

Physiological preparedness and performance of Atlantic salmon *Salmo salar* smolts in relation to behavioural salinity preferences and thresholds

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This study investigated the relationships between behavioural responses of Atlantic salmon *Salmo salar* smolts to saltwater (SW) exposure and physiological characteristics of smolts in laboratory experiments. It concurrently described the behaviour of acoustically tagged smolts with respect to SW and tidal cycles during estuary migration. *Salmo salar* smolts increased their use of SW relative to fresh water (FW) from April to June in laboratory experiments. Mean preference for SW never exceeded 50% of time in any group. Preference for SW increased throughout the course of smolt development. Maximum continuous time spent in SW was positively related to gill Na⁺, K⁺-ATPase (NKA) activity and osmoregulatory performance in full-strength SW (measured as change in gill NKA activity and plasma osmolality). Smolts decreased depth upon reaching areas of the Penobscot Estuary where SW was present, and all fish became more surface oriented during passage from head of tide to the ocean. Acoustically tagged, migrating smolts with low gill NKA activity moved faster in FW reaches of the estuary than those with higher gill NKA activity. There was no difference in movement rate through SW reaches of the estuary based on gill NKA activity. Migrating fish moved with tidal flow during the passage of the lower estuary based on the observed patterns in both vertical and horizontal movements. The results indicate that smolts select low-salinity water during estuary migration and use tidal currents to minimize energetic investment in seaward migration. Seasonal changes in osmoregulatory ability highlight the importance of the timing of stocking and estuary arrival.

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Key words: depth; estuary; migration; movement; residence; salt water.

INTRODUCTION

Many populations of Atlantic salmon *Salmo salar* L. 1758 have been extirpated or currently are in decline (Parrish *et al.*, 1998). Effects of dams in fresh water (FW), pollution and elevated marine mortality all have been cited as drivers of population decline and persistently low levels of abundance in recent years (National Research Council, 2004; Chaput, 2012). These factors are related to the complex suite of physiological,

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morphological and behavioural changes that *S. salar* undergo during smolting (McCormick *et al.*, 1998). The physiological preparation of *S. salar* smolts for hypo-osmoregulation in marine environments has been widely studied from hormonal control of smolting and physiological development of salinity tolerance (Zaugg & Wagner, 1973; Hoar, 1988; McCormick *et al.*, 1995) to behavioural initiation of migration (Zydlewski *et al.*, 2005, 2014; Sykes *et al.*, 2009) and estuary behaviour and survival (Halfyard *et al.*, 2013; Stich *et al.*, 2015). Smolting is physiologically regulated by circannual rhythms in photoperiod and temperature, which cue a suite of endocrine-driven changes to physiology, morphology and behaviour (McCormick *et al.*, 1998). The synchrony of these changes results in annual migrations from FW rivers to the ocean when a threshold set of conditions has been reached. The timing of these migrations is critical for successful ocean entry, and small changes in performance during this period can have major individual (*e.g.* death) and population-level consequences.

High mortality of *S. salar* smolts has been observed during passage through estuaries (Kocik *et al.*, 2009; Holbrook *et al.*, 2011), fjords (Dempson *et al.*, 2011; Thorstad *et al.*, 2012a) and near-coastal waters (Lacroix, 2008; Thorstad *et al.*, 2012b). This mortality recently has been related to physiology, experiences during FW migration and behaviour of individual smolts (Schreck *et al.*, 2006; Halfyard *et al.*, 2013; Stich *et al.*, 2015) as well as predation upon smolts (Hawkes *et al.*, 2013) during the early marine phase of migration. The results of these studies underscore the importance of physiological, morphological and behavioural preparations for successful estuary passage.

The timing of physiological and behavioural smolting and the timing of estuary entrance are instrumental in determining the success of smolts during estuary passage (Thorstad *et al.*, 2012b). Environmental control of smolting results in a physiologically enhanced period for saltwater (SW) entry known as the physiological smolt window (McCormick *et al.*, 1998). The timing of the physiological smolt window overlaps temporally with an ecologically opportune window for SW entry (McCormick *et al.*, 1998). An ecological smolt window can be defined as a period during which temperature (McCormick *et al.*, 1999), abundance of predators (Kocik *et al.*, 2009; Halfyard *et al.*, 2013) and presence of sympatric migrants (Svenning *et al.*, 2005) presumably act together to facilitate increased survival during estuary passage (McCormick *et al.*, 1998). The overlap (match) between physiological and ecological smolt windows confers higher smolt survival during estuary passage than mismatch.

The various neuroendocrine controls of physiological smolting have been investigated in great detail during the past several decades, and it is accepted that multiple endocrine systems are involved with various aspects of smolting (McCormick *et al.*, 1998; McCormick, 2013). One reliable indicator of migratory urge and physiological development of smolts is an enzyme used in ion exchange: gill Na⁺, K⁺-ATPase (NKA) activity (McCormick *et al.*, 1987, 1989). The specific activity of the SW isoform of gill NKA is increased during physiological smolting (McCormick *et al.*, 2009), and differentiation in the cells of gills results in increased abundance of this ion-transport enzyme (McCormick, 2013). Consequently, increased gill NKA activity is a useful indicator of osmoregulatory performance following exposure to SW. Recently, survival of *S. salar* smolts during estuary migration has also been related to activity of gill NKA activity immediately prior to migration (Stich *et al.*, 2015), highlighting the importance of physiological development for successful SW entry.

Although *S. salar* smolts develop features for SW acclimation during migration, the rate at which smolts move through estuaries is controlled at the individual and may relate to variability in the timing of physiological development and environmental conditions experienced. These differences probably manifest during estuary migration through behavioural responses to SW upon estuary entry, and smolts may exhibit specific preferences or movement behaviours as a result of individual variability. Individual variability in behaviour observed during migration (especially vertical distribution of fish), however, may be modified based on trade-offs between ion regulation, energetic demands and predator avoidance. The reasons for vertical movements by post-smolts during migration are still not well understood, although a number of reasons (selection of environmental gradients, predator avoidance and feeding behaviours) have recently been suggested (Davidsen *et al.*, 2008; Plantalech Manel-La *et al.*, 2009; Renkawitz *et al.*, 2012). Furthermore, it has been hypothesized that vertical movements, as well as downstream movement through estuaries, are mediated by diurnal and tidal rhythms (McCleave, 1978; Davidsen *et al.*, 2008) that might result in reduced energetic investment during seaward migration.

Timely information about how migratory success of fish might be affected by synergisms or mismatches between physiology and behaviour during migration could have important implications for ongoing conservation and management efforts surrounding *S. salar*. This is particularly true in light of high marine mortality in recent decades (Mills *et al.*, 2013), much of which is thought to occur during the early marine phase of migration (Friedland *et al.*, 2003), but is often not separately accounted for because marine survival is usually estimated from smolt-to-adult return rates. The goal of this study was to explore the ontogeny of salinity preferences of *S. salar* smolts through laboratory experiments and to use the observed patterns better to understand smolt behaviour during estuary migration from the Penobscot River, Maine, U.S.A. The specific objectives of this study were (1) to determine whether individual variability in selection of FW or SW by *S. salar* smolts in laboratory experiments was related to seasonal timing (ontogeny), gill NKA activity and osmoregulatory performance (measured as change in NKA activity and plasma osmolality) of *S. salar* and (2) to describe the spatial and temporal patterns in depths and salinities used during estuary migration by acoustically tagged Penobscot River smolts with respect to physiological status and tidal influences in a natural system.

