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ORIGINAL PAPER

# Water balance trumps ion balance for early marine survival of juvenile pink salmon (*Oncorhynchus gorbuscha*)

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**Abstract** Smolting salmonids typically require weeks to months of physiological preparation in freshwater (FW) before entering seawater (SW). Remarkably, pink salmon (*Oncorhynchus gorbuscha*) enter SW directly following yolk absorption and gravel emergence at a size of 0.2 g. To survive this exceptional SW migration, pink salmon were hypothesized to develop hypo-osmoregulatory abilities prior to yolk absorption and emergence. To test this, alevins (pre-yolk absorption) and fry (post-yolk absorption) were transferred from FW in darkness to SW under simulated natural photoperiod (SNP). Ionoregulatory status was assessed at 0, 1 and 5 days post-transfer. SW alevins showed no evidence of hypo-osmoregulation, marked by significant water loss and no increase in gill $Na^+/K^+$-ATPase (NKA) activity or $Na^+$:$K^+$:$2Cl^-$ cotransporter (NKCC) immunoreactive (IR) cell frequency. Conversely, fry maintained water balance, upregulated gill NKA activity by 50 %, increased the NKA α1b/α1a mRNA expression ratio by sixfold and increased NKCC IR cell frequency. We also provide the first evidence of photoperiod-triggered smoltification in pink salmon, as fry exposed to SNP in FW exhibited preparatory changes in gill NKA activity and α1 subunit expression similar to fry exposed to SNP in SW. Interestingly, fry incurred larger increases in whole body $Na^+$ than alevins following both SW and FW + SNP exposure (40 and 20 % in fry vs. 0 % in alevins). The ability to incur and tolerate large ion loads may underlie a novel mechanism for maintaining water balance in SW prior to completing hypo-osmoregulatory development. We propose that pink salmon represent a new form of anadromy termed "precocious anadromy".

**Keywords** Smoltification · Ionoregulation · Pink salmon

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## Introduction

Smoltification is a complex suite of behavioural, morphological and physiological changes that prepares anadromous salmonids for the transition from freshwater (FW) to the marine environment (for reviews see Hoar 1976; Folmar and Dickhoff 1980; McCormick and Saunders 1987; Hoar 1988; Boeuf 1993; McCormick 1994). Arguably the most dramatic change associated with this metamorphosis is the acquisition of seawater (SW) tolerance, in which fish switch from a hyper- to hypo-osmoregulatory strategy. To facilitate this remarkable shift, extensive remodelling of the major osmoregulatory organs is triggered by changes in photoperiod up to 2 months prior to seaward migration (Dickhoff et al. 1985; McCormick et al. 1987; Nilsen et al. 2007; Stefansson et al. 2007). The resulting preparation is often characterized by increased gill $Na^+/K^+$-ATPase (NKA) activity, upregulation of the enzyme's α1b/α1a mRNA expression ratio and increased frequency of $Na^+$:$K^+$:$2Cl^-$ cotransporter (NKCC) immunoreactive (IR) cells in the branchial epithelium; all of these endocrine-mediated changes have been shown to vastly

improve SW adaptability (Otto 1971; Boeuf and Harache 1982, 1984; Brauer 1982; Folmar et al. 1982; McCormick et al. 1987; Nielsen et al. 1999; Pelis et al. 2001; Nilsen et al. 2003, 2007; Bystriansky et al. 2006).

Pink salmon are an especially interesting fish in which to study smoltification because they possess the shortest FW residency and smallest body size of any salmonid upon ocean entry. In fact, pink salmon fry migrate seaward directly following yolk absorption and gravel emergence at a size of 0.2 g (Heard 1991; Grant et al. 2009). This life history pattern represents an extreme within salmonids, as most others move to SW after at least 1 year in FW and at sizes between 2 and 30 g (Rounsefell 1958; Clarke 1982; Hoar 1988; Quinn and Myers 2004). Even chum (*Oncorhynchus keta*) and those runs of chinook (*Oncorhynchus tshawytscha*) and sockeye (*Oncorhynchus nerka*) that migrate seaward in the same year of emergence are at least twice as large as pink salmon fry and spend weeks to months in FW prior to ocean entry (Rounsefell 1958; Clarke 1982; Hoar 1988; Quinn and Myers 2004). The osmoregulatory demands of seaward migration are thus particularly challenging to pink salmon for two reasons: (1) a near absence of post-larval FW preparatory time and (2) a high body surface area to volume ratio. These challenges have spurred speculation of a novel strategy where hypo-osmoregulatory development begins before yolk absorption and gravel emergence, perhaps without the usual photoperiod cue (Weisbart 1968; Sullivan et al. 1983; Hoar 1988; McCormick 2009).

