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Acute Toxicity of Salt Cavern Brine on Early Life Stages of Striped Bass (*Morone saxatilis*)

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Abstract
A plan to create solution-mined salt caverns for natural gas storage by discharging brine into the Shubenacadie River estuary poses a potential risk to an "endangered" stock of striped bass. Toxicity of brine made from both salt-core and artificial sea-salt "Instant Ocean" was assessed by 1-h acute toxicity tests at both 19 °C and 12 °C, the typical thermal range in June, post-spawning. The short test duration was justified given the rapid dilution of the brine in the macrotidal estuary. The median lethal concentration (LC50 1 h) 95% confidence intervals of salt-core brine at 19 °C for eggs was 51–60 parts per thousand (ppt); yolk-sac larvae 34–55 ppt; first-feeding stage larvae (6–8 mm total length, TL) 37–44 ppt, and 30–46 ppt for large larvae (14–20 mm TL). Among juveniles, the median lethal concentration was significantly higher compared to larvae: 51–58 ppt for early juveniles (4-cm fork length, FL) and 63–67 ppt for juveniles 12-cm FL. The toxicity of brine made from either Instant Ocean or salt-core was similar. At 12 °C, yolk-sac larvae salinity tolerance was 30% lower than at 19 °C, whereas other life stages exhibited a similar response to 12 °C and 19 °C. The threshold observed effect concentration (TOEC) of the salt-core ranged from 24.4 ppt on large larvae to 59.7 ppt on 12-cm juveniles. In conclusion, a very low direct threat to striped bass is estimated for the discharge of brine into the Shubenacadie River estuary.

Introduction

Salt caverns have been used for storing natural gas since 1944, and by 2017, there were 104 worldwide with 17 under construction (Bays 1963; Cornot-Gandolphe 2018). The caverns are created by "solution mining", where water is injected underground to dissolve the salt, then the brine is pumped out and discharged to the ocean, natural lagoons or lakes, artificial ponds, or into disposal wells (Simpson and Connolly 1982; Crossley 1998; Quintino et al. 2008; Stantec 2014; Lankof et al. 2016; Warren 2016). In Nova Scotia, Canada, Alton Natural Gas Storage LP plans to construct two caverns (each 3.3×10^5 m^3) in the first phase of its development within the Stewiacke Formation, a stratum rich in halite (NaCl) that accrued approximately 344 million years ago (Jutras et al. 2006; Jacques Whitford 2007; MacNeil et al. 2018; T. Church, Alton Gas, personal communication April 2019). Water from the Shubenacadie River estuary will be filtered and pumped 10 km to the cavern site and then saturated brine (up to 260 parts per thousand (ppt), when operating at full capacity) will be returned to a brine pond at the discharge site for slow release during the flood tide and early ebb tide midway along a 200-m long, open-ended constructed channel (T. Church, Alton Gas, personal communication, April 2019; Fig. 1). The Shubenacadie River is macrotidal with a tidal bore, a characteristic of rivers draining into the Inner Bay of Fundy (Lynch 1982; Fig. 1a). The highly turbid tidal water (range 0–28 ppt salinity) will flush the constructed channel at an estimated velocity of 0.7–0.9 m/s, diluting the brine very quickly. Real-time monitoring and discharge control will ensure salinity 5 m from the point of discharge will be no more than 7 ppt above background or exceed 28 ppt (Alton Gas 2016; Fig. 1b). In addition to these safeguards, regulatory agencies requested the present study, because the estuary is a critical habitat for a genetically discrete population of striped bass listed as endangered by COSEWIC (COSEWIC 2012; Duston et al. 2018; Leblanc et al. 2018).

The effects of brine discharge on estuarine ecosystems is undocumented. In the marine environment, brine was toxic to benthic isopods and polychaetes (*Eurydice pulchra*, *Ophelia radiate*) at 60 ppt salinity (LC50 96 h exposure,

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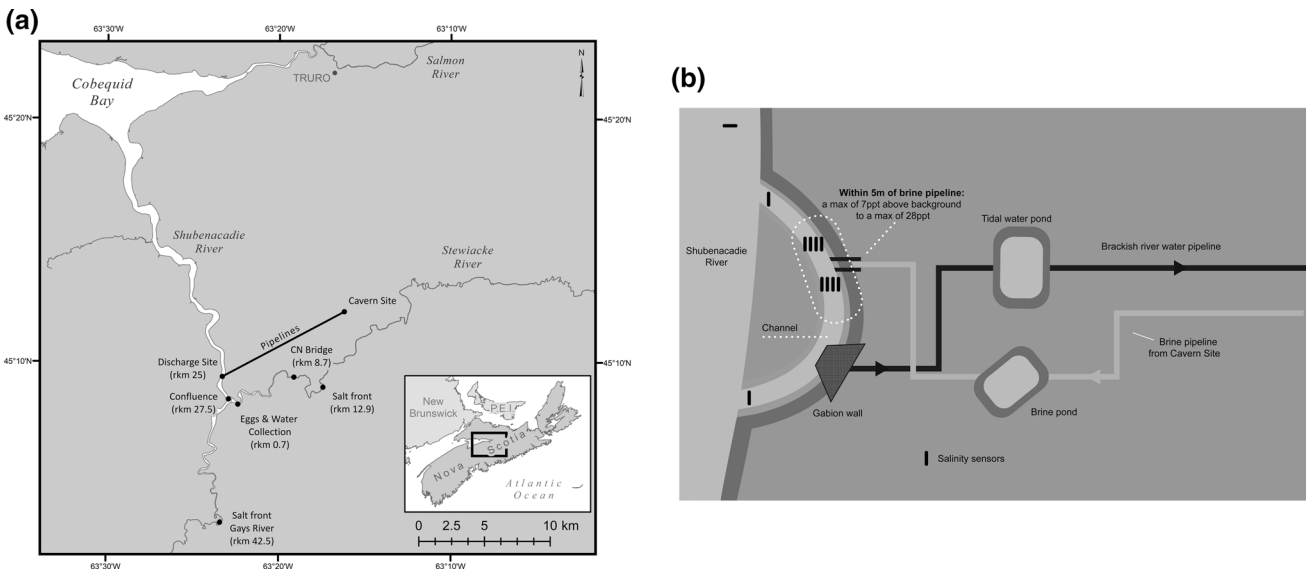

Fig. 1 **a** Shubenacadie-Stewiacke River estuary, Nova Scotia, Canada. The brine discharge site is 25 river kilometers from the mouth of the Shubenacadie River (rkm 25). Eggs and estuary water were collected from the Stewiacke River (rkm 0.7 from the confluence). **b** Details of the brine discharge site, showing the constructed channel (adapted from Alton Gas 2018)

local seawater 36 ppt) and had sublethal effects at 41 ppt (72 h exposure; Quintino et al. 2008). High salinity increases the diffusional influx of ions and osmotic loss of water in aquatic organisms, potentially causing a lethal elevation in the osmolality of the extracellular fluid. Conversely, freshwater is lethal to stenohaline marine fish due to the reversal of these fluxes (Evans 2010). Juvenile and adult stages of euryhaline estuarine fish, such as striped bass, easily cope with large changes in salinity by rapid adjustments in both drinking rate and active transport of ions and water across the gills and gastrointestinal tract (Madsen et al. 1994; Edwards and Marshall 2013; Kültz 2015). The salinity tolerance of the eggs and larval stages of euryhaline species, by comparison, are more uncertain as they undergo ontogenetic changes in their osmoregulatory ability, hence the need for the present study (Varsamos et al. 2005). Shubenacadie striped bass spawn in spring mostly in the Stewiacke River, approximately 5 to 11 km up-estuary from the brine discharge site (Fig. 1a). Fertilization occurs in tidal freshwater, then the early developmental stages travel back-and-forth with the tide over a salinity range of 1–15 ppt in the vicinity of the brine discharge site (Duston et al. 2018; Fig. 1a). Survival and growth of Shubenacadie striped bass was independent of salinity up to 20 ppt among eggs and larvae, and 30 ppt among early juveniles (Cook et al. 2010).