MATERIALS AND METHODS

LABORATORY FISH AND SAMPLING

All fish used in the laboratory study were hatchery-reared, 18 month *S. salar* from the U.S. Fish and Wildlife Service (USFWS), Green Lake National Fish Hatchery (GLNFH) in Ellsworth, Maine, U.S.A. The fish were the progeny of first filial generation (F_1) sea-run adults from the Penobscot River and all fish are reared in FW. Three groups of fish were transported from GLNFH to the University of Maine Aquaculture Research Center, the first on 2 April (presmolts), the second on 6 May (smolts) and the third on 2 June (post-smolts). These groups were used to represent variability in seasonal timing of the Penobscot River smolt run, in which the mean annual peak of estuary arrival date is 9 May (s.d. = 8 days; Stich *et al.*, 2015). Fish were immediately transferred to a circular, 1890l, flow-through holding tank that contained aerated well water within 1° C of the transport tank and were sampled 1 h after transfer. Temperature of the holding tanks was gradually acclimated to reach temperatures identical to those used in salinity choice tanks.

Sampled fish were anaesthetized using a 100 mg l⁻¹ solution of tricaine methanesulphonate (MS-222) adjusted to pH 7.0 with 20 mmol NaHCO₃. For each fish, fork length (L_F , mm) and mass (g) were measured. A non-lethal gill biopsy (four to six filaments) was taken from the front, left gill arch of each fish prior to tagging. After a biopsy was taken, a small incision (c. 1–2 mm) was made offset from the ventral line, 1 cm posterior to the pectoral fins and a PIT tag measuring 2 mm × 12 mm and weighing 0.1 g (Model TX1411L; Destron Fearing; www.destronfearing.com) was gently inserted through the opening for the purpose of individual identification. The incision was small enough that it did not require sutures or glue; tag retention was 100%. All tags and surgical equipments were disinfected in 1% chlorhexidine solution (Bimeda Inc.; www.bimedaus.com). Fish were allowed a minimum of 24 h recovery period in the holding tanks prior to any subsequent behavioural or physiological testing.

After the recovery period, fish were transferred individually to an automated salinity choice tank. Salinity choice tests were conducted in 8 h time blocks to enable the use of a single SW-challenge tank for each time block. For testing, each fish was held in the salinity choice tank for 1 h, after which the fish was immediately transferred to SW (salinity = 35) for 16–24 h in an aerated 100 l tank. After 16–24 h of exposure to full-strength SW, fish were anaesthetized as described above, and a second gill biopsy was taken from the same gill arch of each fish. A blood sample was taken from the caudal vein of each fish using a 1 ml, 25-gauge ammonium-heparinized syringe. After terminal physiological samples were collected, all fish were euthanized by application of a lethal dose (250 mg l⁻¹) of MS-222 adjusted to pH 7.0 with 20 mmol NaHCO₃. The trial period for each group of 60 test smolts lasted about 7 days.

GILL NKA ACTIVITY AND PLASMA OSMOLALITY ASSAY PROCEDURES

Gill biopsies were stored at –80° C in 100 µl sucrose–EDTA–imidazole (SEI) buffer (250 mM sucrose, 10 mM Na₂-EDTA and 50 mM imidazole) for later analysis of gill NKA (enzyme code 3.6.3.9; IUBM 1992) activity (expressed as µmol ADP mg protein⁻¹ h⁻¹) using the method by McCormick (1993). Concentration of nicotinamide adenine dinucleotide (NADH) at 25° C and 340 nm was used to measure kinetic rate of ouabain-inhibitable ATP hydrolysis. Protein concentration in gill samples was determined using the bicinchoninic acid (BCA) method (Smith *et al.*, 1985). Samples from each individual were analysed in triplicate for NKA activity and protein concentration.

Blood samples taken for analysis of plasma osmolality were transferred immediately upon sampling to a 1.8 ml centrifuge tube and centrifuged at 2000 g for 5 min. Plasma was transferred to a 0.6 ml centrifuge tube, immediately frozen on dry ice and stored at –80° C for later analysis. Plasma osmolality was measured with an Advance Instruments Model 3200 freezing-point-depression osmometer (www.aicompanies.com) and was expressed in milliosmolality (mOsm).

SALINITY CHOICE SYSTEM

The Shuttle Box system (Loligo Systems, Inc.; www.loligosystems.com) used to test salinity choice by *S. salar* smolt in the laboratory was previously described by Serrano *et al.* (2010), with minor modifications (*e.g.* smaller tank size and tubing) during this study (Fig. 1). The tank was illuminated from below with two infrared (IR) utility lamps. The movement of individual smolts between compartments of the choice tank was tracked using an IR camera (uEYE USB camera, model UI-164xSE-C, Imaging Development Systems; en.ids-imaging.com) and analysed in ShuttleSoft 2.6.0 (Loligo Systems, Inc.). The salinity choice system and the tanks used for the SW challenge were housed in an isolated, climate-controlled room that was kept at the same temperature as holding tanks and maintained on simulated natural photoperiod. Equipment in this room was controlled through a remote computer system to minimize disturbance to test subjects.

Salt concentration in the choice tank was horizontally stratified using flow such that one compartment contained high-salinity water (HSW) and the other compartment contained low-salinity water (LSW). The mean ± s.d. salinity across all trials was 31.8 ± 3.7 for the HSW compartment and 2.3 ± 3.2 for the LSW compartment. The LSW reservoir tank contained well water that was held constant at c. 10° C throughout the duration of this study and was operated

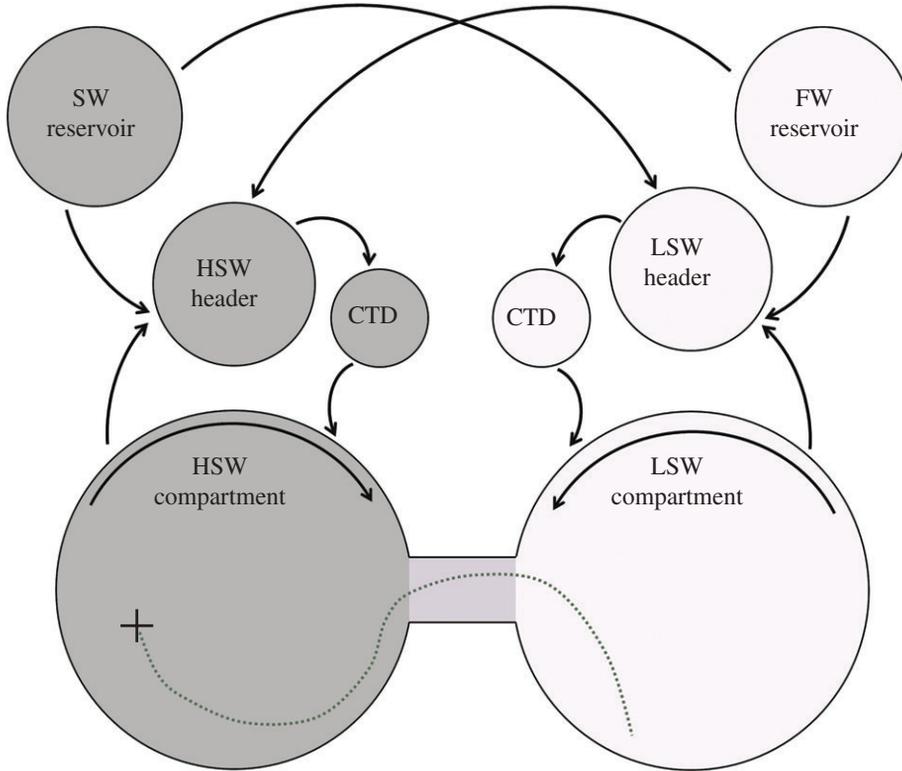


FIG. 1. Schematic drawing of the tank system used to assess salinity choice by hatchery-reared *Salmo salar* smolts in the laboratory. Direction of water flow is indicated (→). , an example of fish track and the + indicates the position of the fish. High-salinity water (HSW; ▒) and low-salinity water (LSW; □) are indicated. CTD, conductivity, temperature, depth sensors.

as flow-through. To achieve homogeneous temperatures ($\pm 1^\circ\text{C}$) between the LSW and HSW reservoir tanks (as well as LSW and HSW header tanks and compartments of the choice tank), well water was constantly run through a 30 m length of cross-linked polyethylene (PEX) tubing for heat exchange, and LSW well water was periodically added to the HSW header tank as the HSW reservoir tank was filled between salinity choice trials. Temperatures in the system were monitored continuously to assure that relatively homogeneous conditions existed in all phases of the experiment.

