Connecting physiological condition with salinity preference behaviour to infer estuary habitat choice in sockeye salmon smolts (*Oncorhynchus nerka*)

by
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B.Sc., Concordia University, 2018

Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Resource Management

in the
School of Resource and Environmental Management
Faculty of Environment

SIMON FRASER UNIVERSITY
Summer 2021

Declaration of Committee

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Abstract

The time period in which juvenile salmon remain in an estuary varies greatly among and within populations, with some individuals passing through estuaries in a matter of hours, while others remain in the estuary for several months. This individual variation in estuary use suggests that there may be underlying differences in individual salmon condition that temporally mitigate the selection of habitat, such as smolt size (fork length, mass, condition factor), stored energy (lipids and proteins), and osmoregulatory function (gill N^+-K^+-ATPase activity, NKA). I investigated the role of physical and physiological condition on the selection of estuarine and ocean habitat by sockeye salmon (*Oncorhynchus nerka*) smolts intercepted at the initiation of their downstream migration from Chilko Lake, Fraser River, B.C.. Since juvenile salmon energetic costs increase with rising salinity, I expected that smolts of lower physiological condition (i.e. low condition factor, poor energetic status and low NKA) would prefer to remain in the freshwater environment of the estuary, while smolts of higher physiological condition would prefer saline waters in the estuary and potentially indicate more rapid ocean entry. Behavioural salinity preference experiments were conducted on unfed smolts (n = 263) held in freshwater at three time intervals during their downstream migration period, representing the expected timing for lake exit, estuary entry, and ocean entry, at 0, 1, and 3 weeks respectively. Smolt condition factor (K), energetic stores and NKA predicted salinity preference behaviour in the estuary and ocean outmigration stages, but not at lake exit. Our results suggest that smolt physiological condition upon reaching the estuary may influence migratory behaviour and habitat selection, providing novel evidence on the temporally dependent interplay of physiology, behaviour and migration in wild juvenile Pacific salmon. As juvenile migratory behaviour is linked to physiological condition, and physiological condition is determined by productivity and competition within the rearing habitat, the importance of estuaries likely varies across years and within a population cohort; thus estuaries may be of heightened importance for wild juvenile salmon in years of poor freshwater growth conditions. These findings support the growing body of evidence on the importance of conserving both rearing habitat for juvenile growth potential, and estuarine habitat for smolt refugia before ocean entry.

Acknowledgements

The Fraser River is the largest river on the west coast of what is now called British Columbia. Every spring, thousands of salmon smolts leave their natal lakes and streams to begin their migration down the Fraser towards the Pacific Ocean. Carried by the waters of the Fraser, these juvenile salmon pass through the territories of over 90 First Nations: broadly, these are the Dakelh, Sekani, Wet'suwet'en, St'atl'imc, Secwepemc, Okanagan, Tsilhqot'in, Nlaka'pamux and Coast Salish (Fraser Basin Council, 2013). These communities have cared for this watershed, and the salmon within it, for over 10,000 years. I am humbled to be a student throughout these regions, and from this experience, I carry a responsibility to respect the lands, waters, rights and title of those who live there.

My research provides an incomplete eco-physiological view of the juvenile salmon migration; Indigenous knowledge is essential to understanding water systems, and the complex ecological relations within them (Atlas et al., 2021; Reid et al., 2021; Stefanelli et al., 2017) and is missing from my research. Future work, especially on cumulative effects of stressors throughout the life cycle, conservation and restoration efforts for watershed connectivity and estuarine habitat, should be guided, informed, and led by Indigenous knowledge holders, elders, and scholars. Indigenous knowledge systems encompass millennia of observations of people, watershed dynamics, and salmon management that is wholly lacking from Western sciences (Bozhkov 2020, Reid 2020, Carothers 2021). In addition, the effects of decisions regarding watersheds and salmon are felt deeply by Indigenous communities, who rely on their relationship with salmon for subsistence, culture, identity and livelihood (Steel 2021, Clark 2018, Voinot-Baron 2020); their voices should be heard first. Echoing the calls of many water scholars, I believe Western science, the "little sister", (Jess Housty Hakai 2020) should be guided by the steady hand of Indigenous Science (Atlas 2020), creating space for "Two Eyed Seeing" (Reid 2020) and for healing severed links within the weave of social-ecological systems of salmon.

I would like to extend the biggest thank you to all those who guided and supported my learning. My supervisors, Dr. Jonathan Moore and David Patterson, for trusting an entomologist to dive into the world of salmon science, welcoming me onto their teams, and for their continuous support throughout this project. Sam Wilson, for her generous guidance on my experimental design and analysis. The E-Watch crew, for teaching me how to be a fisheries biologist: Steve Healy, Kendra Robinson, Lida Nguyen-Dang & Angus Straight. All the incredible Salmon Watersheds lab-mates, for making me feel so very welcome, and surrounded by folks who care. Aimee Houde, for guidance on enzymatic protocols and analysis and answering an endless stream of questions. The extremely tolerant field technicians, Eric Olsen & Brian Hendricks, and the ever-enduring lab technicians, Eugenie Jacobsen, Paul Wilkinson & Emily Yungwirth. Sarah Ouimette, Victoria Chicatun, and Sophia Siedlikowski, true Masters of friendship and science, for ongoing support and proofreading this thesis. For all of my friends and family for supporting me through research, writing, whining, crying, and a global panini, and truly keeping me going. Thank you.

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Estuaries are an intersection of land and sea, where the fast -traveling fresh water from inland, meets the deep, steady salinity of ocean water. These two bodies of water meet, interact, but remain separate, a light freshwater lens above a heavy, saltwater foundation. There is so much to learn from this intersection, from two waters, “two ways” (Muller 2012) meeting and moving in respectful and reciprocal parallel.

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Introduction

Migration is a phenomenon integral to ecosystem dynamics; as individual animals move across ecosystem boundaries, seeking temporally or spatially dispersed resources, they carry with them nutrients, energy and pathogens (Webster et al., 2002). Pacific salmon provide a striking example of the interdependence of migrations and ecosystems, transporting nutrients from the ocean to nutrient-limited inland riparian and forest ecosystems (Naiman et al., 2002), the imprint of which can be seen from space (Brown et al., 2020). Communities, cultures, and industries have formed around the annual migrations of Pacific salmon (B.C. Wild Salmon Advisory Council, 2019). Although people and places depend heavily on the movements of these animals, migration itself is threatened by loss of habitat and habitat connectivity (Wilcove & Wikelski, 2008). Effective conservation of migratory animals, and the ecosystems that they connect, hinges on understanding the mechanisms that underpin migration. Knowing when and why animals use specific habitats requires a holistic understanding of individual behaviour and physiology within migrating populations.

Anadromous Pacific salmon (*Oncorhynchus spp.*) experience some of the most diverse environments and stressors of any aquatic animal, as they migrate through streams, lakes, rivers, estuaries, coastal waters and open oceans and back again to complete their complex life cycle (Healey, 2011; Hodgson et al., 2020). During smolt outmigration, juvenile salmon can experience fluctuations of up to 7˚C in temperature and 32 ppm in salinity in a single day (Idler & Clemens, 1959). Adapting to such contrasting environments requires complex physiological and behavioural changes that determine an individual's performance and subsequent survival. Studies that identify mechanistic links between individual physiology, behaviour and migration is of high priority in fisheries conservation (Horodysky et al., 2015; Lennox et al., 2019). Further, studying the physiology of migration provides an opportunity to assess changes and stressors between habitats, and the potential of these effects to carry-over or accumulate across

different life stages (Hodgson et al., 2019; Weber et al., 2003; Webster et al., 2002). In turn, migration depends on the response of individuals to internal and external cues that trigger this collective, synchronous movement. The sum of individual behaviour and physiology within populations forms the basis of migration (Ramenofsky & Wingfield, 2007).

As the transitionary zone between terrestrial watersheds and the ocean, estuaries form an important habitat along the salmon migratory corridor. As juvenile salmon leave their rearing habitat and swim towards the ocean, estuaries provide a range of salinities, resources and shelter for (i) physiological transition to saline conditions (Iwata & Komatsu, 1984), (ii) juvenile growth (Moore et al., 2016; Morrice et al., 2020), and (iii) refuge from predation (Sheaves et al., 2015) and parasites (Manel-La et al., 2009). Use of estuary habitat is associated with greater life-history diversity (Jones et al., 2014; Reimers, 1971; Simenstad et al., 1982), while the quality of estuarine habitat is correlated to returning adult survival (Magnusson & Hilborn, 2003; Meador & MacLatchy, 2014). Despite the apparent benefits of residing in estuaries, the time period in which juvenile salmon remain in an estuary varies greatly between and within populations, with some individuals passing through estuaries in a matter of days (Carr-Harris et al., 2015; Moore et al., 2016), while others remain in the estuary for several months (Beamish et al., 2016; Birtwell et al., 1987; Levy & Northcote, 1982). This variation in habitat use suggests that there may be underlying differences in individual condition that determine the importance of estuaries as stop-over habitat for juvenile salmon.

Multiple aspects of physical and physiological condition are associated with migration success in juvenile salmon. Smolt size and body condition are strong indicators of swim performance (Bams, 1967; Glova & McInerney, 1977; Taylor & McPhail, 1985a, 1985b) and contribute to migration speed at early ocean entry (Beacham et al., 2014; Freshwater et al., 2018). Juvenile salmon that are smaller and in poor condition can be at greater risk of predation in the ocean (Jeffries et al., 2014; Jonsson et al., 2011; Osterback et al., 2014; Tucker et al., 2016). Downstream migration in smolts is active, and the majority do not

feed, thus smolts rely largely on stored energy from the rearing habitat to fuel migration to the ocean. This energy is derived primarily from lipids, such as triglycerides, as well as protein (Brett, 1995). The amount of stored energy at any given life stage determines the potential for growth and the likelihood of survival in fish (Cunjakl, 1988; Larsen et al., 2000; Post & Parkinson, 2001; Sogard & Olla, 2000; Weber et al., 2003) and provides greater ecological and physiological relevance than traditional length or weight indices of fish condition (Minke-Martin et al., 2018; Trudel et al., 2005).

In addition to size and stored energy, the ability of a smolt to maintain homeostasis with increasing salinity along their migration route depends on the production and activation of osmoregulating enzymes in the gills. Na^+-K^+ ATPase (NKA) is the main enzyme responsible for osmatic regulation of teleost fish (Evans et al., 2005). In order to maintain osmoregulation when transitioning to habitats of increasing salinity, fish gill cells must actively excrete ions from the body into the ocean. NKA does this by creating an electrochemical gradient across the gill epithelial cell membrane that powers active transport of ions out of fish blood plasma and into the ocean. Activity levels of NKA are accurate predictors of downstream migratory behaviour of brown trout (*Salmo trutta*) (Nielsen et al., 2004), and are associated with smolt survival during migration to the ocean in Atlantic salmon (*S. salar*) (Stich, Zydlewski, & Zydlewski, 2015). The influence and interplay of smolt size, energetic stores and osmoregulation (gill NKA activity) on habitat selection during downstream migration is presently unknown and is the focus of this study.

Salinity preference is a commonly used metric to infer estuarine habitat choice (Baggerman, 1960; McInerney, 1963; Otto & McInerney, 1970; Price & Schreck, 2003b; D S Stich et al., 2016; Takei et al., 2013). Observations of tagged coho (*O. kisutch*) and Atlantic salmon found that smolts swim through vertical and horizontal gradients of salinity when passing through estuaries and that individuals vary greatly in occupied salinity and depth through migration (Hedger et al., 2008; Moser et al., 1991; Plantalech Manel-La et al., 2009). Experimental choice tests found that preference for saltwater increases

throughout migration in chum (*O. keta*), pink (*O. gorbuscha*), coho (*O. kisutch*), Chinook (*O. tshawytscha*), and sockeye salmon (*O. nerka*; Baggerman, 1960; McInerney, 1963). In turn, seawater preference decreases with stress (Price & Schreck, 2003b) and disease in Chinook salmon (Price & Schreck, 2003a), and decreases with sea lice infection in pink salmon (Webster et al., 2007). The physiology underlying this behaviour is attributed to seawater tolerance through gill NKA activity, controlled by multiple, interacting hormones, such as cortisol, thyroid hormones, growth hormone, insulin-like growth factor-1, and others (Iwata & Komatsu, 1984; McCormick, 2013; Stich et al., 2016). While the present understanding of the salmon endocrine triggers of downstream migration is complex, an energetic approach to explaining individual variation in smolt behaviour has yet to be undertaken.

I investigated the relationship between smolt physiology and salinity preference at three stages throughout the juvenile downstream migration period: (i) at the on-set of downstream migration from the rearing lake, (ii) 6-10 days after outmigration, when juveniles are expected to reach the Fraser River estuary, and (iii) 21-24 days after outmigration, when juveniles are expected to have fully passed through the influence of the Fraser river estuary and reach the ocean in the Northern Strait of Georgia and Johnstone Strait (Figure 1; Beacham et al., 2014; Clark et al., 2016; Johnson et al., 2019; Preikshot et al., 2012). Using salinity preference experiments to estimate habitat choice, I tested smolts for salinity preference at each of these 3 stages of migration. I formed three possible hypotheses (Table 1):

H_0 Salinity preference is not related to smolt condition (physical and physiological) or time since lake-exit

H_1 Salinity preference is dependent on smolt condition (physical and physiological) or time since lake-exit

H_2 Salinity preference is dependent on smolt condition (physical and physiological) and time since lake-exit

Based on findings from behavioural studies described previously, I predict that saltwater preference will be lowest at river-exit, intermediate at estuary-entry, and highest at ocean-entry [1]. Large body size and/or higher energetic condition of migrating smolts are associated with greater predator avoidance capacity (Glova & McInerney, 1977), prolonged swimming ability (Wilson et al. in press), faster migration (Freshwater et al., 2018), higher salinity tolerance (Conte & Wagner, 1965), and higher survival from smolts to adults (Henderson & Cass, 1991). These observations suggest that smolts of higher physiological condition may be better prepared for the increased predation and swimming capacity required in the ocean life stage. Thus, I predict that saltwater preference will be correlated to traits of increasing fish condition: high body condition (K), high gill NKA activity, and high densities of stored somatic energy (lipid, percent and triglyceride) [2]. This research will help to understand the importance of juvenile fish condition in smolt habitat selection throughout migration.

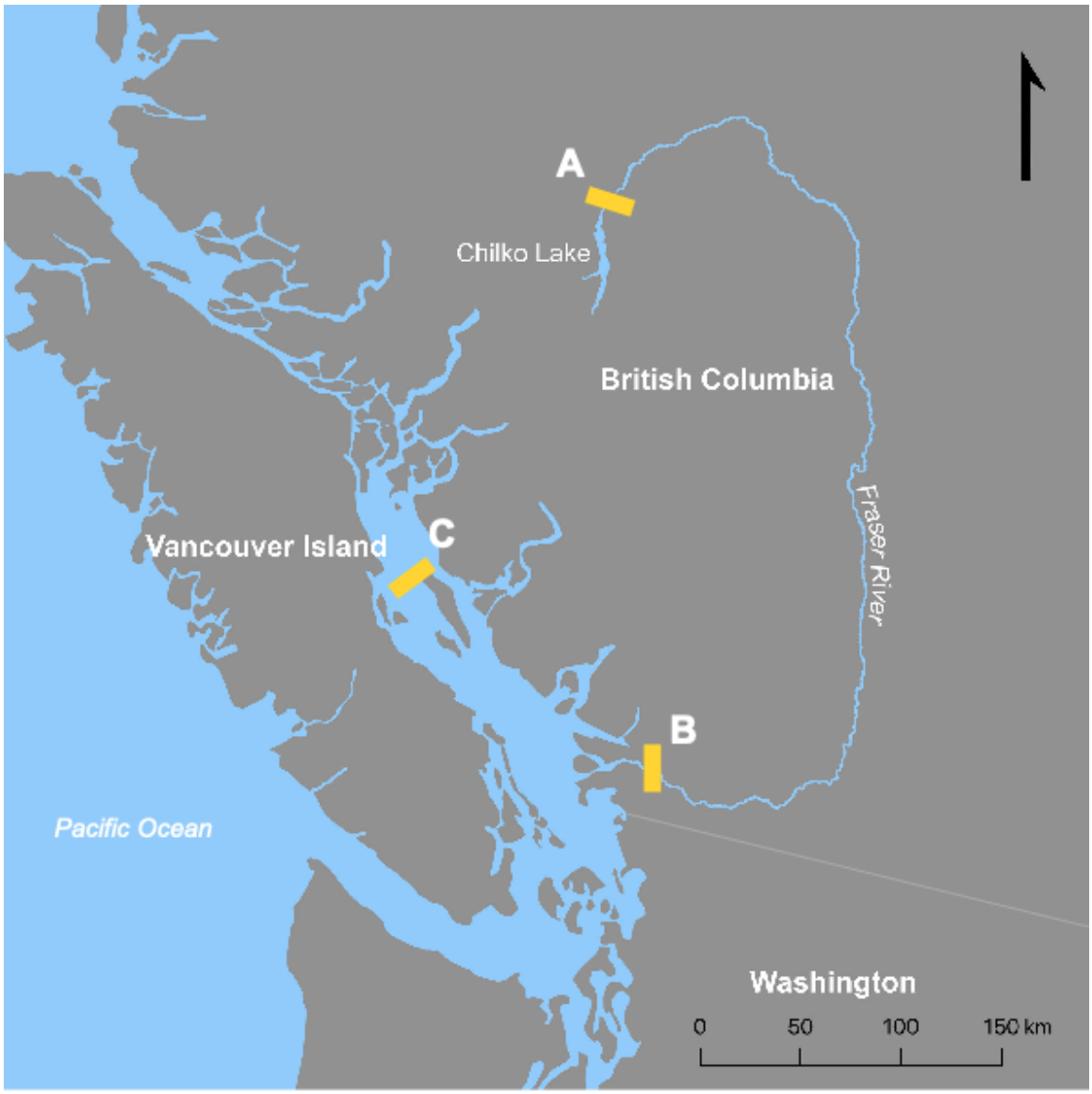

Figure 1. **Map of Chilko sockeye salmon (*O. nerka*) migration route. All smolts for this study were sampled from Chilko lake at the onset of outmigration (A). Smolts were tested for salinity preference at 0, 1, and 3 weeks after lake-exit, corresponding to the locations depicted as A (lake-exit), B (estuary-entry), and C (ocean-entry), respectively.**

Methods

Smolt Collection

This research is focused on the estuary of the largest producer of Pacific salmon in Canada, the Fraser River (Northcote & Atagi, 1997). Our focal population are

sockeye salmon from Chilko Lake (renamed to Tŝilhqox Biny, 11 March 2019, Government of British Columbia & Tŝilhqot'in National Government) located 650 km north of the Fraser River estuary, in the interior of British Columbia, Canada (Figure 1). The smolt outmigration timing of this population is well documented, most recently from tagging and tracking smolts from lake-exit to the Strait of Georgia (Clark et al., 2016); Timing data from this tracking study was used to design the stages of the behavioural experiments. Out-migrating yearling sockeye salmon smolts were collected from Chilko Lake between 22:00 and 4:00 April 30 – May 1, 2019. Smolts were collected by via dip net at a smolt fence and transferred to a river-side transport tank with continual flow-through of aerated river water.

