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ARTICLE

A novel pattern of smoltification in the most anadromous salmonid: pink salmon (*Oncorhynchus gorbuscha*)

Z.S. Gallagher, J.S. Bystriansky, A.P. Farrell, and C.J. Brauner

Abstract: Pink salmon (*Oncorhynchus gorbuscha*) enter seawater earlier and smaller (0.2 g) than any other salmonid following a two-stage salinity tolerance process initiated around yolk-sac absorption and completed with seawater entry. For the first time, this two-stage ontogeny of salinity tolerance was characterized by either holding posthatch pink salmon in fresh water or transferring them to seawater every 2 weeks. A window of salinity tolerance around yolk-sac absorption was evidenced by a period of zero morbidity in seawater compared with 100% morbidity for newly hatched alevins and 25% morbidity for fry (~0.2–0.3 g). Increased hypo-osmoregulatory ability at the time of yolk-sac absorption was indicated in fish held in fresh water under constant photoperiod (12 h light : 12 h dark) and temperature (5 °C) by a switch from catabolic to anabolic growth, increased gill Na^+K^+-ATPase activity and α-1b/α-1a isoform expression, and a plateau in whole-body water content, implying that pink salmon go through a form of smoltification. A large increase in whole-body [Na^+] observed in fresh water at yolk-sac absorption may represent a unique strategy for maintaining water balance once fish enter seawater.

Résumé : Le saumon rose (*Oncorhynchus gorbuscha*) fait son entrée en eau de mer plus tôt et à plus petite taille (0,2 g) que tout autre salmonidé, après un processus d'adaptation à la salinité en deux étapes débutant aux alentours de l'absorption du sac vitellin et se terminant avec l'entrée en eau de mer. Pour la première fois, cette ontogénie en deux étapes de la tolérance à la salinité a été caractérisée soit en maintenant des saumons roses post-éclosion en eau douce ou en les transférant en eau de mer toutes les deux semaines. La présence d'une fenêtre de tolérance à la salinité vers le moment de l'absorption du sac vitellin est indiquée par une période de morbidité nulle en eau de mer, comparativement à une morbidité de 100 % chez les alevins nouvellement éclos et de 25 % pour le fretin (~ 0,2–0,3 g). Une capacité accrue d'hypoosmorégulation au moment de l'absorption du vitellin était indiquée chez les poissons maintenus en eau douce dans des conditions de photopériode (12 h de lumière : 12 h d'obscurité) et de température (5 °C) constantes par le passage d'une croissance catabolique à une croissance anabolique, des augmentations de la Na^+K^+-ATPase branchiale et du rapport d'expression d'isoformes α-1b/α-1a, et un plafonnement de la teneur corporelle globale en eau, ce qui signifie que le saumon rose subit une forme de smoltification. Une importante augmentation de la [Na^+] corporelle globale observée en eau douce au moment de l'absorption du sac vitellin pourrait représenter une stratégie particulière de maintien de l'équilibre hydrique une fois que les poissons entrent en eau de mer. [Traduit par la Rédaction]

Introduction

Pink salmon are the most widely distributed and abundant of the Pacific salmon and by number represent two-thirds of the Pacific salmon caught by Canada, the United States, Russia, and Japan (Heard 1991). Beyond their enormous commercial value to North America, they are also thought of as a valuable indicator of ecosystem health (Brauner et al. 2012). Despite the existence of spawning locations inland, pink salmon typically spawn closer to seawater than do other Pacific salmon (Heard 1991). Compared with other salmonids, the life history of pink salmon is unusual in that they migrate into seawater immediately upon gravel emergence, leaving little preparatory time for the enormous physiological, biochemical, morphological, and behavioural changes associated with smoltification. Thus, by being the smallest of any salmonid that enters seawater (mass ~0.2 g), their large surface area to volume ratio creates significant challenges for water and ion balance. Indeed, a large initial increase in whole-body ions observed following natural seawater entry has led to the suggestion that pink salmon may not be fully prepared for seawater entry while in fresh water (Grant et al. 2009), in contrast with other salmonids whose smolts and are fully prepared for seawater entry. Once in seawater, whole-body ion levels in pink salmon progressively decline in concert with an increase in gill Na^+K^+-ATPase (NKA) activity, which peaks at 8 weeks after seawater transfer (Grant et al. 2009). This suggests that pink salmon may secondarily respond to seawater exposure itself rather than undergoing smoltification.

Smoltification, a complex physiological process that prepares freshwater fish for successful osmoregulation in seawater, is very well studied in anadromous salmon species other than pink and chum salmon. Following a 1- to 2-year residence in fresh water, the classic smoltification process is triggered by a critical size threshold (e.g., >12 cm for Atlantic salmon) (Stefansson et al. 2008; McCormick 2009). Environmental factors also influence the timing of smoltification, with photoperiod being the main environmental cue (Hoar 1988; Stefansson et al. 2008). The size and quantity of gill chloride (Cl^-) cells increase, and ion transporter proteins, such as NKA, $Na^+K^+Cl^-$ cotransporter (NKCC), and cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels are upregulated to increase gill ion excreting capacity in seawater and improve salmon survival (Hoar 1988; Pissam et al. 1988; McCormick 2009). In rainbow trout, an increasing ratio of mRNA expression of the

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α-1b and α-1a subunits of gill NKA signals seawater preparedness (Richards et al. 2003), a pattern also observed in Atlantic salmon smolts (Nilsen et al. 2007; Stefansson et al. 2007).

During smoltification, salinity tolerance is time-limited for salmonid species, termed a smolt window. Outside of this smolt window, hypo-osmoregulation in seawater is reduced (Boeuf and Harache 1982; Hoar 1988; Duston et al. 1991; McCormick et al. 1999; Handleand et al. 2004; Zydlewski et al. 2005; Stefansson et al. 2008; McCormick 2009; Duston et al. 2011), and so premature or delayed entry into seawater can result in an excessive, even lethal, elevation in plasma ions (Conte and Wagner 1965; Boeuf and Harache 1982; Arnesen et al. 2003).

Pink salmon do not display the classic morphological smolt characteristics (e.g., silvering and loss of parr markings on the skin), but possess a greater ability to regulate blood [Na^+] and [Cl^-] following seawater transfer compared with other salmon species of similar size (Weisbart 1968). While it is not known whether pink salmon smolt, there is limited evidence for some preparation for seawater entry, as indicated by an increase in plasma thyroxine (T4), gill NKA activity (Sullivan et al. 1983), and gill NKA α-1b/α-1a mRNA expression ratio (Sackville et al. 2012) in juvenile pink salmon remaining in fresh water at the time of natural outmigration. However, a detailed characterization of the physiological changes associated with salinity tolerance in developing juvenile pink salmon has not previously been conducted and is the objective of this study.