To simulate natural conditions as best possible, eggs and larvae were tested at both 19 °C and 12 °C, because they are the only stages likely to be exposed to these temperatures, and estuary water was used to dissolve salt core samples from the cavern site. Artificial sea-salt was used as reference toxicant. The 1-h test duration, approved by regulators, was conservative, because the brine will be diluted in a few seconds in the constructed channel.

Materials and Methods

Striped Bass Collection and Rearing Procedures

Newly fertilized striped bass (*Morone saxatilis* (Walbaum, 1792)) eggs were collected in May–June 2017 and 2018 from the Stewiacke River estuary approximately rkm 0.7 from the confluence with the Shubenacadie River (Fig. 1a). The pelagic eggs were caught late in the ebb tide (salinity < 2 ppt) with a plankton net (1-mm mesh; Aquatic Research Instruments, Hope, ID), then transported in aerated insulated containers (Igloo Cooler 19-L, Katy, TX) to the lab. Eggs hatched in 2 days in upwelling incubators (Kriesel 100-L, Aquabiotech, Quebec, QC, Canada) in 17–18 °C brackish water (2–4 ppt), a mix of Dal-AC groundwater and 30-µm sand-filtered seawater (~ 30–32 ppt; source: Marine Research Station, NRC, Ketch Harbour, NS). At 3 days post hatch (dph), each cohort of yolk-sac larvae (ca. 4-mm total length, TL) was transferred to a larger tank (1.5-m diameter, 0.25-m water depth, 800-L) at 2–6 ppt and 17–18 °C. Swim-bladder inflation at 5–7 dph was facilitated by removing oil on the water surface by using paper towel, a gentle water spray and turbidity about 150 NTU by addition of porcelain clay (6–50, Dragonfire Pottery and Supplies, Dartmouth, NS, Canada). First feeding was associated with swim-bladder inflation, at which point the temperature was raised to 20 °C. Stage I *Artemia* nauplii (Biomarine Aquafauna, Hawthorn,

CA) were fed up to 12 dph, then stage II nauplii enriched with Algamac 3050 (Biomarine Aquafauna) up to 33 dph. Weaning onto a dry diet began at 30 dph (Gemma 0.3 mm, Skretting, Saint Andrews, NB, Canada), progressing as body size increased to commercial salmonid diets (Nutra 0.5 and 1.0 mm, Skretting; Vita 1.5 and 2.0 mm, Ewos, Surrey, BC, Canada). Rearing tanks from about 15 dph onwards were 500-L volume (1 m diameter) in a small recirculation system (Aquabiotech). Salinity was maintained at 2 ppt until the fish reached approximately 5 cm TL, then freshwater. Photoperiod was L:D 24:0 until August, then simulated natural daylength (Latitude 45 °N). Light intensity at the water surface up to 10 dph was 0 lx, then approximately 30 lx (Light meter 840022, Sper Scientific, Scottsdale, AZ).

Acute Brine Toxicity Tests

Brine (ca. 100 ppt) was prepared from eleven salt core samples each 1-m long (10-cm diameter) extracted in summer 2014 from 883 to 933 m depth at the planned cavern site (Alton, NS; Core hole 08–01, 45°12′04.9″ N, 63°16′11.6″ W). Reference toxicant brine was made from Instant Ocean (Spectrum Brands, Blacksburg, VA). To produce test brine as close as possible to discharge brine, both salts were dissolved in estuary water collected from within 3 km of the discharge point (Fig. 1a). Late in the ebb tide, to avoid salinities > 5 ppt, the highly turbid estuary water was pumped into a 1,400-L tank (82124649, Global Industries, ON, Canada) on a ¾ ton truck using a semi-trash pump (6RLAG-2LST, Red Lion, Oklahoma, OK). At the lab, the water stood for 12 h to allow the sediment to settle, then was pumped (MD12, Danner Supreme, Islandia, NY) from the truck through a one-micron filter (#Bag1, Pentair Aquatic Ecosystems, Miami, FL) into a 1,000-L insulated storage tank (Insulated Container #3501, Xactics Canada, Cornwall, ON, Canada). Brine was produced by mixing 210 L of filtered estuary water with 28 kg of either crushed salt core or Instant Ocean in a 250-L Xactic tank with vigorous aeration (airstone ALR23, Sweetwater, Pentair Aquatic Eco-Systems, Miami, FL). For 24 h, during dissolution, brine was heated to 36 °C ($n = 3$, Top Light Excel 300 W, Rena, Charlotte, NC), then cooled to the test temperature, either 19 °C or 12 °C (chiller: Cyclone AE5DA, AquaLogic, San Diego, CA).

Static non-renewal toxicity tests, 1-h duration, were conducted on six developmental stages: eggs, yolk-sac larvae (3–4 dph), first-feeding larvae (6–7 dph), large larvae (14–20 dph), and early juvenile (ca. 54 dph, 4-cm fork length, FL) and juvenile (ca. 134 dph, 12-cm FL). Each test comprised of six test concentrations and a control at the same salinity as the rearing water (2–6 ppt), each in triplicate. The endpoint was mortality. The 1-h test duration was highly conservative compared to the planned brine dilution rate in the estuary, and provided adequate time to operate the 21 test vessels. Mortalities during the 1-h test were not removed, because they posed no immediate threat to water quality. At each life stage, an initial "range-finder" test was run at 15, 25, 35, 45, 55, and 100 ppt, as agreed with the Department of Fisheries and Oceans and approved by Nova Scotia Environment (NSE 2016). In these "ranger-finder" tests, the time of mortality at the 100-ppt test concentration was recorded. Then, to determine the median lethal concentration (LC50) more accurately, a narrower range of salinities were tested (Table 1). Salinity (based on conductivity and temperature), temperature, oxygen concentration, and pH were recorded with handheld meters (Pro2030, YSI, Yellow Springs, OH; Orion 9107BNMD, Orion Star A121, Thermo Scientific, Beverly, MA). Light intensity at test vessel level was 20 lx for eggs and larvae and 300 lx for juveniles. Fish were transferred without acclimation from their rearing tank to the test vessels. Mortality after the 1-h exposure among swimming stages was assessed in each of the test vessels. Assessing egg mortality required a dissecting microscope; dead eggs have an opaque chorion. The acceptability criterion for each test was mortality in the control of < 30% among eggs and larvae and < 10% among juveniles.