Behavioural experiments were conducted on smolts at three time intervals during their downstream migration period, representing the expected timing for lake exit, estuary entry, and ocean entry, at 0, 1, and 3 weeks respectively (Clark et al., 2016). A random subset of smolts (n = 45) were selected to undergo salinity preference experiments at the lake-side field site to measure salinity preference, energetic reserves and physiology immediately following out-migration (hereafter, lake-exit). The remaining smolts were transported to the ALCAN research facility at Simon Fraser University, Burnaby, B.C., and housed in ambient air temperature, freshwater and natural photoperiod for the duration of the out-migration period (4 weeks following lake-exit, 2 – 29 May 2019). Smolts were randomly placed in one of four 80 gallon holding tanks, each with a maximum density of approximately 15 g m^{-3}. Water quality in holding tanks was maintained at 8.4˚C (CI_{95}[8.25, 8.48]), 98.6 % dissolved oxygen (CI_{95}[98.04, 99.10]), and <0.25 ppm ammonia. Water temperatures are consistent with downstream migration conditions (MacDonald et al. 2018) and are low enough to prevent desmoltification. Nine mortalities were incurred during the four-week holding period; 2 associated with anaesthesia and elastomer tagging and 7 associated with holding.

All smolts were held in freshwater without feeding during the 4 week migration period. Previous work indicates that acclimation to various salinities

does not increase salinity tolerance in smolts (Morgan & Iwama, 1991), nor does fasting impact swim performance (Hvas et al., 2021) until a critical limit of starvation, which smolts in this study did not reach (Wilson et al., n.d.). Observations of empty stomachs of migrating Chilko sockeye smolts caught at lake-exit and the beginning of the estuary, Mission, BC (Chalifour et al., 2019) indicate that this population does not feed extensively during the freshwater migration stage (David Patterson, pers. comms). Freshwater holding experiments on Chilko smolts found that many refused feeding following initiation of migration (Clark et al., 2016), yet holding experiments in saltwater report that Chilko smolts resume feeding after 28 days since lake outmigration (Wilson et al., n.d.). A variable portion (20-40% from 2004 – 2009, 29% in 2014) of juvenile sockeye collected in marine trawls in the Strait of Georgia have empty stomachs (Beamish et al., 2012; Neville et al., 2016) and show gene expression patterns that suggest recent fasting (Houde et al., 2019). To reflect these observations, wild-caught smolts were not fed during the holding period.

Six fish were observed simultaneously in each trial to replicate the natural schooling behaviour of juvenile sockeye salmon, while allowing for determination of individual preference (Webster & Dill, 2007; Tierney et al. 2009). To distinguish individual smolts during the preference test, smolts were tagged with coloured elastomer (Northwest Marine Technology ©). Smolts were anesthetised using an aerated bath of 100 mg l^{-1} tricaine methanesulfonate buffered with 200 mg l^{-1} sodium bicarbonate for a maximum of 3 minutes until no movement occurred when the tail was grasped by an experimenter, while ensuring that operculum beating was regular. Elastomer tags were injected into the base of the anal fin via a 0.33x12.7mm sterile syringe, allowing the elastomer to fill the fin membrane between 1-2 fin rays. This allowed the tag to be seen maximally from both sides of the fish, while limiting fin and tissue damage. Smolts were then allowed to recover in an aerated 20L aquarium recirculating at 7-9˚C. Once equilibrium and swim function were restored, tagged smolts were grouped by tag colour and transferred to labeled containers within holding tanks. Tagging occurred the day before a trial to allow for a minimum of 18 hours for recovery.

Experimental Design

To determine salinity preference of juvenile salmon, I observed the movement of individual smolts between chambers of freshwater (0-2 ppm), brackish water (14-16 ppm) and saltwater (30-32 ppm); the proportion of time spent in each chamber (and its corresponding salinity) was calculated for each fish. Given that all other conditions of the tank were controlled for, I assume that occupancy time is directly correlated to the preference of the individual smolt to the water salinity of the chamber.

The design of the experimental preference tank was modified from previous studies to test the preference for three salinities simultaneously (Baggerman, 1960; Houston, 1957; McInerney, 1963; Price & Schreck, 2003b). Three 20 L glass aquaria were placed side-by-side within a 120 L aquarium (Figure 2). This divided the aquarium into three chambers of equal size, while allowing for a 2-inch gap above the dividing walls for fish to cross between chambers. The outside and bottom of the aquarium was darkened with black plastic to minimize stress imposed on the fish during the experiment. The walls between chambers were left transparent to allow smolts to view conspecifics through the dividing walls, even if smolts were in different chambers. Smolts were tested in groups of six to allow for schooling behavior of sockeye salmon along the horizontal plane (Hoar et al., 1957; Katzman et al., 2010; Tierney et al., 2009; Webster & Dill, 2007), regardless of individual salinity choice. The tank was illuminated uniformly from above using an LED light, and surrounded by shade cloth to prevent visual disturbance. A video camera (GoPro Hero 3 ©) was positioned facing the long side of the aquarium to record fish position within all three chambers throughout the experiment. In addition, an experimenter recorded fish position (chamber and depth) and behavior every ten minutes through viewing slots in the shade cloth.

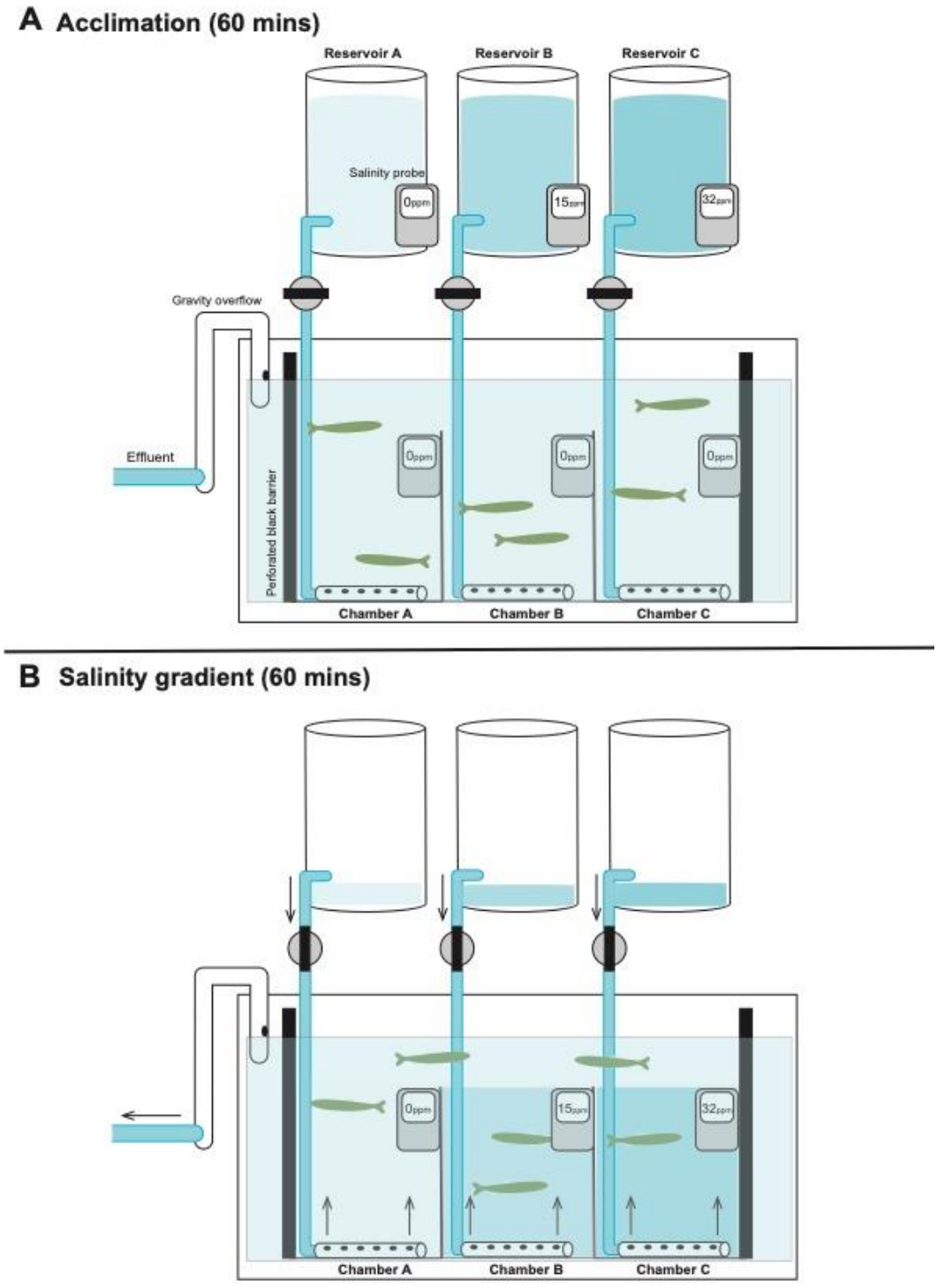

Figure 2. **Experimental tank design to test for salinity preference of juvenile salmon. Smolts were transferred to the experimental set up in aerated freshwater (A) for 1 hour acclimation. A salinity gradient was then formed through bottom-fed fresh, brackish or salt water in respective chambers (B). Salinity preference as chamber occupancy was observed over 1 hour.**

Initially, all three chambers were filled with fresh, aerated water (0.0 -0.2 ppm, 8.0 - 9.7 ˚C, 97.2 - 101.6 % DO, n_{trials} = 42, Figure 2A). Six smolts were randomly selected from holding tanks and transferred to the experimental aquarium, ensuring equally dispersed, randomized placement throughout the three chambers. The smolts were allowed to acclimate to the aquarium and move between the three chambers for 1-hour. Behaviour and position of smolts were recorded during the acclimation period and used to assess chamber preference bias independent of salinity changes.

After the 1-hour acclimation period, a horizontal salinity gradient was imposed in the experimental tank by displacing the water of each chamber with either fresh, brackish, or saltwater (Figure 2B). The order of salinity of each chamber was randomized between each trial to control for any effect of chamber location or order on fish behaviour or movement. Each chamber was filled by separate water reservoirs that fed into the bottom of each chamber taking approximately 5 mins to complete. In this way, fresh, brackish or saltwater could be slowly added in from the bottom of each chamber, displacing the original freshwater from the acclimation period. Instant Ocean (Instant Ocean ©, Spectrum Brands 3001 Commerce St. Blacksburg, VA 24060-6671) was added to filtered, aerated water to make brackish (14-16 ppm) and saltwater (30-32 ppm) and each were added to the respective reservoirs. The freshwater reservoir was filled with filtered, aerated water (0-2 ppm). When the higher-density salt and brackish water is introduced to two of the three chambers, the lower-density freshwater is displaced, forming a halocline. The upper freshwater layer of the halocline serves as a freshwater bridge in which the fish can move freely between chambers throughout the experiment. As water from the reservoirs was added to the chambers, the displaced freshwater was simultaneously drained from the aquarium using a gravity filtration system. Preliminary trials showed that the halocline was stable for over 2 hours and the salinity gradient was maintained even with fish actively moving within the tanks. Salinity probes (Marine Salinity Waterproof Tester ©, HI98319, HANNA Instruments) were fixed within each chamber to monitor salinity and temperature throughout the duration of the

experiments and ensure that haloclines were stable. Once the salinity gradient was imposed and stable, fish position (chamber and depth) and behavior (exploratory or non-exploratory) was recorded every ten minutes for one hour. If a fish was in the freshwater layer above the halocline of a chamber, it was recorded as being in freshwater, regardless of the salinity of the chamber below. Similar experiments have found that salinity preference stabilizes after 40 – 60 minutes (Price & Schreck, 2003b).

Two trials were removed from behavioral analysis. In trial 26A, improper mixing in the reservoir resulted in the saltwater chamber being of equal salinity to the brackish chamber. In trial 21B, one fish became moribund and may have imposed stress on conspecifics of the experiment. In total, 11 smolts out of the 263 tested were removed from behavioural analysis due to experimental failure (Salinity preference behavioural dataset: n = 252).

Physiological Sampling

Following preference trials, smolts were euthanized in tricaine methanesulfonate at a concentration of 400 mg l^{-1}(buffered with 800 mg l^{-1} $NaHCO_3$) until fish operculum beating ceased and reflex response was absent (Neiffer & Stamper, 2009). Immediately after euthanization, smolts were destructively sampled for blood, gill and tissue samples. Blood was extracted via 0.45mm x 10mm sterile heparinized syringes and centrifuged in a centrifuge vial for 5 minutes. Plasma was then extracted via a pipette and both plasma and remaining blood cell were frozen at -80˚C. For gill NKA activity, gill samples were taken from each smolt and stored in SEI Buffer (250 mM sucrose, 20 mM Na_2EDTA, 50 mM Imidazole, pH 7.3). Smolts were then measured for fork length to the nearest mm and body mass (to the nearest 0.01 g). The smolt carcasses were stored in individual whirlpacks and all samples were immediately frozen using dry ice. Blood, gill and carcasses were then transported to the laboratory and stored at -80˚C until analysis.

Measures of Stored Somatic Energy

To estimate the bioenergetics of individual smolts, lipids, protein, water and ash were isolated and measured as proximate constituents following the Bligh and Dyer chloroform extraction method (Bligh & Dyer, 1959). Whole carcasses were thawed at room temperature and re-measured for fork length and mass (to allow for corrections for potential moisture loss associated with freezer storage). Stomachs were retained in samples since all smolts were held without feeding for a minimum of 24 hours before euthanization. In brief, whole thawed carcasses were homogenized in a SPEX SamplePrep 2010 Geno/Grinder (SPEX, Metuchen, NJ) at 1500 rpm in two-minute intervals, for a maximum of 8 minutes. Two replicate samples of 0.3 g +/- 0.015 g of the homogenate were mixed with 1:1:0.48 ratio of methanol, chloroform and water and homogenized to dissolve lipids and protein. Each homogenate mixture was filtered through a Büchner funnel to remove undissolved solids, and the supernatant was decanted into a graduated cylinder. The resulting volume of the lower, more dense layer, comprised of lipid and chloroform was measured, while the upper layer, comprised of water and methanol was removed by aspiration. A 100 µl sub-sample of the lipid-chloroform layer was frozen at at -80^{o}C for triglyceride (TAG) analysis.

Sample percent lipid was calculated by taking the ratio of isolated lipid extracted from the homogenized tissue (g) to the weight of the sample used (g) and multiplying it by the ratio of the volume of chloroform + lipid used to dilute the homogenate (ml) to the volume of chloroform + lipid extracted to isolate the lipid (ml). If replicates differed in percent lipid by more than 20% CV, another sample was taken from the homogenized tissue and the lipid extraction was redone, given that enough homogenate remained. The average **percent lipid** was calculated over all replicates used (range of two - four replicates per sample). Standard deviation and coefficient of variation of the sample percent lipid replicates was reported for each smolt.

A sub-sample of each homogenate was completely dehydrated at 100˚C for 8-24 hours. **Sample percent water** was calculated by first taking the ratio of

the dehydrated sample weight (g) to the homogenized sample weight (g) to get the percent moisture loss, and then taking the difference in percent moisture loss from 100. The dehydrated sample was then heated to 500˚C for 3-4 hours until the sample was fully combusted into ash. **Sample percent carbon** was calculated by the ratio of fully combusted ash sample weight (g) to the homogenized sample weight (g). **Percent protein** was estimated as the percent difference from the sum of percent lipid, water and carbon (Trudel et al., 2005). Energy data from four out of the 263 smolts analyzed were removed from further analysis due to high variance among replicates for lipid, carbon and water estimates (CV> 20%; three from the lake-exit group, and one from the ocean-entry group).

The **energy density (ED)** of each smolt was calculated from lipid (*f*) and protein (*p*) measurements using the following equation (Breck, 2008):

$$ED = fD_f + pD_p$$

where *ED* is expressed in units kJ/g wet weight, *f* is the fraction of lipid measured per smolt (g/g wet weight), D_f is the energetic density of lipids reported for coho salmon (39.54 kJ g^{-1}) (Brett & Groves, 1979; Crossin et al., 2004; Higgs, 1979), *p* is the fraction of protein estimated per smolt (g g^{-1} wet weight), and D_p is the energy density of protein reported for coho salmon (23.64 kJ g^{-1}) (Crossin et al., 2004; Higgs, 1979).

Triglycerides are the major energy storage form of lipids in fish and have high ecological and physiological relevance as indicators of growth potential (Hakanson et al., 1994; Lochmann et al., 1995). A colorimetric method was used to extract percent triglycerides for each smolt. Absorbance was read at 540nm in a FLUOStar Omega © optic fluorescence spectrometer. TAG concentration (mg dL^{-1}) was estimated by the ratio of the blank-corrected absorbance (nm) to the standard slope of each spec plate (nm mg^{-1} dL^{-1}). TAG concentration was converted to the **percent ratio of TAG to lipid** by multiplying TAG concentration (g mL^{-1}) by the total volume of chloroform used for lipid extraction and dividing by the mass of lipid extracted using the Bligh & Dyer method. The **percent ratio of TAG to lipid** represents the density of TAG within lipid stores in individual fish.

Osmoregulatory preparedness: Gill NKA activity

Salinity tolerance is commonly measured as a change in plasma osmolality 24 hours after transfer to seawater (McCormick et al., 2009). Plasma osmolarity is inversely correlated with gill Na^+-K^+-ATPase (NKA) and as such, gill NKA is a common metric to estimate salinity tolerance (Bassett, 2015; Elsner & Shrimpton, 2018; Stich et al., 2016; Zydlewski et al., 2014). Immediately after completion of salinity preference experiments, and following euthanization of smolts, gill samples were clipped from the right gill arch and frozen on liquid nitrogen in SEI buffer to preserve enzyme structure and function. Following the enzymatic assay protocol from McCormick (1993), gill NKA activity was measured as the production of ADP from ATP dephosphorylation. ADP production is coupled with reactions from two other enzymes, pyruvate kinase and lactate dehydrogenase. Pyruvate kinase uses phosphoenolpyruvate and ADP to form pyruvate and ATP, which is then used by lactate dehydrogenate to reduce NADH to NAD+. The rate of disappearance of NADH, which is equimolar to the rate of production of ADP by sodium potassium ATPase obtained from the smolt gill sample, was measured by a FLUOStar Omega © optic fluorescence spectrometer.

Reagent quality was checked prior to each assay by running a standard curve of ADP consumption in the absence of sodium potassium ATPase, in which the acceptable range was -0.17 and -0.2 mOD $nmol^{-1}$. Each gill sample was measured in triplicate. Each replicate was coupled with a sample that was inhibited by 0.5 mM ouabain, which blocks Na^+-K^+-ATPase activity. Assay mixtures of homogenized gill tissue samples and reagents were mixed and immediately measured for change in absorbance at 340 nm and 25˚C. The linear slope of ATP hydrolysis (or the equimolar disappearance of NADH) was calculated over 10 minutes for each sample in Omega BMG Labtech Software © 5.10 R2. This differs from McCormick (1993), who calculated the slope between 3 and 9 minutes of the assay to avoid the beginning and end of the assay where the enzyme rate may be non-linear (increasing at the start to reach maximum rate, or decreasing at the end due to limited remaining reactants). Slopes for all Na^+-K^+-ATPase activity were calculated using the 10 minute protocol, as enzyme

rate over the 10 minute period was observed to be linear; further, the difference in variation and activity between protocols was negligible (see Appendix, Figure A1).

The slope of ATP hydrolysis was then corrected by dividing by the measured ADP extinction coefficient standard curve ($\bar{x}$ = 20.754 mOD nmol^{-1} NADH, *SE* = 0.140, *n* = 3). The enzyme activity of each well was then scaled by the corresponding concentration of protein from each tissue sample using the BCA Protein Assay Kit © (Product No. 23225 from Pierce P.O. Box 117, Rockford, Illinois 61105). Protein assays were measured in triplicate, with bovine serum albumin as the standard, read at 25˚C and 550 nm absorbance. The final metric of NKA activity was determined as the difference between uninhibited and ouabain-inhibited rates of ATP hydrolysis in units of micromoles ADP per milligrams of protein per hour.