Here the ontogeny of salinity tolerance in juvenile pink salmon from the Quinsam River was thoroughly characterized for the first time from hatching through to 26 weeks posthatch (from 568 to 1269 accumulated temperature units (ATU)), a period that bracketed the timing (1000 ATU) of natural gravel emergence when yolk-sac absorption is completed and immediate seaward migration occurs (P. Scott and D. Babchuk, Fisheries and Oceans Canada, Campbell River, British Columbia, personal communication, 2010). Comparisons were made between fish held continuously in fresh water and those transferred and held in seawater on a biweekly basis, which allowed the assessment of changes in salinity tolerance and the determination of whether pink salmon prepare for seawater entry while residing in fresh water.

Materials and methods

Fish sources and husbandry

Approximately 5000 pink salmon eyed eggs were donated by the Quinsam River Hatchery, Campbell River, British Columbia. The Quinsam River is a tributary of the Campbell River, and the hatchery is located a few kilometres from where the two rivers meet and approximately 10 km from the ocean. The eggs had accumulated 487 ATUs before transfer (27 November 2008) to The University of British Columbia (UBC), where they were maintained in Heath trays supplied with recirculating dechlorinated Vancouver city tap water (Na^+, 0.06 mmol·L^{-1}; Cl^-, 0.05 mmol·L^{-1}; Ca^{2+}, 0.03 mmol·L^{-1}; Mg^{2+}, 0.007 mmol·L^{-1}; K^+, 0.004 mmol·L^{-1}; alkalinity, 3.3 mg $CaCO_3$·L^{-1}; hardness 3.55 mg $CaCO_3$·L^{-1}; pH 6.2–6.7; Metro Vancouver 2007) at (5 °C) within a dark, environmentally controlled room. Oxygen levels were maintained at >95% air saturation. Ammonia was measured using a Nutrafin ammonia test kit (unionized ammonia ranged from 0 to 0.0024 ppm at pH 7.6.), and water changes were performed daily.

Hatching started on 11 December and was completed by 14 December. On 11 January 2009, alevins were transferred into two 50 L glass aquaria containing charcoal-filtered, recirculating, dechlorinated fresh water (see above) that was changed three times each week and maintained at 5 °C on a 12 h light : 12 h dark photoperiod. Although it is known that both temperature and photoperiod are important cues for smoltification in other species (as is abrupt exposure to light in juvenile pink salmon fry; Sackville et al. 2012), temperature and photoperiod do not always covary (i.e., warm vs. cold years). Given that we were rearing fish over such a long period, we decided to simplify our experimental design and use a constant 12 h light : 12 h dark photoperiod and 5 °C. We chose these conditions because they represent the natural photoperiod and temperature that pink salmon could often experience at swim-up. Further studies should investigate the influence of changes in both temperature and photoperiod throughout development to simulate the natural environment; however, this was beyond the scope of the present study.

Comparisons with previous work are facilitated by characterizing the developmental stages as both the number of weeks posthatch and the ATU (e.g., W0; 568 describes fish that were 0 weeks posthatch and at 568 ATU). Fish possessing yolk are referred to as alevins, and after full absorption of the yolk sac as fry. The first feeding of powdered trout chow (Bio-vita Bio-Oregon, Longview, Wash., USA) occurred on 23 February 2009, when fish had absorbed approximately 90% of their yolk (W10; 924 ATU; Fig. 1). Fish were fed once or twice daily ad libitum, but food was withheld for approximately 18 h prior to sampling.

Experimental protocol and sampling

Continuous freshwater exposure

Every 2 weeks from 14 December 2008 (W0; 568 ATU) to 14 June 2009 (W26; 1479 ATU; Fig. 1), 20–30 fish from the freshwater holding tank were terminally sampled (see below) for measurements of whole-body wet and dry mass (used to calculate % body water content); whole-body [Na^+] (N = 10 fish); gill NKA α-1a and α-1b mRNA isoform expression; and gill NKA enzyme activity (N = 10 fish aged W0–W4 (568–709 ATU) or N = 20 fish aged W6–W26 (779–1479 ATU)). Fish aged W0 and W2 (568 and 639 ATU, respectively) were too small for gill sampling, and fish were not sampled on W22 (1408 ATU).

