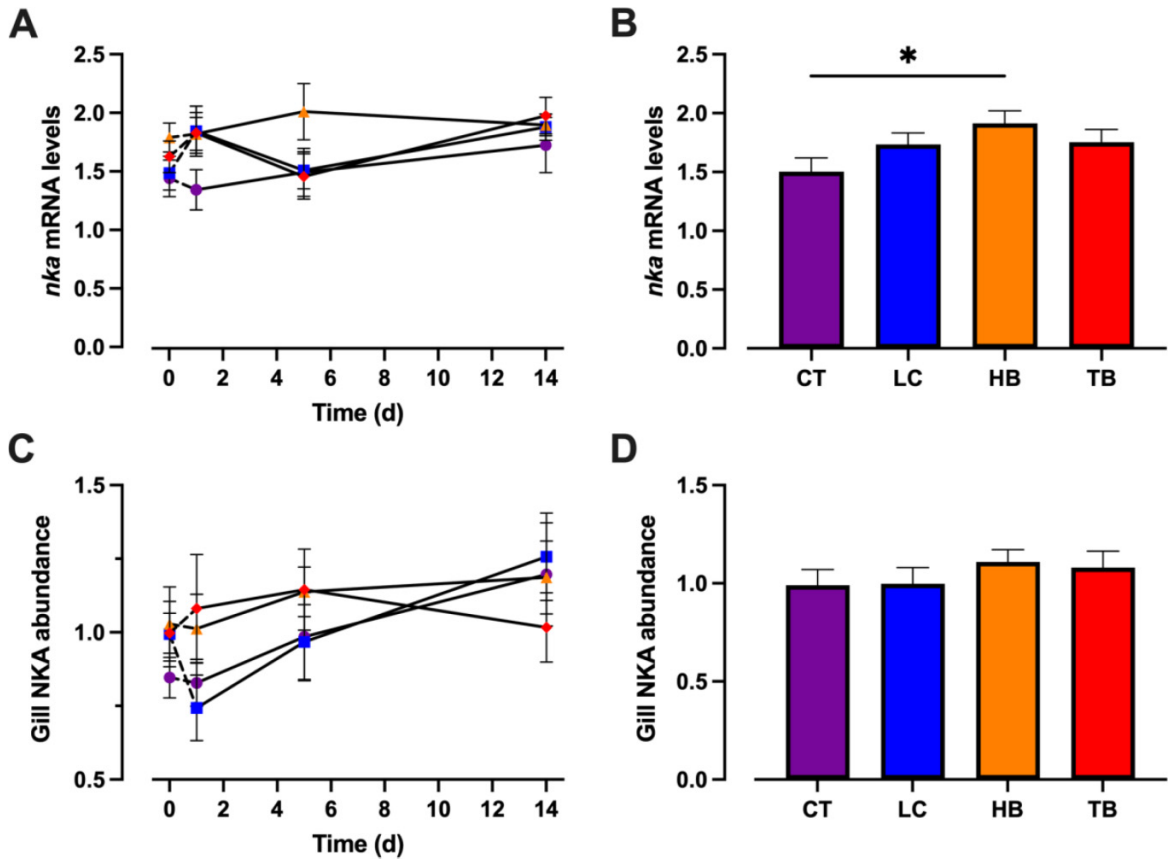


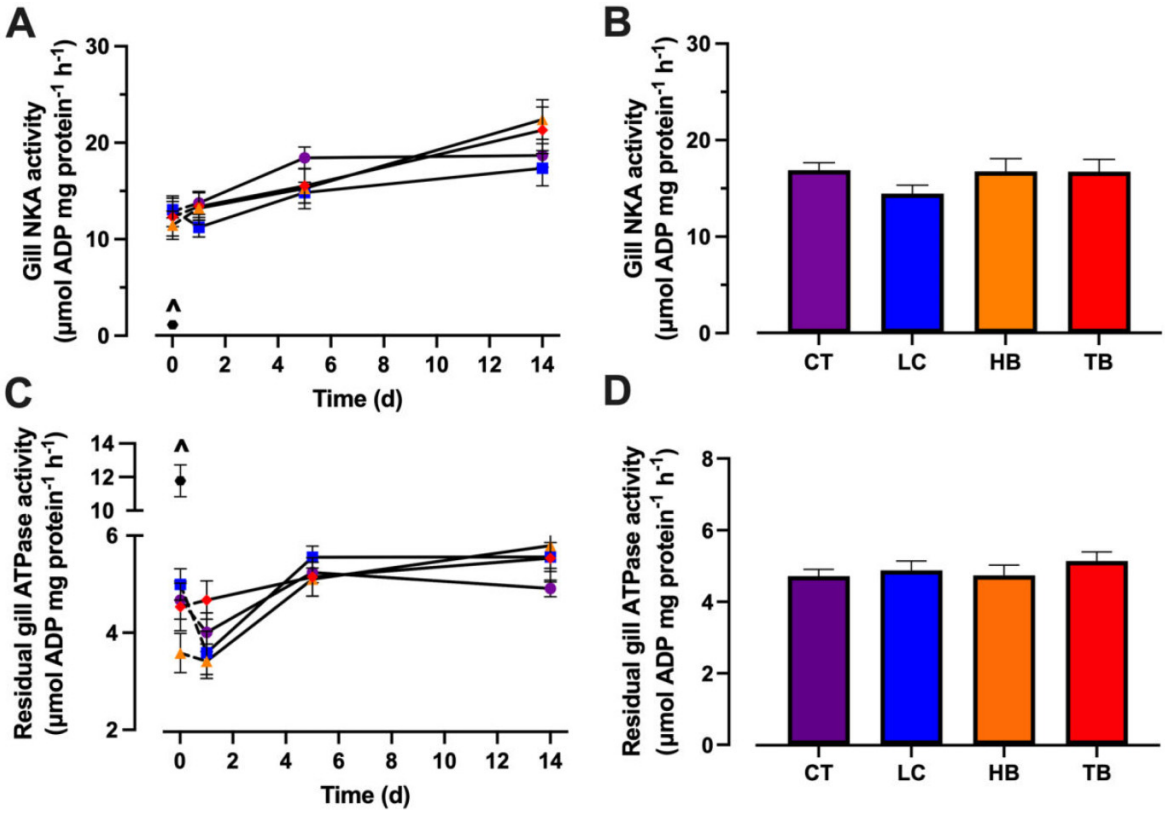
Table S11). Residual gill ATPase activity did not differ among populations on FW day 0 (one-way ANOVA, $p = 0.08$, Fig. 7C). After SW exposure, residual gill ATPase activity was affected by time ($p < 0.01$) but not population ($p = 0.57$) or population × time ($p = 0.19$, Fig. 7C). CT and LC lamprey had a 14% and 28% decrease on day 1 from initial FW levels, respectively, while other landlocked lamprey were relatively unchanged (TB increased 3%, HB decreased 5%). Following day 1, residual gill ATPase activity increased in LC and HB lamprey over time (LC1–LC5: $p < 0.01$, LC1–LC14: $p < 0.01$, HB1–HB5: $p < 0.01$, HB1–HB14: $p < 0.01$). Residual gill ATPase activity did not differ among population SW marginal means (Fig. 7D). Residual gill ATPase activity did not show a population effect ($p = 0.07$), but there were significant time ($p < 0.01$) and population × time ($p = 0.01$) effects in FW (Figs. S8A and S8B).

Discussion

The present study shows that there are minor differences in osmoregulatory capacity between landlocked and anadromous populations of sea lamprey. Survival was lower in a landlocked population within the first 2 days of exposure to 35 ppt. Plasma ions and gill NKCC1 protein abundance were elevated in upper Great Lakes populations compared to anadromous sea lamprey after exposure to 30 ppt over a 2 week time course. Gill *ncca* mRNA levels were higher in all landlocked populations in FW and remained higher throughout the SW time course.

We assessed SW tolerance of landlocked and anadromous sea lamprey populations by monitoring survival after direct exposure to either 30 or 35 ppt. Mortality occurred in the first 2 days after direct exposure to SW, yet no population showed <60% survival over time in 30 ppt in our preliminary study (Fig. 1A) and all populations had >97% survival in our main experiment (Table 2). Our results contrast with those of Mathers and Beamish (1974) who found that landlocked sea lamprey from Lake Ontario had 50% mortality at 26 ppt and 100% mortality at 34 ppt. Plasma osmolality of Lake Ontario sea lamprey in 34 ppt was much higher (400 mOsm·kg^{-1}) compared to HB and TB lamprey (~265 mOsm·kg^{-1}) in the