EXPERIMENTAL DESIGN FOR LABORATORY STUDY

Salinity choice was observed in three discrete groups of *S. salar* during spring 2014 (presmolts, smolts and post-smolts) using *c.* 60 fish for each group. Half of the fish in each group were tested during daylight hours and half during hours of darkness (using local sunrise and sunset times; 44.9°N) to test if salinity choice was related to daylight. Starting salinity (LSW or HSW) was alternated between compartments of the choice tank, and diel timing (light or dark), such that half of the fish tested at night and half of the fish tested during daylight hours started in HSW. Fish always entered the choice tank on the same side to account for possible tank effects on the selection of compartments. This resulted in an initial sample size of *c.* 15 fish in each level of a full-factorial design (group \times starting salinity \times diel timing). During the first SW challenge (five presmolts), salinity of 40 was used, but it was determined that this concentration was too high as it resulted in 100% mortality. All subsequent SW challenges were conducted at salinity of 35.

LABORATORY DATA ANALYSIS

Two behavioural responses of *S. salar* were measured in the salinity choice tank as indices of SW choice: proportion use of the HSW compartment and maximum continuous time spent in the HSW compartment. The proportion of time spent in the HSW compartment of the salinity choice tank (preference) was calculated as the amount of time that each fish occupied the HSW compartment of the choice tank, divided by the total duration of the trial. Maximum continuous time spent in the HSW compartment (residence) was calculated for each fish based on consecutive relocations within the HSW compartment of the salinity choice tank. For all fish exposed to SW challenge, physiological performance was assessed in two ways. First, change in gill NKA activity was calculated as the difference in gill NKA activity at tagging and gill NKA activity following SW challenge as an indicator of upregulation of gill NKA activity. Second, plasma osmolality was measured directly.

The effects of treatment group (presmolt, smolt and post-smolt), daylight (night = 0, day = 1), starting compartment (LSW = 0, HSW = 1), time after tagging, initial gill NKA activity, change in gill NKA activity (Δ NKA) and plasma osmolality on each of the response variables were tested using generalized linear models (GLMs; Montgomery *et al.*, 2006) in R 3.1.0 (R Core Team; www.r-project.org). Preference is on a binary scale (0, 1) and as such a logit transformation $\{\log [p / (1 - p)^{-1}]\}$ was used for the analysis of this response. Because of the wide range of variability in residence and because most of the values were small (*i.e.* right skewed), residence was analysed assuming a negative binomial error structure and the GLMs for the models used a ln-link function. Approximation of a variance inflation factor (\hat{c}) indicated that model structures were appropriate for the analyses conducted ($\hat{c} \approx 1.00$; Montgomery *et al.*, 2006). The final sample size used for each group during analysis of behaviour in the salinity choice tank and performance during SW challenge was 53 (presmolts), 60 (smolts) and 56 (post-smolts).

Competing models of preference and residence were constructed to test the effects of explanatory variables on each of the behavioural responses described above. An information-theoretic approach using AIC corrected for sample size (AIC_c; Burnham & Anderson, 2002) was used for model selection. Covariate effects were considered to be statistically significant if 95% c.i. for the estimated regression coefficient did not overlap zero. Effects are reported as mean (95% c.i.). ANOVA was used to test for differences in gill NKA activity, change in gill NKA activity following SW challenge and plasma osmolality between treatment groups. Tukey–Kramer pair-wise comparisons of means were used to determine group-level differences at $P < 0.05$ (Zar, 1999). Central tendency of physiological measurements for each group is reported as a median.

COLLECTION OF FIELD DATA

The Penobscot Estuary (Fig. 2) runs *c.* 45 km from the mouth of the estuary (river kilometre, rkm 0) to the head of tide (rkm 45; Haefner, 1967). The water column in the estuary is uniformly fresh at the head of tide (Imhoff & Harvey, 1972), considerable mixing of FW and SW in the middle of the estuary between rkm 30 and 0 (Fig. 1; Seiwel, 1932), and stratification of FW and SW occurs in the lower estuary and bay (Imhoff & Harvey, 1972). Given the vertical stratification within the Penobscot Estuary based on temperature and salinity gradients (Haefner, 1967), depths at which fish migrate dictate exposure of individual smolts to elevated salinities. Due to the prominent influence of salinity gradients in the physical and chemical characteristics of the Penobscot Estuary, depth used by smolts probably exposes them to different salinities.

Ten hatchery-reared *S. salar* smolts from GLNFH were acoustically tagged with depth-sensing transmitters on 24 April and tracked during migration from release at the head of tide (rkm 43.5) in the Penobscot Estuary to Penobscot Bay. Fish were tagged using previously described methods (Holbrook *et al.*, 2011; Stich *et al.*, 2014). Smolts were anaesthetized using a 100 mg l⁻¹ solution of MS-222 buffered to pH 7.0 (using 20 mmol NaHCO₃), and L_F (mm) and mass (g) were measured. Mean \pm s.d. L_F of acoustically tagged smolts was 192 \pm 12 mm, and mean mass was 70 \pm 13 g. A non-lethal gill biopsy (four to six filaments) was taken from the front, left gill arch of each fish prior to tagging. Individual biopsies were stored and assayed using procedures described above for gill NKA. For each fish, a 1 cm incision was made offset from the ventral line and 1 cm posterior to the pelvic fins. An acoustic tag was inserted and the incision was closed with two interrupted knots using 4-0 absorbable vicryl sutures (Ethicon; www.ethicon.com). Smolts were allowed to recover for 1 week before release. All tagged

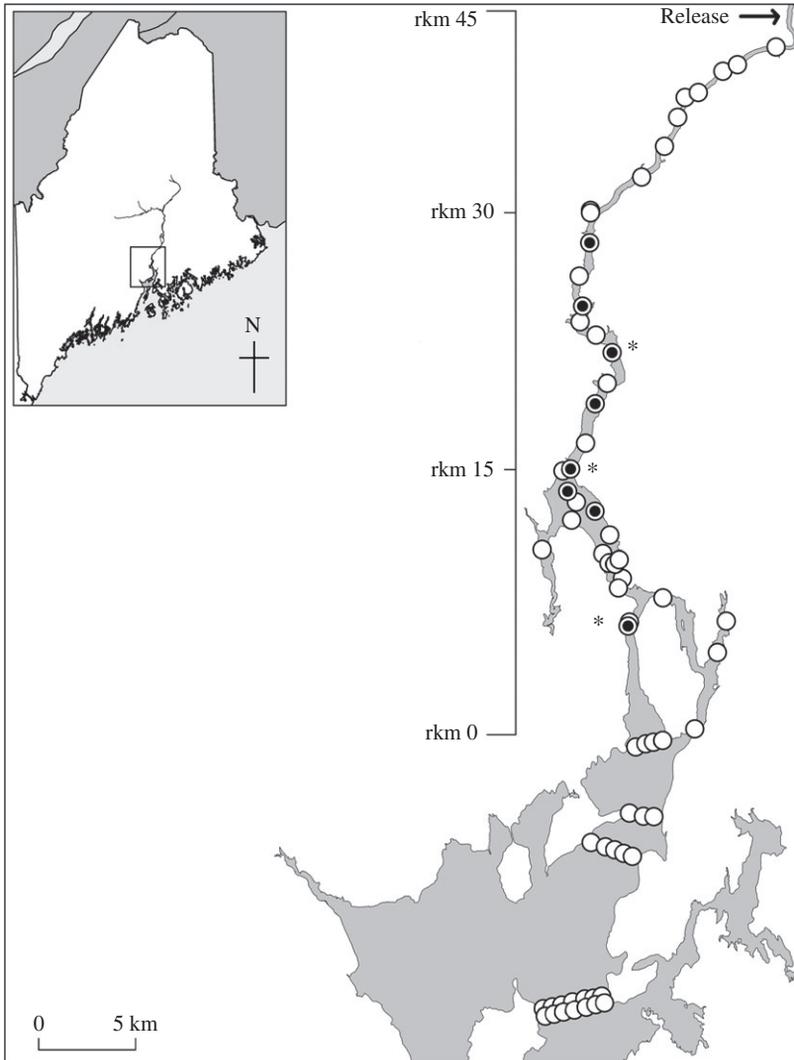


FIG. 2. Map showing the release location of tagged smolts and the location of VR2-W acoustic receivers and corresponding river kilometre (rkm) of deployment. Acoustic receiver locations (O) and locations at which a conductivity, temperature and depth sensor (CTD) were deployed on the river bottom (●) are indicated. Receiver locations at which CTDs were also deployed at the top the water column are also indicated (*).

smolts were released at the same time. Smolts were tagged with model V9P-6L tags (Amirix Vemco Ltd; www.vemco.com). Tags were 39 mm in length, 9 mm diameter and weighed 2.2 g in water, with estimated battery life of 60 days.