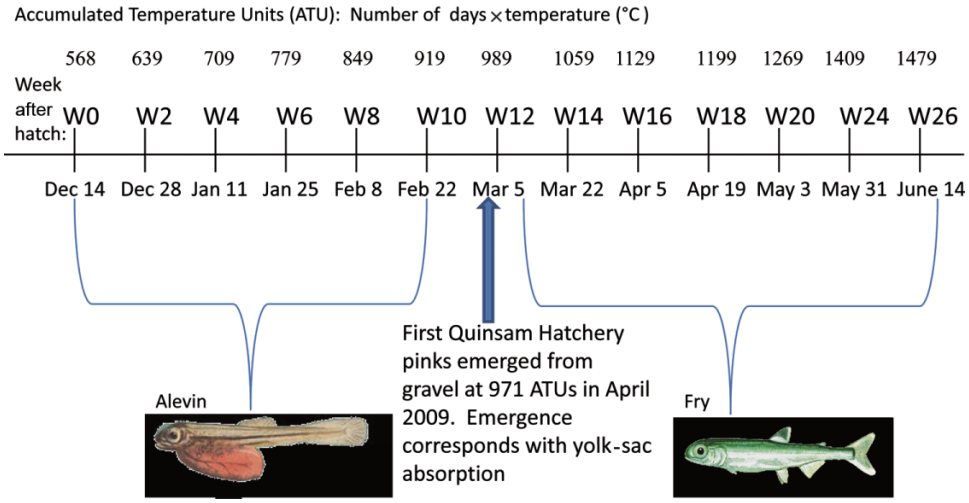
Seawater transfers

Fish were transferred from fresh water to 100% seawater (Instant Ocean, 33.1 ± 0.27 ppt, 5 °C; in a 50 L glass aquarium equipped with a charcoal filter) every 2 weeks from 14 December 2008 through to 3 May 2009 (Fig. 1). The cleaning and feeding protocol of fish held in seawater was similar to that in freshwater tanks, where oxygen, ammonia, and pH were measured multiple times per week. The first three transfers each involved 195 fish (W0–W4, 568–709 ATU; 14 December – 11 January), and the subsequent eight transfers each involved 245 fish (W6–W20, 779–1269 ATU; 25 January – 3 May). After transfer, fish were maintained in seawater and monitored daily for 8 weeks with moribund fish being immediately euthanized and counted. Fish were deemed moribund when they had lost equilibrium. Live fish were terminally sampled (see below) at 24 h, 5 days, 2 weeks, 4 weeks, and 8 weeks following seawater transfer for measurement of whole-body wet and dry mass (N = 10 fish); whole-body [Na^+] (N = 10 fish); gill NKA α-1a and α-1b mRNA isoform expression (N = 10 fish); and NKA enzyme activity (N = 10 W0–W4 fish or N = 20 W6–W20 fish. Of the collected samples, those chosen for gill NKA analysis were based upon the results for salinity tolerance (% morbidity and whole-body water content data) and corresponded to before, at, and after yolk-sac absorption (W4, W12, W18, and W20; 709, 989, 1199, and 1269 ATU, respectively). The measurements included gill NKA α1 mRNA isoform expression and NKA enzyme activity (with the exception of W4).

Fish sampling protocol

Fish were individually euthanized in a receptacle containing a lethal dose of buffered tricaine methanesulfonate (1.0 g L MS-222; Syndel Laboratories, Vancouver, British Columbia, Canada). Once euthanized, individual fish were rinsed with deionized water, blotted dry, transferred to a preweighed 15 mL polystyrene tube, weighed, and then either dried at 65 °C to constant mass and used

Fig. 1. A timeline of fish sampling and seawater transfers relative to accumulated temperature units (ATUs), weeks posthatch (W), and the corresponding calendar date. Fish were sampled from fresh water every 2 weeks beginning at hatching on 14 December 2008, when fish were defined as 0 weeks posthatch (W0), through to 14 June 2009, 26 weeks posthatch (W26). No fish were sampled from fresh water on 17 May 2009, and therefore one freshwater control group (W22) is missing. Fish were transferred to seawater every 2 weeks from 14 December 2008 (W0) until 3 May 2009 (W20) and were sampled at 24 h, 5 days, and 2, 4, and 8 weeks following seawater transfer. Fish were held at a constant temperature of 5 °C, whereas temperature was more variable at the Quinsam River Hatchery, which explains the disparity between the calendar dates at which fish reached approximately 1000 ATU (March vs. April).



for whole-body [Na^+] measurement or frozen immediately in liquid nitrogen inside a 1.5 mL Eppendorf tube and stored at −80 °C for later measurement of gill NKA activity and mRNA isoform expression levels.

Analytical techniques

Determination of whole-body percent water content and [Na^+]

Percent whole-body water content was calculated from wet and dry mass. Dried fish were digested at 65 °C in 1 mol·L^{-1} nitric acid (10× the wet mass of the fish) to determine whole-body [Na^+] (Grant et al. 2009; Sackville et al. 2011). Fish were dissociated with a metal spatula to aid the 48 h digestion process, after which the tubes were vortexed and the contents were allowed to settle at room temperature overnight. The supernatant was then analyzed for [Na^+] on a flame atomic absorption spectrophotometer (Spectra AA-220FS; Varian, Mulgrave, Victoria, Australia) and then standardized to dry body mass for whole-body [Na^+].

Gill NKA enzyme activity

Gill NKA enzyme activity was measured following the method described by Gibbs and Somero (1989) by measuring the difference in phosphate released from the gill homogenates with and without the NKA-specific inhibitor ouabain (final concentration 1 mmol·L^{-1}). Gills were dissected from the frozen fish on ice and homogenized on ice in buffer (pH 7.5, 150 mmol·L^{-1} sucrose, 10 mmol·L^{-1} EDTA, 50 mmol·L^{-1} imidazole, 0.1% sodium deoxycholate) using a glass homogenizer. NKA activity levels have previously been measured from frozen tissue (Gibbs and Somero 1989; Bystriansky and Schulte 2011). The homogenate was centrifuged for 1 min (4 °C) at 5000*g* to remove insoluble material, and the supernatant was used in the assay of gill NKA activity. Protein concentration of the homogenate was measured using the Bradford method (Bradford 1976). All samples were run in triplicate, and the mean value for each fish was used in the statistical analyses.

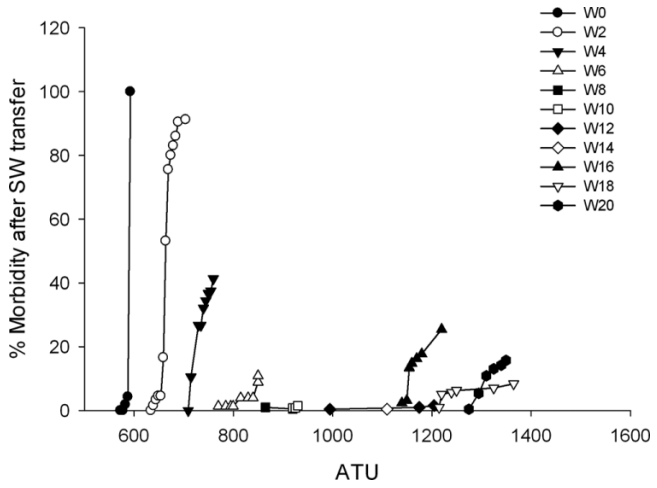
Gill mRNA expression

Expression studies followed protocols described in Bystriansky et al. (2006) and Bystriansky and Schulte (2011). Briefly, a guanidine thiocyanate method (Chomczynski and Sacchi 1987) was used to extract total RNA from gill samples using TriZol isolation reagent (Invitrogen, Carlsbad, Calif., USA). The isolated total RNA concentration and purity was determined spectrophotometrically. All total RNA samples used were found to be of very high purity (260:280 absorbance ratio > 1.8). RNA quality and concentration was further confirmed by running 2 μg on an agarose gel (1%). First-strand cDNA was synthesized from 2 μg of total RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems Inc., Foster City, Calif., USA). Quantitative reverse transcriptase (RT)–PCR (qRT–PCR) was conducted using an ABI Prism 7000 sequence analysis system (Applied Biosystems Inc.). Each PCR tube contained 1 μL of cDNA, 150 pmol of each primer, and 150 pmol of the universal SYBR green master mix (Applied Biosystems Inc.) (Bystriansky et al. 2006; Bystriansky and Schulte 2011). Forward and reverse primers used for each gene were isoform specific and tested to ensure that they amplified only a single target gene. Primer sequences used were as follows: EF1-α forward: 5′-GAGACCCATTGAAAAGTTCGAGAAG-3′, EF1-α reverse: 5′-GCACCCAGGCATACTTGAAAG-3′; NKA α-1a forward: 5′-GGCCGGCGAGTCCAAT-3′, NKA α1-a reverse: 5′-GAGCAGCTGTCCAGGATCCT-3′, NKA α-1b forward: 5′-CTGCTACATCTCAACCAACAACATT-3′, NKA α-1b reverse: 5′-CACCATCACAGTGTTCATTGGAT-3′. Multiple RNA samples that had not been reverse transcribed were also analyzed to determine potential genomic DNA contamination. These samples were randomly chosen and subjected to qRT–PCR with each primer pair. The observed ratio of amplification was found to be at most 1:1400 compared with the same normally reverse-transcribed sample, suggesting that genomic contamination was negligible. For each gene sampled, the relative quantity of mRNA was normalized to an endogenous gill reference (EF1-α; elongation factor (EF)) and expressed relative to the mean value for pink salmon aged W4 (709 ATU), which was the earliest freshwater value measured (Bystriansky et al. 2006; Bystriansky and Schulte 2011). EF1-α expression was found to remain stable for all experimental groups used in this study, suggesting it was a suitable reference gene.