Readings of ATP hydrolysis above 0.5 mOD nmol^{-1} were denoted as out of acceptable range and were removed from NKA activity calculations for that sample (McCormick et al., 2009). Readings of ATP hydrolysis in ouabain-inhibited samples that exceeded the corresponding rate of uninhibited samples were denoted as measurement error and were also removed from NKA calculations for that sample. Variation in measurements of NKA activity came primarily from three sources: (i) variation among replicates of spectrometry readings of uninhibited ATP hydrolysis, (ii) variation among replicates of spectrometry readings of ouabain-inhibited ATP hydrolysis, and (iii) variation among replicates of spectrometry readings of BCA protein concentration assays. To account for variation in all sources, I calculated the coefficient of variation for each sample from the pooled variance as follows:

$$s_p^2 = \frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2 + (n_3 - 1)s_3^2}{n_1 + n_2 + n_3 - 3}$$

Where the subscripts 1, 2, and 3 refer to the three sources of variation listed previously, *n* is the sample size and *s* is the variance for each variable. Eight gill samples out of the 263 produced high variation among replicates (CV>20%) and were removed from further analysis.

Behavioural Analysis

Salinity preference was calculated for individual smolts as the salinity of the chamber in which the smolt was observed more than 50% of the time after the salinity gradient was established (for a total experimental duration of 60 minutes). For the majority of trials, the final chamber occupied was also the chamber with the longest occupancy (see Appendix B, Figure B3). If a smolt spent 50% of the experimental time in each of two chambers, salinity preference was determined as the salinity of the occupied chamber at the end of the trial. The standard deviation of salinity preference was calculated using the Wald normal approximation interval for a binary outcome; in this case, the binary outcome is preferred v. non-preferred chamber (Wallis, 2013).

$$s = \frac{\sqrt{p(1-p)}}{n}$$

Where *n* is the sample size (6 observations per fish per trial), *p* is the proportion of observations in the preferred chamber, and *s* is the standard deviation of the proportion of observations in the preferred chamber. Some fish were observed to remain in the chamber in which they were initially placed, and thus did not explore the experimental tank. For these fish, salinity preference could not be calculated with certainty, as they may not have moved from the chamber they were placed in due to salinity preference or because they did not explore the other chambers and thus were unaware of the other choices. Fish that did not move chambers throughout the acclimation or experimental stages of trials were not included in analyses of salinity preference (see Appendix B).

Smolt Condition Analysis

263 smolts were tested for salinity preference. Two trials (12 smolts) were removed due to experimental failure, resulting in 252 smolts from successful behavioural trials. Four smolts were removed due to high variance in energetic analysis. The final dataset of salinity preference behaviour coupled with energetic

profiles includes 248 smolts, of which 133 were non-exploratory and 115 were exploratory.

To test for differences in size, mass and physiological condition between outmigration stages, I compared group distributions using a Type II ANOVA and Tukey *post hoc* test in the anova_test and tukey_hsd functions in the rstatix package of R version 4.0.2 (Kassambara, 2020b). Normality of variables was assessed visually using the ggqqplot function in the ggpubr package (Kassambara, 2020a), while heteroscedasticity of residuals was visually assessed using the plot function of the base package (R Core Team, 2018).

Salinity Preference Analysis

I tested the hypothesis that salinity preference of smolts is dependent on their physiological condition, gill development, and the stage of their outmigration. The possible values for the dependent variable, salinity preference, are ordered by increasing salinity: freshwater (approximately 2ppm) < brackish water (approx. 15 ppm) < saltwater (approx. 32 ppm). The choice of occupying a chamber is inherently dependent on the previous chamber occupied, as well as the other chambers. This lack of independence violates the assumptions of ANOVA and MANOVA (Bruzzone & Corley, 2011). Ordinal logistic regression accounts for this dependency by considering the probability of the choice of a salinity as well as all other salinities through cumulative log odds (Bilder & Loughin, 2014).

$$logit(P(Y \leq j) = log\left(\frac{P(Y \leq j)}{1 - P(Y \leq j)}\right)$$

Where *Y* is the ordered response variable of salinity preference with levels *j* =1 (freshwater, ~2 ppm), 2 (brackish, ~15 ppm), and 3 (salt water, ~ 32 ppm). For each category of Y (preferring freshwater, brackish, or saltwater), the parameter β_{j0} changes, but the slope for each unit of a given explanatory variable remains constant. There is no equation for the proportional log odds of the highest category of salinity preference, saltwater (*j* = 3 = *J*), as the probability

of observing saltwater preference, $P(Y \leq salt)$, inherently includes brackish and freshwater, and so $P(Y \leq salt) = 1$.

$$P(Y \leq j) = \frac{\exp\left(\beta_{j0} + \beta_1 X_1 + \cdots + \beta_p X_p\right)}{1 + \exp\left(\beta_{j0} + \beta_1 X_1 + \cdots + \beta_p X_p\right)}$$

The probability for observing category *j* is:

$$P(Y = j) = P(Y \leq j) - P(Y \leq j - 1)$$

For each of the three experimental salinities in this study, the probability of observing preference is:

$$P(Y = freshwater) = \frac{\exp\left(\beta_{j0} + \beta_1 X_1 + \cdots + \beta_p X_p\right)}{1 + \exp\left(\beta_{j0} + \beta_1 X_1 + \cdots + \beta_p X_p\right)} - 0$$

$$P(Y = brackish) = \frac{\exp\left(\beta_{j0} + \beta_1 X_1 + \cdots + \beta_p X_p\right)}{1 + \exp\left(\beta_{j0} + \beta_1 X_1 + \cdots + \beta_p X_p\right)} - P(Y = freshwater)$$

The probability for category *J* is the difference between 1 and the probability of the second last category (in this case, preferring brackish water):

$$P(Y = saltwater) = 1 - P(Y = freshwater) - P(Y = brackish)$$

The odds ratio (*OR*) was calculated for each explanatory variable (*X*) as:

$$OR = \exp(c\beta)$$

Where *c* is set as one standard deviation of a given continuous explanatory variable, and as *c* = 1 for categorical variables. β is the slope parameter for a given explanatory variable.

Ordinal logistic regression assumes that the dependent variables are inherently ordered, the absence of multicollinearity between independent variables, and proportional odds between each pair of outcome groups (Bilder & Loughin, 2014). The salinity preference experiment was designed to limit the choice of occupation to three chambers of increasing salinity, thus ordering the dependent variable as freshwater, brackish water or saltwater preference. Due to high collinearity among proximate constituents of somatic energy, energetic

density and condition variables, I ran each variable as separate predictors of salinity preference. The assumption of proportional log odds was tested for each model following Bilder & Loughin (2014) (Suppl. Table 1). The only exception is predicting salinity preference by NKA activity in the river outmigration stage: in this group, only one smolt chose saltwater, and that smolt displayed high NKA activity; when tested, this results in a predicted logit of negative infinity. Although this variable violates the assumption of proportional odds, I included it in the model for the river outmigration group as the it reflects the true biological nature of the data and transformations failed to be useful. When predicting salinity preference by NKA activity in the estuary and ocean outmigration groups, and across all three outmigration groups, the proportional odds assumption holds. I used the function polr() in the R package MASS (Ripley, 2020; Venables & Ripley, 2002) to predict the ordinal outcome of salinity preference by measurements of smolt physiological condition for each outmigration stage separately. The assumption of normality and homoscedasticity of variables and residuals were verified via visual assessment for all models.

Smolts were held in freshwater and unfed for up to 4 weeks after lake-exit as previously justified. To account for the innate decline in smolt condition (mass, condition factor, lipid and protein stores) with holding time, I used the residuals of the linear model of smolt condition by day of year as relative smolt condition to predict salinity preference across outmigration stages. The residuals of smolt condition by date thus account for daily changes in physiological and physical condition under the assumption that no feeding occurred during the downstream migration to the ocean. I detected a decrease in fork length throughout the experimental period (n = 115, Intercept = 87.25 mm, β = -1.49 x10^{-1} mm day^{-1}, SE = 0.059 mm day^{-1}, p = 0.01). To account for this size-bias, I included fork length as a variable in all iterations of the full model.

When testing for salinity preference across outmigration stages, I formed three possible hypotheses that I tested using ordinal logistic regression models (Table 1). Models were selected by the second-order bias correction of Akaike's Information Criterion (AIC_c), recommended for comparison of models of sample

sizes where *n* per *K* ≤ 40. AIC_c values were calculated with the function confset() in the package AICcmodavg (Burnham et al., 2011; Mazerolle, 2020), using the following equation:

$$\mathrm{AICc} = -2\log(L(\hat{\theta})) + 2K + \frac{2K(K+1)}{n-K-1}$$

Where *n* is the sample size, -2 $\log(L(\hat{\theta}))$ is the log likelihood estimates of the model parameters, and *K* is the number of parameters fitted by the regression model. The top model represents the most parsimonious model which minimizes information loss.

Table 1. Experimental hypotheses and model parameters. Variables of physical condition are fork length, mass and condition factor (K). Variables of physiological condition are % lipid, % protein, % triglyceride (TAG), and energy density (ED).

	Hypothesis	Model(s)
H_0	Salinity preference is not related to smolt condition (physical and physiological) or time since lake-exit	preference ~ 1
H_1	Salinity preference is dependent on either smolt condition (physical and physiological) or time since lake-exit	preference ~ physical condition preference ~ physiological condition preference ~ outmigration stage
H_2	Salinity preference is dependent on both smolt condition (physical and physiological) and time since lake-exit	preference ~ physical condition + outmigration stage preference ~ physiological condition + outmigration stage preference ~ physical condition + physiological condition + outmigration stage

Results

I tested the hypothesis that smolt outmigration behaviour is dependent on smolt physical and physiological condition and outmigration stage. Chilko sockeye smolts were tested for salinity preference and coupled with physiological profiles (*n* = 248) across three stages of outmigration: at the on-set of downstream migration from the rearing lake (lake-exit group, *n* = 45), 6-10 days after outmigration, when juveniles are expected to reach the Fraser River estuary (estuary-entry group*, n* = 120), and 21-24 days after outmigration, when juveniles are expected to have fully passed through the estuary and reach the ocean (ocean-entry group, *n* = 83). Physiological profiles of smolts include physical condition (Fulton's condition factor, K), physiological condition (% lipid, % protein, % triglyceride, energy density, and gill NKA activity).

Smolt behaviour through downstream migration

Salinity preference behaviour varied over time, consistent with the natural transition from river to estuary to ocean (Figure 3, Table 2). At the onset of lake outmigration, the majority of exploratory smolts preferred freshwater (83%), and very few smolts selected brackish (13%) or saltwater (4%). A shift in salinity preference occurred at estuary-entry, where brackish water preference (37%) became equal to freshwater preference (37%), and saltwater preference increased to 26% of exploratory smolts. This general trend of preference held when tested at ocean-entry, two weeks later, with 43% of exploratory smolts preferred freshwater, 31% preferred brackish, and 26% preferred saltwater.

Out of the 248 smolts in which both salinity preference and physiological profiles were obtained, 54% were non-exploratory (*n* = 133). Non-exploratory smolts, those that did not move from the initial chamber in which they were placed, were not included in salinity preference models, as preference cannot be accurately determined without exploration or knowledge of alternatives (see Methods for further justification). Predictors of non-exploratory behaviour are analyzed in detail in Appendix B and are briefly summarized here. The general

temporal trend of salinity preference was similar regardless of exploratory behaviour (Table 2, Figure 3). The duration of time that wild smolts were held since capture (*ie*. the number of days since lake-exit) did not change the proportion of fish that exhibited exploratory behaviour (Appendix B, Figure B1), nor was there sufficient evidence of physical or physiological differences between exploratory and non-exploratory smolts (Appendix B, Figure B2). Inclusion of non-exploratory smolts in salinity preference models resulted in the same variables of smolt condition selected for best model fit, and the same directionality between variables of smolt condition and preferred salinity (Appendix B, Figure B3).

Table 2. Salinity preference and exploratory behaviour of smolts through outmigration experiment. The total number of exploratory smolts that preferred freshwater, brackish water, or saltwater are listed for each outmigration stage, with mean standard deviation of the proportion of time in preferred chamber denoted in brackets. Non-exploratory smolts are those that did not swim between chambers and thus have a standard deviation of zero. The most preferred salinity is highlighted for each stage and behaviour in bold.

Outmigration stage (days since lake outmigration)	Behaviour	Salinity Preference *n* (SD)		
		Freshwater	Brackish water	Saltwater
Lake-exit (1)	All	**37** (0.03)	6 (0.08)	2 (0.1)
	Exploratory	**25** (0.05)	4 (0.13)	1 (0.19)
	Non-exploratory	**12** (0)	2 (0)	1 (0)
Estuary-entry (6-10)	All	44 (0.03)	**45** (0.04)	31 (0.03)
	Exploratory	**16** (0.09)	**16** (0.1)	11 (0.08)
	Non-exploratory	28 (0)	**29** (0)	20 (0)
Ocean-entry (21-24)	All	**31** (0.07)	28 (0.07)	24 (0.05)
	Exploratory	**18** (0.13)	13 (0.14)	11 (0.10)
	Non-exploratory	13 (0)	**15** (0)	13 (0)

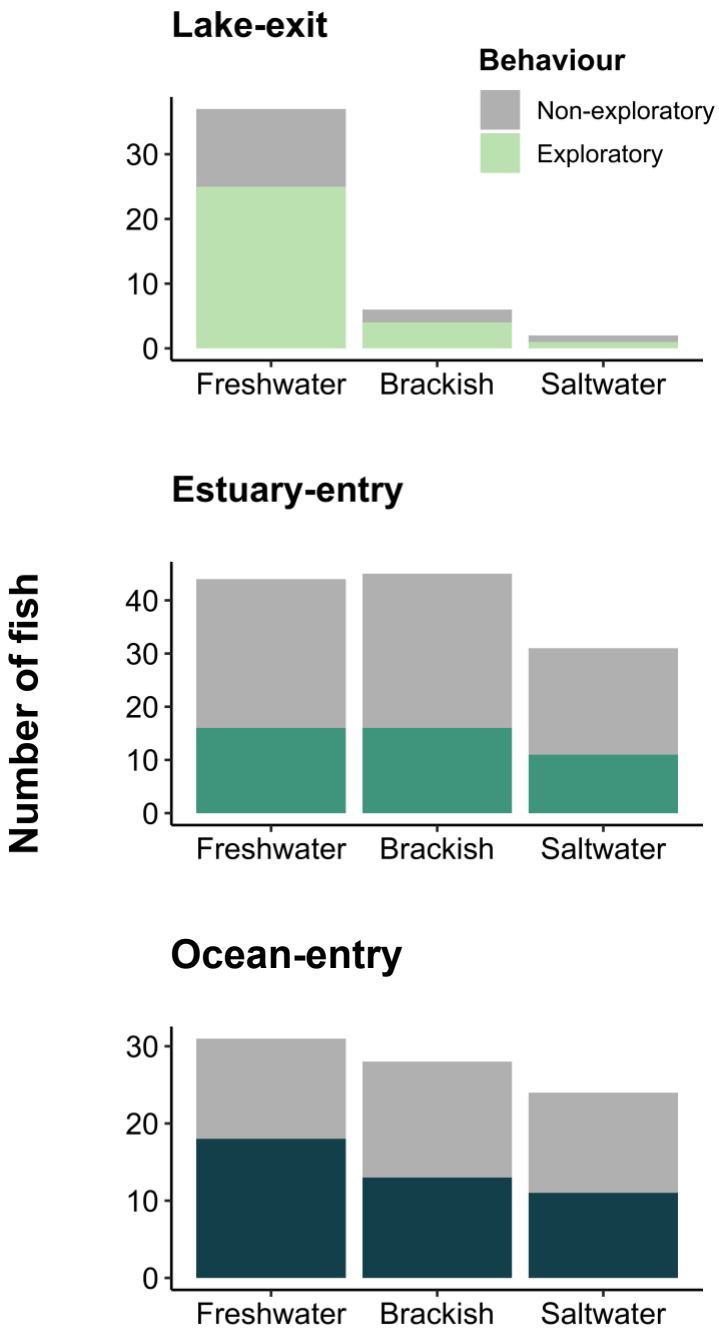

Figure 3. **Total number of smolts preferring freshwater, brackish or saltwater at lake-exit, estuary-entry, and ocean-entry. Non-exploratory fish are shaded in grey, while exploratory smolts are colored by migration stage.**

Smolt physiology through downstream migration

Smolt physical and physiological condition generally declined throughout the smolt outmigration window (May 1-24, 2019). Smolts that were tested at lake-exit were on average larger (*M* = 88 mm fork length, *SD* = 5 mm) than smolts tested at ocean-entry (*M* = 84 mm, *SD* = 5 mm, Table 3). Over the 3-week, freshwater, non-feeding migration period, smolts declined by 0.97 g in mass, 0.08 in condition factor (K), 1.01% lipid, 1.03% protein, 17% triglyceride (TAG), and 0.64 kJ g^{-1} energy density (ED); conversely, percent water increased by 2.04 %. The only physiological condition parameter that did not show a significant change throughout the smolt outmigration window was gill NKA, with an overall average activity of 8.4 µmol ADP mg $protein^{-1}$ hr^{-1} (SD = 3.0, *n* = 110, Figure 3).

Size-metrics (fork-length, mass, K) showed weak-moderate correlations (Pearson's $r < \pm 0.5$) with physiological variables (% water, % lipid, % protein, ED, %TAG). This held across outmigration stages (Suppl. Figure 1). Of the physiological variables measured, percent water was highly correlated to % lipid (Pearson's $r < -0.68$) and ED (Pearson's $r < -0.73$). Gill NKA did not correlate strongly with any of the size- or energetic-metrics assessed (Pearson's $r < 0.5$).