Salmo salar smolts were tracked during migration using an array of stationary VR2-W acoustic receivers (Amirix Vemco Ltd) deployed prior to tagging in cooperation between the U.S. Geological Survey (USGS) Maine Cooperative Fish and Wildlife Research Unit, the University of Maine and the National Oceanic and Atmospheric Administration (NOAA). The array extended from the head of tide (rkm 43.5) to Penobscot Bay (rkm -15) and included 66 acoustic receivers (Fig. 2). Receivers deployed in the Penobscot Estuary were moored to 45 kg concrete anchors on the river bottom, and in the bay receivers were tethered 10 m below the surface.

Where necessary, multiple receivers were deployed to achieve adequate coverage in a given location. Detections at receivers within these locations were pooled as a single site for analyses.

Data storage conductivity, temperature and depth loggers (DST-CTD, Starr-Oddi; www.starr-oddi.com) were deployed at eight receiver locations in the estuary in configurations of either one or two loggers (Fig. 2). In locations at which one CTD was used, the logger was attached to an acoustic receiver mooring near the bottom of the water column. Where multiple DST-CTDs were deployed at a receiver location, one CTD was deployed in the top 2 m of the water column and the other CTD was deployed about 0.5 m from the bottom.

ANALYSIS OF FIELD DATA

Patterns in depth use during migration were investigated using data from acoustic tags. To determine if depth use was related to the presence of SW in the estuary, the relationship between depth use and distance from the mouth of the estuary was estimated using a generalized linear mixed-effects model (GLMM; Zuur *et al.*, 2009). Individual fish identity was included as a random effect on the intercept because an unequal number of measurements were collected for each fish at each location. A quadratic term was used to describe the relationship between depth use and estuary location. A GLMM with an individual random effect was used to test whether depth used by individual fish was related to tidal cycles (incoming or outgoing) for the week during which fish moved through the estuary. The link function used in each of these models was the inverse Gaussian, because the response (depth) was continuous but constrained to be greater than zero (*i.e.* fish could not occupy negative depths within the water column).

The probabilities of encountering varying salinities at a given rkm in the estuary were modelled using logistic regression and salinity data for the locations with CTDs in the lower estuary (Fig. 2). The probabilities of encountering salinities greater than concentrations from 0 to 15 (0, 5, 10 and 15) were used to predict the presence of SW in the Penobscot Estuary. For each SW concentration examined, water of that concentration was considered to be present (1) if a CTD-recorded salinity was greater than or equal to the concentration. If salinity equal to or greater than the concentration was not recorded at a site, SW greater than the corresponding concentration was considered to be absent (0). Salinity of 10 was assumed to be near isosmotic. From each salinity used, the probability that SW concentrations greater than each salinity existed at each receiver location was estimated from a logistic regression model. These probabilities were then plotted and used to assess average position of the salt wedge in the Penobscot Estuary visually and qualitatively compare salinity gradients to changes in fish depth and movement rate.

Travel time through the FW reaches of the estuary from release (rkm 43.5) to the first recorded SW (rkm 30) was compared with gill NKA activity of smolts using simple linear regression. Similarly, the relationship between gill NKA activity and SW travel time was tested using simple linear regression. A significance level of $\alpha < 0.05$ was used for both tests. Finally, vertical and horizontal movements of fish were plotted against date and tidal cycles to assess potentially interesting patterns in behaviour related to residency time in FW and tidal cycles in SW. Differences in depth use during day and night were not investigated, because tidal and diurnal cycles were confounded over the week of the field study. In addition, the laboratory experiment did not indicate effects of diurnal cycle on salinity preference or residence.

RESULTS

LABORATORY EXPERIMENT

Proportional use of the HSW compartment of the salinity choice tank (preference) by fish was related to development and time after handling (Table I). Presmolts used the SW compartment of the choice tank significantly less (mean \pm s.d. = 0.37 ± 0.09) than post-smolts (0.44 ± 0.09), and smolts were intermediate in their preference for SW [0.41 ± 0.13 ; Fig. 3(a)]. Preference for SW increased with time after handling. Fish tested at the end of each treatment group (*c.* 1 week) increased proportional use

TABLE I. Model-selection statistics for generalized linear models (GLM) used to test the effects of treatment group (early, middle or late), daylight (dark = 0; light = 1), starting salinity (low-salinity water = 0; high-salinity water = 1), gill Na^+ , K^+ -ATPase activity (NKA; $\mu\text{mol ADP mg protein}^{-1} \text{h}^{-1}$) at tagging, change in NKA following saltwater (SW) challenge (ΔNKA), plasma osmolality (osmolality) and time after tagging (handling) on proportional use of salt water (preference) by hatchery-reared *Salmo salar* smolts in salinity choice tanks. Symbols in table are defined as number of parameters (k), corrected AIC (AIC_c), the difference in AIC_c between the best model and the i th model (Δ_i), and the relative probability that the i th model is the best model in the candidate set (w_i)

Model	k	AIC_c	ΔAIC_c	w_i
Group + daylight + handling	6	237.01	0.00	0.10
Group + handling	5	237.43	0.42	0.08
Group + daylight + salinity + handling	7	237.51	0.50	0.08
Group + salinity + handling	6	237.88	0.87	0.07
Group + daylight + handling + osmolality	7	238.59	1.58	0.05
Group + daylight + handling + NKA	7	238.95	1.95	0.04
Group + daylight + salinity + handling + osmolality	8	238.99	1.98	0.04
Group + daylight + handling + ΔNKA	7	239.14	2.13	0.04
Group + daylight + salinity + handling + NKA	8	239.34	2.33	0.03
Group + handling + NKA	6	239.44	2.43	0.03

of the SW compartment by about 10% (3–17%) compared with fish tested 24 h after tagging.

The maximum continuous time spent in the HSW compartment of the salinity choice tank (residence, measured in s) by *S. salar* was related to development, daylight, handling, gill NKA activity, change in gill NKA activity (ΔNKA) following SW challenge and plasma osmolality (Table II). Residence in the HSW compartment of the salinity choice tank was greater in smolts [mean \pm s.d. (286 \pm 482 s)] than in presmolts (97 \pm 89 s) or post-smolts (123 \pm 131 s); Fig. 3(b)]. Fish occupied the HSW compartment of the salinity choice tank for longer continuous periods during darkness than during daylight hours, but the mean difference was just 17 s (1–51 s). Smolts tested at the beginning of each treatment group exhibited increased residence, but the difference of 13 s (4–14 s) was not significant at the test level ($\alpha = 0.05$). Fish with higher gill NKA activity at tagging exhibited greater residence compared with fish with lower gill NKA activity. Fish with the highest gill NKA activity continually resided in the HSW compartment 9 min (2–35 min) longer on average than fish with the lowest gill NKA activity. Similarly, *S. salar* with greater ΔNKA and plasma osmolality had greater residence in HSW than fish that performed more poorly during SW challenge, although ΔNKA was a better predictor of residence than was plasma osmolality (Table II). Fish that exhibited the greatest ΔNKA activity continuously occupied the SW compartment of the salinity choice tank for 80 s (22–223 s) longer than fish with lowest ΔNKA across all groups. Similarly, when ΔNKA was not included as an explanatory variable, fish that had the highest plasma osmolality following SW challenge exhibited reduced residence compared with fish that had the lowest plasma osmolality, a difference of 76 s (2–163 s).