Statistical analyses

All data are presented as means ± standard error of the mean for 8–10 fish unless otherwise specified. Statistical pairwise comparisons (*t* test) between fish developing in fresh water and those

Fig. 2. Percent morbidity following transfer from fresh water to seawater (SW) in pink salmon at different stages of development defined as accumulated temperature units (ATUs). Groups of developing pink salmon were transferred to SW every 2 weeks, where W in the legend refers to weeks posthatch. Thus, W2 refers to 2 weeks posthatch. Yolk absorption in the fish represented below was complete by 989 ATU. Pink salmon from the same cohort but maintained at Quinsam River Hatchery emerged at 971 ATU. (See Fig. 1 for further details.)



transferred to seawater were matched to the closest sample time in fresh water. Thus, fish sampled after 24 h or 5 days in seawater were compared with the freshwater value obtained at the time of seawater transfer, while fish that had been held for 2, 4, and 8 weeks in seawater were compared with the time-matched (same calendar date) freshwater-held fish. To compare groups in fresh water as they developed, one-way analysis of variance (ANOVA) followed by a Holm–Sidak post hoc test was used to compare groups when significance was found with ANOVA. All statistical analyses were performed using SigmaStat (version 10), and results with a p value < 0.05 were considered significant.

Results

Morbidity after seawater transfer

Most alevins transferred into seawater at 0 weeks posthatch (W0; 568 ATU) exhibited morbidity within 5 days, while those transferred at 2 weeks posthatch (W2; 639 ATU) exhibited morbidity in 2 weeks (Fig. 2). Survival in seawater continued to improve with time posthatch. At 4 weeks posthatch (W4; 709 ATU), seawater transfer resulted in 27% morbidity after 5 days and 41% morbidity after 2 weeks (Fig. 2), whereas at 4 weeks posthatch (W4; 709 ATU), 59% had survived in seawater for 4 weeks, at which time they were terminally sampled. Therefore, starting at 4 weeks posthatch, the majority of fish could survive for 4 weeks after an abrupt seawater transfer, which we term seawater tolerance.

At 6–14 weeks posthatch (W6; 779 ATU through to W14; 1059 ATU), survival approached 100% following seawater transfer (Fig. 2), increasing from 89% survival for 17 days at 6 weeks posthatch to 98% survival for 8 weeks at 14 weeks posthatch. However, at 16, 18, and 20 weeks posthatch (W16–W20; 1129–1269 ATU), a modest rate of morbidity that never exceeded 25% returned for seawater transfers (Fig. 2).

Percent whole-body water content

In fresh water, percent whole-body water content increased significantly with development, increasing from 62% at hatching to ~82% by 14 weeks posthatch (W14; 1059 ATU), where it stabilized through to the end of the experiment and complete yolk-sac absorption (~1000 ATU). This developmental change in percent whole-body water content of freshwater fish required time-matched statistical comparisons with seawater transfer and a grouping relative to time in seawater (24 h, 5 days, and 2, 4, and 8 weeks) (Fig. 3*b*).

With the exception of seawater transfer shortly following hatching when morbidity was high and rapid, percent whole-body water content was remarkably similar between seawater transfers and freshwater fish. Fish transferred at 568 and 639 ATU (W0, W2) experienced a large significant reduction in percent whole-body water content following 5 days in seawater (Fig. 3*b*). Figure 3*b* illustrates other instances (W8, W14, W16, W20; 849, 1059, 1129, 1260 ATU) when percent whole-body water content increased significantly following 5 days in seawater.

Wet and dry mass

While wet mass in fresh water almost tripled during the 26-week experiment, this apparent growth was not always realized as an increase in dry mass (Fig. 3*c*). Indeed, dry mass reached a nadir at 0.028 g in fish aged 14 weeks posthatch (W14; 1059 ATU; Fig. 3*d*), decreasing progressively from 0.055 g in recently hatched alevins (W0; 568 ATU) before regaining dry mass, which peaked at 0.08 g at W24 (1409 ATU). The initial loss of dry mass was compensated by a gain in water content, and the gain in dry mass after 1100 ATU reflected true anabolic growth, which again coincided with complete yolk-sac absorption.

Following seawater transfer, wet mass rarely differed from the freshwater values and similarly increased with ATU. However, seawater transfer resulted in significantly smaller but not permanent decreases in wet mass at 568 to 990 ATU (W0–W12) compared with the freshwater fish (Fig. 3*c*). Seawater transfer after 1100 ATU sometimes slightly delayed but never prevented anabolic growth, as revealed by increased dry mass (Figs. 3*c*, 3*d*).

Whole-body sodium levels

Freshwater fish increased whole-body [Na^+] by more than fivefold between hatching (79.3 μmol Na^+·(g dry mass)$^{-1}$ at W0; 568 ATU) and complete yolk-sac absorption (427.7 μmol Na^+·(g dry mass)$^{-1}$ at W12; 989 ATU) (Fig. 4*a*). Whole-body [Na^+] continued to increase but at a reduced rate through to 24 weeks posthatch (W24; 1409 ATU), when it had reached 566.4 μmol Na^+·(g dry mass)$^{-1}$ (Fig. 4*a*) and declined slightly to 546.7 μmol Na^+·(g dry mass)$^{-1}$ 2 weeks later (W26; 1479 ATU) (Fig. 4*a*).