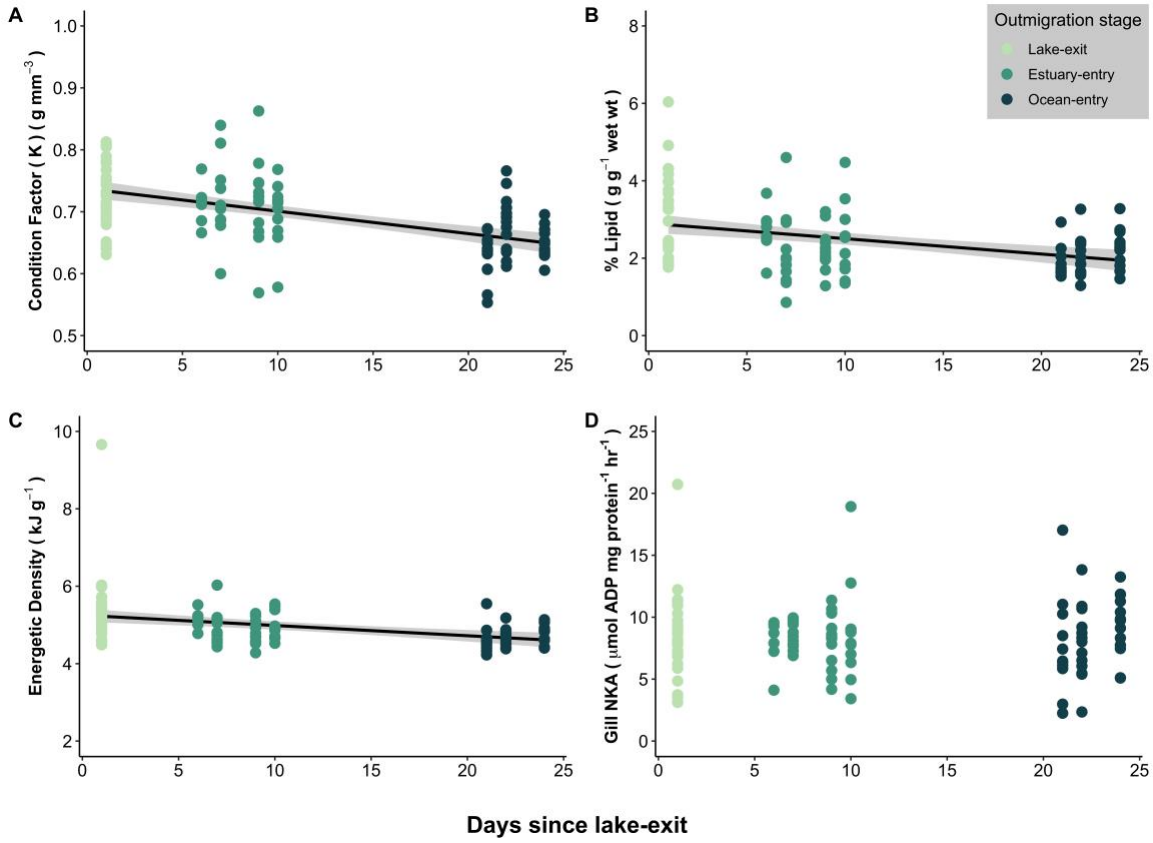

Figure 4. **Temporal changes in physical and physiological smolt condition through outmigration experiments. Significant linear models are shown for for α = 0.05.**

Table 3. Smolt physical and physiological condition throughout each outmigration stage of salinity preference experiments. Means within each stage are reported with standard deviation in parenthesis. Results are from linear regressions of each condition variable by day since lake outmigration. The slope (β) indicates the estimated rate of change in condition per day, and the intercept indicates the model estimate for condition at lake outmigration. Models that were significant to α = 0.05 are shown in bold (P).

Condition variable	Mean within outmigration stage (SD)			*n*	Intercept	β	*SE*	*P*
	River	Estuary	Ocean					
Fork length (mm)	88 (5)	85 (6)	84 (5)	115	87.25	-0.149	0.059	**0.01**
Wet Mass (g)	4.92 (0.90)	4.50 (0.96)	3.95 (0.81)	115	4.93	-0.045	0.009	**<0.001**
Fulton's K (10^5 g mm^{-3})	0.73 (0.05)	0.71 (0.06)	0.65 (0.04)	115	1.17	-0.004	0.001	**<0.001**
Water Density, % (g g^{-1} wet weight)	79.42 (3.57)	80.63 (1.06)	81.46 (0.96)	108	79.63	+0.086	0.022	**<0.001**
Lipid Density, % (g g^{-1} wet weight)	3.09 (1.03)	2.34 (0.82)	2.08 (0.48)	108	7.67	-0.040	0.009	**<0.001**
TAG Density, % (g g^{-1} Lipid)	33 (18)	36 (27)	16 (8)	108	38.50	-0.944	0.215	**<0.001**
Protein Density, % (g g^{-1} wet weight)	17.38 (3.33)	16.92 (0.67)	16.35 (0.76)	108	17.36	-0.046	0.020	**0.02**
Energetic Density (kJ g^{-1})	5.33 (0.92)	4.93 (0.36)	4.69 (0.28)	108	5.25	-0.026	0.006	**<0.001**
Gill NKA activity (µmol ADP mg protein^{-1} hr^{-1})	8.63 (3.33)	8.34 (2.56)	8.36 (3.16)	109	8.48	-0.005	0.032	0.9

Physiological predictors of smolt behaviour through downstream migration

The most parsimonious models for predicting salinity preference for all smolts included variables of gill NKA activity, outmigration stage, fork length, and energetic variables(relative lipid, protein, TAG and energy density; AIC_c [199.03 - 200.94], $\Delta AIC_c < 2$, Suppl. Table 5). In all of these models, osmoregulatory function (gill NKA activity) and outmigration stage (lake-exit, estuary-entry,

ocean-entry) predicted salinity preference throughout the smolt outmigration window, after accounting for smolt size (FL), and relative smolt somatic energy. While all four of these models (relative lipid, protein, TAG and energy density) are equivalent in fit, for brevity, the values I report are for the model that includes relative lipid density. Smolts with higher gill NKA activity showed increased preference for brackish or saltwater (β = 0.153, *SE* = 0.07, p = 0.03), given smolt size and relative lipid density. For every 1 SD increase in gill NKA activity (SD = 2.97 µmol ADP mg protein^{-1} hr^{-1}, n = 104), the odds of choosing brackish or saltwater (≥15ppm) increase by 58% (*OR* = 1.58, 95% CI [1.04, 2.39]). Smolts tested during estuary-entry showed increased preference for brackish or saltwater (β = 2.281, *SE* = 0.61, $p < 0.001$), where the odds of choosing brackish or saltwater increase by 9.79 times compared to those tested at lake-exit (95% CI [2.94, 32.61]). Likewise, smolts tested during ocean-entry showed increased preference for brackish or saltwater (β = 2.038, *SE* = 0.62, $p < 0.001$), where the odds of choosing brackish or saltwater increase by 7.67 times compared to those tested at lake-exit (95% CI [2.3, 25.64]). Models that included relative lipid density, protein density, energetic density and TAG density as variables of somatic energy all showed equal strength in contributing to model fit, but there was insufficient evidence to suggest that individual somatic energy variables predicted salinity preference, after accounting for gill NKA activity and outmigration stage ($p > 0.05$).

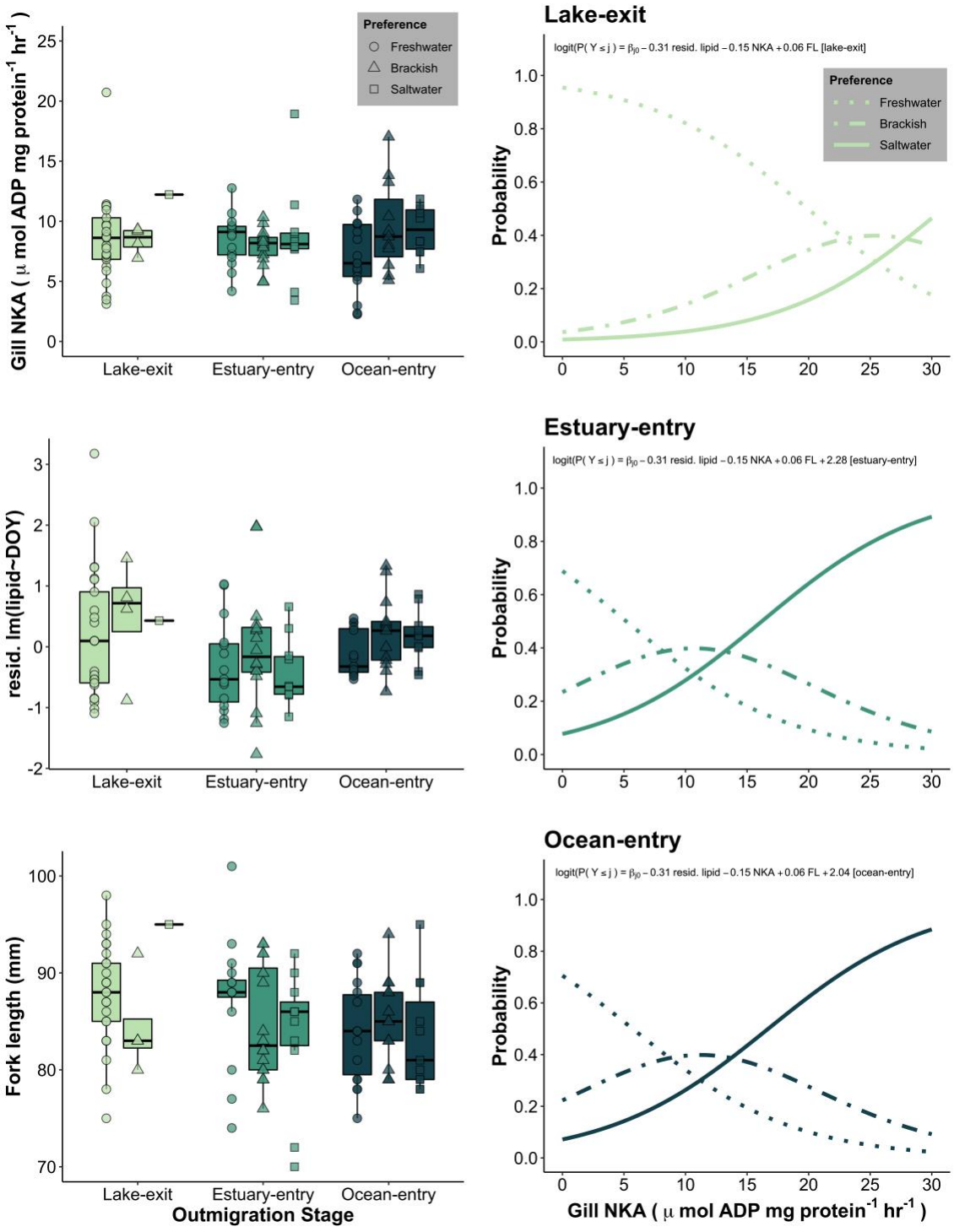
Gill NKA (μ mol ADP mg protein-1 hr-1)
Preference
Freshwater
Brackish
Saltwater
Lake-exit
Estuary-entry
Ocean-entry
Lake-exit
logit(P(Y ≤ j) = βj0 – 0.31 resid. lipid – 0.15 NKA + 0.06 FL [lake-exit]
Probability
resid. lm(lipid~DOY)
Estuary-entry
logit(P(Y ≤ j) = βj0 – 0.31 resid. lipid – 0.15 NKA + 0.06 FL + 2.28 [estuary-entry]
Fork length (mm)
Outmigration Stage
Ocean-entry
logit(P(Y ≤ j) = βj0 – 0.31 resid. lipid – 0.15 NKA + 0.06 FL + 2.04 [ocean-entry]
Gill NKA (μ mol ADP mg protein-1 hr-1)

Figure 5. Predicted probabilities for preferring freshwater (dotted line), brackish (dot and dash line) or saltwater (solid line) depending on smolt physiology in the lake-exit, estuary-entry and ocean-entry outmigration stages. Probabilites were calculated for gill NKA activity while holding other variables constant at their respective mean values in the lake-exit ($M_{resid.\ lipid}$ = 0.23, M_{FL} = 88 mm), estuary-entry ($M_{resid.\ lipid}$ = -0.23, M_{FL} = 85 mm) and ocean-entry outmigration stages ($M_{resid.\ lipid}$ = 0.07, M_{FL} = 84 mm).

Smolt physical condition (K), gill activity (NKA) and energy stores (TAG and ED) predicted salinity preference behaviour in the estuary and ocean outmigration stages, but not in the river, when outmigration stages were each run separately (Suppl. Tables 2, 3, 4). In the estuary outmigration stage, the best model for predicting salinity preference included Fulton's K, gill NKA activity, and TAG (Suppl. Table 3). After accounting for smolt osmoregulatory ability and energy stores, higher values of K increased the likelihood of preference for brackish or saltwater (β = 23.06, *SE* = 8.38, *p* = 0.01). For every 1 SD increase in Fulton's K (SD = 0.06 g mm^{-3}), the odds of choosing brackish or saltwater (≥ 15 ppm), increase by 3.68 times (*OR* = 3.68, 95% CI [1.45., 9.28], Figure 4A). After accounting for smolt gill activity and smolt physical condition, smolts with higher percent TAG showed decreased preference of brackish or saltwater (β = -0.031, *SE* = 0.02, *p* = 0.04). For every 1 SD increase in percent TAG (SD = 24.3 g g^{-1} lipid) the odds of choosing brackish or saltwater (≥15ppm) decrease by 53% (*OR* = 0.47, 95% CI [0.23, 0.95], Figure 4B).

In the ocean outmigration stage, the top model for predicting salinity preference included energetic density and gill NKA activity (Suppl. Table 4). After accounting for smolt physical condition and osmoregulatory ability, smolts with higher densities somatic energy showed increased preference for brackish or saltwater (β = 3.272, *SE* = 1.46, *p* = 0.02). For every 1 SD increase in energetic density (SD = 0.28 kJ g^{-1}), the odds of choosing brackish or saltwater (≥15ppm) increase by 2.49 times (*OR* = 2.49, 95% CI [1.12, 5.52], Figure 4C). In the lake-exit outmigration stage, the null model produced the lowest AIC_c; none of our

variables of physical or physiological condition contributed significantly to model fit (Table A2).

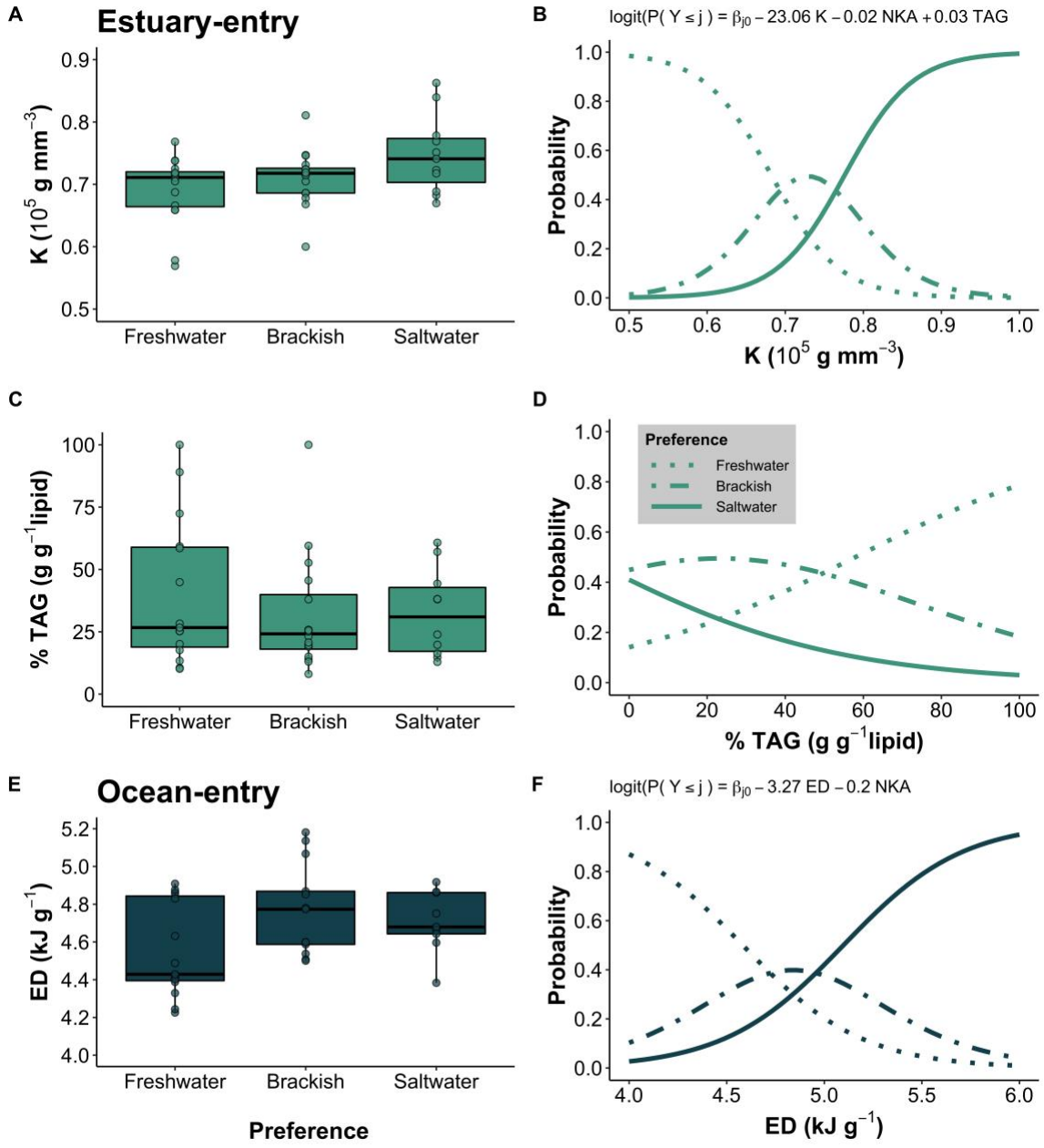

Figure 6. **Preference of freshwater (dotted line), brackish (dot and dash line) or saltwater (solid line) depending on smolt physiology at estuary-entry (A-D) and ocean-entry (E, F). In the estuary model (B, D), probabilites were calculated for values of Fulton's condition factor (K) and TAG density while holding other variables constant at their respective mean values (M_K = 0.71 g mm^{-3}, M_{NKA} = 8.3 µmol ADP mg $protein^{-1}$ hr^{-1}, M_{TAG} = 34.9 g g^{-1} lipid). In the ocean model (F), probabilites were calculated for values of energetic density (ED) while holding other variables constant at their respective mean values (M_{NKA} = 8.4 µmol ADP mg $protein^{-1}$ hr^{-1}).**

Table 4. Salinity preference model summaries for smolts tested within the estuary-entry, ocean-entry and all outmigration stages (from lake-exit to ocean-entry). Models were selected by lowest AIC_c. Odds ratio and 2.5% and 97.5% confident interals are shown for coefficients scaled by 1 SD increase/decrease of each explanatory variable. The t value shows the Wald statistic.

Outmigration Stage	Predictor	*β*	*SE*	*t* value	*p*	OR	2.5% CI	97.5% CI
Estuary-entry	Fulton's K (10^5 g mm^{-3})	23.058	8.375	2.753	**0.01**	3.68	1.45	9.28
	Gill NKA activity (µmol ADP mg $protein^{-1}$ hr^{-1})	0.022	0.132	0.164	0.87	1.06	0.55	2.05
	TAG Density, % (g g^{-1} Lipid)	-0.031	0.015	-2.090	**0.04**	0.47	0.23	0.95
Ocean-entry	Gill NKA activity (µmol ADP mg $protein^{-1}$ hr^{-1})	0.196	0.108	1.822	0.07	1.86	0.95	3.61
	Energetic Density (kJ g^{-1})	4.900	2.097	2.336	**0.02**	3.92	1.25	12.33
All stages (Lake-exit to ocean-entry)	Resid.(Lipid Density ~ DOY)	0.307	0.262	1.170	0.24	1.28	0.85	1.94
	Gill NKA activity (µmol ADP mg $protein^{-1}$ hr^{-1})	0.153	0.072	2.134	**0.03**	1.58	1.04	2.39
	Fork length (mm)	-0.056	0.038	-1.485	0.14	0.72	0.47	1.11
	Estuary-entry	2.281	0.614	3.716	**<0.001**	9.79	2.94	32.61
	Ocean-entry	2.038	0.616	3.310	**<0.001**	7.67	2.30	25.64

Discussion

Migrations of animal populations depend on individual physiological and behavioural adaptations. Research on the smolt migration has focused either on behaviour (Clark et al., 2016; Furey et al., 2016; Katzman et al., 2010; Melnychuk et al., 2010; Moser et al., 1991; Neville et al., 2016) or physiology (Houde et al., 2019; Shrimpton et al., 2005; Stefansson et al., 2003; Stefansson et al., 2012; Wilson et al., n.d.), but rarely on the interplay of the two (Baggerman, 1960; Stich et al., 2016; Stich et al., 2015). I investigated how physiology and salinity preference behaviour change throughout the juvenile sockeye salmon migration period, from lake-exit to ocean-entry. Using behavioural experiments coupled with physiological measurements from smolts, I found that behaviour is both dependent on the stage of the smolt outmigration (i.e., the number of days since lake-exit), as well as the physiological condition of migrating smolts. Smolt traits of physiological condition such as osmoregulatory preparedness (gill NKA activity), body condition (Fulton's K) and stored energy (triglyceride and energetic density) are important predictors of behaviour. In addition, the physiological factors that best describe smolt behaviour change in importance throughout the smolt outmigration window.