Test vessel design and volume increased with fish size. Eggs were tested in a set of four tissue culture plates (6-wells, each 15.5 mL, Falcon, Corning Life Sciences, Oneota, NY), following Kupsco et al. (2017). Using a small spoon, 20 eggs were placed in each well containing 10 mL of test solution. Yolk-sac and first-feeding larvae were tested in 30-mL plastic mesh baskets (Café Cup, Spark Innovators, Fairfield, NJ). Three replicate baskets were secured with tape in each of seven food-grade, white, plastic 2-L containers (QS21, Ropak, Springhill, NS, Canada) filled to the brim with the test solution. Using a small plastic spoon, 20 larvae were counted into 25 mL of rearing water in a 90-mL specimen cup (02-1104, Fisher Scientific, Ottawa, ON, Canada). Then, the entire contents were gently poured into each basket; the brine in the 2-L container was stirred briefly to homogenize the test solution. To account for the small dilution due to addition of rearing water, salinity was measured after each test, and this value was used for LC50 estimation. The same procedure was used to transfer 20 large larvae to their test vessels, a 2-L container (QS21) filled with 1.5 L of test solution. Ten juveniles, both size classes, were transferred using an aquarium dip net from a bucket containing rearing water into 15 L of test solution in each of twenty-one 26-L clear plastic totes (ClearView Latch, Sterilite, Townsend, MA). Oxygen saturation was > 90% in all tests; additional aeration was not needed.

The median lethal concentration (LC50 1 h) was estimated using the Trimmed Spearman-Kärber Method with the "tsk" package in R software (Hamilton et al. 1977, 1978; Stone 2015). The trim level with the lowest

Table 1 Summary of the test conditions for the early life stages of striped bass

Test type	Static non-renewal
Test duration	1 h
Temperature	19 °C and 12 °C (eggs and larvae), 19 ºC (juveniles)
Light quality	Fluorescent lamps
Light intensity	20 lx (eggs–larvae), 300 lx (juvenile)
Photoperiod	Not applicable
Test vessel size	15-mL well (eggs), 30-mL cup (yolk-sac and first-feeding larvae), 2-L container (large larvae), 26-L tote (juvenile)
Test solution volume	10 mL (eggs), 2 L (yolk-sac and first feeding larvae), 1.5 L (large larva), 15 L (juvenile)
Renewal of test solutions	No renewal
Age of test organisms	Eggs to 143 days post hatch
N° organisms per test vessel	20 (eggs and larvae), 10 (juvenile)
N° replicate vessels per concentration	3
N° organism per concentration	60 (eggs and larvae), 30 (juvenile)
Feeding regime	No food
Test vessel cleaning	Cleaning not required
Test solution aeration	No
Dilution water	1-µm filtered Stewiacke River estuary water
Test concentrations	Initial: 15, 25, 35, 45, 55, and 100 ppt Eggs: 30, 40, 50, 60, 70, and 80 ppt Yolk-sac larvae: 45, 50, 55, 60, 65, and 70 ppt First-feeding larvae: two sets of six concentrations from 25 to 70 ppt Large larvae: four sets of six concentrations from 25 to 70 ppt Early juvenile: two sets of six concentrations from 30 to 70 ppt Juvenile: three sets of six concentrations from 30 to 80 ppt
Endpoint	Mortality
Volume of brine (100 ppt) required	Variable depending on life stage, maximum 170 L per test on juvenile
Test acceptability criterion	≥ 70% survival at the control (eggs and larvae) ≥ 90% survival at the control (juvenile)
Chemical	Salt core samples
Reference toxicant	Instant Ocean

standard deviation was selected. Egg mortality among controls (14–21%) was corrected by the Henderson–Tilton equation (Henderson and Tilton 1955). The difference in the LC50 between two tests was considered statistically significant if their respective 95% confidence intervals did not overlap. To compare the effect of the type of brine (SC or IO) at 19 °C, a two-way ANOVA (life stage and salt source) was conducted using the Proc Mixed model with Bonferroni adjustment (SAS 9.4, Institute Inc., Cary, NC). Yolk-sac larvae were excluded from this analysis, because only a single datum was available for the IO brine. A second two-way ANOVA (life stage and temperature) was conducted to compare the effect of temperature (12 °C or 19 °C) when SC brine was used. The "No Observed Effect Concentration" (NOEC) was estimated for each test comparing the survival at the different test concentrations with the control by an ANOVA, Proc Mixed model with Bonferroni adjustment (SAS). In the cases where the normality requirement was not met, the Kruskal–Wallis test was conducted in SAS. The "Threshold Observed Effect Concentration" (TOEC) was determined for each test condition, based on the geometric mean of the NOECs (EC 2005). To analyze the difference between two TOECs (geometric means), the NOECs were log transformed and a two-sample *t* test was conducted in SAS (Zar 2014). The number of pairwise comparisons was reduced from 120 to 55, because four test conditions only had a single datum and one test condition had zero valid NOEC values. The slope of the survival curves for each life stage and test condition was calculated by a linear regression between highest test concentration with 100% survival and the lowest test concentration with 0% survival, including all the intermediate points (SAS). Then each pair of slopes were compared by Student's *t* test.

The chemical composition of the dilution water (1-µm filtrated estuary water) and brine samples were evaluated by Standard Water Analysis and Total Metals (AGAT Laboratories, Dartmouth, NS). Due to ion interference, not all the samples were analyzed to the same detection limit (Gros 2013). Parameters were standardized to the lowest Reported Detection Limit possible that allowed a comparison between

samples. Of the 31 water-quality parameters measured, only 19 are reported here; the others were considered irrelevant to the study. Of the total metals, 12 of 26 analyzed are presented; the concentration of the others was considered too low to warrant inclusion. Halite (%) of each sample was calculated from the proportion of sodium chloride relative to the total ionic composition. Salinity (ppt) of each sample was calculated from the chloride concentration (Wooster et al. 1969). The difference in the mean concentration of each parameter between brine made from either salt core or Instant Ocean was analyzed by a two-sample *t* test using SAS. All protocols were reviewed and approved by the Faculty's Animal Care and Use Committee (ACUC 2016-52, 2017-44, 2018-45).

Results

Water Quality

The chemical composition of the dilution water exhibited some variation due to natural fluctuations in the tide and freshwater run-off (see Shubenacadie River estuary water at Tables 2 and 3). Nevertheless, it was consistently very hard (mean 398 mg/L), well buffered (total alkalinity 40 mg/L), oligotrophic (nitrite < 50 mg/L, ammonia 0.17 mg/L), and slightly saline (total dissolved solids (TDS) 1858 mg/L, 1.6 ppt; Table 2). After filtration to 1 micron, the water remained turbid (210 NTU); true color 26 TCU (Table 2). Total metals were highly correlated with turbidity, indicating they were bound to particulates and biologically inactive.