Laboratory analyses indicated significant differences between treatment groups (presmolts, smolts and post-smolts) of *S. salar* in gill NKA activity prior to tagging

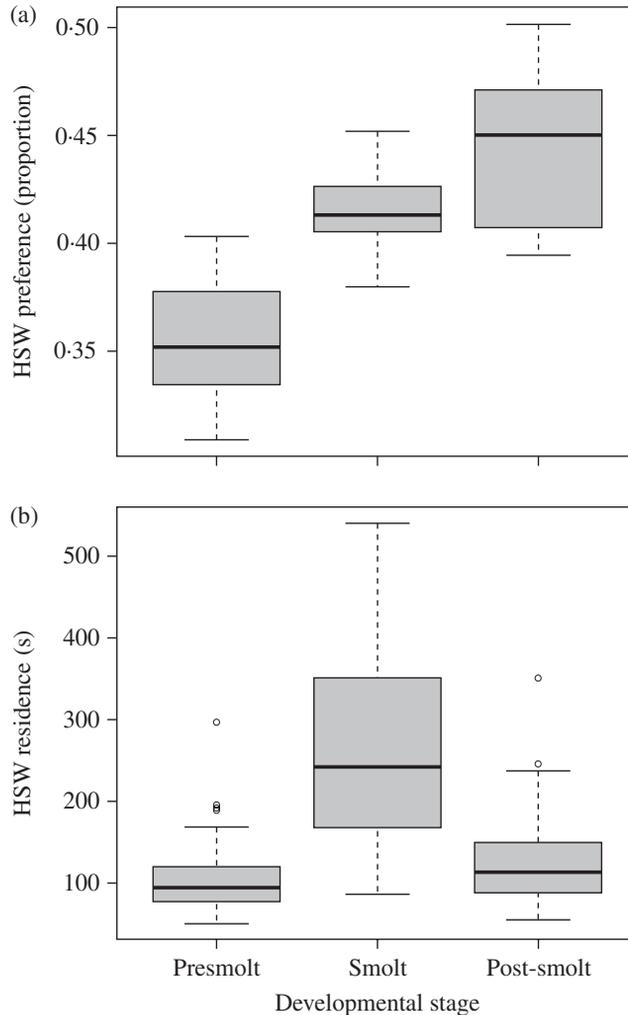


FIG. 3. Differences in (a) proportional use of high-salinity water (HSW) in salinity choice tank ('preference') and (b) maximum continuous time in HSW compartment of salinity choice tank (residence) by hatchery-reared *Salmo salar* smolts tested early in the smolt run (3–9 April), in the middle of the smolt run (7–15 May) and late in the smolt run (4–10 June). \square represents 25–75% c.i. and — in the centre of boxes are medians. --- indicates 95% c.i.; \circ , outlier observations.

(ANOVA, $F_{2,166} = 31.21$, $P < 0.001$), change in NKA activity over the trial (ANOVA, $F_{2,166} = 4.28$, $P < 0.001$) and plasma osmolality (ANOVA, $F_{2,166} = 40.97$, $P < 0.001$) following SW challenge (Fig. 4). Median gill NKA activity was significantly lower in presmolts (3.11) than smolts (5.78), and post-smolts had gill NKA activity (3.66) intermediate to presmolts and smolts [Fig. 4(a)]. Similarly, presmolts increased NKA activity (1.41) significantly more than smolts (0.16), and post-smolts were intermediate in observed upregulation of gill NKA activity following SW challenge [0.51; Fig. 3(b)]. Congruent with changes in gill NKA activity following SW challenge, plasma osmolality (mOsm) was significantly higher in presmolts (377) after

TABLE II. Model-selection statistics for generalized linear models (GLM) used to describe the maximum continuous time spent in salt water (SW) compartment of salinity choice tank (residence) by *Salmo salar* smolts. Model-selection statistics and symbols used for explanatory variables are defined as in Table I

Model	k	AIC _c	Δ AIC _c	w_i
Group + daylight + handling + NKA + Δ NKA	7	2008.47	0.00	0.15
Group + daylight + salinity + handling + NKA + Δ NKA	8	2008.71	0.25	0.13
Group + daylight + NKA + Δ NKA	6	2009.10	0.63	0.11
Group + daylight + salinity + handling + NKA + osmolality + Δ NKA	9	2009.23	0.77	0.10
Group + daylight + handling + NKA + osmolality + Δ NKA	8	2009.33	0.87	0.10
Group + daylight + NKA + osmolality + Δ NKA	7	2009.45	0.99	0.09
Group + daylight + salinity + NKA + osmolality + Δ NKA	8	2009.50	1.04	0.09
Group + daylight + salinity + NKA + Δ NKA	7	2009.53	1.07	0.09
Group + NKA + osmolality + Δ NKA	6	2011.57	3.10	0.03
Group + salinity + NKA + osmolality + Δ NKA	7	2012.32	3.86	0.02

SW challenge than in smolts (326) or post-smolts [356; Fig. 4(c)]. Plasma osmolality was also significantly higher in post-smolts than in smolts.

Of the fish exposed to a 24 h SW challenge, two (4%) mortalities were observed in the presmolt group. This result was assumed to be due to reduced osmoregulatory capacity in those fish because it represented only two of 55 (<4%) total fish remaining in the presmolt group and two of eight (25%) of the fish in that specific SW-challenge trial. In the presmolt treatment group, 13 fish (24%) exhibited moribund behaviour and physical appearance (indicated by loss of equilibrium and lack of movement) following SW challenge, and 10 (18%) of the post-smolts similarly appeared to be moribund. Most fish that displayed signs of imminent mortality had extremely high (>400 mOsm) plasma osmolality, and mean gill NKA activity of these fish ($2.9 \mu\text{mol ADP mg protein}^{-1} \text{h}^{-1}$) was lower than the remaining fish ($4.38 \mu\text{mol ADP mg protein}^{-1} \text{h}^{-1}$) that did not show such signs (t -test, $t = 4.49$, d.f. = 40, $P < 0.001$). There were no mortalities in the smolt group, and none of those fish exhibited the obvious signs of distress following SW challenge that were observed in fish from the presmolt and post-smolt groups.

FIELD STUDY

Logistic regression models used to estimate salinity throughout the estuary fit the data well ($\hat{c} \approx 1.00$), and indicated low probability of encountering SW at receiver locations until c . rkm 20, at which point salinity increased gradually until full-strength SW was present in the mouth of the estuary at rkm 0 [Fig. 5(a)]. The slopes of the individual regression lines and the spread of these lines in the middle estuary indicated a gradual increase in mean salinity until full-strength SW was reached at the mouth of the estuary [Fig. 5(a)].