Fig. 7. Gill ATPase activity of juvenile sea lamprey transferred from freshwater to seawater. (A) Time course changes in gill NKA activity in Connecticut River (CT, purple circles), Lake Champlain (LC, blue squares), Hammond Bay (HB, orange triangles), and Thunder Bay (TB, red diamonds) sea lamprey (*Petromyzon marinus*) exposed to 30 ppt seawater (SW). Freshwater (FW) larval sea lamprey gill NKA activity is shown with a black hexagon. (B) SW population marginal mean (data from all individuals exposed to SW) of gill NKA activity from two-way ANOVA results. (C) Time course changes in residual (ouabain-insensitive) gill ATPase activity in CT, LC, HB, and TB sea lamprey exposed to 30 ppt SW. FW larval sea lamprey gill residual ATPase activity (ouabain-insensitive) is shown in black. (D) SW population marginal mean of residual gill ATPase activity from two-way ANOVA results. Values are means (±SEM, $n = 5$–10). Dashed line in time course figures indicates that fish previously in freshwater (Time 0) have been exposed to SW, which occurred within 1 h after day 0 sampling. A caret indicates significant differences between larvae and all populations of juvenile sea lamprey ($p < 0.01$).



present study. There are a number of differences in the approaches in the two studies, including gradual salinity acclimation (2 ppt per day) and the use of previously fed juveniles in the study by Mathers and Beamish (1974), which may have altered their osmoregulatory physiology. Gradual acclimation has been shown to increase survival compared to direct transfer to SW in a number of fish species (Nordlie 2009), so the lower survival and higher plasma osmolality observed by Mathers and Beamish (1974) compared to the present results is somewhat surprising. Feeding on an isosmotic meal likely decreases ion uptake mechanisms, such as increased posterior NKA activity (Barany et al. 2020), because the osmotic load is lower in the intestinal tract. However, the impact of prior feeding on subsequent SW tolerance in either landlocked or anadromous sea lamprey is currently unknown.

Plasma chloride levels and osmolality revealed differences in the inherent physiological capacity of all four populations over 2 weeks after exposure to 30 ppt. Landlocked sea lamprey have higher plasma chloride concentrations compared to anadromous sea lamprey in SW, while a similar trend was observed in the plasma osmolality response, though not statistically significant. After exposure to 30 ppt, plasma chloride levels increased in all populations over time, and TB and HB lamprey had greater plasma ion levels compared to CT lamprey. The plasma chloride levels observed in this study are similar to those observed by Reis-Santos et al. (2008) and Barany et al. (2020) in sea lamprey exposed to 30 ppt for 2 weeks, while the plasma osmolality values found in this study are comparable to those found by Shaughnessy and McCormick (2020) in sea lamprey. Similarly, plasma osmolality was approximately 275 mOsm·kg^{-1} after 90 min in 21 ppt in European river lamprey (*Lampetra fluviatilis*, an anadromous parasitic species, Brown et al. 2005), which is comparable to 257–265 mOsm·kg^{-1} observed in all populations of this study after SW exposure. An increase in plasma ions in FW accompanies metamorphosis in sea lamprey (Reis-Santos et al. 2008, which may help prepare juveniles by reducing the osmotic load that occurs after exposure to SW) and juveniles of both landlocked and anadromous populations showed similar increases in FW in the present study.

We measured hematocrit to see if there were any differences in red blood cell dynamics among populations in FW and following exposure to SW. All populations experienced a decrease in hematocrit immediately after salinity exposure. Hematocrit of landlocked populations remained low throughout the SW exposure while the hematocrit levels of the anadromous lamprey returned to baseline levels by the end of the experiment. Hematocrit has previously been shown to decrease in sea lamprey juveniles after SW acclimation (Reis-Santos et al. 2008; Barany et al. 2020). The strong correlation between hematocrit and osmolality indicates that the observed decreases in hematocrit in SW may have been due to osmotically induced shrinkage of red blood cells. We hypothesize that the reduced hematocrit during SW exposure may limit the respiratory and swimming capacity of sea lamprey, and that this phenomenon will be lasting longer in landlocked sea lamprey.