Salt core (SC) brine and Instant Ocean (IO) brine were similar in mean chloride concentration, conductivity, TDS and calculated salinity (Table 2). Salt core brine was significantly higher than IO brine in calculated halite (94 vs. 81%), sodium (53 vs. 40 mg/L), and uranium (1.7 vs. 0.4 µg/L) and was significantly lower in magnesium (−98%), potassium (−97%), boron (−91%), hardness (−89%), strontium (−84%), total alkalinity (−78%), barium (−75%), sulphate (−71%), molybdenum (−51%), calcium (−44%), nickel (−30%), and pH (−5%; Tables 2 and 3).

In test solutions at 19 °C, the pH of the SC brine was significantly lower than IO brine (mean ± SE, 7.52 ± 0.10 vs. 8.07 ± 0.05), but dissolved oxygen was similar (98 ± 1% vs. 97 ± 1%). Tests at 12 °C were conducted with SC brine only, the parameters were similar to those at 19 °C, pH 7.64 ± 0.05, and dissolved oxygen 100 ± 1%.

Table 2 Mean (SE) water quality parameters of the dilution water ($n = 9$), salt core brine ($n = 7$), Instant Ocean brine ($n = 7$), and 5th and 95th percentile of the natural range in the Shubenacadie River at the discharge site, rkm 25, over 20 tidal cycles between November 2014 and August 2016. Salinity and halite were calculated from the parameters analyzed in the lab. Brine was prepared by dissolving 28 kg of salt in 210 L of the dilution water (1-µm filtrered estuary water)

Parameter	Unit	RDL	Shubenacadie River Estuary	Dilution water	Salt core brine	Instant Ocean brine
pH			6.72–7.61	7.81 ± 0.06	7.48 ± 0.05	7.89 ± 0.03*
Hardness	mg/L		38.2–3630	398 ± 132	2360 ± 282	21,514 ± 979*
Total alkalinity	mg/L	5	8–95	40 ± 5	43 ± 3	201 ± 40*
Bicarbonate alkalinity (as $CaCO_3$)	mg/L	0.1	8.0–95.0	39.9 ± 4.7	43.4 ± 3.3	201.1 ± 40.1*
Carbonate alkalinity (as $CaCO_3$)	mg/L	10	< 10	< 10	< 10	< 10
Nitrite as N	mg/L	50	< 50	< 50	127 ± 5	93 ± 24
Ammonia as N	mg/L	0.03	< 0.03–0.18	0.17 ± 0.06	0.17 ± 0.04	1.14 ± 0.51
True color	TCU	5	14–92	26 ± 4	23 ± 7	17 ± 2
Turbidity	NTU	0.1	18.6–1356	210 ± 57	119 ± 35	322 ± 116
Chloride	mg/L	1	16–11,640	894 ± 321	72,886 ± 4974	65,571 ± 2828
Conductivity	µS/cm	1	152–12,620	3335 ± 1154	150,143 ± 9478	137,429 ± 3884
Calculated total dissolved solids	mg/L	1	77–20,210	1858 ± 646	128,571 ± 7600	123,143 ± 3826
Sulphate	mg/L	2000	< 2000–2347	< 2000	2800 ± 790	9746 ± 272*
Sodium	mg/L	0.1	9.5–5951	608 ± 226	52,915 ± 2973*	40,371 ± 1546
Potassium	mg/L	0.1	1.3–222	24.0 ± 8.4	52.3 ± 12.1	1511 ± 51*
Calcium	mg/L	0.1	10.4–267	46.2 ± 10.4	798 ± 135	1429 ± 59*
Magnesium	mg/L	0.1	2.5–720	68.6 ± 26.0	89.0 ± 33.4	4356 ± 231*
Calculated salinity	ppt	0.1	0.03–21.0	1.6 ± 0.6	132 ± 9	119 ± 5
Calculated halite	%		28.2–82.2	80.7 ± 4.9	93.5 ± 1.7*	81.0 ± 1.2

RDL report detection limit

*Significant difference between the salt core brine and the Instant Ocean brine ($P < 0.05$; two-sample *t* test)

Table 3 Natural range of total metals in the Shubenacadie River at the discharge site, rkm 25, 5th and 95th percentile over 20 tidal cycles between November 2014 and August 2016. Mean (SE) dilution water ($n = 9$), salt core brine ($n = 7$), and Instant Ocean brine ($n = 7$). All units are µg/L. Brine was prepared by dissolving 28 kg of salt in 210 L of dilution water (1-µm filtred estuary water)

Parameter	RDL	Shubenacadie River estuary	Dilution water	Salt core brine	Instant Ocean brine
Aluminum	5	578–26,975	3531 ± 1005	3203 ± 637	2617 ± 1109
Barium	5	8–146	26.1 ± 3.4	34 ± 4	134 ± 10*
Boron	5	15–2354	252 ± 92	1444 ± 313	15,343 ± 951*
Copper	1	2–20	5 ± 1	64 ± 23	73 ± 11
Iron	50	837–36,575	3894 ± 974	4970 ± 860	3832 ± 1389
Lead	0.5	1.4–23.2	4.4 ± 0.9	9.2 ± 1.6	7.5 ± 3.4
Molybdenum	2	< 2–9	3 ± 0	5 ± 1	10 ± 0*
Nickel	2	3–29	7 ± 2	45 ± 3	64 ± 9*
Silver	0.1	< 0.1–0.1	0.3 ± 0.1	4.0 ± 3.6	0.3 ± 0.1
Strontium	5	70–4165	569 ± 163	5069 ± 608	30,857 ± 1327*
Uranium	0.1	0.1–2.3	0.4 ± 0.1	1.7 ± 0.3*	0.4 ± 0.1
Vanadium	2	4–41	19 ± 3	468 ± 69	399 ± 40

RDL report detection limit

*Significant difference between the salt core brine and the Instant Ocean brine ($P < 0.05$; two-sample t test)

Salinity Tolerance

Survival curves for five of the six life stages had a similar shape. The exception was eggs, which exhibited a significantly lower slope in the three test conditions (Fig. 2). The slope of eggs, first-feeding larvae, large larvae, and juvenile did not vary between the test conditions ($P > 0.05$). The slope of the survival curves of yolk-sac larvae, by contrast, was significantly higher in SC brine at 19 °C (sudden acute mortality; Fig. 2a) than at 12 °C (Fig. 2c; $P = 0.044$). Finally, the slope of the survival