All (100%) of the acoustically tagged smolts survived migration from the release site to the ocean during the course of the field study, based on relocation of all individuals at the mouth of the Penobscot Bay. Minimum residency time in the estuary

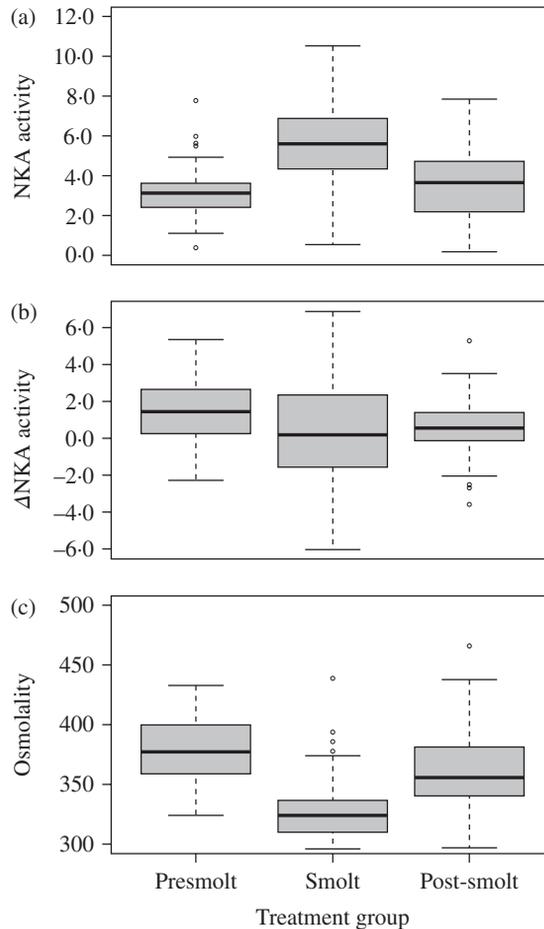


FIG. 4. Differences in (a) Gill Na^+ , K^+ -ATPase activity (NKA; $\mu\text{mol ADP mg protein}^{-1} \text{h}^{-1}$), (b) change in NKA activity following saltwater challenge and (c) plasma osmolality for hatchery-reared *Salmo salar* smolts that were tested in salinity choice tanks early in the smolt run (3–9 April), in the middle of the smolt run (7–15 May) and late in the smolt run (4–10 June). represents 25–75% c.i. and in the centre of boxes are medians. indicates 95% c.i.; \circ , outlier observation.

was 1 day (one fish), and maximum residency time in the estuary and bay was 7 days (two fish).

The overall mean \pm s.d. depth used by *S. salar* smolts during passage through the Penobscot Estuary was 5.9 ± 3.2 m. Depth use by migrating smolts was highly variable until the fish reached uniform SW conditions downstream of rkm 10 in the lower estuary [Fig. 5(b)]. Smolts became increasingly surface oriented during migration from the head of the estuary to the mouth of the bay. Upon reaching full-strength SW in the lower estuary near rkm 10, variability in the depths used by individual smolts decreased and mean depth used in SW was shallower than in FW or in the mixing zone of the Penobscot Estuary, despite that mean maximum channel depth increased [Fig. 5(b)]. The quadratic relationship between estuary rkm and depth used by smolts indicated a rapid decrease in depth used, as well as in the variability of depth used, when smolts

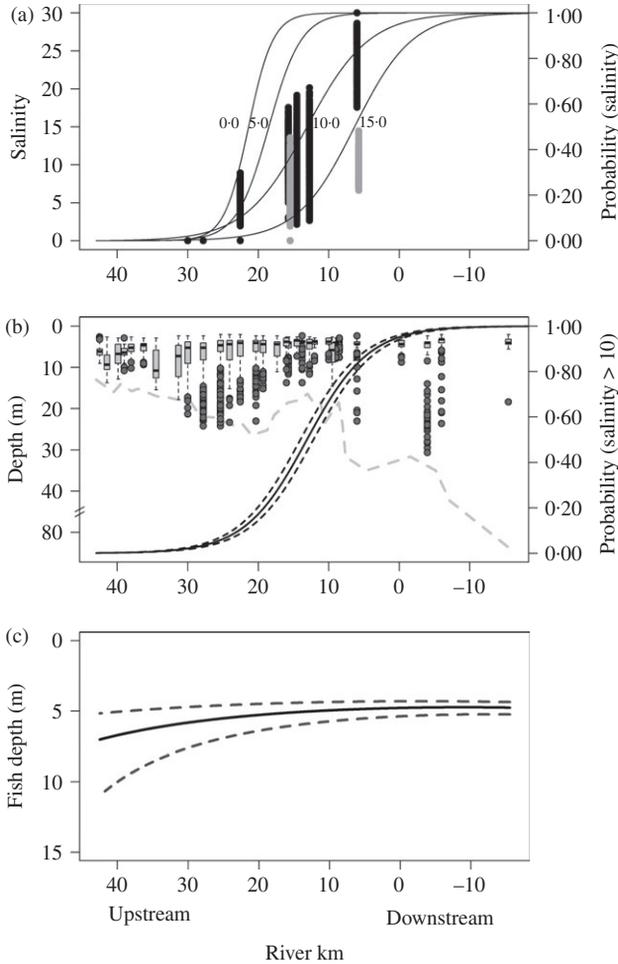


FIG. 5. (a) Predicted salinity, (b) observed fish depth and (c) modelled fish depth in the Penobscot Estuary. (a) Observed salinities at top (●) and bottom (●) of the water column at conductivity, temperature and depth (CTD) locations on the primary y-axis; —, modelled probabilities that mean salinity exceeds 0, 5, 10 or 15 at a given location in the estuary. (b) Box plot of depths used by *Salmo salar* during migration plotted against probability that mean \pm 95% C.I. Salinity (— and - - -) in the estuary was >10 at acoustic receiver locations, and mean maximum channel depth (· · ·) is shown. □ represents 25–75% C.I., ▤ in the centre of the boxes are median depth, ○ are outliers and ▭ indicates 95% C.I.

initially encountered the salt wedge in the middle estuary [Fig. 5(c)]. Estimated depth use from the regression model appeared to agree well with the mean observed depths (Fig. 5). Despite that smolts became increasingly surface oriented during downstream migration, fish continued to make some use of the deepest parts of the water column for the full length of the estuary. Several smolts were observed to use water as deep as 30 m in the estuary [Fig. 5(b)].

The relationship between depths used and location within the estuary was related to the presence or absence of SW [Fig. 5(a)]. When SW was absent in the upper estuary, depths used by smolts was highly variable and no obvious pattern in depth used between locations was apparent. Similarly, fish were located deepest in areas of the estuary with

low probability of encountering SW in the water column [Fig. 5(a)] until they reached full-strength SW.

Travel time through FW from the release site (rkm 43.5) to rkm 30 (where salinity > 0 was first detected by CTDs) was inversely related to gill NKA activity of individual smolts (simple linear regression, $R^2 = 0.53$; $F_{1,8} = 9.204$, $P < 0.05$). Total travel time from rkm 30 to the mouth of the estuary, however, was not related to gill NKA activity (simple linear regression, $R^2 = 0.03$; $F_{1,8} = 0.265$, $P > 0.05$). Based on the examination of individual plots of horizontal and vertical movements, the differences observed in FW were a result of station-holding behaviour, whereby fish remained in a given location over multiple tidal cycles (Fig. 6). When fish exhibited station-holding behaviour, they generally occupied deeper water than when actively migrating, although vertical movements apparently related to tidal cycles were observed. When holding station in FW, fish tended to rise into the water column late during incoming tides or early during outgoing tides, and if they did not make seaward movement on that tidal cycle, they moved deeper later in the outgoing tide. None of the fish that exited the estuary within 3 days exhibited station-holding behaviour in FW reaches of the estuary.

Upon arrival at estuary reaches in which LSW (0–5) was present (*c.* rkm 30), all smolts began to display directional reversals in movement (*i.e.* movement upstream), a behaviour that ceased after entry of SW > 10 near rkm 10 in the estuary, regardless of the time spent in FW reaches. Mean \pm s.d. number of reversals in these reaches was 2.7 ± 1.3 , and the number of migratory reversals ranged from one to five for the fish used in this study. This behaviour appeared to correlate well with tidal cycle, as upstream movements generally occurred on incoming tides, and downstream movements occurred on outgoing tides in virtually all observations (Fig. 6).

The tidal patterns in horizontal movements through the estuary were accompanied by corresponding changes in vertical movements. Fish were nearer to the surface while mobile than when holding position. Furthermore, fish became more surface oriented while embarking on movement and they increased depth as they terminated movement. Smolts were generally located deeper on incoming tides than on outgoing tides (*t*-test, $t = 2.8129$, d.f. = 10 783, $P < 0.05$). Once in the lower estuary (downstream or rkm 30), fish always initiated station-holding behaviour at the end of an outgoing tide; continuing to hold horizontal position until the end of the subsequent incoming tide (Fig. 6).