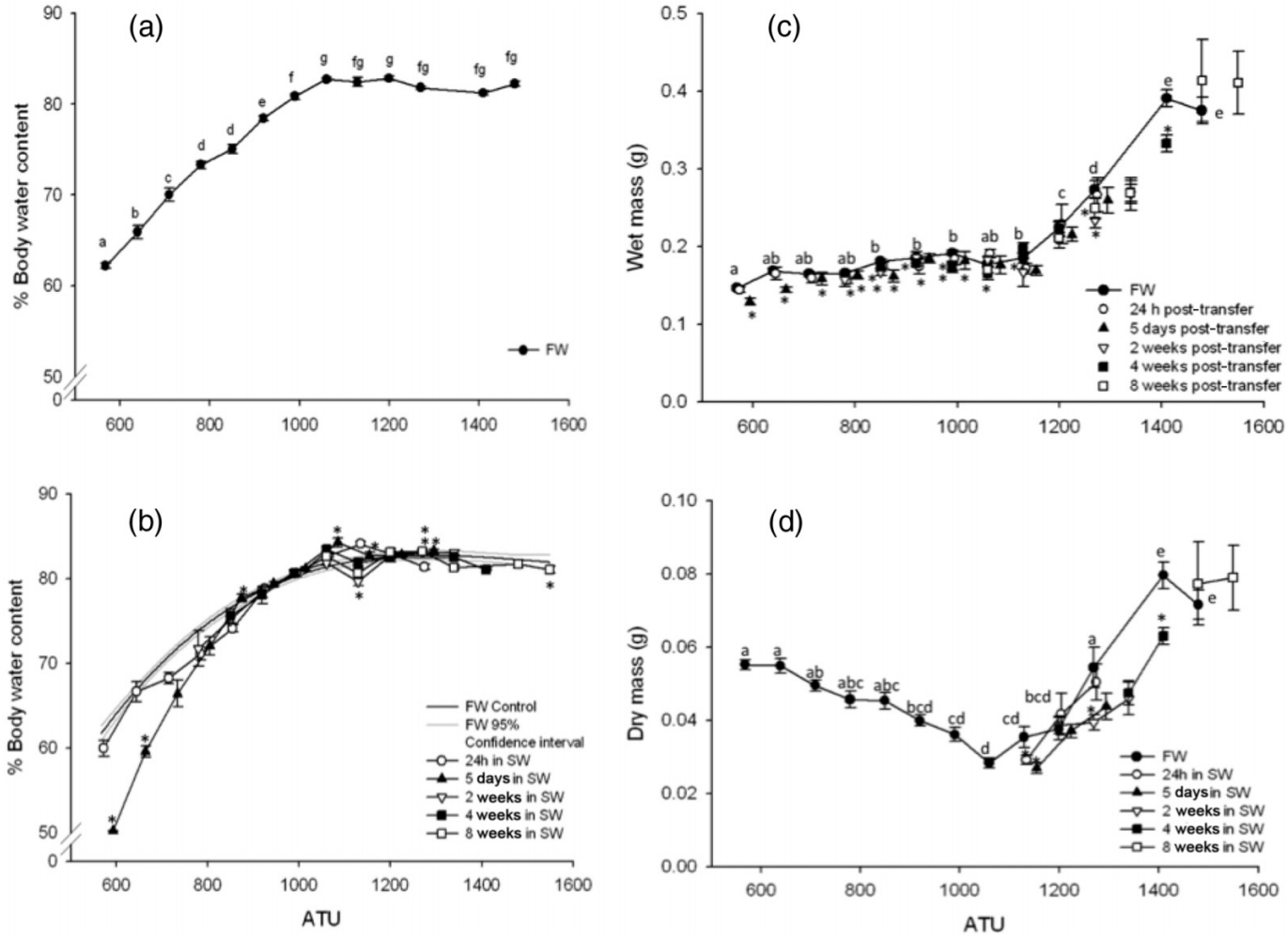
Following seawater transfer, whole-body [Na^+] rarely differed from the freshwater values until 8 weeks after seawater transfer (W6–W20; 779–1269 ATU), when whole-body [Na^+] was between 489.8 and 590.2 μmol Na^+·(g dry mass)$^{-1}$, regardless of when (and at what age) they were transferred (Fig. 4*b*). Fish that had been in seawater for 8 weeks displayed higher levels of whole-body [Na^+] than time-matched freshwater counterparts, with the exception of those transferred at 18 and 20 weeks posthatch (W18, W20; 1199, 1269 ATU), which had a similar whole-body [Na^+] as time-matched freshwater fish (Fig. 4*b*). Significant increases in whole-body [Na^+] following 24 h or 5 days in seawater were only observed in fish transferred to seawater 16 weeks posthatch (W16; 1129 ATU) and 18 weeks posthatch (W18; 1199 ATU) (Fig. 4*b*).

The plateau in [Na^+] along with the nadir in dry mass, the plateau in wet mass, and a stable level of water content all occurred around 1000 ATU, which corresponded with the completion of yolk-sac absorption.

Gill NKA activity and isoform expression in fresh water

In fresh water, gill NKA activity varied between 8.0 and 16.6 μmol ADP·(mg protein)$^{-1}$·h^{-1}, tending to increase over time without reaching statistical significance (Fig. 5). Gill NKA α-1a isoform expression relative to EF1-α started around 0.5–1.0, but significantly increased initially at 1100 ATU and secondarily to ~2.0 at 1409 ATU (W24) (Fig. 6*a*). Gill NKA α-1b isoform expression almost tripled from its initial value of 1.0 at 1100 ATU. Thereafter, gill NKA α-1b

Fig. 3. (*a*, *b*) Changes in percent whole-body water content in pink salmon at different stages of development defined as accumulated temperature units (ATUs) and held (*a*) continuously in fresh water (FW) or (*b*) following different durations of seawater (SW) exposure (24 h, 5 days, or 2, 4, or 8 weeks) in fish that were transferred from FW to SW every 2 weeks following hatching (see Figs. 1 and 2). In panel (*b*), a regression line (R^2 = 0.94) with 95% confidence intervals describing the FW controls presented in panel (*a*) is included for comparison with SW transfer values owing to the pronounced effect of development on percent whole-body water content. (*c*, *d*) Changes in wet and dry mass in pink salmon at different stages of development defined as accumulated temperature units (ATUs) held continuously in FW or following different durations of SW exposure (24 h, 5 days, or 2, 4, or 8 weeks). (*d*) Only fish that were transferred from FW to SW at 16, 18, and 20 weeks posthatch (W16, 1129 ATU; W18 1199 ATU; W20, 1269 ATU) are shown. Different letters indicate statistically significant differences ($p < 0.05$). An asterisk (*) indicates a significant ($p < 0.05$) difference from the initial FW control fish (24 h and 5 days) or a significant difference from time-matched FW control fish (2, 4, and 8 weeks). No FW control exists at 1339 ATU (W22). Symbols indicate mean values ± standard error of the mean (N = 10).



isoform expression decreased and remained around 1.0 until 1479 ATU (W26) (Fig. 6*b*).