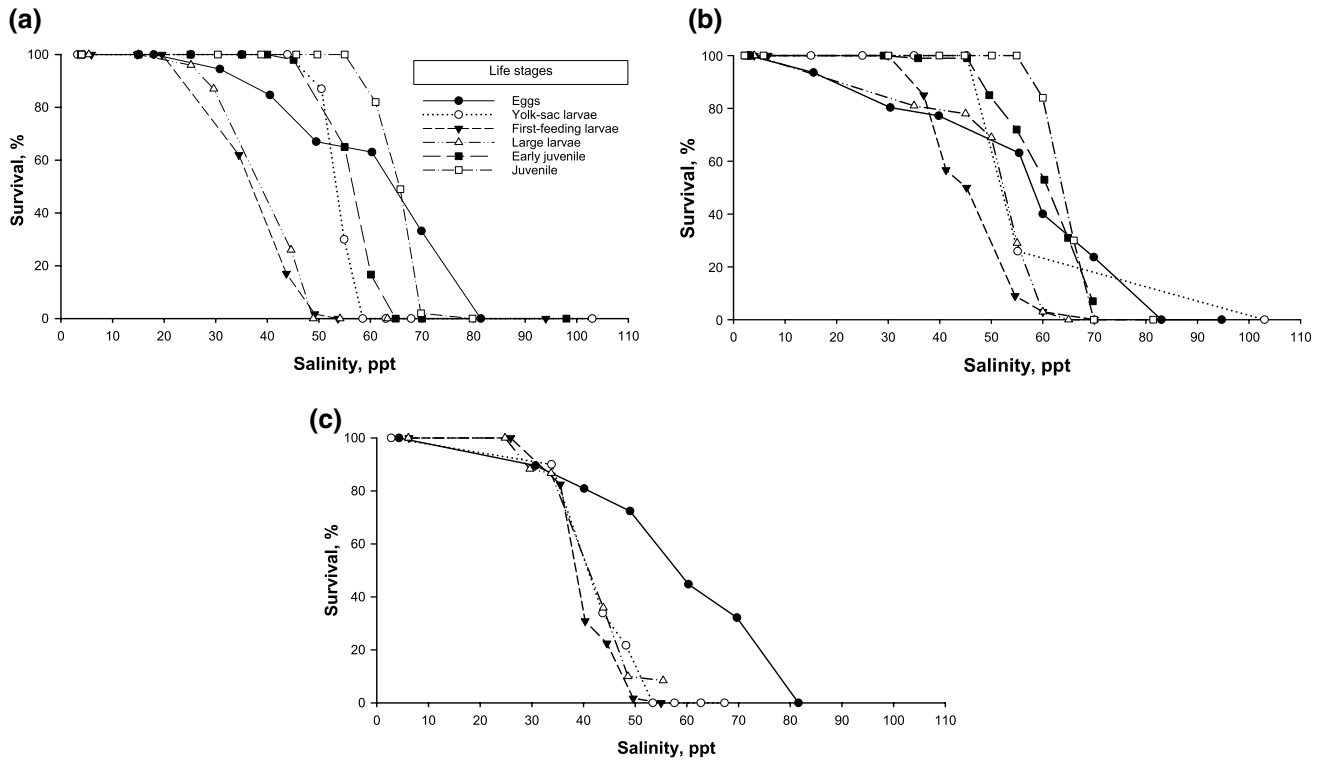

Fig. 2 Mean survival of striped bass early life stages exposed for 1-h to salt core (SC) brine at 19 °C (**a**), Instant Ocean brine at 19 °C (**b**), and SC brine at 12 °C (**c**). The life stages were: eggs, yolk-sac larvae (3–4 days post-hatch, dph); first-feeding larvae (6 dph); large larvae (14–15 dph); early juvenile (57 dph); and juvenile (130–143 dph)

curve of early juveniles was higher in IO brine 19 °C (Fig. 2b) than in SC brine 19 °C (Fig. 2a; $P = 0.038$).

The TOEC following 1-h exposure ranged from 25.8 to 59.7 ppt depending on stage of development, brine type, and temperature (Table 4). In SC brine at 19 °C, large larvae and eggs were the most vulnerable life stages (TOEC 24.4 and 30.8 ppt, respectively; Table 4). Early juveniles were significantly less tolerant than the 12-cm juveniles (TOEC 45.1 vs. 59.7 ppt, respectively; Table 4). In IO brine at 19 °C and SC brine 12 °C, there was no significant difference among the life stages (Table 4).

Median Lethal Concentration

The mean of the median lethal concentration (LC50 1 h) at 19 °C estimated for the six developmental stages ranged between 34 and 65 ppt, with similar values for both SC and IO brine (Fig. 3). Salinity tolerance was highest among egg and juvenile stages and lowest among larval stages (Fig. 3). Salinity tolerance at 12 °C among eggs was similar to 19 °C, but among yolk-sac and first-feeding larvae was reduced significantly at the lower temperature (Fig. 3).

Salt core brine toxicity (LC50 1 h) on eggs at 19 °C was 54.3 ± 0.0 ppt (mean ± SE; tests 1 and 2; Table 5). Yolk-sac larvae (3–4 dph) tolerance to SC brine was similar to eggs; mean LC50 was 53.5 ± 1.5 ppt with a significant difference between tests 5 and 6 (Table 5). First-feeding larvae (6 dph)

Table 4 Threshold-observed-effect concentration (TOEC) of brine made from either salt core (SC) or Instant Ocean (IO) after 1-h exposure on six early life stages of striped bass (0 to 143 days post hatch, dph) at 19 °C or 12 °C

Test condition	Eggs	Yolk-sac	First-feeding	Large larvae	Early juvenile	Juvenile
SC 19 °C	30.8	46.6^{AB}	41.4^{ABC}	24.4	45.1^{BC}	59.7^{A}
IO 19 °C	42.1^{AB}	45.2	29.6^{ABC}	40.0^{ABC}	46.3^{AB}	58.0^{AB}
SC 12 °C	30.7	Ind.	25.8^{C}	31.5^{BC}	–	–

TOEC corresponds to the geometric mean of the no-observed-effect concentration (NOEC) at each test condition. TOECs sharing the same letter are not significantly different (two-sample *t* test, α 0.05). TOECs without a letter had a single NOEC