Whether a smolt was tested at river-exit, estuary-entry, or ocean-entry had the strongest effect on the likelihood a smolt would prefer brackish or saltwater, after accounting for smolt physiology and size. This result is consistent with the stage of outmigration expected in the wild and provides support for the experimental approach taken. In this study, smolts were captured after lake-exit and held in a laboratory setting lacking variable environmental cues associated with out-migration behaviour such as changes in water temperature (Clark et al., 2016; Sykes & Shrimpton, 2010; Zydlewski et al., 2005), increasing photoperiod (Baggerman, 1960; McCormick et al., 2002), and changing river flow rate (Katzman et al., 2010; Sykes & Shrimpton, 2010). Despite the lack of environmental triggers of migration, I found that preference for brackish and saltwater increased at estuary-entry and was maintained through ocean-entry.

Changes in salinity preference of smolts throughout downstream migration are conserved even in laboratory conditions (Baggerman, 1960; McInerney, 1963; Otto & McInerney, 1970; Stich et al., 2016). This temporal dependence of migration behaviour, observed without environmental cues, is mainly attributed to physiological changes associated with osmoregulation (Baggerman, 1960; Stich et al., 2016; Sykes & Shrimpton, 2010; Zydlewski et al., 2005). In support of this, I found that salinity preference behaviour was best explained when physiological variables, notably gill osmoregulatory activity, stored energy, and physical condition, were included with outmigration stage as predictors.

Osmoregulation in smolts is an important component of salinity preference behaviour throughout the downstream migration. Salinity tolerance occurs prior to lake outmigration as a rapid, energetically demanding upregulation of gill NKA (Hoar, 1976; Iwata et al., 1990; McCormick, 2013), that remains relatively stable throughout the freshwater migration (Bassett et al., 2018). Thus, gill NKA activity levels are useful indicators for how prepared a smolt is for ocean-entry, and can provide insight on habitat selection through-out seaward migration. If salinity preference behaviour was solely driven by osmoregulation, I would expect a strong positive relationship between gill NKA activity and saltwater preference. Indeed, I found that gill NKA activity is a strong predictor of salinity preference throughout the downstream migration period, and that smolts with higher gill NKA activity are more likely to choose brackish or saltwater, supporting observations in Atlantic smolts (Stich et al., 2016; Stich et al., 2015). While my findings support the importance that osmoregulatory preparedness is an important component of migratory behaviour, as gill NKA activity was included as a variable in all top selected models, variables of stored energy and physical condition showed stronger effects on the salinity preference of migrating smolts.

In addition to smolt osmoregulatory preparedness, stored energy plays an essential role in smolt physiological transition and behaviour during outmigration. Increased metabolism is a signature of smoltification, where lipids are required for energetic demands of active swimming downstream (Fontaine & Baraduc, 1955; Hoar, 1976). Lipids are also linked to osmoregulation through changing

composition to make cell membranes less permeable to water loss and ion gain (Fontaine & Baraduc, 1955; Hoar, 1976). I did not find that lipid density was a strong predictor of smolt migration behaviour alone. However, accounting for lipid density, in addition to gill NKA activity and size, greatly improved the accuracy of predicting salinity preference across migration stages. Generally, smolts with higher lipid density were more likely to choose brackish and saltwater. Lipid depletion is also found in Atlantic smolts migrating from rivers to the ocean (Stefansson et al., 2003). These results suggest that smolts of both higher gill NKA activity and higher lipid stores may choose to enter the ocean habitat earlier than smolts of lower NKA activity and stored energy. The synergistic effect of osmoregulation and energetic condition may have important implications to the use of downstream habitats that vary in salinity and foraging opportunity.

At different stages of the smolt outmigration, different physiological variables were more important for predicting salinity preference behaviour**.** Physical condition (K, a ratio of mass to length) of smolts was most important for predicting behaviour at estuary-entry, yet at ocean-entry, energetic density (ED) was the best predictor. Previous work on migrating Chilko sockeye smolts found that swim performance increased with smolt K, and survival decreased when fasting smolts dropped below 3.47MJ/kg (Wilson et al., n.d.). I found that smolts of higher condition factor at estuary-entry are more likely to choose brackish or saltwater, while smolts of lower K are more likely to choose freshwater. Likewise, smolts of higher ED at ocean-entry are more likely to choose brackish or saltwater, while smolts of lower ED are more likely to choose freshwater. The trend in salinity preference observed suggests that smolts of lower physical (K) and energetic condition may choose to remain in the less saline waters of the estuary, rather than continue into full strength sea water.

Physical condition (K), not size, of smolts best explained migratory habitat choice behaviour. Smolt size is a strong indicator of migratory speed (Freshwater et al., 2019), coastal residency (Freshwater et al., 2019), and estuarine habitat use (Chalifour et al., 2020; Levings et al., 1983; Moore et al., 2016). Smolt size is also associated with higher predator avoidance capacity (Glova & McInerney,

1977), higher salinity tolerance (Conte & Wagner, 1965), and higher survival from smolts to adults (Henderson & Cass, 1991). Field observations in Chinook salmon found that larger smolts were predominantly found in more saline, predator-exposed, eelgrass habitat (Chalifour et al., 2020; Levings et al., 1983). Further, marine sampling of Chilko sockeye smolts found that smaller individuals rear in the Strait of Georgia for longer, while larger individuals pass through the strait rapidly (Beacham et al., 2014; Freshwater et al., 2019; Healey, 2011). Although smolt size and mass is a component of K, I did not find strong correlations between them. While I did not find evidence that fork length of smolts predicted salinity preference, physical condition (K), contributed strongly to habitat choice. K was also found to be a stronger predictor of swim performance than length or mass in migrating Chilko sockeye smolts (Wilson et al., n.d.). At estuary-entry, using the broad measurement of K was a better predictor of smolt behaviour than size or energetics independently. This may be because K is a coarse measure that can inherently encompass variation across a suite of important physiological variables, such as morphometrics, muscle mass and lipid content.

At estuary-entry, smolt physical condition (K) best predicted salinity preference, but at ocean-entry, energetic density (ED) became the strongest indicator of habitat choice. The shift in importance from a physical metric to an energetic metric of condition at ocean-entry is likely related to the diminishing energetic reserves at this late stage in outmigration. Migrating Atlantic smolts are shown to be 'energy deficient' from freshwater to early-ocean entry (Stefansson et al., 2003; Stefansson et al., 2012), while coho smolts sampled at early-ocean entry show gene expression indicative of fasting (Houde et al., 2019). Without significant feeding during active downstream migration, smolts rely on the energy that was stored before leaving the rearing habitat. In this study, smolts tested at ocean-entry had an average energetic density of 4.69 kJ g^{-1} This is close to the 3.47 kJ g^{-1} critical energy threshold for swim performance observed in Chilko sockeye smolts (Wilson et al., n.d.). Increased energy is also required for osmoregulation in marine environments. The metabolic rate of Chinook fry is

highest in saline water compared to freshwater (Morgan & Iwama, 1991). At ocean-entry, the amount of stored lipids is positively correlated to survival of juvenile Chinook (Burrows, 1969; Higgs et al., 1992). At this late stage of the smolt migration, energetic reserves would be of dire importance, and would very likely influence smolt behaviour to seek environmental conditions that impose the least energetic cost.

Osmoregulatory ability was independent of other physiological parameters, such as size, physical condition and energy stores. The independence of size and osmoregulatory function is also noted in Chilko sockeye smolts sampled in previous years (Bassett et al., 2018) and Atlantic smolts (Whalen et al., 1999). Work on coho salmon found that larger smolts develop higher gill NKA activities than smaller smolts, but this relationship diminishes throughout the outmigration window (Zaugg & McLain, 1972); Gill NKA activity was not correlated with energetic variables, such as lipid density, energetic density or triglyceride density. A smolt in high osmoregulatory condition may also be in poor energetic condition. Prior to lake-exit, and as an indicator of smoltification, gill NKA activity increases while lipid density declines (Larsen et al., 2000). After outmigration, I observed a linear decline in energy stores, but no significant change in gill NKA activity. Gill NKA activity remained stable through the experimental period that spanned the downstream migration from lake to estuary to ocean. This in-laboratory finding aligns with field observations of Chilko sockeye smolts intercepted throughout the freshwater stages of migration, where gill NKA activity remains relatively stable from lake-exit to pre-estuary entry (Bassett et al., 2018). The same trend was found in migrating Atlantic smolts where gill NKA activity was stable from freshwater to estuary-entry, and only increased once smolts entered the ocean (Stefansson et al., 2003). Smolt condition, therefore, should be assessed using multiple traits of condition specifically related to the adaptations required for the life stage in question. As seen in this study, one trait or condition metric does not encompass the full breadth of physiological status in smolts with respect to understanding critical movement behaviours such as salinity preference.

Although ranges of smolt physiology align with those of outmigrating salmon measured for lipid, protein densities and gill NKA activity (Bassett et al., 2018; Stefansson et al., 2003; Stefansson et al., 2012), some surprising findings of smolt migration physiology remain as outliers. I found evidence of protein catabolism during the freshwater, non-feeding downstream migration. Decreases in protein densities from freshwater to the ocean were also seen in migrating Atlantic smolts (Stefansson et al., 2003). I saw a surprising decrease in fork length of randomly sampled smolts over holding time. As all fish had completed smoltification upon initial capture at lake-exit, morphological changes associated with smolting (Hamon & Foote, 2000) is unlikely, but possible. Triglyceride (TAG) density, the dominant form of stored lipid energy in vertebrates, showed an opposite effect on salinity preference than overall lipid density. Smolts with higher densities of TAG were more likely to prefer saltwater at estuary-entry, though the probability only reached 50% at extremely high, and potentially unrealistic, densities of TAG (when over half of all lipid stores were TAG). My estimates of TAG densities were well above densities reported for the same population in the previous year (Wilson et al., n.d.). This study also found that TAG densities were not the strongest predictors of smolt swim performance and survival (Wilson et al., n.d.). The shift in behaviour at extremely high TAG densities should be noted cautiously, yet warrants further investigation on the role of TAG in migration energetics.

Limitations and recommendations for further study

The estuary environment encompasses a suite of gradients that a migrating smolt experiences, such as food abundance, predation risk, temperature, depth, flow rate, and turbidity (Manel-La et al., 2009). A salinity gradient is one very simplified way to represent an estuary, and choice experiments are one simplified way to infer habitat choice. Future work should attempt combinations of multiple variables of the estuarine environment, such as turbidity, temperature and salinity preference. One of the larger assumptions of this study was that smolts do not feed during the downstream migration period,

and although supported by field observations of empty stomachs in migrating wild smolt (Beamish et al., 2012; Neville et al., 2016) and gene expression indicative of fasting at ocean-entry (Houde et al., 2019), the effects of starvation and migration stage in this experiment are inherently coupled. An effect of feeding on salinity preference behaviour is worth investigating further. This could incorporate other behavioural metrics such as consumption rate in different salinities to show growth potential in regards to habitat choice within estuaries and coastal regions (see Webster & Dill, 2007).

Physiological and behavioural assays commonly produce high individual variation. In anticipation of this, I chose to prioritize sample size over experimental duration to increase predictive power. Handling and predator stress is known to decrease saltwater preference in Chinook smolts (Price & Schreck, 2003b) and this stress effect may account, in part, for the high freshwater preference observed in my 2 hour experiments. Extending preference observational experiments to 24 hours at each migration stage (the approximate duration of Chilko sockeye transit through the estuary) would allow for recovery from handling stress. In addition, it would allow for comparison of diurnal and nocturnal differences in smolt migration behaviour, as has been noted for Chilko sockeye at lake-exit (Clark et al., 2016), and salmonid smolts in other systems (Quinn, 2018).

Gill NKA activity is one broad metric for estimating salinity tolerance in teleosts (McCormick et al., 2009). Gill NKA activity measured in freshwater did not correlate to activity when measured later in saltwater in Atlantic smolts (Zydlewski & Zydlewski, 2012). This is likely because the gill NKA enzyme has two isoforms which alternate expression in different salinities; the α1a subunit is expressed highly in freshwater, and the α1b subunit is expressed in saltwater (McCormick et al., 2009; Richards et al., 2003; Stefansson et al., 2012). In this study, all smolts were held in freshwater and exposed to saline water in choice experiments for up to one hour, so differentiating between gill NKA isoforms would not likely alter results. Coupling salinity preference and tolerance experiments throughout smolt migration would mitigate this assumption, and

provide a powerful link between behaviour, physiology and survival (see Stich et al., 2016).

This study was conducted on a population of sockeye from Chilko lake, and these behavioural trends may be adaptations that are not transferable to other populations, species or watersheds. Sockeye is a species that does not typically reside extensively in estuaries; however many populations show variation from the typical 1-2 year lake residency, where individuals enter the estuary and coastal waters as fry (Quinn, 2018). This is known as a 'sea-type' or 'ocean-type' life history. While this life-history strategy may be rare, Harrison Lake ocean-type juvenile sockeye represented 27.9% and 27.6% of total sockeye production in the Fraser River in 2009 and 2011, respectively (Beamish et al., 2016). Non-Harrison ocean-type sockeye made up 1.5% of smolts in the Strait of Georgia from 2014 to 2016 (Freshwater et al., 2018). Mechanistic understanding of juvenile sockeye estuary use is lacking, and this may be especially important for populations with ocean-type life history that may use estuaries as nursery habitat, such as the population from Harrison Lake (see Hodgson et al., 2020). Future work should focus on comparing salinity preference with ocean-type sockeye populations or other species of salmon known to occupy estuaries for longer periods, such as Chinook or coho (Chalifour et al., 2019, 2020).

Conclusion

Salinity preference tests conducted throughout the juvenile salmon migration period suggest that smolt physiological condition upon reaching the estuary has the potential to influence migratory behaviour and habitat selection. These results provide novel evidence on the temporally dependent interaction between physiology, behaviour and migration in juvenile Pacific salmon. With the knowledge that many physiological factors undergo changes during outmigration (McCormick, 2013), our findings further highlight the importance of assessing multiple systems in smolt physiology to explain migratory behaviour.

As juvenile migratory behaviour is linked to physiological condition, and physiological condition is determined by productivity and competition within the rearing habitat, the importance of estuaries likely varies across years and within a population cohort; thus estuaries may be of heightened importance for wild juvenile salmon in years of poor freshwater growth conditions. Conservation of behavioural diversity in the use of estuaries can act as a buffer, enhancing the resilience of populations to environmental change (Flitcroft et al., 2018). These findings support the growing body of evidence on the importance of conserving both rearing habitat for juvenile growth potential, and estuarine habitat for smolt refugia before ocean entry.

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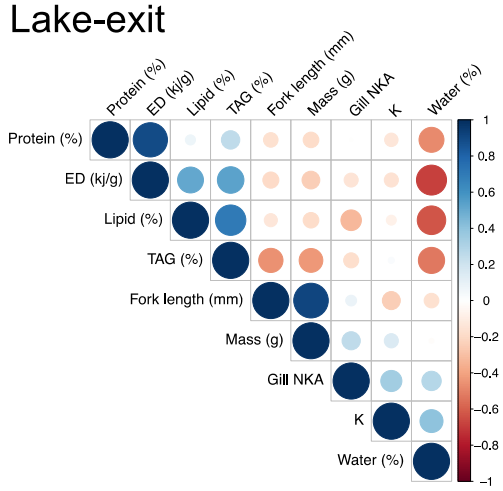
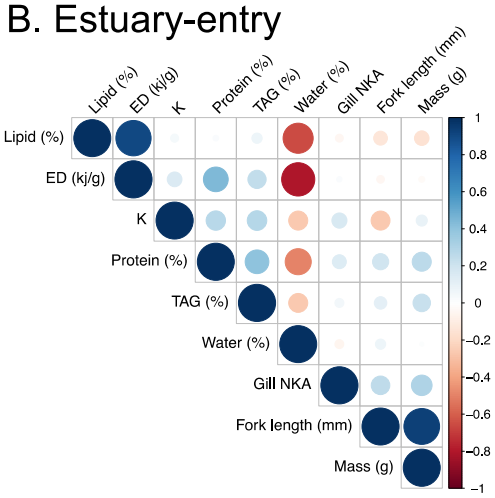
Supplementary Materials

Correlations between physiological variables of smolt condition

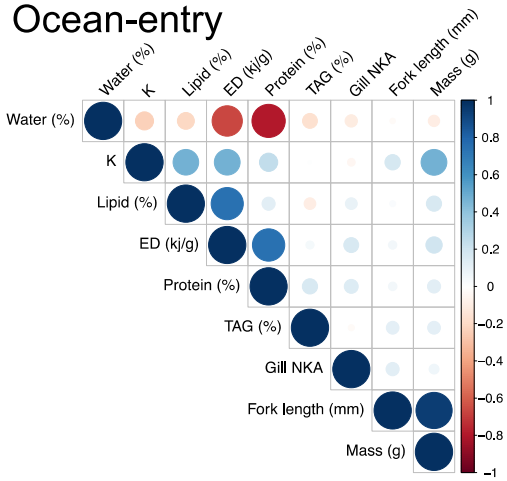
A. Lake-exit



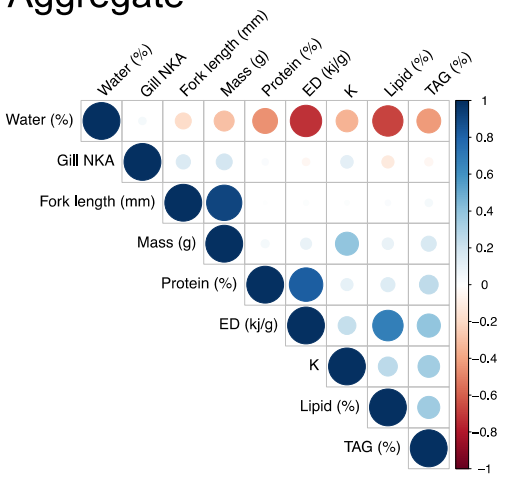
B. Estuary-entry



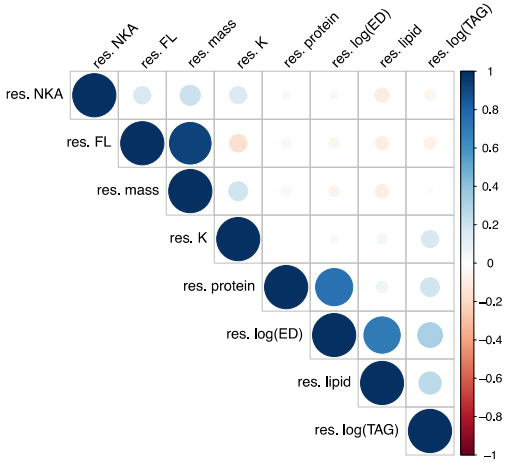
C. Ocean-entry



D. Aggregate



E. Aggregate – residuals



Suppl. Fig. 1. Correlation matrix between physiological variables of Chilko sockeye smolts sampled throughout outmigration window (May-June): at lake-exit (A), estuary-entry(B), and ocean-entry(C). Variables from smolts sampled across all outmigration stages were pooled for aggregate correlations (D), and residuals of mass and energetic variables were corrected for linear temporal decline (E). Energetic density is denoted as ED and Fulton's condition factor as K (10^5 g mm^{-1}). Positive correlations are shown in blue and negative correlations in red. Color intensity and the size of the circle are proportional to the correlation coefficients.