Despite this exceptional life history, smoltification and the development of SW tolerance in pink salmon remains largely unstudied. Previous work shows that larval pink alevins fail to survive SW exposure prior to yolk absorption, but exhibit signs of plasma ion regulation and possess a higher $LT_{50}$ than other Oncorhynchids (Weisbart 1968). These results suggest larval hypo-osmoregulatory development, but extra-branchial mitochondrion rich cells (MRC's) as seen on the yolk sac of chum alevins (Kaneko et al. 1995) could explain this effect. Further confounding interpretation is the observation that fry experience a doubling of whole body $Na^+$ and $Cl^-$ content that coincides with naturally timed SW entry post-yolk absorption (Grant et al. 2009). The reported elevation in whole body ion levels persists until gill NKA activity peaks 2 months later. This period of acclimatization greatly exceeds the hours to days reported for other salmonids (*O. keta*, *O. mykiss*, *O. kisutch*, *S. salar*; Black 1951; Miles and Smith 1968; Leray et al. 1981; Prunet and Boeuf 1985) and suggests hypo-osmoregulatory capacity may be underdeveloped upon naturally timed ocean entry. Furthermore, the majority of the increase in gill NKA activity occurs after SW entry (Honma 1982; Grant et al. 2009), also suggesting compensatory rather than preparatory changes. Evidently, when and how SW tolerance and hypo-osmoregulatory ability of juvenile pink salmon develops remains unclear.

The objective of this study was thus twofold: (1) To determine if pink salmon develop adult-like hypo-osmoregulatory abilities as larval alevins before yolk absorption; and (2) identify the physiological differences pre- and post-yolk absorption critical to SW survival. To address these issues, ionoregulatory status of larval alevins (pre-yolk absorption) and post-larval fry (post-yolk absorption) was measured before and after 5 days of SW exposure.

## Materials and methods

### Experimental animals

Pink salmon alevins aged 500 accumulated thermal units (ATU's) were transported from Seymour River Hatchery to the University of British Columbia on 28th February 2008. Fish were separated and held in two 90 L static glass aquaria filled with charcoal-filtered dechlorinated Vancouver city tap water ($[Na^+]$, 0.17 mM; $[Cl^-]$, 0.21 mM; hardness, 30 mg $L^{-1}$ as $CaCO_3$; pH 5.8–6.4) and maintained at a temperature of 3.5 °C in total darkness to mimic natural rearing conditions. Fish were treated in accordance with the University of British Columbia animal care protocol #A07-0055.

### Transfer protocol

Fish aged 540 ATU's are referred to as alevins. Alevins possess an externally visible yolk sac and do not naturally emerge from gravel at this stage in development to enter SW (M. Casselman; personal communication, Seymour River Hatchery). Fish aged 600 ATU's are referred to as fry. Fry no longer possess an externally visible yolk sac and naturally emerge from gravel at this stage in development to enter SW.

Alevins and fry were randomly transferred to new 90 L static glass aquaria filled with either charcoal-filtered freshwater (FW; 3.5 °C; composition listed above) or 100 % seawater [SW; 3.5 °C; 32.0 g $L^{-1}$, prepared in dechlorinated Vancouver city tap water with Instant Ocean sea salt mix (Cincinnati, USA)]. Transfer tanks were maintained on a simulated natural photoperiod (SNP) of 12L:12D to mimic conditions during gravel emergence in the wild. Fish were sampled from the respective FW holding tanks pre-transfer (pre-transfer FW) and from FW- and SW-treatment tanks under SNP (FW + SNP, SW + SNP) at 1 and 5 days post-transfer. Five days was chosen as a sampling point to provide enough time for measurable changes in mRNA and protein expression to occur, while also being short enough to minimize potential SW-induced mortality. Each sampling

consisted of 43 fish ($n = 10$ for wet/dry mass and whole body [$Na^+$] and [$Cl^-$]; $n = 10$ for gill $Na^+/K^+$-ATPase activity; $n = 10$ for drinking rates; $n = 8$ for gill $Na^+/K^+$-ATPase α1 subunit mRNA expression; $n = 5$ for immunofluorescence microscopy). Due to a limited number of fish, alevins were not subjected to the FW + SNP treatment and immunohistochemical staining was only performed on pre-transfer FW fish and SW + SNP fish 5 days post-transfer. Drinking rates were only measured in SW + SNP treatment groups because they were expected to be very low or absent in FW.