The gill NKA α-1b/α-1a isoform expression ratio in fresh water changed significantly over time (Fig. 6*c*), increasing significantly from its initial value of 2.6 at 709 ATU to a peak four to five times higher, between 900 and 1100 ATU. Thereafter, the ratio declined significantly to the initial level or lower (Fig. 6*c*). Thus, the peak in the gill NKA α-1b/α-1a isoform expression ratio corresponded with the completion of yolk-sac absorption.

Gill NKA activity and isoform expression after seawater transfer

Gill NKA activity was unaffected by seawater transfer at 990 ATU, but almost doubled at 1269 ATU when compared with the time-matched freshwater control (Fig. 5). Seawater transfer always significantly decreased gill NKA α1-a isoform expression by 0.1–0.2 from initial values of between 0.4 and 1.4 (Fig. 6*a*) regardless of the life stage at transfer (W4, W12, W18, W20; 709, 989, 1199, 1269 ATU; Fig. 6*a*). In contrast, seawater transfer always significantly increased gill NKA α-1b isoform expression to between 2.7 and 4.1 regardless of life stage at transfer (W4, W12, W18, W20; 709, 989, 1199, 1269 ATU; Fig. 6*b*). The ratio continued to always increase with time in seawater such that after 2 weeks in seawater, increases of 8- to 30-fold were evident: from 2.6 to 60.2 at W4 (709 ATU), from 12.1 to 95.0 at W12 (989 ATU), from 2.1 to 61.0 at W18 (1199 ATU), and from 2.3 to 47.4 at W20 (1269 ATU) (Fig. 6*c*). Thus, seawater transfer stimulated the gill NKA α-1b/α-1a isoform expression ratio the greatest at the time of complete yolk-sac absorption.

Discussion

This study fully characterized the development of salinity tolerance in juvenile pink salmon from hatching to 20 weeks post-hatch using morbidity and recognized measures of smoltification (NKA activity and isoform expression). The normal developmental changes associated with freshwater residence resulted in toler-

Fig. 4. Changes in whole-body [Na^+] in pink salmon at different stages of development defined as accumulated temperature units (ATUs) held (*a*) continuously in fresh water (FW) or (*b*) following different durations of seawater (SW) exposure (24 h, 5 days, or 2, 4, or 8 weeks) in fish that were transferred from FW to SW every 2 weeks following hatching. In panel (*b*), FW values from panel (*a*) are included (with letters indicating statistical differences omitted for clarity) for comparison with SW transfer values owing to the pronounced effect of development on whole-body Na^+. No FW control exists at 1059 ATU (W14) or 1339 ATU (W22) (see Fig. 3 for further details).

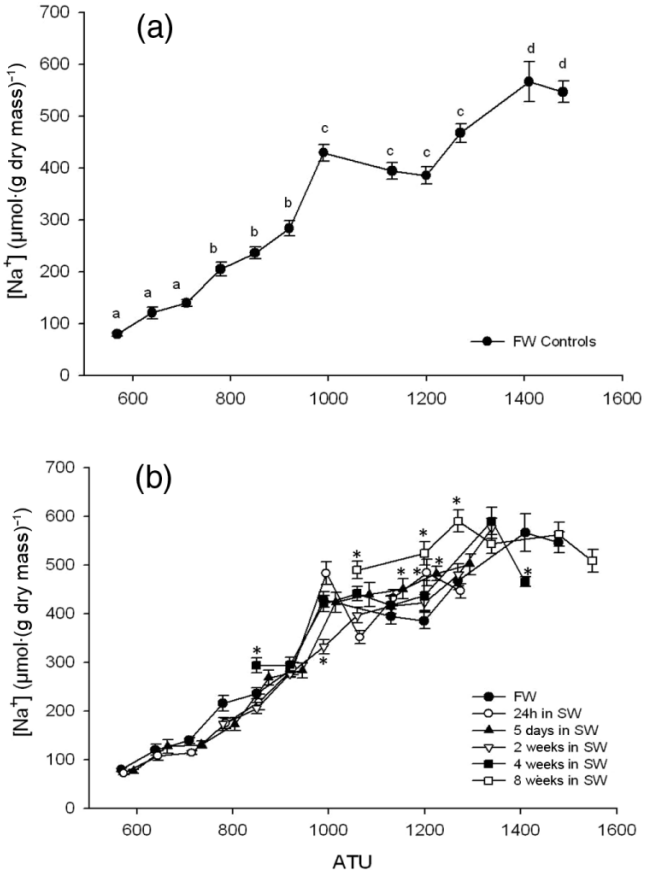
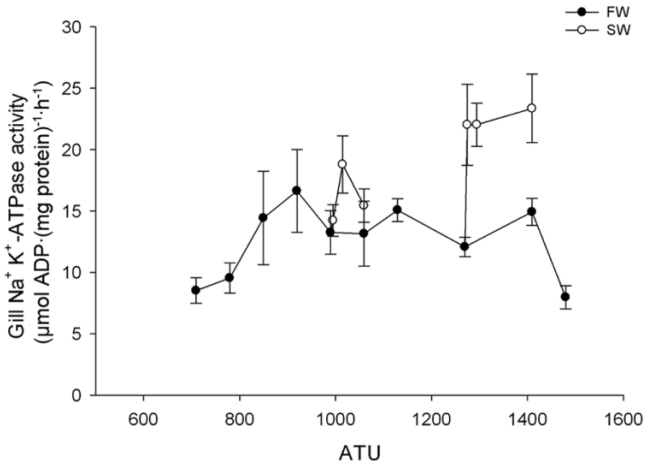


Fig. 5. Gill Na^+K^+-ATPase (NKA) activity (µmol ADP·(mg protein)$^{-1}$·h^{-1}) of fish in fresh water (FW) and those transferred to seawater (SW) at 12 and 20 weeks posthatch (W12, W20; 989, 1269 ATU). NKA activity represents mean ± standard error of the mean; $N = 8$ except at 4 weeks posthatch (W4; 709 ATU; $N = 3$) and 8, 14, 16, and 20 weeks posthatch (W8, W14, W16, W20; 849, 1059, 1129,1260 ATU; $N = 4$). No statistically significant differences exist between NKA values from FW-held fish. An asterisk (*) indicates a significant ($p < 0.05$) difference between SW and time-matched FW fish.