Parallel Odds Model

Suppl. Tab. 1. Results for tests of assumption of proportional odds for top salinity preference models. Response variable is ordered as preferred salinity, where freshwater < brackish < saltwater. The null hypothesis states that the slope of the logistic regression of the predictor by the response variable is equal among each category (freshwater, brackish, and saltwater preference).

Outmigration stage	Explanatory Variables	Resid. diff.	df	p-value
Estuary-entry	K + ATP + TAG	1.41	3	0.70
Ocean-entry	ED + NKA	0.43	2	0.81
	K + ED + NKA	1.38	3	0.71
	mass + ED + NKA	1.01	3	0.80
	FL + ED + NKA	1.95	3	0.58
All stages (from lake-exit to ocean entry)	resid. lipid + NKA + outmig. stage + FL	1.55	5	0.91
	resid. log(ed) + NKA + outmig. stage + FL	0.48	5	0.99
	resid. log(TAG) + NKA + outmig. stage + FL	0.54	5	0.99
	resid. protein + NKA + outmig. stage + FL	0.80	5	0.98

Model Selection

Suppl. Tab. 2. Model Selection for predicting salinity preference by physical and physiological condition in the lake-exit outmigration stage.

Candidate Model Explanatory Variables	AIC_c	ΔAIC_c	Model Likeli.	AIC_c Wt	Least Likeli.	Cum. Wt
1 (null model)	36.18	0.00	1.00	0.11	-16.02	0.11
K	36.53	0.36	0.84	0.09	-15.13	0.20
TAG	37.77	1.59	0.45	0.05	-15.74	0.25
lipid	37.95	1.77	0.41	0.05	-15.84	0.30
protein	38.08	1.91	0.39	0.04	-15.90	0.34
NKA	38.10	1.92	0.38	0.04	-15.91	0.39
K + TAG	38.14	1.97	0.37	0.04	-14.84	0.43
FL	38.23	2.05	0.36	0.04	-15.97	0.47
mass	38.26	2.09	0.35	0.04	-15.99	0.51
ED	38.31	2.13	0.34	0.04	-16.02	0.55
K + lipid	38.34	2.16	0.34	0.04	-14.93	0.58
K + protein	38.69	2.51	0.28	0.03	-15.11	0.61
K + ED	38.70	2.52	0.28	0.03	-15.11	0.65
K + NKA	38.72	2.55	0.28	0.03	-15.13	0.68
mass + TAG	39.47	3.29	0.19	0.02	-15.50	0.70
TAG + NKA	39.55	3.38	0.18	0.02	-15.54	0.72
lipid + NKA	39.62	3.45	0.18	0.02	-15.58	0.74
FL + TAG	39.95	3.77	0.15	0.02	-15.74	0.76
mass + lipid	40.00	3.83	0.15	0.02	-15.77	0.77
protein + NKA	40.07	3.89	0.14	0.02	-15.80	0.79
FL + lipid	40.10	3.92	0.14	0.02	-15.81	0.80
FL + protein	40.17	3.99	0.14	0.02	-15.85	0.82
FL + NKA	40.18	4.00	0.14	0.02	-15.85	0.83
mass + protein	40.23	4.05	0.13	0.01	-15.88	0.85
mass + NKA	40.27	4.10	0.13	0.01	-15.90	0.86
ED + NKA	40.29	4.11	0.13	0.01	-15.91	0.88
K + NKA + TAG	40.36	4.18	0.12	0.01	-14.82	0.89
FL + ED	40.40	4.22	0.12	0.01	-15.96	0.91
mass + ED	40.45	4.28	0.12	0.01	-15.99	0.92
K + NKA + lipid	40.52	4.35	0.11	0.01	-14.90	0.93
K + NKA + protein	40.93	4.75	0.09	0.01	-15.11	0.94
K + NKA + ED	40.94	4.76	0.09	0.01	-15.11	0.95
mass + NKA + TAG	41.41	5.23	0.07	0.01	-15.35	0.96
FL + NKA + TAG	41.77	5.60	0.06	0.01	-15.53	0.97
mass + NKA + lipid	41.81	5.63	0.06	0.01	-15.55	0.97
FL + NKA + lipid	41.82	5.65	0.06	0.01	-15.55	0.98
FL + NKA + protein	42.19	6.01	0.05	0.01	-15.74	0.99
mass + NKA + protein	42.30	6.12	0.05	0.01	-15.79	0.99
FL + NKA + ED	42.42	6.24	0.04	0.00	-15.85	1.00
mass + NKA + ED	42.52	6.34	0.04	0.00	-15.90	1.00

Suppl. Tab. 3. Model Selection for predicting salinity preference by physical and physiological condition in the estuary-entry outmigration stage.

Candidate Model Explanatory Variables	AIC_c	ΔAIC_c	Model Likeli.	AIC_c Wt	Least Likeli.	Cum. Wt
K + NKA + TAG	84.17	0.00	1.00	0.55	-36.82	0.55
K + TAG	87.34	3.16	0.21	0.11	-39.50	0.66
K + NKA + protein	88.61	4.44	0.11	0.06	-39.04	0.72
K + NKA + ED	88.88	4.71	0.09	0.05	-39.18	0.77
K + NKA + lipid	89.07	4.89	0.09	0.05	-39.27	0.82
K + ED	90.40	6.23	0.04	0.02	-41.03	0.84
K+ lipid	90.46	6.29	0.04	0.02	-41.06	0.87
K+ protein	90.53	6.36	0.04	0.02	-41.09	0.89
K + NKA	90.65	6.48	0.04	0.02	-41.16	0.91
K	92.39	8.22	0.02	0.01	-43.10	0.92
TAG + NKA	92.40	8.23	0.02	0.01	-42.03	0.93
FL + TAG + NKA	92.98	8.80	0.01	0.01	-41.22	0.94
ED + NKA	93.82	9.65	0.01	0.00	-42.73	0.94
lipid + NKA	93.82	9.65	0.01	0.00	-42.74	0.95
protein + NKA	93.82	9.65	0.01	0.00	-42.74	0.95
TAG	93.91	9.74	0.01	0.00	-43.86	0.95
FL + NKA + protein	94.41	10.24	0.01	0.00	-41.94	0.96
mass + NKA + TAG	94.42	10.24	0.01	0.00	-41.94	0.96
FL + NKA + lipid	94.45	10.28	0.01	0.00	-41.96	0.96
FL + NKA + ED	94.46	10.28	0.01	0.00	-41.96	0.97
protein	94.52	10.35	0.01	0.00	-44.16	0.97
ED	94.69	10.52	0.01	0.00	-44.24	0.97
lipid	94.69	10.52	0.01	0.00	-44.25	0.98
FL + TAG	94.91	10.73	0.00	0.00	-43.28	0.98
FL + protein	95.32	11.14	0.00	0.00	-43.49	0.98
FL + NKA	95.59	11.42	0.00	0.00	-43.63	0.98
FL + lipid	95.63	11.46	0.00	0.00	-43.65	0.98
FL + ED	95.69	11.52	0.00	0.00	-43.68	0.99
mass + NKA + protein	95.70	11.53	0.00	0.00	-42.59	0.99
mass + NKA + ED	95.72	11.55	0.00	0.00	-42.60	0.99
mass + NKA + lipid	95.73	11.55	0.00	0.00	-42.60	0.99
mass + TAG	95.95	11.78	0.00	0.00	-43.81	0.99
mass + protein	96.38	12.21	0.00	0.00	-44.02	0.99
mass + lipid	96.65	12.48	0.00	0.00	-44.15	0.99
FL	96.66	12.48	0.00	0.00	-45.23	1.00
mass + ED	96.66	12.49	0.00	0.00	-44.16	1.00
NKA	96.66	12.49	0.00	0.00	-45.23	1.00
1	97.36	13.19	0.00	0.00	-46.63	1.00
mass + NKA	97.71	13.53	0.00	0.00	-44.69	1.00

mass	98.47	14.30	0.00	0.00	-46.14	1.00

Suppl. Tab. 4. Model Selection for predicting salinity preference by physical and physiological condition in the ocean-entry outmigration stage.

Candidate Model Explanatory Variables	AIC_c	ΔAIC_c	Model Likeli.	AIC_c Wt	Least Likeli.	Cum. Wt
ED + NKA	72.42	0.00	1.00	0.23	-32.01	0.23
K + NKA + ED	72.85	0.42	0.81	0.18	-31.11	0.41
mass + NKA + ED	73.15	0.73	0.69	0.16	-31.26	0.57
FL + NKA + ED	73.51	1.08	0.58	0.13	-31.44	0.70
lipid + NKA	74.86	2.44	0.30	0.07	-33.22	0.77
protein+ NKA	75.87	3.44	0.18	0.04	-33.73	0.81
mass + NKA+ lipid	76.02	3.60	0.17	0.04	-32.70	0.84
FL + NKA + lipid	76.07	3.65	0.16	0.04	-32.72	0.88
K + NKA + lipid	76.26	3.84	0.15	0.03	-32.82	0.91
FL + NKA+ protein	77.08	4.66	0.10	0.02	-33.23	0.94
mass + NKA + protein	77.46	5.04	0.08	0.02	-33.42	0.96
K + NKA + protein	78.09	5.67	0.06	0.01	-33.73	0.97
TAG + NKA	78.44	6.01	0.05	0.01	-35.01	0.98
FL + NKA + TAG	79.67	7.24	0.03	0.01	-34.52	0.99
mass + NKA + TAG	80.21	7.79	0.02	0.00	-34.79	0.99
K + NKA + TAG	80.54	8.12	0.02	0.00	-34.96	0.99
ED	83.04	10.62	0.00	0.00	-38.41	1.00
NKA	83.58	11.16	0.00	0.00	-38.68	1.00
K + ED	84.29	11.86	0.00	0.00	-37.96	1.00
FL + NKA	85.05	12.63	0.00	0.00	-38.34	1.00
mass + ED	85.11	12.69	0.00	0.00	-38.37	1.00
FL + ED	85.18	12.76	0.00	0.00	-38.41	1.00
protein	85.37	12.95	0.00	0.00	-39.58	1.00
mass + NKA	85.48	13.06	0.00	0.00	-38.56	1.00
K + NKA	85.58	13.16	0.00	0.00	-38.61	1.00
lipid	86.08	13.65	0.00	0.00	-39.93	1.00
FL + protein	87.43	15.01	0.00	0.00	-39.53	1.00
mass + protein	87.47	15.05	0.00	0.00	-39.55	1.00
K + protein	87.52	15.10	0.00	0.00	-39.58	1.00
K + lipid	88.01	15.59	0.00	0.00	-39.82	1.00
FL + lipid	88.22	15.80	0.00	0.00	-39.93	1.00
mass + lipid	88.22	15.80	0.00	0.00	-39.93	1.00
TAG	88.69	16.27	0.00	0.00	-41.24	1.00
K + TAG	90.69	18.27	0.00	0.00	-41.16	1.00
FL + TAG	90.83	18.40	0.00	0.00	-41.23	1.00
mass + TAG	90.83	18.41	0.00	0.00	-41.23	1.00
1 (null model)	94.57	22.14	0.00	0.00	-45.23	1.00

K	96.53	24.11	0.00	0.00	-45.17	1.00
FL	96.65	24.23	0.00	0.00	-45.23	1.00
mass	96.66	24.24	0.00	0.00	-45.23	1.00

Suppl. Tab. 5. Model selection for predicting salinity preference by physical condition, physiological condition and outmigration stage.

Candidate Model Explanatory Variables	AIC_c	ΔAIC_c	Model Likeli.	AIC_c Wt	Least Likeli.	Cum. Wt
resid. lipid + NKA + outmig. stage + FL	199.03	0.00	1.00	0.34	-92.33	0.34
resid. log(ED) + NKA + outmig. stage + FL	199.19	0.16	0.92	0.31	-92.41	0.65
resid. log(TAG) + NKA + outmig. stage + FL	200.39	1.36	0.51	0.17	-93.01	0.83
resid. protein + NKA + outmig. stage + FL	200.39	1.36	0.51	0.17	-93.01	1.00
NKA + outmig. stage + FL	210.03	11.00	0.00	0.00	-98.88	1.00
resid. log(ED) + outmig. stage + FL	213.35	14.32	0.00	0.00	-100.54	1.00
resid. lipid + outmig. stage + FL	213.88	14.85	0.00	0.00	-100.81	1.00
resid. protein + outmig. stage + FL	214.48	15.45	0.00	0.00	-101.11	1.00
resid. log(TAG) + outmig. stage + FL	214.57	15.54	0.00	0.00	-101.16	1.00
resid. K + outmig. stage + FL	220.53	21.50	0.00	0.00	-104.14	1.00
NKA + FL	222.06	23.03	0.00	0.00	-106.97	1.00
outmig. stage	224.31	25.28	0.00	0.00	-108.10	1.00
outmig. stage + FL	224.44	25.41	0.00	0.00	-107.13	1.00
resid. mass + outmig. stage	226.07	27.03	0.00	0.00	-107.94	1.00
resid. log(TAG) + FL	228.28	29.25	0.00	0.00	-110.08	1.00
resid. log(ED) + FL	228.34	29.31	0.00	0.00	-110.11	1.00
resid. protein + FL	228.58	29.55	0.00	0.00	-110.23	1.00
resid. lipid + FL	228.63	29.60	0.00	0.00	-110.25	1.00
resid. mass + FL	230.08	31.05	0.00	0.00	-110.98	1.00
resid. K + FL	233.92	34.89	0.00	0.00	-112.90	1.00
FL	237.25	38.22	0.00	0.00	-115.59	1.00
1 (null model)	239.22	40.19	0.00	0.00	-117.59	1.00

Appendix A.

Validity of methods for estimating N^+-K^+-ATPase activity from gill tissue samples of juvenile sockeye salmon (*Oncorhynchus nerka*)

Introduction and Methods

The full methodology of estimating gill NKA activity is detailed in the main methods of this thesis, following McCormick (1993). The methods used in this thesis differed slightly from the original protocol in two ways: (1) The linear slops of ATP hydrolosis was calculated over the full 10 minutes, rather than only beginning the rate calculation after the first 3 minutes of enzyme activity and stopping after 9 minutes have passed; and (2) due to technical constraints of lab access due to the COVID-19 pandemic, gill sample assays were run in two batches, one in the fall of 2019, and one in the fall of 2020. Here, I test for variation in NKA values due to deviations from the original protocol.

The linear slope of ATP hydrolysis (or the equimolar disappearance of NADH) was calculated over 10 minutes for each sample in Omega BMG Labtech Software 5.10 R2. This differs from McCormick (1993), who calculated the slope between 3 and 9 minutes of the assay. I compared a randomized subsample of assays (n = 104) between those calculated over 6 minutes or 10 minutes using paired t-tests functions in the rstatix package of R version 4.0.2 (Kassambara, 2020b).

To account for the potential error due to freezer time between samples run in 2019 and those in 2020, I compared NKA and CV of a subset of samples that were ran both in 2019 and 2020 in a paired t-test (n = 61). For the full gill dataset, 348 samples were run in 2019 and 84 were run in 2020. All samples were run in randomized order. In this full datatset, I tested for an effect of days in freezer on the CV of gill NKA values using a linear model.

Results

Calculation of slope of gill NKA activity over 10-minutes

There is no strong evidence that the coefficient of variation differed between protocols ($\bar{x}_{6minute}$ = 9.61, $SD_{6minute}$ = 12.28, $\bar{x}_{10minute}$ = 8.92, $SD_{10minute}$ = 11.87, $t(102)$ = -1.018, p = 0.31, d = -0.1). Estimations of Na^+-K^+-ATPase activity was slightly higher in the 6 minute protocol ($\bar{x}_{6minute}$ = 8.99, $SD_{6minute}$ = 2.76) than the 10 minute protocol ($\bar{x}_{10minute}$ = 8.52, $SD_{10minute}$ = 2.55), but the effect size was small ($t(102)$ = -4.781, p < 0.001, d = -0.5).

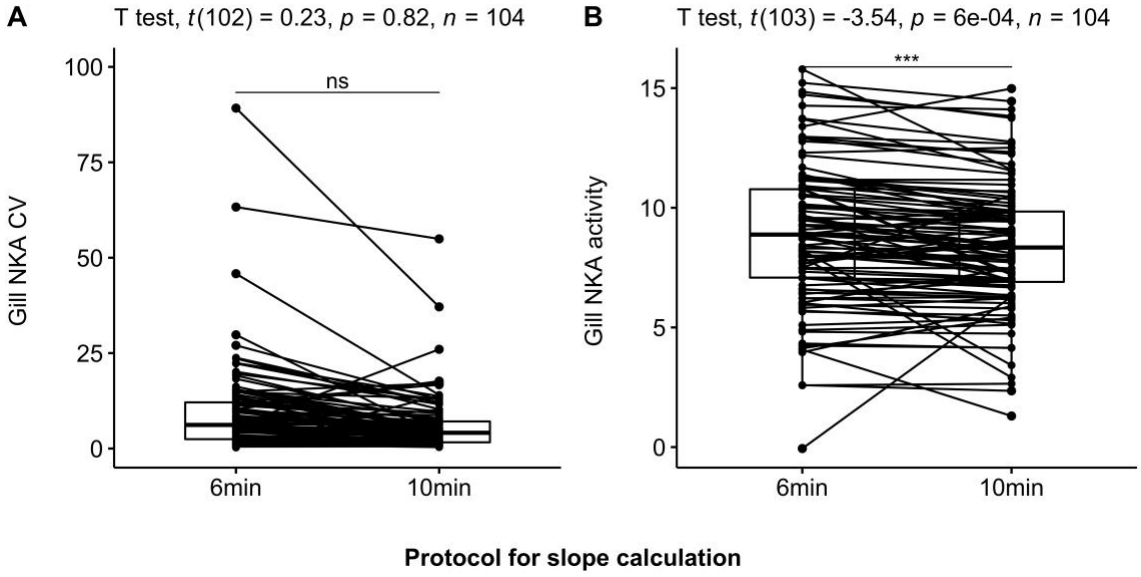


Figure A1. Comparison of NKA values and coefficient of variance (CV) resulting from slope calculations over 6 minutes and 10 minutes from the same gill sample.

Duration of time frozen at -80˚C on estimates of gill NKA activity

The absolute difference in NKA values between 2019 and 2020 samples ranged from 0 - 9.94 µmol ADP mg protein^{-1} hr^{-1}, with a mean absolute difference of 2.64 µmol ADP mg protein^{-1} hr^{-1}. Gill NKA values from the same fish that were run in 2019 did not significantly differ from NKA values that were run in 2020, t(60) = 1.768, p = 0.08 (Figure A2). The absolute difference in CV between 2019 and 2020 samples ranged from 0.2 - 49.25 %, with a mean absolute difference in CV of 12.23 %. Gill samples from the same fish that were run in 2020 had significantly lower variance than those ran in 2019, t(58) = 2.070, p = 0.04, indicating improved technical skill from previous experience running the assays. NKA values measured in 2019 showed a linear increase in activity over the duration of time in freezer, with a daily increase in activity of 0.04 µmol ADP mg protein^{-1} hr^{-1} (Figure A3); This trend was not reflected in the samples that were run in 2020.