### Body mass and whole body [$Na^+$] and [$Cl^-$]

Fish were anaesthetized with a lethal dose of buffered tricaine methanesulfonate (0.2 g $L^{-1}$ MS-222; Syndel Laboratories, Vancouver, BC, Canada), rinsed in de-ionized water, blotted dry and weighed. Fish were dried in pre-weighed 15 mL polystyrene tubes at 65 °C until no further reduction in mass was observed (~2 days). Dried fish were then digested in 2 mL of 1 M nitric acid for 3 days at 65 °C and the supernatant subsequently analysed for $Na^+$ and $Cl^-$ content. Supernatant [$Na^+$] was measured using flame atomic absorption spectroscopy (Spectra AA-220FS; Varian, Mulgrave, VIC, Australia) and standardized to wet and dry body mass for whole body [$Na^+$]. Supernatant [$Cl^-$] was measured using the colorimetric mercuric thiocyanate method (Zall et al. 1956) and standardized to wet mass for whole body [$Cl^-$].

### Drinking rates

Drinking rates were measured using a technique similar to that employed by Gonzalez et al. (2005). Ten fish were individually placed in static polyethylene chambers containing 60 mL of aerated SW. To minimize disturbance, trials were completed in darkness and fish were permitted a 1-h chamber acclimation period. This acclimation period was deemed sufficient as alevins and fry were almost entirely motionless in chambers at trial onset and not observed to be hyperventilating. Furthermore, many studies with larval fishes forego an acclimation period altogether (Tytler et al. 1990; Fuentes and Eddy 1996; Lin et al. 2001). At trial onset, 90 μCi of [$^3$H]-polyethylene glycol (PEG-4000; American Radiolabeled Chemicals Inc., St. Louis, MO, USA) was added to the system and 5 mL of water sampled. Following 4 h of exposure, fish were lethally anaesthetized as described above and an additional 5 mL water sample was taken. Fish were rinsed thrice in de-ionized water to remove residual PEG, blotted dry and weighed. Fish were digested with 2 mL of 10 % perchloric acid for 48 h at 65 °C, then homogenized, vortexed and left to settle. One-mL aliquots of clear supernatant and trial water samples were analysed for radioactivity using a liquid scintillation counter (LSC-2000; Beckman-Coulter Inc., Fullerton, CA, USA). Water uptake was determined by dividing total counts per fish by the specific activity (μCi/ml $H_2O$) of reference water samples. Drinking rates were calculated by expressing total water uptake relative to fish mass and [$^3$H]polyethylene glycol exposure time. Drinking rates are reported as mL $H_2O$ $kg^{-1}$ $h^{-1}$.

### Gill $Na^+/K^+$-ATPase activity

Whole gills were removed following terminal anaesthetization (as described above) and stored at −80 °C. A modified version of the method outlined by McCormick (1993) was used to determine gill NKA activity. This method couples ouabain-sensitive ATP hydrolysis to the oxidation of NADH by way of pyruvate kinase and lactate dehydrogenase. Briefly, whole frozen gills were homogenized on ice in SEI buffer (250 mM sucrose, 10 mM EDTA, 50 mM imidazole; pH 7.3), centrifuged at 5,000*g* for 1 min and the supernatant removed. Supernatant ATPase activity was measured spectrophotometrically in the presence and absence of ouabain (1 mM), and activity was taken as the difference between these conditions. Protein concentration was measured using the bicinchoninic acid method (Sigma-Aldrich) and bovine serum albumin standards. NKA activity is reported as μmol ADP mg $protein^{-1}$ $h^{-1}$. All samples were run in triplicate within 2 h of homogenization.

### Gill mRNA expression

Quantitative real-time PCR (qRT-PCR) was used to estimate gill NKA α1-subunit isoform expression. Isoform-specific primers identical to those designed by Richards et al. (2003) were employed.

Total RNA was extracted from whole frozen gills using the Invitrogen TRIzol Reagent, and RNA concentrations were subsequently determined spectrophotometrically. First-strand cDNA was synthesized from 2 μg of total RNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). All qRT-PCR reactions were performed on an ABI Prism 7000 sequence analysis system (Applied Biosystems) as follows: 1 cycle of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each PCR reaction contained 1 μl of cDNA, 4 pmol of each primer and Universal SYBR green master mix (Applied Biosystems) in a total volume of 21 μl. Melt curve analyses were performed following each reaction to confirm the presence of only a single reaction product, and negative-control reactions were performed with non-reverse-transcribed RNA to determine the level of genomic contamination (which was subsequently shown to be negligible).

A randomly selected control sample was used to develop a standard curve relating threshold cycle to cDNA amount for each primer set. Results were expressed relative to these

standard curves and mRNA amounts normalized relative to EF-1α, Ub and total RNA. All samples are expressed relative to the pre-transfer FW alevin group and each was run in duplicate with a co-efficient of variation <10 %.