V-type H^+-ATPase is an apical transporter that has been hypothesized to be coupled with a Na^+ channel to create a gradient for Na^+ uptake in some teleost fish (Ferreira-Martins et al. 2021). V-type H^+-ATPase has been localized using immunohistochemistry in the larval ionocyte and FW juvenile ionocyte, but not the SW juvenile ionocyte of sea lamprey (Sunga et al. 2020), and it has also been localized using immunohistochemistry in the gills of the adult pouched lamprey (*Geotria australis*, Choe et al. 2004). These localization studies help elucidate the role of V-type H^+-ATPase in FW stages of lamprey, underlying the importance of Na^+ uptake in FW. Our results show initial FW population differences, with HB having higher *h⁺-atpase* mRNA levels compared to anadromous lamprey. After SW exposure, HB maintained higher *h⁺-atpase* mRNA levels compared to anadromous lamprey. While we saw no apparent reduction after SW exposure, lower *h⁺-atpase* mRNA levels were observed in adult anadromous sea lamprey acclimated transferred from FW to brackish water (25 ppt; Ferreira-Martins et al. 2016), and V-type H^+-ATPase β subunit protein levels were downregulated in juvenile anadromous sea lamprey exposure to SW (Reis-Santos et al. 2008). Landlocked alewife had higher mRNA levels of gill *h⁺-atpase* transcripts compared to the anadromous population shown using RNA-seq methods (Velotta et al. 2017). The results of our study indicate that there may be positive selection for V-type H^+-ATPase utilized for ion uptake throughout the life cycle of landlocked sea lamprey.

There is evidence that the apically located NCC is involved in ion uptake in some euryhaline fishes, transporting Na^+ and Cl^- into the ionocyte from the FW environment (Edwards and Marshall 2012). In species where NCC is present, exposure to SW decreases NCC mRNA and protein abundance (Hiroi et al. 2008; Takei et al. 2014). Barany et al. (2021) showed that gill *ncca* (the major isoform present in sea lamprey gill) mRNA levels were lower in SW-acclimated juvenile sea lamprey compared to FW-acclimated lamprey, and Ferreira-Martins et al. (2016) showed a downregulation of gill *ncca* in adult lamprey when transferred from FW to brackish water. Our results also show clear differences among populations, where all landlocked populations had significantly higher *ncca* mRNA levels compared to anadromous lamprey in FW. Following SW exposure, there were rapid decreases of *ncca* mRNA levels in all populations, but levels remained higher in landlocked populations compared to anadromous lamprey throughout the experiment. These results suggest that there is positive selection for maintaining higher levels of gill *ncca* mRNA levels in landlocked populations compared to anadromous populations after metamorphosis, which was also seen in landlocked alewife populations using RNA-seq methods (Velotta et al. 2017). If elevated levels of *ncca* mRNA are reflective of higher protein levels, the inability to reduce this FW ion transporter after SW exposure may have compromised the ion regulatory ability of landlocked sea lamprey as observed in our study.

NKCC1 is located on the basolateral membrane of the SW-type ionocyte in sea lamprey gill where it is utilized for chloride secretion (Shaughnessy and McCormick 2020; Ferreira-Martins et al. 2021). In the present study, there were no population differences in FW or SW *nkcc1* mRNA levels. There was, however, a time and population effect at the protein level in SW. Landlocked populations had significantly greater NKCC1 protein abundance compared to anadromous lamprey. SW-acclimated juvenile lamprey had 70% higher *nkcc1* mRNA levels compared to FW-acclimated juvenile lamprey (Shaughnessy and McCormick 2020), while adult sea lamprey had a two-fold increase in gill *nkcc1* mRNA levels after transfer from FW to 25 ppt (Ferreira-Martins et al. 2016). Landlocked Atlantic salmon had significantly elevated gill *nkcc1* mRNA levels when exposed to 34 ppt SW for 96 h while the anadromous population did not alter transcription of *nkcc1* (Nilson et al. 2007). Landlocked alewife had four-fold lower gill *nkcc1* mRNA levels compared to the anadromous population after exposure to 30 ppt (Velotta et al. 2014), and *nkcc1* RNA-seq transcript patterns were significantly lower in landlocked than anadromous alewife populations (Velotta et al. 2017). In our study, we see a significant positive correlation between plasma chloride levels and NKCC1 protein abundance, which are both higher in landlocked populations. While correlative, this relationship indicates that the elevated NKCC1 abundance in SW may be a response to the higher levels of plasma ions experienced by the landlocked populations.