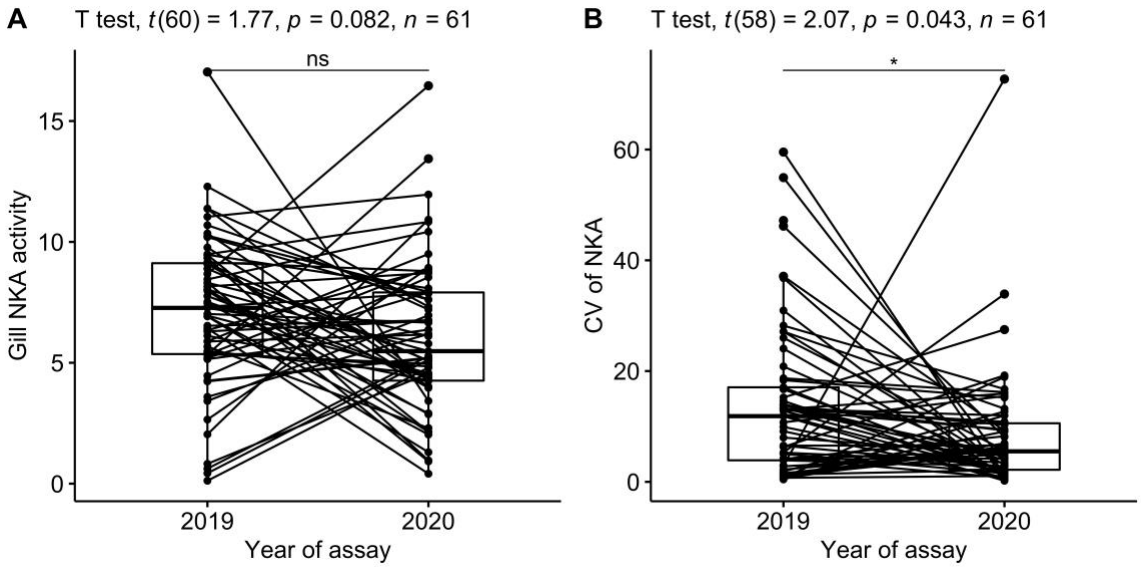


Figure A2. Comparison of NKA values and coefficient of variance (CV) between gill samples from the same fish run in 2019 and 2020.

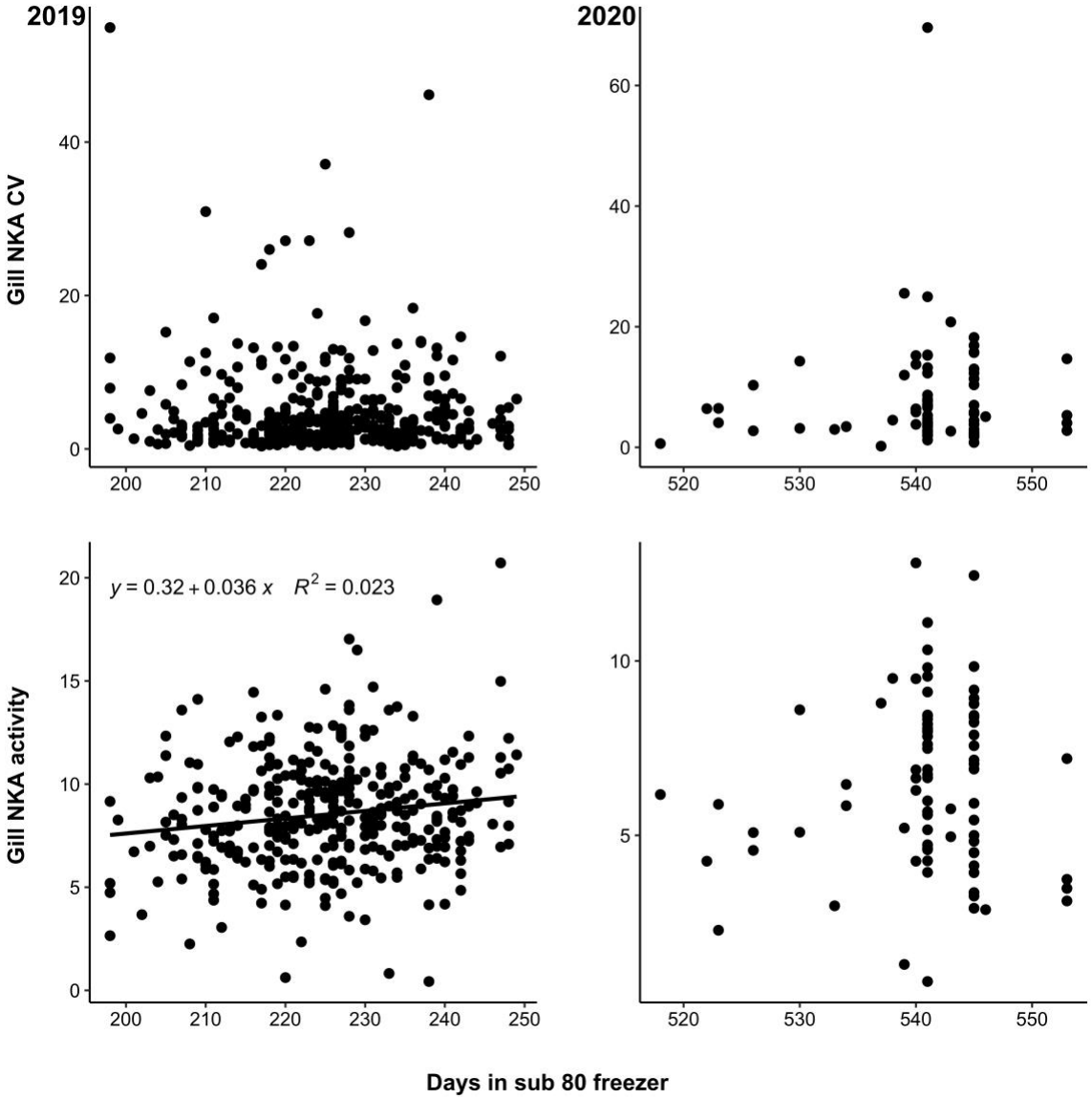


Figure A3. Effect of duration of time frozen at -80˚C on gill NKA coefficient of variation (CV, top) and NKA activity (bottom) for those that were analyzed in 2019 (left) and 2020 (right).

Conclusion

The difference in variation and activity between protocols was negligible. There was a detectable effect of freezer time, but only on samples that were frozen for less than a year. It appears that a freeze effect dampens over time in freezer, and is likely non-linear. The daily frozen rate of change in gill NKA activity (0.04 μmol ADP mg protein^{-1} hr^{-1}) was very small relative to the average gill NKA activity (8.4 μmol ADP mg protein^{-1} hr^{-1}). From these results, gill samples from 2019 and 2020 were pooled for analysis, and slopes for all Na^+-K^+-ATPase activity reported in this thesis were calculated using the 10-minute protocol. Final NKA values for smolt gills that were re-ran were selected from the year (2019 or 2020) with the lowest CV and the highest number of viable replicates.

Appendix B.

Analysis of non-exploratory behaviour in juvenile sockeye salmon (*Oncorhynchus nerka*)

Introduction

Behavioural studies provide a useful companion to physiological thresholds as a more ecologically relevant indictor of response. The tolerance of an individual to a stressor or change in condition may not represent the conditions an individual would experience in the wild, as mobile animals may simply avoid the stressor and move to another location of acceptable conditions. Understanding the mechanisms that drive behaviour in wild salmon has implications for population dynamics, proper care in aquaculture and accuracy in stock assessment (Fréon et al., 1993).

Exploratory behavior is when an individual engages with a novel stimuli or environmental condition (Greenberg & Mettke-Hofmann, 2001). This behaviour may result in a new source of food or un-occupied habitat, or it may not be an acceptable resource. Further, exploratory behaviour may put the individual at higher risk of predation from exposure in a novel environment. The costs and benefits of exploration are relative to fish condition, previous experience, and the life-stage of the fish.

Non-responsiveness in laboratory conditions is a common observation in behavioural studies. While this may be a behavioural trait due to the temperament of individual fish (Réale et al., 2007), it may also be related to common factors of laboratory studies, such as transport, handling, and acclimation to novel laboratory holding conditions. Non-responsiveness may also be due to physical traits of fish, such as size and physiology.

In the experiments of this thesis, I only determined salinity preference for smolts that had explored the experimental chambers. This ensured that fish (1) were physically capable of swimming and thus representative of the population,

(2) could navigate the experimental tank and access the other chambers, and (3) had knowledge of the other salinities in which to make a choice to occupy or not.

Nearly half of all fish tested (47%) did not move from the original chamber in which they were placed; these were categorized as non-exploratory and were not included in models to predict salinity preference. However, non-exploratory fish may not have moved from the chamber because the resulting salinity was within their preference. To evaluate the assumptions of my salinity preference models, I tested whether exploratory behaviour was a factor of (1) the duration of time in captivity, or (2) physiological condition. In addition, I tested whether inclusion or exclusion of non-exploratory smolts in salinity preference models resulted in different outcomes.

Methods

Observations of exploratory behaviour were taken during salinity preference experiments at three stages of outmigration: (1) lake-exit, (2) estuary-entry, and (3) ocean-entry. The full experimental methodology is outlined in the main methods of this thesis. Briefly, six smolts were placed in an experimental aquarium divided into three chambers. The conditions of the aquarium were kept constant for a one-hour acclimation period. After one-hour, a salinity gradient was imposed across the aquarium, where each chamber was filled from the bottom with either freshwater (~2 ppm), brackish water (~15 ppm) or saltwater (~32 ppm), displacing the freshwater of the acclimation period. The response of the fish to this environmental change was observed as movement between chambers. If a fish failed to move between chambers during the acclimation period and after the salinity gradient was imposed, it was deemed non-exploratory.

Temporal variation in exploratory behaviour

There may be an effect of holding time on willingness to explore an experimental chamber. Exploration behaviour during salinity preference trials was tested

against holding time (days since capture at outmigration), smolt size and energetics. To test for an effect of holding time on exploratory behaviour of wild smolts, I used a binomial logistic regression to predict the likelihood of exploratory (1) or non-exploratory behaviour (0) from the duration of time that smolts were held in the aquatic facility. The model was run using the glm function of the stats package of R version 3.5.2 (R Core Team, 2018), with the following equation:

$$log\left(\frac{p}{1-p}\right) = \beta_0 + \beta(Days\ since\ outmigration)$$

Physiology of exploratory and non-exploratory smolts

There may be a relation between fish physiology (overall condition) and willingness to explore experimental chambers. I compared fork length, wet mass and Fulton's K between exploratory (*n* = 121) and non-exploratory (*n* = 142) fish using separate t-tests for each dependent variable in the stats package of R version 3.5.2 (R Core Team, 2018). Normality and heteroscedasticity of residuals were verified visually and quantitatively using the ncvTest function from the package car (Fox et al., 2018) of R version 3.5.2 (R Core Team, 2018). Energetic variables of non-exploratory fish were only available for smolts from those tested on day 1 of outmigration (river outmigration group, *n* = 45). I compared % lipid, % protein, energetic density, and % TAG of lipid between exploratory (n = 29) and non-exploratory fish (*n* = 16) using separate t-tests. Normality and heteroscedasticity of residuals were verified visually and quantitatively, as above. Percent lipid was square root transformed and % TAG of lipid was log transformed to correct for positive skew.

Metrics of salinity preference

I compared differences in preferred salinity if assigned as salinity of chamber occupied for more than 50% of experimental trial (>30 mins) or as last chamber occupied at end of experimental trial (at 60 mins).

Inclusion of non-exploratory smolts in salinity preference models

I followed the same method of analysis as described in the main thesis methods to predict salinity preference by smolt physiology at each of the migration stages. Energetic measurements for non-exploratory smolts were only available for the lake-exit migration stage. Values for TAG, % lipid, and gill NKA were log transformed prior to analysis to meet assumptions of normality.

Results

Is there an effect of holding time on exploratory behaviour?

Out of the 252 smolts tested for salinity preference overall, 47% were non-exploratory (n = 119). At the onset of lake outmigration, 68% of smolts tested were exploratory. 6-10 days after lake outmigration, 36% of smolts were exploratory. 21-24 days after lake outmigration, 51% of smolts were exploratory. Days since outmigration failed to explain the likelihood of exploratory behaviour (β =-2.96 x10^{-3}, SE = 0.015, z = -0.194 p = 0.85).

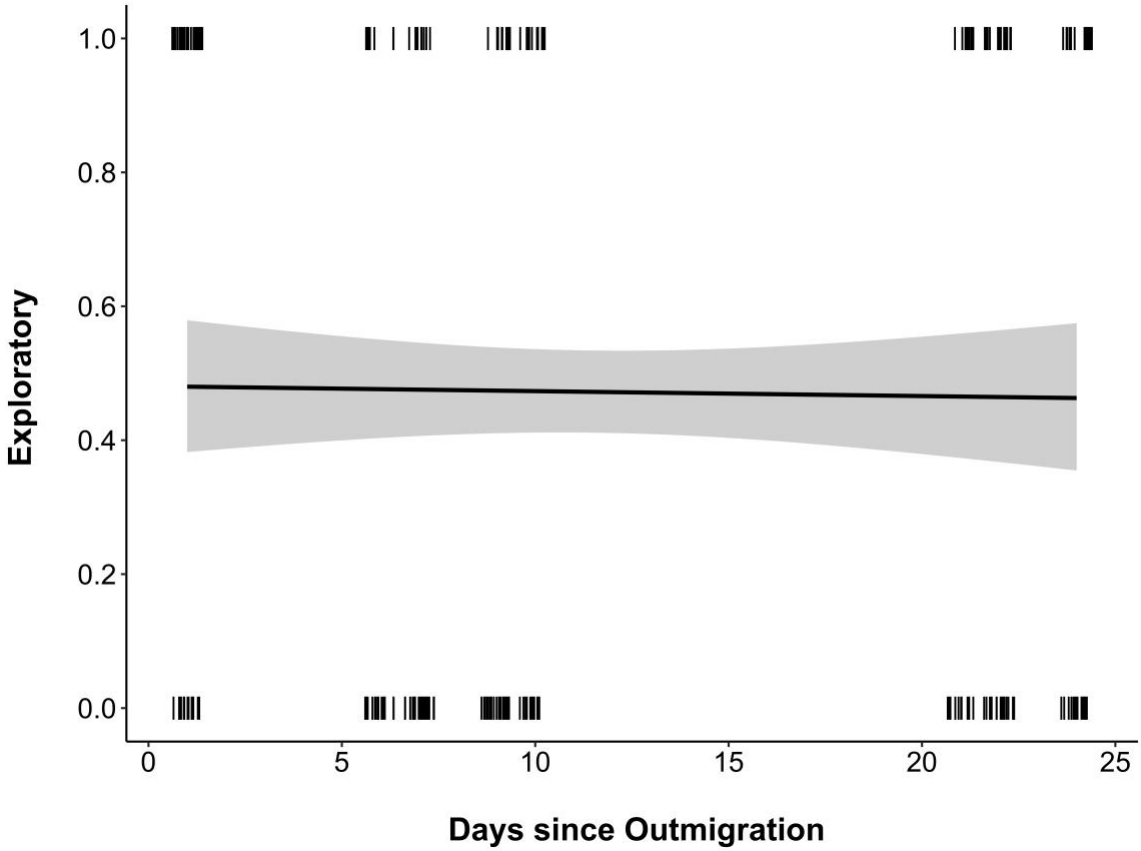


Fig. B1. **Exploratory behaviour is not explained by days since outmigration. Smolts in the river treatment group were tested 1 day after outmigration. Smolts in the estuary treatment group were tested 6-10 days after outmigration. Smolts in the estuary treatment group were tested 21-24 days after outmigration.**

Are there differences in physiology between exploratory and non-exploratory smolts?

I did not find sufficient evidence that exploratory or non-exploratory smolts differed in condition (Figure A5). Fork length ($t(242) = 0.103$, $SE = 1.606$, $p > 0.1$), wet mass ($t(243) = 0.301$, $SE = 0.118$, $p > 0.1$) and Fulton's Condition Factor ($t(250) = -0.734$, $SE = 0.008$, $p > 0.1$) did not significantly differ between exploratory and non-exploratory fish. Energetic variables were only available for comparison between exploratory and non-exploratory fish in the first outmigration group (river, $n = 45$). Log of percent lipid ($t(43) = 0.871$, $SE = 0.108$, $p = 0.39$), percent protein ($t(43) = 1.103$, $SE = 0.325$, p = 0.276), energetic density ($t(43) = 0.521$, $SE = 0.267$, p = 0.605), and percent TAG (g/g lipid, $t(43) = -0.482$, $p >$

0.1), were not significantly different between exploratory (n = 29) and non-exploratory (n = 16) fish. Gill NKA activity did not differ between exploratory and non-exploratory smolts ($t(243) = -0.052$, $SE = 0.348$, $p > 0.1$).

Tab. B1. Smolt size, weight and physiological condition throughout each outmigration stage of salinity preference experiments. Means within each stage are reported with standard deviation in parenthesis. Results are presented for exploratory and non-exploratory (NE) smolts separately, from linear regressions of each condition variable by day since lake outmigration. The slope (β) indicates the estimated rate of change in condition per day, and the intercept indicates the model estimate for condition at lake outmigration. Models that were significant to $\alpha = 0.05$ are shown in bold (P).

Condition variable	Behaviour	Mean within outmigration stage (SD)			n	β_0	β	SE	P
		River	Estuary	Ocean					
Fork length (mm)	E	88 (5)	85 (6)	84 (5)	115	87.25	-0.149	0.059	**0.01**
	NE	86 (4)	85 (6)	86 (5)	133	85.51	+0.014	0.060	0.8
Wet Mass (g)	E	4.92 (0.90)	4.50 (0.96)	3.95 (0.81)	115	4.93	-0.045	0.009	**<0.001**
	NE	4.79 (0.62)	4.58 (0.97)	4.14 (0.79)	133	4.82	-0.030	0.010	**<0.01**
Fulton's K (10^5 g mm^{-3})	E	0.73 (0.05)	0.71 (0.06)	0.65 (0.04)	115	1.17	-0.004	0.001	**<0.001**
	NE	0.74 (0.04)	0.73 (0.05)	0.65 (0.06)	133	1.36	-0.005	0.001	**<0.001**
Gill NKA activity (µmol ADP mg protein^{-1} hr^{-1})	E	8.63 (3.33)	8.34 (2.56)	8.36 (3.16)	109	8.48	-0.005	0.032	0.9
	NE	8.26 (2.34)	7.90 (2.47)	9.44 (2.43)	131	7.42	+0.086	0.028	**<0.01**

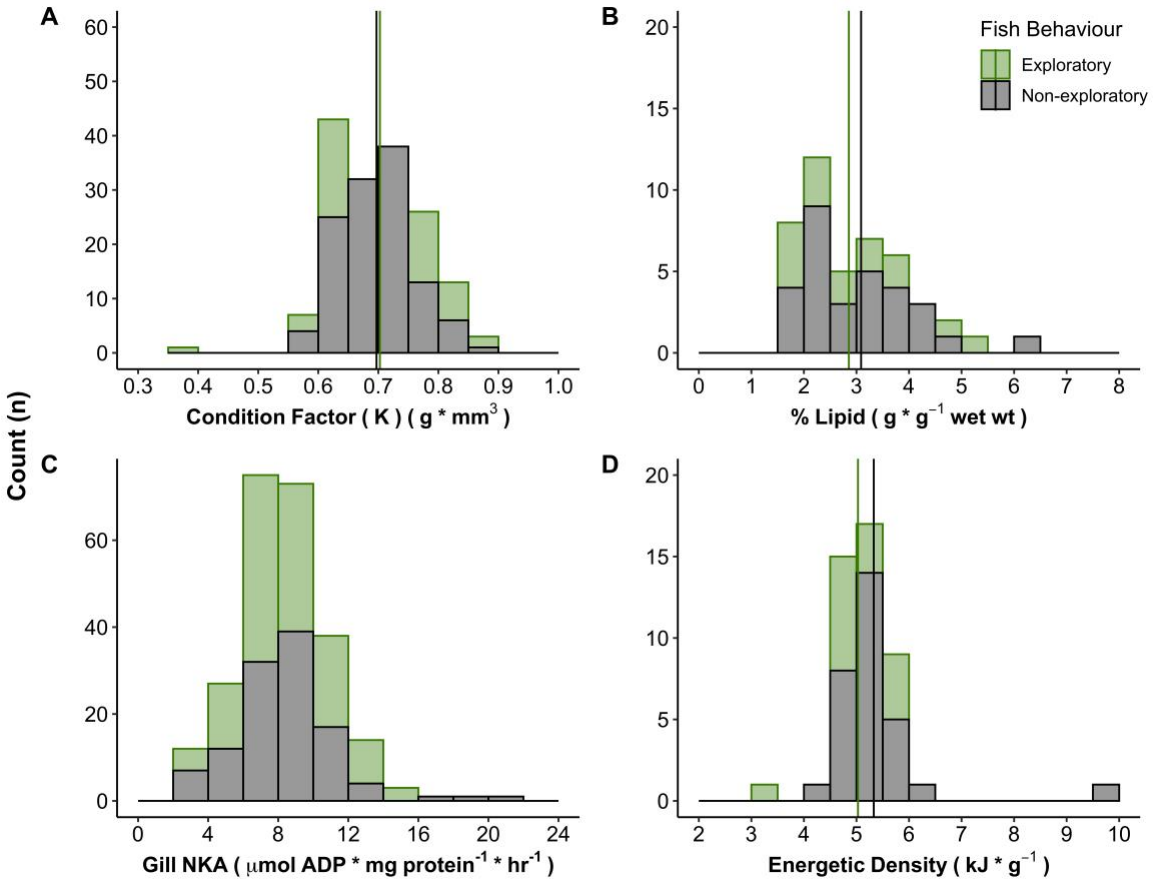


Fig. B2. Physiological condition distributions of exploratory (green) and non-exploratory (grey) smolts from salinity preference experiments. Values for Fulton's Condition Factor (K) and gill Na^+ K^+ ATPase (NKA) activity are shown for all experimental smolts (*n* = 252), while values for lipid, and energetic density (ED) are from smolts at the onset of lake outmigration (*n* = 45). Means for each group are shown as solid lines.