DISCUSSION

Salinity choice in the laboratory study (measured as preference and residence) was successfully related to an established measure of physiological preparedness for SW entry (gill NKA activity: McCormick *et al.*, 2012; McCormick, 2013) as well as physiological performance following SW exposure (indicated by Δ NKA and demonstrated by plasma osmolality). As expected from the results of previous work (Zaugg & McLain, 1970; Duston & Saunders, 1995), a developmental shift in gill NKA activity was observed, and *S. salar* tested during May (*i.e.* smolts) had greater gill NKA activity than presmolts or post-smolts. A corresponding increase was observed in the osmoregulatory performance of *S. salar* during the course of development, as indicated by the fact that smolts had lower plasma osmolality and upregulated gill NKA activity was less in response to SW exposure than either presmolts or post-smolts. These results are consistent with the results of a large body of work regarding osmoregulatory capacity during smolt development, summarized by McCormick (2013).

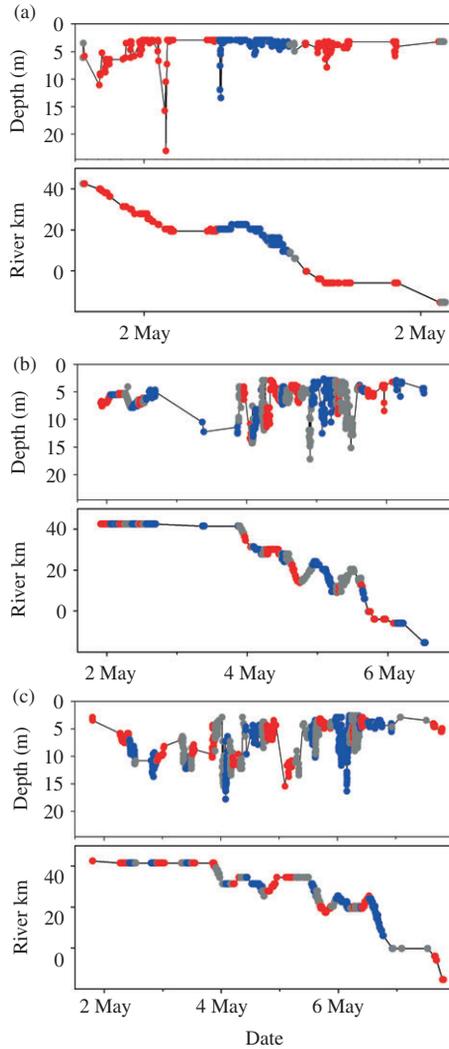


FIG. 6. Representative plots of vertical (depth) and horizontal (river km) movements of (a–c) three *Salmo salar* smolts during estuary migration with respect to tidal cycles for each observation (●, ebb tide; ●, flood tide; ●, slack tide).

The behavioural responses to SW by the post-smolt group in the salinity choice tank indicate a general correspondence with some potential decoupling of the behaviours late in the ecological smolt window with respect to the timing of the physiological optimum for SW entry. Physiological controls of salinity preferences (Iwata *et al.*, 1990) and SW orientation (Otto & McInerney, 1970) in salmonids may differ from physiological controls of the actual behavioural preference for SW. For example, whereas behavioural preferences for downstream movement and salinity preference are probably controlled primarily by release of thyroid hormone mediated by photoperiod (Iwata *et al.*, 1990), physiological salinity tolerance is controlled by a suite of hormones and peptides, including thyroid hormones, cortisol, adrenocorticotrophic hormone,

growth hormone, insulin-like growth factor-1 and possibly others (McCormick, 2013).

An ontogenetic increase in preference was observed during the course of the laboratory experiment. Residency in the HSW compartment of the salinity choice tank also increased until the assumed peak of the run, but was less in the post-smolt group than the smolt group. Similarly, measurements of physiological performance increased until the assumed peak of physiological smolt development and subsequently were lower in the post-smolt group. In contrast, preference for SW continued to increase even after physiological performance of smolts in SW began to decrease. These results could have important implications for the seasonal timing of smolt runs and effects of migration delays behind dams. Early and late-migrating smolts tend to have reduced estuary survival relative to smolts that migrate during the peak of physiological smolt window (Stich *et al.*, 2015), and estuary survival has now been related to gill NKA activity (Stich *et al.*, 2015). It is possible that continued preference for SW late in the run, combined with reduced SW tolerance (measured as plasma osmolality or Δ NKA activity) could result in lower estuary survival of fish migrating late in the physiological smolt window. Delays such as those at dams (Keefer *et al.*, 2012) have the potential to reduce survival of *S. salar* in the later part of smolt runs (Marschall *et al.*, 2011), possibly because fish might retain similar preferences for SW despite reduced osmoregulatory capacity from temperature-related loss of smolt characteristics (McCormick *et al.*, 1999).

It is unknown whether this behaviour has direct implications for fitness in the wild or if it represents behavioural plasticity that might allow for corresponding flexibility in the period of overlap between ecological and physiological smolt windows. This result may indicate a reason for strong relationships between timing of estuary arrival and smolt survival observed in the wild (Thorstad *et al.*, 2012b), and suggests that strong optimizing selection might occur on natural variability in physiological preparedness, resulting in temporally narrow, synchronous migrations that last only weeks (McCormick *et al.*, 1998), such as has been observed in the Penobscot River (Stich *et al.*, 2015). Alternatively, behavioural preferences of smolts for salinity may be highly plastic compared with physiological underpinnings of salinity tolerance (Hutchings, 2011), and as a result promote potential behavioural adaptability to changing environmental conditions driving physiological development from year to year. This might have implications for the adaptability of smolts to changing climate in the future, as greater flexibility in behavioural SW preferences could promote resilience to changes in environmental releasers of migratory behaviour (Zydlowski *et al.*, 2014).

It is notable that *S. salar* in the laboratory study never exhibited a demonstrable preference for SW (fish in all groups never spent >50% of time in HSW), even at the assumed peak of physiological development. Smolts at all developmental stages avoided SW, despite the fact that gill NKA activity and SW-challenge performance measurements indicated that fish were fully competent for transition into SW based on previous research (Duston & Saunders, 1990). While this has the potential to be an artefact of the tank design used in the laboratory study, it was consistent with what was observed during migration in the Penobscot Estuary. Similar avoidance of SW previously has been observed in migrating *S. salar* smolts that use the upper, FW layers of the water column for migration (Renkawitz *et al.*, 2012).

Depths used by smolts during estuary migration indicated a pattern consistent with salinity influence in the estuary. Depth use was highly variable in the upper estuary until

fish encountered SW. Upon reaching water with elevated salinity, fish moved closer to the surface. These results indicate that migrating fish made use of the entire water column during passage of FW reaches, but concentrated in the top of the water column, where salinities were lower, upon reaching SW in the lower estuary. Although fish continued to make excursions into deeper (and higher salinity) water in the lower estuary as previously observed in the Penobscot Estuary (Renkawitz *et al.*, 2012), the frequency with which fish made vertical movements and the duration of these movements appeared to be greatly reduced in reaches of the lower estuary where mean salinity exceeded concentrations that were isosmotic (salinity *c.* 10). The mean depth during estuary migration was deeper than has previously been described for this species (Plantalech Manel-La *et al.*, 2009; Renkawitz *et al.*, 2012; Thorstad *et al.*, 2012a). This is expected because previous studies have examined vertical movements of post-smolts in fjords and coastal bays, where availability of FW is restricted to upper layers of the water column and fish depth was observed to decrease substantially upon reaching full-strength SW in the present study.