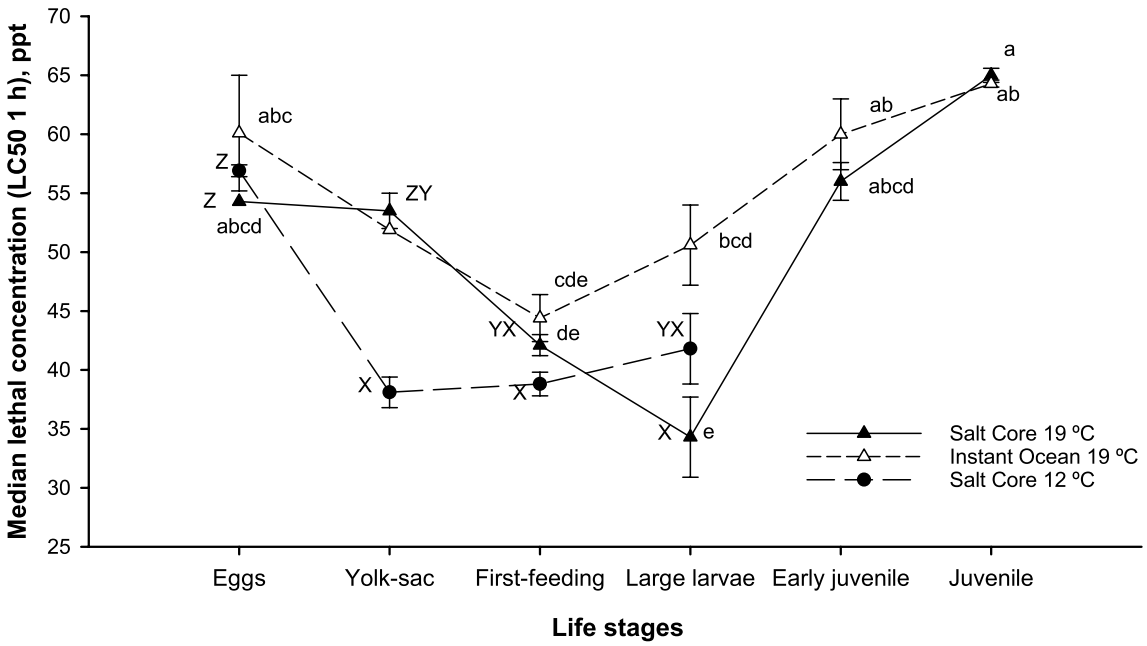

Fig. 3 Median lethal concentration (LC50 1 h) of brine made from either salt core or Instant Ocean on early life stages of striped bass: eggs, yolk-sac larvae (3–4 days post-hatch, dph), first-feeding larvae (6–8 dph), large larvae (14–20 dph), early juvenile (43–57 dph), and juvenile (130–143 dph) exposed to 19 °C and 12 °C. Mean of the LC50 by stage and test condition. Error bar = SE of the mean. Means sharing the same lowercase letter are not significantly different between life stage and brine type at 19 °C. Means sharing the same capital letter are not significantly different between life stage and temperature when exposed to SC brine. One-way ANOVA with Bonferroni adjustment (α 0.05)

Table 5 Median lethal concentration (LC50 1 h) of brine made from either salt core (SC) or Instant Ocean (IO) on six early life stages of striped bass (0 to 143 days post hatch, dph) at 19 °C

Test	Stage	Age, dph	Size	Salt	LC50	95% CI	SD	Trim
1	Eggs	0		SC	54.2^{EFG}	51.0–57.4	1.6	0.1
2	Eggs	0		SC	54.3^{EFG}	51.3–57.2	1.5	0.1
3	Eggs	0		IO	65.0^{HIJ}	61.3–68.7	1.9	0.1
4	Eggs	0		IO	55.2^{EFG}	52.9–57.5	1.2	0.1
5	Yolk-sac larvae	3	5.0	SC	52.0^{E}	50.9–53.2	0.6	0.5
6	Yolk-sac larvae	4	6.0	SC	55.0^{F}	54.7–55.4	0.2	0.5
7	Yolk-sac larvae	3	5.0	IO	51.9^{E}	50.9–52.9	0.5	0.5
8	First-feeding larvae	6	6.5	SC	41.2^{C}	40.1–42.4	0.6	0.5
9	First-feeding larvae	6	6.5	SC	42.9^{C}	42.3–43.6	0.3	0.0
10	First-feeding larvae	8	9.0	IO	42.4^{C}	41.1–43.7	0.7	0.4
11	First-feeding larvae	8	6.1	IO	45.9^{D}	45.1–46.8	0.4	0.5
12	Large larvae	14	7.4	SC	30.9^{A}	30.3–31.4	0.3	0.5
13	Large larvae	15	10.3	SC	37.7^{B}	36.6–38.9	0.6	0.0
14	Large larvae	12	9.0	IO	48.3^{D}	47.4–49.3	0.5	0.3
15	Large larvae	15	11.0	IO	46.1^{D}	44.4–47.7	0.8	0.1
16	Large larvae	20	12.0	IO	57.3^{G}	56.9–57.7	0.2	0.2
17	Early juvenile	57	38.4	SC	54.4^{EFG}	51.1–57.6	1.6	0.5
18	Early juvenile	57	38.4	SC	57.6^{G}	56.8–58.3	0.3	0.5
19	Early juvenile	43	23.0	IO	60.3^{H}	58.6–62.0	0.8	0.0
20	Early juvenile	52	41.0	IO	51.8^{E}	50.4–53.2	0.7	0.0
21	Early juvenile	57	44.0	IO	62.0^{H}	60.5–63.5	0.8	0.1
22	Early juvenile	59	41.8	IO	66.0^{IJ}	64.0–68.0	1.0	0.5
23	Juvenile	130	121.0	SC	65.7^{IJ}	64.7–66.6	0.5	0.0
24	Juvenile	131	126.5	SC	65.5 ^{J}	64.9–66.0	0.3	0.5
25	Juvenile	143	124.0	SC	63.7^{I}	62.7–64.7	0.5	0.0
26	Juvenile	127	129.7	IO	64.2^{I}	63.7–64.8	0.3	0.5
27	Juvenile	130	121.0	IO	64.8^{IJ}	63.8–65.9	0.6	0.0
28	Juvenile	143	124.0	IO	63.9^{I}	63.5–64.3	0.2	0.2

Size (mm) is total body length for larvae and fork length for juveniles. The Spearman-Kärber Method was used to determine LC50, lower and upper 95% confidence intervals (95% CI), standard deviation of the LC50 estimate (SD), and trim level. Tests sharing the same letter are not significantly different, based on 95% CI overlap

were significantly more sensitive to SC brine than both eggs and yolk-sac stages; their mean LC50 was 42.1 ± 0.9 ppt (tests 8 and 9; Table 5). Large larvae 14–15 dph were the least tolerant of all developmental stages; their mean LC50 was 34.3 ± 3.4 ppt (tests 12 and 13; Table 5). The tolerance of early juveniles to SC brine was similar to eggs and yolk-sac larvae; mean LC50 was 56.0 ± 1.6 ppt (tests 17 and 18; Table 5). The larger 12-cm juveniles (130–143 dph) were even more tolerant; the mean LC50 was 65.0 ± 0.6 ppt (tests 23 to 25; Table 5).

Instant Ocean brine toxicity at 19 °C was similar to SC brine at 19 °C. Among eggs, mean ± SE LC50 1 h was 60.1 ± 4.9 ppt compared with 54.3 ppt in SC brine (tests 3 and 4; Table 5). Among yolk-sac larvae (3 dph), the LC50 of IO brine was 51.9 ppt (test 7; Table 5), similar to the 53.5 ppt for SC brine. First-feeding larvae (8 dph) mean LC50 in IO brine was 44.4 ± 2.0 ppt compared with 42.1 ppt in SC brine. Large larvae (12–20 dph) LC50 in IO brine ranged widely from 46.1 to 57.3 ppt in four tests (14–16; Table 5), the overall mean was 50.6 ± 3.4 ppt. Early juveniles, 4-cm long, also exhibited significant variability in their LC50s. The overall mean was 60.0 ± 3.0 ppt (tests 19–22; Table 5), but among large juvenile the LC50s were consistent, mean 64.3 ± 0.3 ppt (tests 26–28; Table 5).