### Immunofluorescence microscopy

Following immersion fixation overnight in 4 % paraformaldehyde in phosphate-buffered saline at 4 °C, alevins and fry were transferred to 70 % ethanol for storage and shipping to Portugal. Samples were then dehydrated in an ethanol series, cleared in xylene and infiltrated and embedded in paraffin (Type 6, Richard-Allen Scientific, Kalamazoo MI). Paraffin sections (5 μm) were collected onto 3-aminopropyltriethoxysilane (Sigma) coated slides, completely air dried and de-waxed in xylene. Sections were processed for immunofluorescence microscopy as described in Wilson et al. (2007). Antigen retrieval was performed by pretreating sections with 0.05 % citraconic anhydride (Namimatsu et al. 2005) for 30 min at 100 °C followed by 1 % SDS/PBS for 5 min at room temperature (Brown and Breton 1996) to improve immunoreactivity. Double labelling was performed using the rabbit anti-peptide $Na^+/K^+$-ATPase polyclonal antibody αRbNKA (Ura et al. 1996; Wilson et al. 2007), with the mouse monoclonal NKCC (clone T4; Lytle et al. 1995). Peptide pre-absorption and isotyped monoclonal antibody (J3 clone) were used as respective negative controls. Mouse and rabbit antibodies were detected with goat anti-mouse Alexa Fluor 568 and 488 antibodies, respectively (Invitrogen, Carlsbad CA). The corresponding differential interference contrast (DIC) image was also captured for tissue orientation. Double-labelled sections were viewed on a Leica DM 6000B epifluorescence microscope and images captured using a digital camera (Leica DFC 340 FX). Optimal exposure settings were predetermined and all images captured under these settings. Plates were assembled in Adobe Photoshop CS2 and brightness and contrast adjusted while maintaining the integrity of the data. Two plates were prepared for each individual sampled. NKA and NKA/NKCC colocalized IR cells were counted for each plate and the ratio of NKA/NKCC to NKA IR cells determined. The ratios of NKA/NKCC to NKA IR cells were used to quantify differences in the proportion of NKCC IR cells between treatments. NKCC/NKA IR cells are representative of SW MRC's, and NKA IR cells representative of FW MRC's.

### Statistical analyses

Data are expressed as means ± SEM. Ratios from the immunofluorescence microscopy cell counts were analysed with a one-way nested analysis of variance (ANOVA). A one-way ANOVA was used to analyse remaining alevin data and a two-way ANOVA for remaining fry data. The Holm–Sidak post hoc test was subsequently applied for pairwise comparisons when effects were found to be significant. All data passed tests for normality and homogeneity of variance. All statistical analyses were conducted with Sigmastat (version 3.0, Systat Software Inc., San Jose, CA, USA), and a significance level of $P < 0.05$ was used throughout.

## Results

### Alevins

Alevin wet body mass decreased significantly by 8 and 15 % at 1 and 5 days post-SW + SNP exposure, respectively ($P < 0.05$, Fig. 1a). However, no significant change in alevin dry body mass occurred post-transfer ($P = 0.545$, Fig. 1b), indicating that reductions in wet mass were due to water loss.

Whole body [$Na^+$] of alevins increased significantly by 15 % relative to the pre-transfer FW control at 5 days post-SW + SNP exposure when expressed as a function of wet mass ($P < 0.05$, Fig. 1c); however, whole body [$Na^+$] remained unchanged when expressed as a function of dry mass ($P = 0.996$, Fig. 1d), indicating the increase in % [$Na^+$] relative to wet mass was due to dehydration and not net $Na^+$ influx. Whole-body [$Cl^-$] expressed as a function of wet mass increased by 35 % relative to the pre-transfer FW control at 1 day post-SW + SNP exposure and remained elevated at 50 μmol g wet $mass^{-1}$ for trial duration ($P < 0.001$, Fig. 1e). This change in [$Cl^-$] exceeded the 15 % change expected for dehydration alone as described above for $Na^+$, indicating that about half of the increase in [$Cl^-$] was due to net influx.

Gill NKA activity of alevins did not change significantly following SW + SNP exposure ($P = 0.742$, Fig. 1f), but significant differences in α1 subunit mRNA expression were observed (Fig. 2). Expressed relative to total RNA, α1a decreased by 60 % by day 5 ($P < 0.001$, Fig. 2b) while α1b remained unchanged ($P = 0.199$, Fig. 2a). As a result, the α1b/α1a ratio increased nearly fourfold from 0.59 to 2.17 ($P < 0.001$, Fig. 2c). A similar pattern in the α1b/α1a ratio was observed when expressed relative to the control genes EF-1α and Ub (Table 1) even though significant decreases in both of these control genes were observed ($P < 0.05$, Table 1).