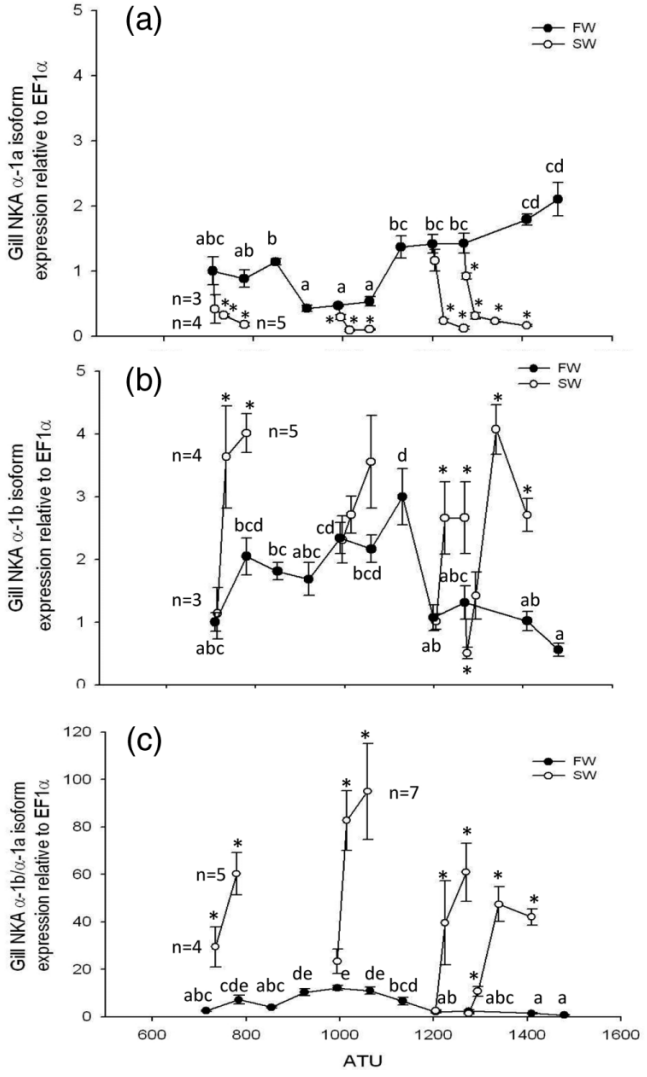
ance (no morbidity) to an abrupt seawater transfer that coincided with the completion of yolk-sac absorption (~1000 ATU), a time that corresponds with emergence and out-migration. This development of salinity tolerance was accompanied by physiological changes, including increased whole-body water content, increased gill NKA α-1b/α-1a mRNA isoform expression, and increased gill NKA enzyme activity that characterize smoltification in other species. Therefore, we conclude that pink salmon can indeed undergo the hypo-osmoregulatory changes associated with smoltification while in fresh water. Prior to this discovery, it was unknown whether pink salmon emerge from gravel redds completely ready for seawater life by undergoing appropriate physiological adjustments to their hypo-osmoregulatory capabilities. Further support for a smoltification process is the observation of a secondary series of physiological changes in freshwater pink salmon after yolk absorption, namely a decreased expression of α-1b/α-1a and survival in seawater. Taken together, these results imply a window of increased hypo-osmoregulatory ability as reported for other salmonid species (Hoar 1988; McCormick 2009).

While pink salmon displayed some characteristics of a smoltification window when held in fresh water, it is equally clear that this process differs from that of other anadromous salmon studied to date, in that pink salmon are not fully prepared for life in seawater. An abrupt transfer into full-strength seawater acted as a profound environmental stimulus to further induce smolt-like physiological changes, in particular a nearly eightfold increase in α-1b/α-1a gill NKA isoform expression following 2 weeks in seawater in fish transferred near 1000 ATU. Furthermore, gill NKA activity continues to increase for almost 8 weeks following natural seawater entry (Grant et al. 2009). Thus, even those "most ready" for seawater need to make adjustments once in seawater and are likely undergoing de novo synthesis of α-1b and suppressing α-1a expression following seawater entry.

This different lifestyle is undoubtedly related to the small size and abbreviated development of pink salmon on emergence and out-migration. For example, at hatching, the pink salmon alevins used here weighed a mean of 0.146 g, which is typical for this species (Honma 1982; Beacham 1991; Heard 1991), but do not develop scales and start mounting immune responses to sea lice until they reach ~0.7 g (Jones et al. 2008). By complete yolk-sac absorption, fry averaged 0.192 g, which lies within the normal size for migrant pink salmon fry throughout its Pacific range (0.13–0.26 g), as reported by Heard (1991) and others (Honma 1982; Higgs et al. 1985; Beacham 1991; Varnavsky et al. 1991, 1993). We also discovered that developing pink salmon possess a catabolic life phase between 570 and 1000 ATU, characterized by the consumption of yolk (lipid) and decreased dry mass, but increased wet mass associated with water accumulation (Fig. 3*a*). Consequently, wet mass is an unreliable indicator of somatic growth in developing pink salmon until yolk-sac absorption. The anabolic life phase (both wet and dry mass increased) that followed yolk-sac absorption was characterized by a growth rate of ~1.72% body mass·day^{-1} between 1100 and 1400 ATU, a rate lower than previously reported (Grant et al. 2009) when pink salmon were reared at a higher temperature (10 °C relative to 5 °C in this study) over a longer period.