At 12 °C, the mean ± SE LC50 1 h of the SC brine on eggs was 56.9 ± 0.5 ppt (tests 29 and 30; Table 6), similar to 19 °C, 54.3 ppt (Table 5). By contrast, yolk-sac larvae (3–4 dph) salinity tolerance was reduced considerably at 12 °C vs. 19 °C, 38.1 ± 1.3 ppt (tests 31 and 32; Table 6) versus 53.5 ppt. First-feeding larvae (7 dph) LC50 was independent of temperature: 38.8 ± 1.0 ppt at 12 °C (tests 33 and 34; Table 6) and 42.1 ppt at 19 °C. Finally, large larvae (16–20 dph) exhibited better survival at 12 °C than at 19 °C; 41.8 ± 3.0 ppt (tests 35 and 36; Table 6) versus 34.3 ppt (Table 5).

Table 6 Low temperature (12 °C). Median lethal concentration (LC50 1 h) of brine made from salt core on early life stages of striped bass (0 to 20 days post hatch, dph)

Test	Stage	Age, dph	Size	LC50	95% CI	SD	Trim
29	Eggs	0		57.4^{FG}	54.6–60.3	1.5	0.0
30	Eggs	0		56.4^{F}	53.2–59.5	1.6	0.0
31	Yolk-sac larvae	3	6.0	39.4^{ABC}	38.5–40.2	0.4	0.5
32	Yolk-sac larvae	4	6.0	36.8^{A}	33.8–39.7	1.5	0.0
33	First-feeding larvae	7	7.0	39.7^{ABCD}	38.9–40.5	0.4	0.0
34	First-feeding larvae	7	7.0	37.8^{A}	37.0–38.6	0.4	0.5
35	Large larvae	16	10.5	38.0^{A}	36.9–39.1	0.6	0.3
36	Large larvae	20	11.5	44.8^{E}	43.7–45.9	0.6	0.5

Size (mm) is total body length. The Spearman-Kärber Method was used to determine LC50, lower and upper 95% confidence intervals (95% CI), standard deviation of the LC50 estimate (SD), and trim level. Tests sharing the same letter are not significantly different, based on 95% CI overlap

Timing of Mortality in 100-ppt Brine

Yolk-sac larvae (3 dph, 5-mm TL) exposed to 100-ppt SC brine began to die after 3 min, and 100% mortality was reached at 5.5 min (Fig. 4). First-feeding larvae (6 dph, 6.5-mm TL) reached 50% mortality at 4 min and 100% at 5.75 min (Fig. 4). Large larvae (13 dph, 10-mm TL, exposed to IO brine) mortality started around 3.5 min and reached 100% at 12 min (Fig. 4). Early juveniles (4 cm FL) started to die after 10 min, mortality was 20% at 13 min, and reached 100% at 20.5 min (Fig. 4). Juveniles (12 cm FL) in 100-ppt brine started to die at 9 min and reached 100% mortality at 19.5 min (Fig. 4).

Discussion

The engineering design considers that at 5 m from the discharge, the salinity will be no higher than 7 ppt above background and never higher than 28 ppt (Fig. 1b). After the water leaves the mixing channel, it will be quickly fully mixed with the river water. Based on these design criteria, the discharge of brine into the Shubenacadie River estuary poses a very low direct threat to striped bass, because the TOECs for five of the six early life stages were higher than the 28-ppt threshold at the normal spring water temperature (18–20 °C). Large larvae were the most sensitive, but even their TOEC was 24.4 ppt. In colder conditions (12 °C), the TOECs were closer to the 28-ppt threshold, but the quick dilution in the estuary reduces the threat observed in the 1-h test exposure. Further safeguards include the 24-days cessation of brine release following detection of the first

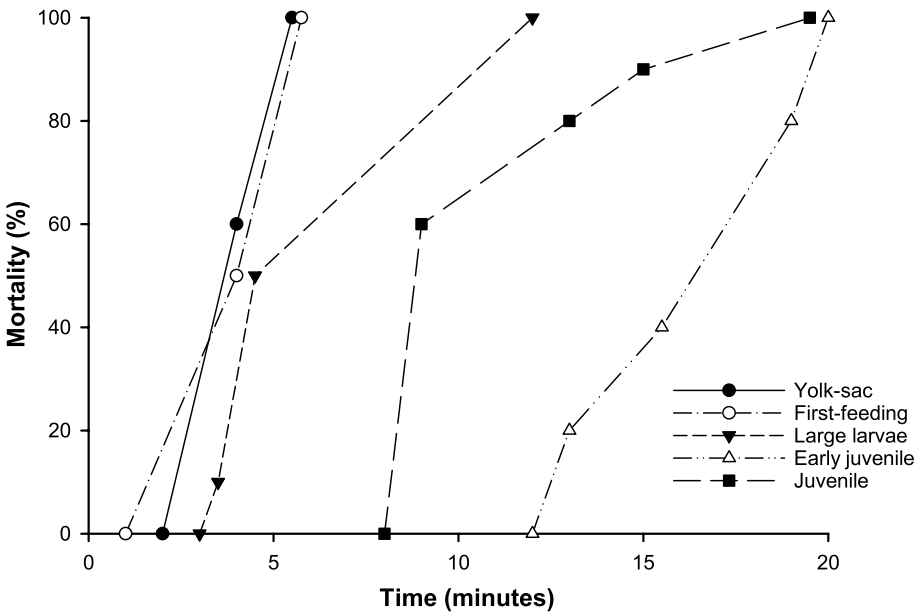

Fig. 4 Mortality rate (%) of striped bass early life stages exposed to 100-ppt salt core brine at 19 °C, except large larvae expose to Instant Ocean brine: yolk-sac larvae (3 days post-hatch, dph); first-feeding larvae (6 dph); large larvae (13 dph); early juveniles (57 dph); and juveniles (129 dph)

striped bass egg, and the compulsory 20-ppt maximum at 5 m distance from point of discharge when eggs and larvae are present after the 24-days cessation. The significant changes in salinity tolerance from egg to juvenile can be attributed to the ontogenic changes in the osmoregulatory mechanisms, a common characteristic among euryhaline teleosts. Ionic composition differences between the two sources of salt were associated with the salt core brine being relatively more toxic to eggs and large larvae, but the other four stages were unaffected. Low temperature is recognized as a lethal factor for yolk-sac stage striped bass larvae and was confirmed by the greater toxicity of the brine at 12 °C compared with 19 °C.