Gill NKA was immunolocalized to branchial chloride cells in a pattern indicative of basolateral tubular system staining. NKCC was colocalized to a subpopulation of NKA immunoreactive (IR) cells with a similar cellular distribution, although there was also some immunoreactivity with chondrocytes in the gill. The ratio of NKA/NKCC to NKA IR cells in the gill epithelium did not differ significantly between the FW pre-transfer fish (0.61 ± 0.13) and those exposed to SW + SNP for 5 days (0.84 ± 0.17; $P = 0.217$, Fig. 3).

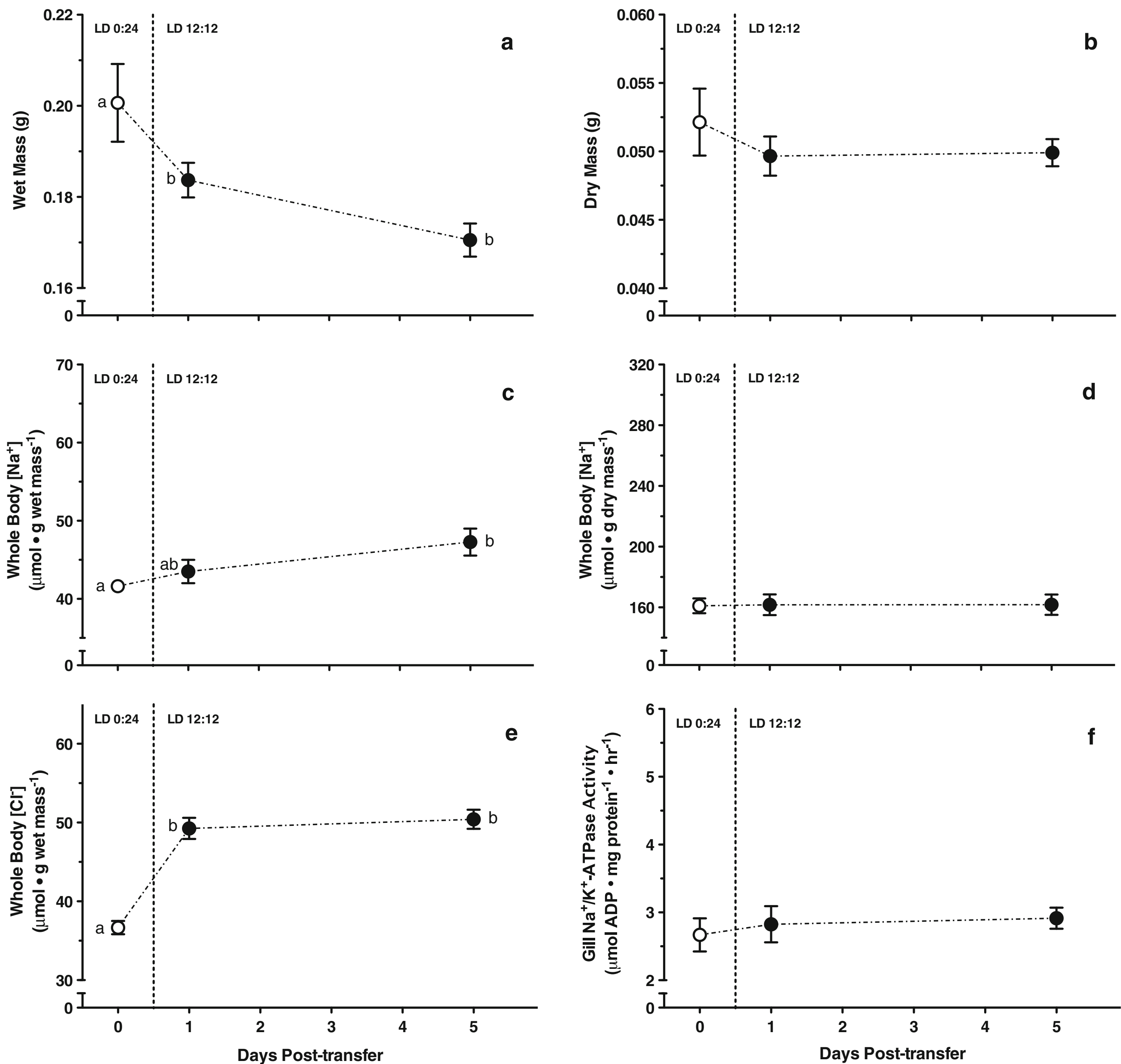


**Fig. 1** Wet mass (**a**), dry mass (**b**), whole body [$Na^+$] relative to wet and dry mass (**c**, **d**, respectively), whole-body [$Cl^-$] relative to wet mass (**e**) and gill $Na^+/K^+$-ATPase activity (**f**) of pink salmon alevins. *Open circles* represent alevins reared in freshwater under total darkness (pre-transfer FW), and *closed circles* represent alevins simultaneously exposed to seawater and a simulated natural photoperiod (SW + SNP). Values are means ± SEM ($n = 10$); *letters* that differ indicate statistically significant differences ($P < 0.05$)

Alevin drinking rates were 1.92 ± 0.09 and 1.75 ± 0.17 mL $kg^{-1}$ $h^{-1}$ at 1 and 5 days post-SW + SNP transfer, respectively. Rates did not differ significantly ($P = 0.353$).