Pink salmon populations vary in the distance they spawn from the ocean, with some populations spawning in the intertidal zone and others hundreds of kilometres from the ocean (Beacham 1991). Pink salmon with longer seawater migrations will have an increased freshwater residency period and may differ from coastal populations in the timing of the development of increased

Fig. 6. Changes in gill Na^+K^+-ATPase (*a*) α-1a, (*b*) α-1b, and (*c*) ratio of α-1a/α-1b mRNA isoform expression relative to EF1-α in pink salmon at different stages of development defined as accumulated temperature units (ATUs). The first data presented in all panels is 4 weeks posthatch (W4; 709 ATU; *N* = 3) with subsequent values obtained every 2 weeks in fresh water (closed circles in (*a*), (*b*), and (*c*)). In (*a*), (*b*), and (*c*), data are presented for fish transferred to seawater (open symbols where fish were sampled at 24 h, 5 days, and 2 and 4 weeks) at 4, 12, 18, and 20 weeks posthatch (W4; 709 ATU, W12; 989 ATU, W18; 1199 ATU and W20; 1269 ATU). Values in (*a*) and (*b*) are expressed relative to W4 fish (709 ATU). No freshwater control exists at 1339 ATU (W22). Symbols indicate means ± standard error of the mean (*N* = 8, unless otherwise indicated on graph; see Fig. 3 for further details).



hypo-osmoregulatory ability. Pink salmon from the Quinsam hatchery would be considered a coastal spawning population, as they spawn less than 10 km from the ocean (Beacham 1991) and they would reach the estuary within a day or two, once released from the hatchery (D. Babchuk, Fisheries and Oceans Canada, Campbell River, British Columbia, personal communication, 2012). We suggest that yolk-sac absorption is a useful life stage to indicate the time when coastal pink salmon are ready to out-migrate and display the peak in seawater tolerance and possibly the greatest potential for seawater entry to trigger physiological changes that further enhance hypo-osmoregulatory ability. Emergence of pink salmon fry from gravel redds generally occurs around complete yolk-sac absorption, typically between 900 and 1000 ATU (Bams 1972; Bailey et al. 1980; Heard 1991). Pink salmon from the same cohort used here, but held at the Quinsam River Hatchery, started emerging at 971 ATU in 2009 (P. Scott, Fisheries and Oceans Canada, Campbell River, British Columbia, personal communication, 2009) when yolk-sac absorption was mostly completed. Here, the window of salinity tolerance likewise occurred at about 1000 ATU, concurrent with complete yolk-sac absorption and the onset of exogenous feeding (W10; 924 ATU).

Physiologically, seawater tolerance appears to be associated with an improved ability to maintain water balance following seawater entry and may be mediated by increased gill NKA activity, which reached 14.4 μmol ADP·(mg protein)$^{-1}$·h^{-1} in fresh water in 8-week posthatch fish (W8; 849 ATU), around the time of nearly 100% seawater survival. This level of gill NKA activity is similar to previous reports for pink salmon smolts (Grant et al. 2009; Webster et al. 2007; Sackville et al. 2011) and chum (Iwata et al. 2010), but about half the 25–30 μmol ADP·(mg protein)$^{-1}$·h^{-1} measured in Atlantic salmon smolts (Nilsen et al. 2007; Stefansson et al. 2007). An increased α-1b/α-1a gill mRNA expression ratio is also a good index of seawater tolerance in pink salmon, with declines after yolk-sac absorption demarcating the decay of the window of salinity tolerance. This finding is consistent with studies of smolting Atlantic salmon in fresh water (Bystriansky et al. 2006; Nilsen et al. 2007; Stefansson et al. 2007).

Another trait apparently unique to pink salmon is the pattern of increasing whole-body [Na^+] in freshwater pink salmon. Developmental increases in whole-body ion levels have been previously reported in Atlantic salmon held in fresh water, but not nearly to the same degree (Rombough and Garside 1984). Following hatching, whole-body [Na^+] in Atlantic salmon increased 3.5-fold to 220 μmol·(g dry mass)$^{-1}$ around the time of yolk-sac absorption from 64.4 ± 7.1 μmol·(g dry mass)$^{-1}$ (compared with 79.3 ± 3.6 μmol·(g dry mass)$^{-1}$ for pink salmon; Rombough and Garside 1984). Here the increase in whole-body [Na^+] was nearly fivefold (to 427.7 μmol·(g dry mass)$^{-1}$. How this dramatic increase in whole-body [Na^+] in pink salmon is controlled (i.e., upregulation of influx or reduction in efflux) and whether it is associated with preparation for seawater entry is unknown, but it would seem to create a more favorable osmotic gradient between the animal and its seawater environment, facilitating maintenance of water balance (Sackville et al. 2012), a topic clearly worthy of further investigation. Consistent with this idea is the discovery of very little difference in whole-body [Na^+] for pink salmon in seawater or fresh water near yolk-sac absorption. Conversely, it has been suggested that completion of smoltification in Atlantic salmon is associated with a loss of hyperosmoregulatory ability and that migration to seawater occurs when fish are no longer suited for life in fresh water (Langdon and Thorpe 1985). Plasma osmolality or ion content after seawater transfer is often used to measure a fish's readiness for seawater (24 h seawater challenge; Blackburn and Clarke 1987); however, plasma could not be readily obtained in 0.15–0.30 g juvenile pink salmon. Instead, whole-body [Na^+] was measured here, which has been previously used as a proxy for ionoregulatory status following direct transfer to seawater in pink salmon (Grant et al. 2009; Sackville et al. 2011, 2012).

Pink salmon were held at a constant photoperiod and temperature to employ a simple experimental approach that would allow for physiological changes that occur throughout development to be observed. It is possible that developmental changes associated with seawater tolerance would be magnified if pink salmon were exposed to a naturally changing photoperiod and temperature regime, which should be the focus of future experiments. The natural environment is of course far more complex than the laboratory environment employed here. Also, fish were reared in Heath trays and tanks as opposed to gravel, obviating the natural experience of gravel emergence, yolk-sac absorption, initiation of feeding, and exposure to the natural changes in light. Therefore,

additional factors to those demonstrated here are likely potential cues of the smoltification process we characterized here. For example, a short photoperiod followed by lengthening photoperiod is typically a powerful trigger for smolting (Clarke and Hirano 1995), but not necessarily in other early ocean entry fish (chum and ocean chino; Clarke et al. 1989). All the same, Seymour River Hatchery pink salmon without a visible external yolk sac increased gill NKA enzyme activity within 24 h after being transferred from complete darkness to a 12 h light : 12 h dark photoperiod in fresh water (Sackville et al. 2012).

The finding that pink salmon possess a preparatory phase during which they exhibit heightened salinity tolerance is in line with the results of previous studies (Sullivan et al. 1983; Sackville et al. 2012). However, this study clearly defines the window of salinity tolerance that occurs at yolk-sac absorption and provides evidence that pink salmon go through a process of smoltification. Pink salmon that enter seawater around the time of normal gravel emergence and yolk-sac absorption are the most prepared for seawater, but they still use seawater entry to further upregulate their hypo-osmoregulatory machinery after out-migration, as was reported by Grant et al. (2009). The large increase in whole-body [Na^+] that occurs at yolk-sac absorption in fresh water may represent a novel pattern related to maintaining water and ion balance that is associated with seawater entry at such a small size, which has been referred to as "precocious anadromy" (Sackville et al. 2012), an observation clearly worthy of further investigation.

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