The use of the upper water column may occur for a number of reasons other than or in addition to salinity preferences such as decreased energetic cost of migration (Moore *et al.*, 1998), pelagic feeding (Renkawitz & Sheehan, 2011), selection of warmer temperatures (Steffansson *et al.*, 2003) or avoidance of deep-water predators (Hvidsten & Lund, 1988). The agreement between laboratory and field studies, as well as the physical-chemical data collected, suggests that salinity preferences may also influence the depth of water occupied by smolts due to the presence of LSW in the top few metres with a net seaward flow during spring. Periodic excursions to greater depths are probably not related to salinity preference, but rather feeding behaviour or surface-predator avoidance, as previously has been suggested (Renkawitz *et al.*, 2012). Based on these results, smolts in this and other systems may use the FW layers of estuarine and coastal systems during migration when those layers are available, after which the fish adopt a surface-oriented disposition during coastal and ocean phases of migration, probably for other reasons.

Movement rate from the release location at the head of tide to the middle estuary (rkm 30, where SW was first present) was related to gill NKA activity of individual *S. salar* smolts. Conversely, transit time from rkm 30 to the mouth of the estuary was not related to gill NKA activity. Previous research has failed to detect differences in estuarine movement rates based on gill NKA activity in the Penobscot Estuary (Stich *et al.*, 2015), but that study did not attempt to separate movement rates based on location of SW in the estuary. It appears that *S. salar* behaviourally prepared for SW entry by prolonging their residency time in the upper estuary prior to entering SW, and not through behavioural acclimation after entry into SW, as previously has been speculated (Halfyard *et al.*, 2012, 2013). In fact, gill NKA activity explained more than half ($R^2 = 0.53$) of the variation in travel time from release to the middle estuary (rkm 30), and virtually no support was found for a relationship between transit time through SW and gill NKA activity in this study. The exact mechanisms and environmental cues controlling this relationship currently are not well understood, but might involve both internal (*e.g.* circulating hormone levels) and external (*e.g.* temperature, current velocity or olfaction) cues. The relationship between traverse time in the upper estuary and gill NKA activity could have important implications for optimizing stocking decisions regarding *S. salar* smolts with respect to seasonal timing, as well as timing of tidal cycles. For example, stocking of fish before they are physiologically prepared

might result in prolonged station-holding behaviours and increased exposure to predators.

Salmo salar at various developmental stages of smolting are generally capable of tolerating exposure to full-strength SW for short periods (McCormick, 2013). But, fish early and late in the smolt window might experience greater mortality during estuary migration due to reduced osmoregulatory performance (Duston & Saunders, 1990) in conjunction with stressors (Price & Schreck, 2003a) and other agents of osmotic perturbation during chronic exposure to SW (Handeland *et al.*, 1996; McCormick *et al.*, 2005; Zydlewski *et al.*, 2010). In the laboratory study, this was indicated by low-level mortalities in the presmolt group, as well as observations of moribund presmolts and post-smolts following 24 h exposure to SW. In contrast, differences in plasma osmolality were observed between moribund fish and fish that showed no change in physical disposition. Based on the relationships between osmoregulatory preparedness, performance and behavioural choices in the laboratory experiment, it is expected that downstream-migrating *S. salar* might mediate behaviour prior to or during estuary migrations to minimize osmoregulatory perturbation. This could occur through behavioural selection of LSW (Price & Schreck, 2003b), reduction of energetic investment in movement with respect to prevailing currents (McCleave, 1978), reduced exposure to predators through selection of specific depths during day or night (Moore *et al.*, 1995) or some combination of these behaviours (Ibbotson *et al.*, 2006).

All migrating smolts exhibited reversals in movement direction during migration through the Penobscot Estuary, a behaviour observed in other systems (Kocik *et al.*, 2009; Dempson *et al.*, 2011; Halfyard *et al.*, 2013). The locations at which fish in the present study reversed movement direction always occurred within a 20 km section of the estuary, between rkm 30 and rkm 10. This section of the estuary also appeared to be the area in which SW was first encountered by smolts. While the fish observed in this study displayed only minimal numbers of directional reversals (maximum = 5) compared with other studies (*e.g.* maximum of 10 in Halfyard *et al.*, 2012), it appears possible that this behaviour might promote osmoregulatory capacity through behavioural acclimation to SW (Halfyard *et al.*, 2012); however, this would in theory occur at the cost of increased exposure to predators and other sources of osmoregulatory perturbation (McCormick *et al.*, 1998). As a result of the trade-off between increased osmoregulatory capacity and increased predation, the fitness benefits and associated adaptive value of this behaviour appear dubious if related to SW acclimation. More likely, reversal of movement direction was related to minimizing energetic costs of movement by moving in synchrony with tides and surface currents (Gibson, 2003), as suggested by plots of fish movements with respect to tide. Tidal current speeds in excess of sustained swimming capacity have been observed in the lower Penobscot Estuary (McCleave, 1978), supporting the hypothesis that observed reversal of movement direction might be related to tides rather than SW acclimation.

The apparent tidal modulation of acoustically tagged smolts in the Penobscot Estuary was indicative of selective tidal stream transport (STST; Forward & Tankersley, 2001). STST is a behavioural tactic that reduces energetic costs of migration for marine animals (Forward & Tankersley, 2001), and several examples exist in a number of invertebrate (Queiroga, 1998; Forward *et al.*, 2003; Criales *et al.*, 2011) and fish species (Barbin, 1998; McCleave & Arnold, 1999; Kelly & Kimley, 2012), including adult sockeye salmon *Oncorhynchus nerka* (Walbaum 1792) (Levy & Cadenhead, 1995). Changes in depth by *S. salar* smolts in the Penobscot Estuary suggested that fish were

located deeper during incoming tides than outgoing tides, a behaviour that would result in increased, rather than decreased exposure to SW during tidal cycles as would be expected from the laboratory results. Although tidal movements have been observed in estuarine and coastal waters by *S. salar* smolts and post-smolts (McCleave, 1978; Lacroix *et al.*, 2005), this behaviour has not previously been linked to STST due to incomplete information on individual behaviour during migration. This vertical pattern does, however, typify behaviours involved in STST (Gibson, 2003) used in reducing energetic expenditures of swimming against strong currents because current speeds are reduced near boundary layers (*i.e.* estuary bottom) compared with the upper water column. The vertical and horizontal movements of *S. salar* smolts in the Penobscot Estuary appear to support the hypothesis that STST is used by these fish to minimize energetic expenditures during early marine migration. Because all of the tagged smolts in this study demonstrated some apparent use of STST during migration through the middle estuary, and because all fish exhibited directional reversal of movements regardless of gill NKA activity, it appears reasonable that the vertical and horizontal movements of *S. salar* smolts through the lower estuary are based on minimizing energetic cost, and not physiological acclimation for SW entry. There may be, however, important interactions between osmoregulatory preparedness and osmotic perturbation incurred by increased energetic costs of migrating against currents that might manifest in terms of the duration and number of migratory reversals undertaken by individual smolts (Halfyard *et al.*, 2012), a hypothesis that merits further investigation.

This study has supported a number of existing hypotheses about *S. salar* smolt behaviour and corroborates the findings of previous research. Observations of migratory behaviour of smolts during estuary migration showed intriguing evidence for tide-related movement patterns and clear patterns in depth use with respect to salinity gradients through a 45 km estuary. The results of both the laboratory study and the field study suggested that, when given the choice, smolts generally use FW in greater relative proportion than SW. The ability of smolts at various developmental stages to enter and remain in SW, however, was related to an important enzyme used as an indicator of osmoregulatory capacity, as well as the ultimate physiological disposition of fish. The use of information from laboratory studies to guide investigation of interesting trends in estuary behaviour helped uncover important spatial and temporal trends in behaviour that otherwise might have gone un-noticed. Given the observation of a gradual salt wedge in the Penobscot Estuary with respect to distance and the advanced mediation of behaviour by smolts to prolong SW entrance in the wild, future experiments might investigate the mechanisms by which smolts make behavioural decisions about SW entry with respect to physiology and minimal detectable salinities. Such research could be particularly informative for decisions about where and when hatchery-reared *S. salar* smolts are stocked in recovery efforts.

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