Previous studies have established that gill NKA transcription, protein abundance, and activity increase during metamorphosis in association with increased salinity tolerance in sea lamprey (Reis-Santos et al. 2008; Ferreira-Martins et al. 2016; Shaughnessy and McCormick 2020). All populations had elevated levels of gill NKA activity (~10-fold higher) compared to larvae, and there was no population effect observed on any of these parameters of NKA in the present study. Salinity did not have an effect on mRNA or protein abundance, but gill NKA activity increased in all populations over time after SW exposure. The strongly elevated gill NKA activity in all populations compared to larvae indicates that metamorphosis-related increases in mechanisms for salt secretion are occurring in all populations. A capacity for further physiological acclimation was observed as all populations increased the activity of this key ion transporter over time after SW exposure. These results contrast with differences seen in Atlantic salmon, where higher levels of NKA protein abundance and activity have been found in anadromous relative to landlocked populations (Nilsen et al. 2007; McCormick et

al. 2019). Similarly, Velotta et al. (2014) found that gill NKA activity in landlocked alewife was 38% lower than anadromous alewife. We also observed that juveniles in all populations had higher levels of plasma chloride and osmolality compared to larvae (Fig. 2; Beamish et al. 1978; Reis-Santos et al. 2008), providing further evidence of osmoregulatory changes related to metamorphosis in both landlocked and anadromous sea lamprey.

Differences in osmoregulatory ability between landlocked and anadromous populations have been found in several anadromous species. Norwegian landlocked Atlantic salmon from Lake Byglandsfjord ("Blege" strain), which have been isolated from the anadromous population for ~9000 years, showed 60% survival of the landlocked strain compared to 100% survival of the anadromous strain in the first 2 weeks after exposure to 34 ppt (Nilsen et al. 2003). McCormick et al. (2019) observed 83% survival of landlocked Atlantic salmon compared to 100% survival of anadromous Atlantic salmon, which have been separated for ~14 000 years. The development of landlocked populations in alewife is likely the result of construction of dams in the last 300–400 years (Palkovacs et al. 2008). Landlocked alewife had 60% survival compared to 86%–100% survival in the anadromous population in the first 24 h of exposure to 30 ppt (Velotta et al. 2014). Evolution of osmoregulatory traits can occur relatively rapidly in some species; Divino et al. (2016) found a lower survival after SW exposure in a population of threespine stickleback (*Gasterosteus aculeatus*) that had colonized FW over the course of three generations. Threespine stickleback may be a special case in which short generation times and high evolvability of traits associated with FW survival have allowed for rapid development of FW populations (Hohenlohe et al. 2012).

One possible reason for why we see no major difference in metamorphic increases in gill NKA and only very small differences in SW tolerance may be due to the pleiotropic effects of hormones during metamorphosis (Dantzer and Swanson 2017). A hallmark of endocrine-controlled metamorphosis is that a single hormone (or hormone interactions) can affect a large number of physiological and morphological changes during metamorphosis that may be difficult to change rapidly in response to selection pressures that act differentially on various traits. Metamorphosis in sea lamprey and other vertebrates (e.g., amphibians) involves a number of complex physiological and morphological changes that are under endocrine control, leading to the adult stage and ultimately reproduction (Denver 2013). All lamprey species (FW nonparasitic, FW parasitic, and anadromous parasitic) undergo metamorphosis after which adult reproduction can occur. In sea lamprey, thyroid hormone levels decrease prior to the initiation of metamorphosis (Manzon and Manzon 2017). 11-Deoxycortisol and prolactin-like hormone have been shown to increase transiently during sea lamprey metamorphosis and drive osmoregulatory changes (Shaughnessy et al. 2020; Gong et al. 2022), while transcription of growth hormone, growth hormone receptor, and prolactin receptor increase from early stages of metamorphosis to late stages (Gong et al. 2020, 2022). The parr–smolt transformation of Atlantic salmon may be less complex and pleiotropic in nature compared to the more complex metamorphoses of sea lamprey and amphibians. It has been shown in Atlantic salmon that circulating levels of both growth hormone and cortisol that drive osmoregulatory changes are lower in landlocked populations whereas the thyroid axis which drives morphological and behavioral changes do not differ (Nilson et al. 2008; McCormick et al. 2019). We have shown in this study that all populations of sea lamprey show osmoregulatory changes associated with metamorphosis, thus the pleiotropic effects of hormones may have a role in limiting the ability of selection to reduce SW physiological traits in landlocked sea lamprey, especially over the short time frames during which these populations have likely been separated (~200 years). The conservation of the hormonally controlled metamorphosis leads to the conservation of SW tolerance, and the benefits of metamorphosis and the development of parasitism in sea lamprey (rapid growth and reproduction) likely outweigh the constitutive costs of the SW tolerance traits. This relationship suggests that the SW-adaptive traits will persist for long periods of time and constrain the independent evolution and increasing the time for evolution to act on these SW traits ("evolutionary lag", Lahti et al. 2009; Dantzer and Swanson 2017).