Is there a discrepancy between metrics of salinity preference?

The majority of the time, the last chamber occupied by the fish was also the chamber in which the fish spent >50% of the experimental time (Figure B3). At lake-exit, the last chamber occupied for each fish tested (n = 48) was also the chamber most occupied by each fish. Interestingly, the result for individual salinity preference changes slightly depending on the metric used for fish tested at estuary-entry and ocean-entry. At estuary-entry, a small proportion of fish (5%) that spent more than 50% of the experiment in freshwater changed chambers at

the end of the experiment to end in saline water. Likewise, a small proportion of fish (2%) that spent the most time in saltwater changed chambers at the end of the experiment to reside in brackish water. At ocean-entry, I saw the highest variation in determining salinity preference between the two metrics, where all chambers showed a minor proportion (<20%) of fish switching at the end of the experiment. Overall, using the salinity of the final chamber as opposed to the salinity of the longest occupied chamber would have increased the number of fish that prefer brackish water by 8% (reducing freshwater preference by 5% and saltwater preference by 3%).

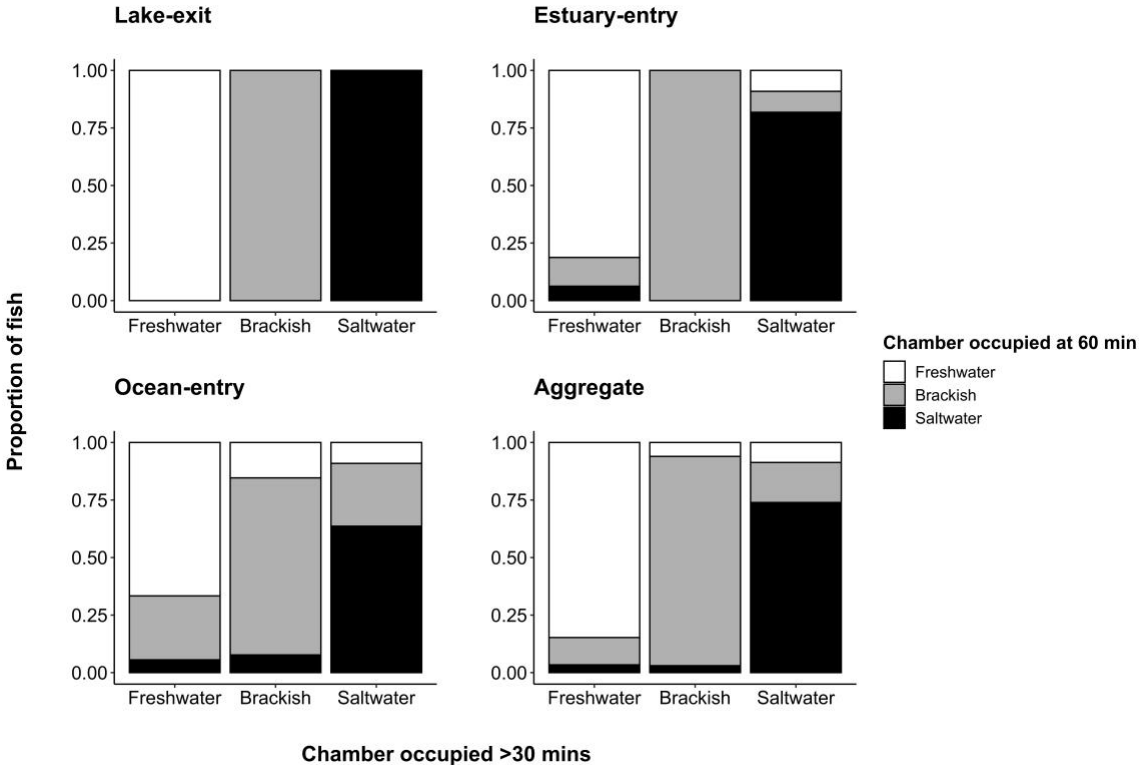


Fig. B3. **Difference in preferred salinity if assigned as salinity of chamber occupied for more than 50% of experimental trial (>30 mins) or as last chamber occupied at end of experimental trial (at 60 mins).**

Does inclusion of non-exploratory smolts alter the outcome of salinity preference models?

Including non-exploratory smolts in salinity preference analysis for lake-exit, estuary-entry, and ocean-entry increased sample sizes from 30 to 45 fish, 43 to 120 fish, and 42 to 83 fish, respectively. Similar trends of salinity preference were seen for exploratory and nonexploratory fish throughout migration stages (see Table 2 in main body of text). At lake-exit, freshwater was the most preferred salinity, regardless of behaviour. At estuary-entry, there was relatively equal preference for fresh and brackish water among exploratory and non-exploratory smolts. At ocean entry, the majority of exploratory smolts preferred freshwater, while most non-exploratory smolts preferred saltwater.

At lake-exit, the null model was selected as the most parsimonious model to predict salinity preference for the dataset including non-exploratory smolts ($\underline{n}$ = 45). This is the same result when only exploratory smolts were included in the analysis. At estuary-entry, the top model for predicting salinity preference among exploratory and non-exploratory smolts (n = 120) included K and gill NKA (AIC_c =251.48, Table B2). When only exploratory smolts were included in model selection, the top model included K, gill NKA and also TAG. Energetics were unavailable for non-exploratory smolts at this stage and so the effect of TAG on salinity preference is undetermined. Smolts (exploratory and non-exploratory) with increasing condition (K and gill NKA) showed increased preference for saltwater (Figure B4, Table B3). This is the same trend when only exploratory smolts were included in the analysis.

At ocean-entry, gill NKA was the top model for predicting salinity preference in exploratory and non-exploratory fish (AIC_c =172.11, Table B2). When only exploratory smolts were included in model selection, the top model included gill NKA and energetic density (ED) as predictors. Energetics were unavailable for non-exploratory smolts at this stage and so the effect of ED on salinity preference is undetermined. Smolts (exploratory and non-exploratory) with increasing condition (ED and gill NKA) showed increased preference for

saltwater (Figure B4, Table B3). This is the same trend when only exploratory smolts were included in the analysis.

Table B2. Model selection for predicting salinity preference by physical and physiological condition in the each outmigration stage. All models include exploratory and non-exploratory smolts.

Migration Stage	Candidate Model Expl. Variables	AICc	ΔAICc	Model Likeli.	AICc Wt	Least Likeli.	Cum. Wt
Lake-exit	1	55.21	0.00	1.00	0.12	-25.56	0.12
	k	56.25	1.04	0.59	0.07	-25.03	0.19
	log(NKA)	56.90	1.69	0.43	0.05	-25.36	0.25
	log(lipid)	56.99	1.78	0.41	0.05	-25.40	0.30
	fl	57.14	1.93	0.38	0.05	-25.48	0.34
	log(TAG)	57.18	1.97	0.37	0.05	-25.50	0.39
	ed	57.19	1.98	0.37	0.05	-25.51	0.43
	prot	57.26	2.05	0.36	0.04	-25.54	0.48
	mass	57.30	2.09	0.35	0.04	-25.56	0.52
	k + log(lipid)	58.19	2.98	0.23	0.03	-24.94	0.55
	k + ed	58.23	3.02	0.22	0.03	-24.96	0.58
	k + prot	58.23	3.02	0.22	0.03	-24.96	0.60
	k + log(NKA)	58.24	3.03	0.22	0.03	-24.97	0.63
	k + log(TAG)	58.28	3.07	0.22	0.03	-24.99	0.66
	log(lipid) + log(NKA)	58.47	3.26	0.20	0.02	-25.08	0.68
	log(TAG) + log(NKA)	58.84	3.64	0.16	0.02	-25.27	0.70
	ed + log(NKA)	58.87	3.66	0.16	0.02	-25.28	0.72
	fl + log(NKA)	58.88	3.67	0.16	0.02	-25.29	0.74
	prot + log(NKA)	58.98	3.78	0.15	0.02	-25.34	0.76
	fl + log(lipid)	59.00	3.79	0.15	0.02	-25.34	0.78
	mass + log(NKA)	59.02	3.81	0.15	0.02	-25.36	0.79
	mass + log(lipid)	59.10	3.89	0.14	0.02	-25.40	0.81
	fl + ed	59.20	3.99	0.14	0.02	-25.45	0.83
	fl + log(TAG)	59.21	4.01	0.13	0.02	-25.45	0.84
	fl + prot	59.24	4.03	0.13	0.02	-25.47	0.86
	mass + log(TAG)	59.28	4.07	0.13	0.02	-25.48	0.88
	mass + ed	59.30	4.09	0.13	0.02	-25.50	0.89
	mass + prot	59.37	4.17	0.12	0.02	-25.53	0.91
	k + log(NKA) + ed	60.22	5.01	0.08	0.01	-24.88	0.92
	k + log(NKA) + log(TAG)	60.27	5.06	0.08	0.01	-24.90	0.93
	k + log(NKA) + prot	60.27	5.06	0.08	0.01	-24.90	0.94
	k + log(NKA) + lipid	60.33	5.13	0.08	0.01	-24.93	0.95
	fl + log(NKA) + lipid	60.87	5.66	0.06	0.01	-25.20	0.95
	fl + log(NKA) + ed	60.94	5.73	0.06	0.01	-25.24	0.96
	mass + log(NKA) + lipid	60.95	5.74	0.06	0.01	-25.24	0.97
	fl + log(NKA) + log(TAG)	60.95	5.74	0.06	0.01	-25.24	0.97
	mass + log(NKA) + log(TAG)	60.98	5.78	0.06	0.01	-25.26	0.98
	fl + log(NKA) + prot	61.02	5.81	0.05	0.01	-25.28	0.99
	mass + log(NKA) + ed	61.03	5.82	0.05	0.01	-25.28	0.99
	mass + log(NKA) + prot	61.14	5.93	0.05	0.01	-25.34	1.00
Estuary-entry	k + log(NKA)	251.48	0.00	1.00	0.87	-121.68	0.87
	k	255.50	4.02	0.13	0.12	-124.72	0.99

	log(NKA)	261.81	10.32	0.01	0.00	-127.87	0.99
	fl + log(NKA)	263.57	12.09	0.00	0.00	-127.73	1.00
	mass + log(NKA)	263.62	12.14	0.00	0.00	-127.75	1.00
	1	264.52	13.04	0.00	0.00	-130.24	1.00
	fl	266.22	14.74	0.00	0.00	-130.08	1.00
	mass	266.45	14.97	0.00	0.00	-130.19	1.00
Ocean-entry	log(NKA)	172.11	0.00	1.00	0.38	-83.00	0.38
	k + log(NKA)	172.59	0.48	0.79	0.30	-82.21	0.68
	fl + log(NKA)	173.56	1.45	0.48	0.18	-82.69	0.86
	mass + log(NKA)	174.18	2.07	0.36	0.14	-83.00	1.00
	1	185.52	13.41	0.00	0.00	-90.74	1.00
	k	185.99	13.88	0.00	0.00	-89.95	1.00
	mass	186.84	14.72	0.00	0.00	-90.37	1.00
	fl	187.48	15.37	0.00	0.00	-90.69	1.0

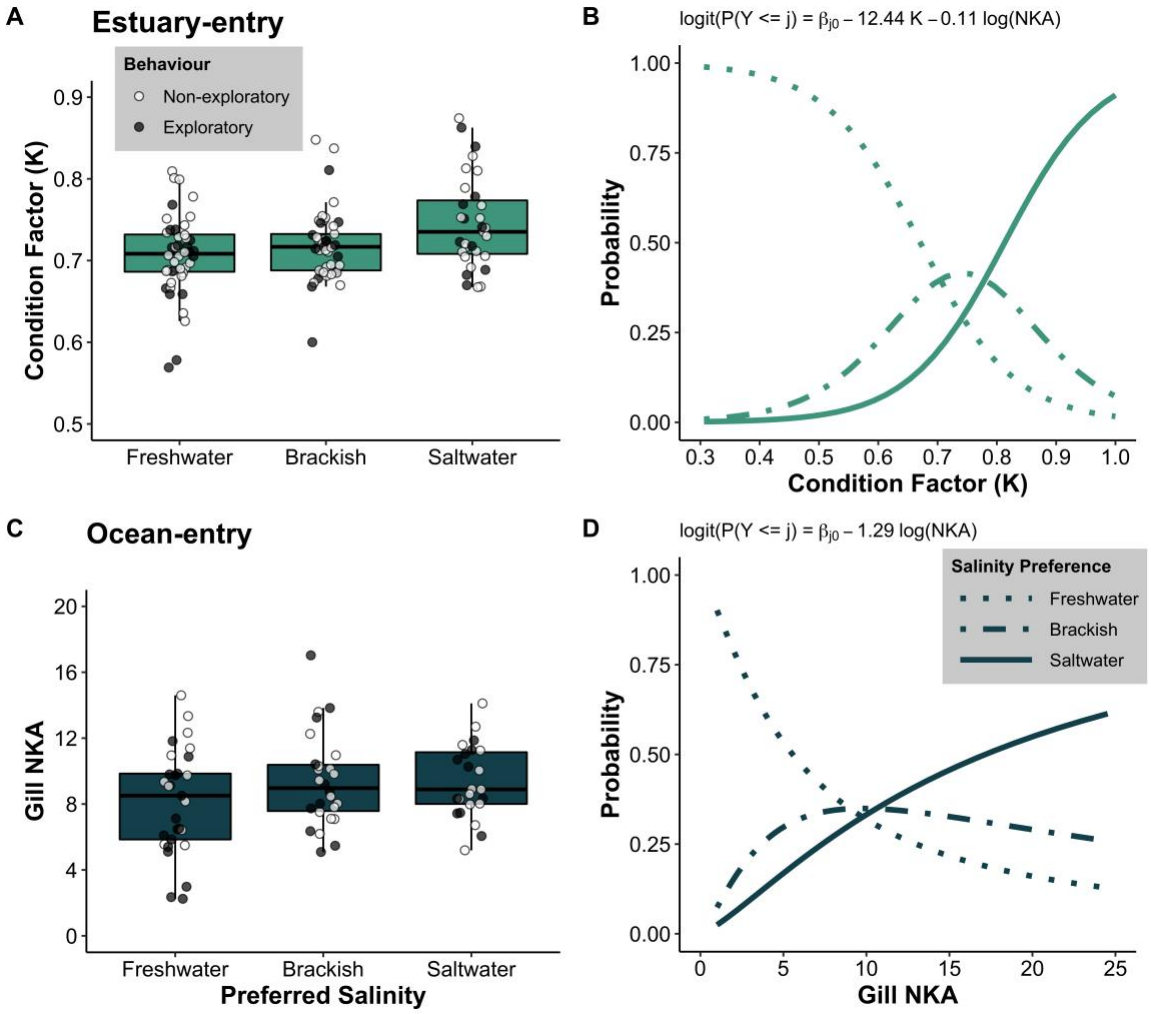


Figure B4. **Preferred salinity by smolt condition factor (A) and gill NKA activity (C). White and black circles indicate non-exploratory and exploratory smolts, respectively. Predictive models of salinity preference depending on smolt physiology at estuary-entry (A,B) and ocean-entry (C,D). Linetype indicates freshwater (dotted line), brackish (dot and dash line) or saltwater (solid line). In the estuary model (B), probabilites were calculated for values of Fulton's condition factor (K) while holding gill NKA at mean (M_{NKA} = 7.8 µmol ADP mg protein^{-1} hr^{-1}. The probability distribution for gill NKA is not shown as the effect size is small (β = 0.11).**

Table B3. Salinity preference model summaries for smolts tested within the estuary-entry, ocean-entry and all outmigration stages (from lake-exit to ocean-entry). All models include exploratory and non-exploratory smolts. Models were selected by lowest AIC_c. Odds ratio and 2.5% and 97.5% confident interals are shown for coefficients scaled by 1 SD increase/decrease of each explanatory variable. The t value shows the Wald statistic. The null model was selected for lake-exit and is not shown.

Outmigration Stage	Predictor	*β*	SE	*t* value	*p*	OR	2.5% CI	97.5% CI
Estuary-entry	Condition Factor (K)	12.441	3.700	3.363	0.00	1.89	1.31	2.75
	log(NKA)	0.108	0.545	0.198	0.84	1.04	0.73	1.48
Ocean-entry	log(NKA)	1.294	0.622	2.082	0.04	1.62	1.03	2.54

Conclusion

I did not find sufficient evidence that exploratory or non-exploratory behaviour in smolts was explained by the duration of time in freshwater captivity. Further, exploratory and non-exploratory smolts did not differ in physiological condition. While there may be other important physiological parameters not assessed in this study, these results suggest that exploratory behaviour is most likely driven by individual temperament to risk, and not physiology or time in captivity. To determine salinity preference, I did not find evidence to suggest that the metric used would alter overall results. For the majority of fish, the last chamber occupied was also the chamber with the longest occupancy. Trends in salinity preference throughout migration by exploratory and non-exploratory smolts are congruent. When pooled, results for model selection and the effect of smolts physiology on salinity preference were similar in variables selected and direction of effect. Inclusion or exclusion of non-exploratory smolts did not change the overall findings of this study: Smolts of higher condition generally chose water of higher salinity, while smolts of lower condition preferred freshwater. Overall, non-

exploratory behaviour was non explained by holding time or fish condition, and exclusion of non-exploratory behaviour did not change model results.