### Fry

No significant changes in wet or dry mass were observed in fry following FW + SNP and SW + SNP exposure ($P = 0.210$, Fig. 4a, b), indicating that, unlike alevins, water balance was maintained in SW.

Whole body [$Na^+$] expressed as a function of wet body mass increased significantly by 11 and 35 % relative to the pre-transfer FW control at 1 and 5 days post-SW + SNP exposure, respectively ($P < 0.001$, Fig. 4c). This post-transfer increase exceeded that observed in alevins by more than two-fold. Additionally, values for fry 5 days post-SW + SNP exposure were significantly greater than time-matched FW + SNP values by approximately 20 % ($P < 0.001$, Fig. 4c). Whole body [$Na^+$] expressed as a function of dry body mass also increased significantly by approximately 20 and 40 % relative

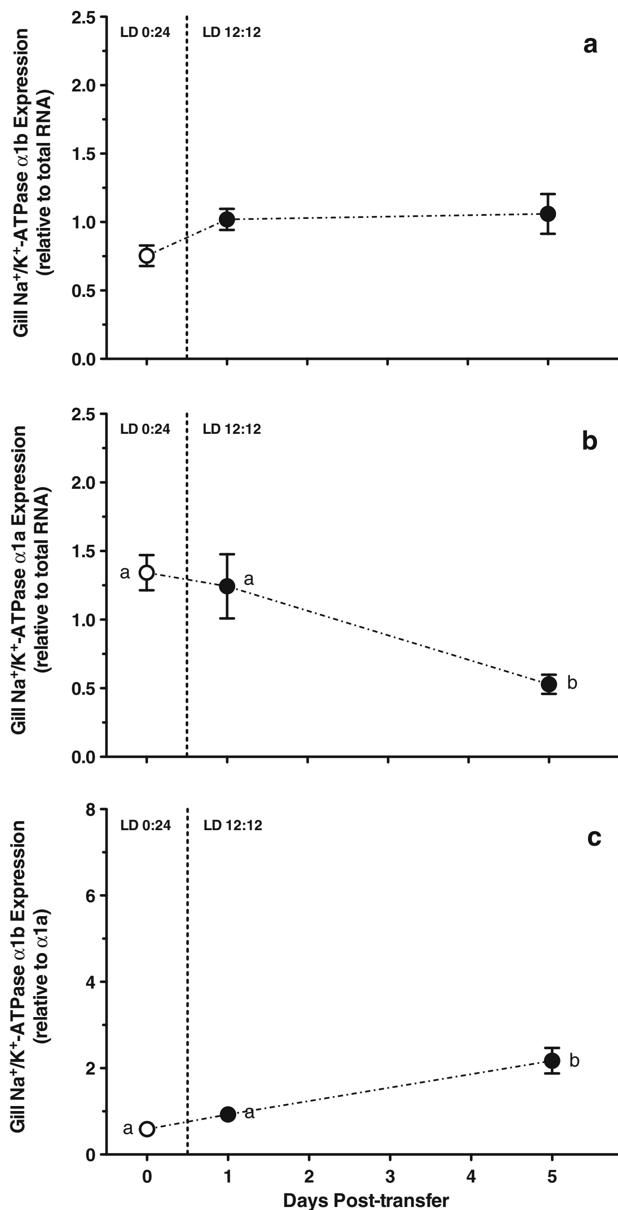


**Fig. 2** Gill $Na^+/K^+$-ATPase α1b mRNA expression relative to total RNA (**a**), α1a mRNA expression relative to total RNA (**b**), and α1b mRNA expression relative to α1a (**c**) of pink salmon alevins. See legend of Fig. 1 for further details ($n = 6$–$10$)

to the pre-transfer FW control at 5 days post-FW + SNP and SW + SNP exposure, respectively ($P < 0.001$, Fig. 4d). Furthermore, SW + SNP values significantly exceeded those for FW + SNP at day 5 by approximately 15 % ($P < 0.05$, Fig. 4d). These results indicate a net $Na^+$ influx for fry in both SW + SNP and FW + SNP treatments, a response that differs from alevins. Whole body [$Cl^-$] expressed as a function of wet mass in SW + SNP fry increased significantly above pre-transfer FW and time-matched FW + SNP values to a plateau of approximately 50 μmol $g^{-1}$ wet mass for trial duration ($P < 0.001$, Fig. 4e), exhibiting a pattern more closely resembling that observed in alevins.