Eggs were highly tolerant to the brine due to a largely impermeable vitelline epithelium and ionocytes (aka mitochondria-rich cells, chloride cells), specialized ion pumping cells that protect the developing embryo in all teleosts examined (Guggino 1980; Rombough 2007). Ionocytes become functional at gastrulation, the developmental stage of the eggs tested, maintaining the osmotic homeostasis of the extracellular fluid (Guggino 1980; Kaneko et al. 2008). From fertilization through blastulation, by comparison, salinity tolerance is lower since the ionocytes are absent. Among all striped bass stocks, spawning and water hardening occurs in freshwater, and survival is greatly reduced if they are exposed to brackish water as low as 2 ppt (Turner and Farley 1971). In the Shubenacadie River estuary, the risk of exposure of newly fertilized eggs the brine discharge is very low. Following spawning in the Stewiacke River in freshwater, the pelagic eggs are transported down-estuary on the ebb-tide at approximately 2 km/h (Duston, unpublished data), reaching the brine discharge site in approximately 3–4 h, by which time they are fully hardened. Moreover, any threat is further reduced, because brine discharge will be stopped for 24 days after detection of the first egg, and when larvae are present the allowable upper salinity threshold 5 m from the point discharge will be 20 ppt (Alton Gas 2015; DFO 2016; NSE 2016). From fertilization to hatch, approximately 48 h, the eggs are transported up- and down-estuary with the tide, distributed mostly between 0.5 and 15 ppt in the main channel of the estuary (Duston et al. 2018). Despite this distribution pattern, the upper salinity tolerance appears to be greater than 15 ppt, because survival to hatch was independent of salinity up to 20 ppt, and reduced significantly only at 30 ppt (Cook et al. 2010). Salinity tolerance of eggs from U.S. stocks, by comparison, appears to be lower than Shubenacadie River stock (Lal et al. 1977; Winger and Lasier 1994; Cook et al. 2010).

Yolk-sac larvae (3–4 dph, 5.5 mm TL) salinity tolerance at 19 °C was similar to eggs, because they share the same impermeable epithelium and associated ionocytes (Hirai et al. 2000). The subsequent decrease in salinity tolerance around the first-feeding stage, confirming Winger and Lasier (1994), is associated with the yolk-sac decreasing in both size and surface area, reducing its capacity for osmoregulation (Rombough 2007). The rudimentary gills and gastrointestinal tract begin to contribute to ion and water balance at first-feeding in all teleosts studied but are unable to fully compensate for the loss of yolk-sac osmoregulatory capacity (Rombough 2007). By 11 dph, striped bass gill filaments have functional ionocytes, which begin to actively excrete sodium (Na^+) and chloride (Cl^-; Hirai et al. 2002). Also, larval marine fish commence drinking saltwater and extract water across the intestinal wall, actively excrete Na^+ and Cl^- at the gills and magnesium (Mg^{2+}) and sulfate (SO_4^{2-}) via the primitive urinary system (Guggino 1980; Varsamos et al. 2005; Edwards and Marshall 2013). The increase in salinity tolerance through the larval stage reported here was likely due the increase in density of ionocytes on the skin and gill filaments quantified from 11 to 41 dph by Hirai et al. (2002). Transformation from larva to juvenile occurs during this period, around 25–36 mm TL in striped bass, associated with acquisition of definitive organs and adult morphology and structures (Otwell and Merriner 1975; Lal et al. 1977; Hardy 1978).

The metamorphosis from larva to juvenile can temporarily reduce osmoregulatory capacity in some species (Varsamos et al. 2005); but at its completion, the juveniles are better adapted to tolerate changes in salinity, as demonstrated by the 4- and 12-cm long striped bass exhibiting a LC50 1 h of 54–66 ppt (Fig. 3), and surviving in 100 ppt SC several minutes longer than larvae (Fig. 4). Similarly, survival and growth of Shubenacadie River early juveniles (6–9 cm FL) was independent of salinity between 1 to 30 ppt (Cook et al. 2010), and 114-dph juveniles from other stocks easily tolerated transfer to full seawater (34 ppt; Lal et al. 1977). When juvenile striped bass are transferred directly from freshwater to seawater (30 ppt), the structure of their ionocytes is modified quickly to maintain osmotic balance (King and Hossler 1991). The ability of juvenile striped bass to tolerate rapid increases in salinity is due to a high abundance of gill Na^+/K^+/ATPase and $Na^+/K^+/2Cl^-$ cotransporter that are "dormant" in freshwater but are activated immediately when the fish are exposed to seawater (Madsen et al. 1994; Tipsmark et al. 2004). Moreover, the rapid response is facilitated by insulin-like growth factor 1 and 2 and "epidermal growth factor" receptors present in the gill lamellae of striped bass are absent in other teleosts (Madsen et al. 2007; Tipsmark et al. 2007).

Differences in the ion composition between the salt core brine (SC) and the Instant Ocean brine (IO), specifically the very low potassium (K^+) and magnesium (Mg^{2+}) in the salt core, had no effect on the relative toxicity of the two brines at 19 °C in most of the life stages (Fig. 3). Moreover, the mixing of the brine with estuary water in the constructed channel will be very rapid, homogenizing the ions. Salt core

composition is dictated by the long-term oscillation of the ocean ionic make-up over the past 600 My (Horita et al. 2002; Lowenstein et al. 2003; Holt et al. 2014). There is no evidence the very low levels of either K^+ or Mg^{2+} in the SC brine would pose a threat to aquatic organisms. The K^+ concentration in the SC brine was fourfold higher than the lower-lethal level to larval Gulf killifish (*Fundulus grandis*), and Mg^{2+} concentration was 30% greater than the lower-level that interfered with Na^+/K^+-ATPase activity (Fisher et al. 2013, 2015). Potassium ions are essential for ionocyte function and needed for the ion exchange by the Na^+/K^+-ATPase and $Na^+/K^+/2Cl^-$ cotransporter (Fisher et al. 2013). Magnesium and Ca^{2+} to a greater extent serve to reduce the ionic permeability of the epithelium. Their deficiency produced an osmotic shock following transfer of teleosts to hypertonic media due to a rapid influx of Na^+ and dehydration (Lemm et al. 1993; Dolomatov et al. 2012; Fisher et al. 2015).

The interaction between temperature and salinity is important for the survival of striped bass early life stages, with a low of 12 °C identified as a lethal factor among U.S. stocks (Otwell and Merriner 1975; Morgan et al. 1981; Rutherford and Houde 1995). Poor egg survival at 12 °C in the Chesapeake Bay, estimated by Rutherford and Houde (1995), was in contrast to the similar acute salinity tolerance of eggs at 12 and 19 °C (Fig. 3). The vulnerability of yolk-sac larvae to low temperature, as evidenced by the 28% decrease in the median lethal brine concentration at 12 versus 19 °C (Fig. 3), supports data on U.S. stocks (Dey 1981; Morgan et al. 1981; Rutherford and Houde 1995). The cold tolerance of yolk-sac larvae of Shubenacadie stock, however, appears to be superior to U.S. stocks (Cook et al. 2010). Among feeding larvae, the independence in salinity tolerance between 12 and 19 °C reported here contrasts with reduced survival at 10 to 14 versus 16 °C in a 6-day trial in Instant Ocean (Cook et al. 2010). The longer exposure may have caused more disruption to osmoregulation than our 1-h tests because of delayed effects on Na^+/K^+/ATPase, an integral component of the salt pump (Donaldson et al. 2008).

In conclusion, the discharge of brine from the salt cavern in the mixing channel should not present any direct threat to the early life stages of striped bass, because it will be diluted to no more than 28-ppt (or 20 ppt when eggs or larvae are present) at 5 m from the point of release due to the high mixing and dilution due to the macrotidal conditions in the estuary.

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