Within Petromyzontiformes, there have been multiple instances of evolution of paired species in which a parasitic and anadromous species gave rise to an FW-resident nonparasitic species (Docker 2009; Docker and Potter 2019). Some FW-resident lamprey species appear to have maintained their SW-type ionocyte despite diverging from the respective anadromous species 10 000–130 000 years ago: western brook lamprey (*Lampetra richardsoni*), which diverged from the river lamprey (*Lampetra ayresii*, Youson and Beamish 1991); European brook lamprey (*Lampetra planeri*), which diverged from the European river lamprey (Bartels et al. 2015); and American brook lamprey (*Lethenteron appendix*), which diverged from the anadromous parasitic Arctic lamprey (*Lethenteron camtschaticum*, Docker et al. 1999; Bartels et al. 2011). However, there are four known FW lamprey species do not produce the SW-type ionocyte during metamorphosis. Two *Ichthyomyzon* species, silver lamprey (*Ichthyomyzon unicuspis*) and chestnut lamprey (*Ichthyomyzon castaneus*) have pavement and FW-type ionocytes but no SW-type ionocytes (Bartels et al. 2012, 2015). Bartels et al. (2015) proposed that the SW-type ionocyte may have been lost in this genus due to its long separation from their ancestral anadromous species as indicated by its basal position among the Northern Hemisphere lamprey phylogeny (Lang et al. 2009). The Ukrainian brook lamprey (*Eudontomyzon mariae*) is a FW nonparasitic species that does not produce the SW-type ionocyte, but its origin, phylogenetic placement, and evolutionary history are still unclear (Bartels et al. 2017; Docker and Potter 2019). Finally, the least brook lamprey (*Lampetra aepyptera*), a FW nonparasitic lamprey that does not produce an SW-type ionocyte, is considered one of six relict species that has no obvious ancestral parasitic species (Docker 2009; Docker and Potter 2019). Molecular clock data estimate that least brook lamprey was derived from European river lamprey >2 million years ago (Docker et al. 1999; Martin and White 2008). Thus, it appears that significantly greater time of separation (>10 000 years) of anadromous and landlocked forms allows for relaxed selection on SW traits to result in the loss of SW ionocytes. The

evidence of high NKA activity and survival in 30 ppt in all sea lamprey populations in this study is indicative of the presence of the SW-type ionocyte and SW tolerance, supporting a short period of separation. Given the highly quantitative ability to compare NKA activity and abundance, it would be valuable to compare this parameter in these and other lamprey species pairs.

Future studies with sea lamprey populations from Lake Ontario and Finger Lakes could help elucidate the relaxed selection patterns on SW osmoregulatory traits as these lakes provide a possible transition between anadromous and landlocked populations in the Great Lakes. In addition, further work to uncover the effects of positive selection on FW osmoregulation in landlocked populations would be beneficial for understanding the breadth of traits that have been impacted by FW invasion in sea lamprey. These traits could also be compared to species pairs that have been separated for even long periods of time. Finally, one way to assess the impacts of hormonal pleiotropy on metamorphosis and SW tolerance would be through a comparative analysis using paired lamprey species (anadromous and FW resident) to examine circulating hormone levels, receptor abundance and affinity, and/or pathway regulators between anadromous and FW-resident lamprey species (Dantzer and Swanson 2017).

Relaxed selection on SW traits will likely not occur quickly due to the effects of hormonal pleiotropy involved in the control of sea lamprey metamorphosis. Based on known paired species, it may take thousands of years for relaxed selection to remove all traits associated with SW survival in landlocked sea lamprey. We did not find lower levels of SW ion transporters as would be expected with relaxed selection acting over thousands of years. We did find small but detectable differences in ionoregulatory capacity between landlocked and anadromous sea lamprey, providing evidence that is consistent with a relatively recent sea lamprey invasion of the Great Lakes. However, we did see a difference in the transcriptional levels of FW ion transporters after exposure to SW, indicating that positive selection on FW traits may be acting more quickly than relaxed selection on SW traits as predicted by evolutionary theory (Lahti et al. 2009).

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Competing interests

The authors declare there are no competing interests.

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Supplementary material

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