While alevins failed to upregulate gill NKA activity, fry significantly increased gill NKA activity following both SW + SNP and FW + SNP exposure at 1 and 5 days post-transfer relative to the pre-transfer value ($P = 0.012$ and <0.001 for SW + SNP, $P = 0.004$ and 0.048 for FW + SNP, Fig. 4f). The gill NKA α1b/α1a mRNA ratio expressed relative to total RNA also exhibited similar changes after FW + SNP and SW + SNP exposure (Fig. 5c), increasing from 0.79 to 2.84 by day 5 in FW + SNP ($P < 0.05$) and 5.32 by day 5 in SW + SNP ($P < 0.001$, Fig. 5c). This ratio is more than twice that reported for SW + SNP alevins, but does follow a similar trend. As in alevins, the expression ratio appears to be driven largely by significant decreases in α1a ($P < 0.001$, Fig. 5a), as α1b did not change significantly (Fig. 5b). When expressed relative to the control genes EF-1α and Ub (Table 2), similar patterns in the α1b/α1a ratio were observed, but as with the alevin trials, significant decreases in both of these control genes were observed ($P < 0.05$, Table 2).

Gill NKA and NKCC of fry was immunolocalized in a pattern similar to that of alevins. Salinity transfer did not appear to alter this pattern. However, the ratio of NKCC/NKA to NKA IR cells increased significantly from 0.90 ± 0.09 to 1.43 ± 0.09 at 5 days post-SW + SNP transfer ($P < 0.05$, Fig. 6).

Fry drinking rates were 1.82 ± 0.16 and 1.73 ± 0.06 mL $kg^{-1}$ $h^{-1}$ at 1 and 5 days post-SW + SNP transfer, respectively. Rates did not differ significantly among fry ($P = 0.570$) or when compared with alevins ($P = 0.276$).

## Discussion

This study does not support the hypothesis that pink salmon develop adult-like hypo-osmoregulatory abilities prior to yolk absorption and gravel emergence. This conclusion is

**Table 1** Expression of gill $Na^+/K^+$-ATPase α1a and α1b mRNA relative to elongation factor 1α (EF-1α) and ubiquitin (Ub) in pink salmon alevins

| Treatment | α1a/EF-1α | α1b/EF-1α | EF-1α/total RNA | α1a/Ub | α1b/Ub | Ub/total RNA |
|---|---|---|---|---|---|---|
| Pre-transfer FW | $1.30 \pm 0.10^a$ | $0.76 \pm 0.10^a$ | $1.04 \pm 0.06^a$ | $1.59 \pm 0.17^a$ | $0.94 \pm 0.14^a$ | $0.89 \pm 0.08^a$ |
| SW + SNP day 1 | $1.12 \pm 0.07^a$ | $1.01 \pm 0.13^a$ | $1.09 \pm 0.15^a$ | $1.30 \pm 0.14^{a,b}$ | $1.13 \pm 0.13^a$ | $0.93 \pm 0.07^a$ |
| SW + SNP day 5 | $0.74 \pm 0.07^b$ | $1.52 \pm 0.19^b$ | $0.71 \pm 0.06^b$ | $0.90 \pm 0.11^b$ | $1.82 \pm 0.21^b$ | $0.61 \pm 0.07^b$ |

Fish were held in freshwater in darkness (pre-transfer FW) and then simultaneously exposed to seawater and a simulated natural photoperiod (SW + SNP). Data are means ± SEM; letters that differ indicate statistically significant differences

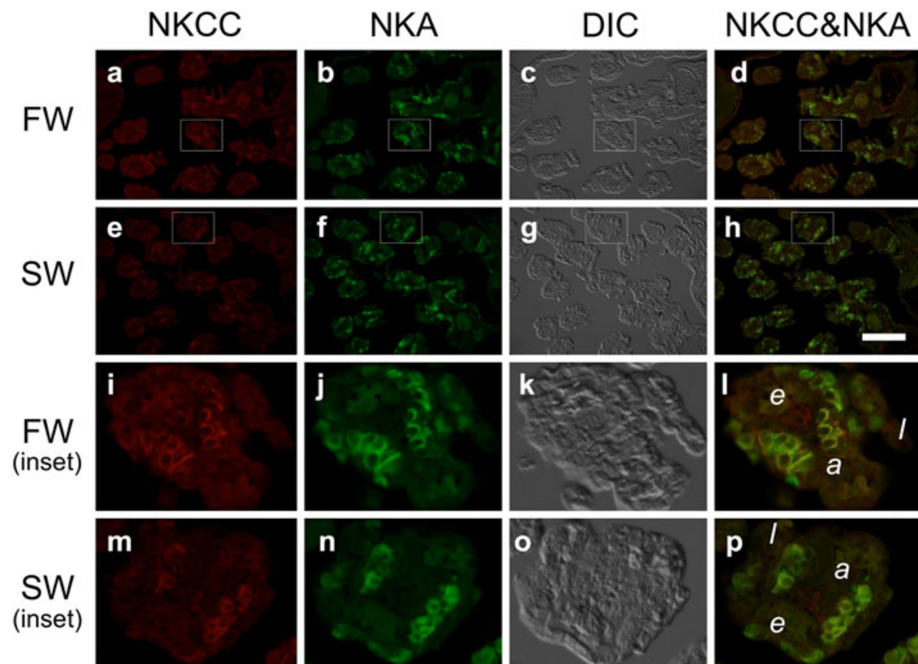


**Fig. 3** Immunofluorescent localization of $Na^+$:$K^+$:$2Cl^-$ cotransporter (NKCC; **a**, **e**, **i**, **m**), $Na^+/K^+$-ATPase (NKA; **b**, **f**, **j**, **n**), co-localization of these transporters (**d**, **h**, **l**, **p**), and differential interference contrast images (DIC; **c**, **g**, **k**, **o**) of gill sections of pink salmon alevins kept in freshwater in darkness (**a**–**d**, **i**–**l**) or acclimated to seawater for 5 days under a simulated natural photoperiod (**e**–**h**, **m**–**p**). The high magnification insets (**i**–**l**, **m**–**p**) show similar cellular distributions of NKA and NKCC. Frequency of NKCC immunoreactive cells does not differ between treatments ($P = 0.217$; see "Results"). *Scale bar* **a**–**h** 500 µm; **i**–**p** 100 µm

clearly supported by the failure of larval alevins to maintain ion and water balance following SW exposure, the lack of upregulation in gill NKA activity and the absence of an increase in the proportion of NKCC IR cell in the branchial epithelium. Furthermore, the key difference between larval alevins and post-larval fry critical to SW survival appears to be the maintenance of water balance, for which fry seemingly sacrifice $Na^+$ balance. We also provide novel evidence that photoperiod may trigger smolt-like increases in gill NKA activity of pink salmon fry post-yolk absorption.

## Do larval alevins develop adult-like hypo-osmoregulatory ability?

Larval pink salmon alevins showed no evidence of adult-like hypo-osmoregulatory ability following SW exposure. Gill NKA activity did not increase as seen in post-larval stages (Honma 1982; Grant et al. 2009); levels remained low and comparable to those previously reported for FW pre-smolt salmonids (Richards et al. 2003; Madsen et al. 2009), and the proportion of NKCC IR cells in the branchial filament epithelium did not increase. A 35 % increase in body [$Cl^-$] persisted 5 days post-SW entry, indicating a failure to maintain ion balance [the closely related chum regains [$Cl^-$] balance 24 h post-transfer (Black 1951)], and the reduction in wet mass despite no change in dry mass clearly indicates a failure to maintain water balance.

The measured drinking rate of $\sim$1.8 ml $kg^{-1}$ $h^{-1}$ for alevins transferred to SW + SNP equals that reported for fry and is comparable to that recorded for other SW teleosts (1.25 for *Oreochromis mossambicus*, 2–4 for *S. salar*; Sardella et al. 2004, Fuentes and Eddy 1997). However, alevins still dehydrated, suggesting that sufficient water uptake may not be occurring. Several points further support this interpretation. First, the method employed to determine drinking rate estimates water ingested into the gastrointestinal tract and not that absorbed across the gut wall. Thus, this method is not a true measure of water uptake. Second, the primary source of $Na^+$ uptake in SW salmonids is by active transport at the gut to facilitate water absorption (Smith 1930; Kirschner et al. 1974; Grosell 2006), yet [$Na^+$] did not increase relative to dry mass. Finally, the lack of increase in gill NKA activity and NKCC IR cells suggests that other changes typical of SW acclimation (i.e. intestinal remodelling for water uptake) may not have taken place, as they often co-occur (Collie and Bern 1982; Loretz et al. 1982). Thus, the reported drinking rates may represent an ineffective behavioural response to the SW + SNP transfer.

## Keys to post-larval fry SW survival

In contrast to alevins, fry appear to hypo-osmoregulate at the whole animal level following SW exposure. This is expected, as SW migration occurs naturally at this point in pink salmon development. The induction of compensatory measures is indicated by a significant increase in gill NKA activity, an increased proportion of NKCC IR cells in the branchial epithelium and a significant increase in the NKA α1b/α1a mRNA expression ratio. Furthermore, the lack of change in wet and dry body mass, coupled with a significant increase in whole body [$Na^+$], suggests that water balance is maintained by drinking. Maintaining water balance thus appears to be a critical difference between alevins and

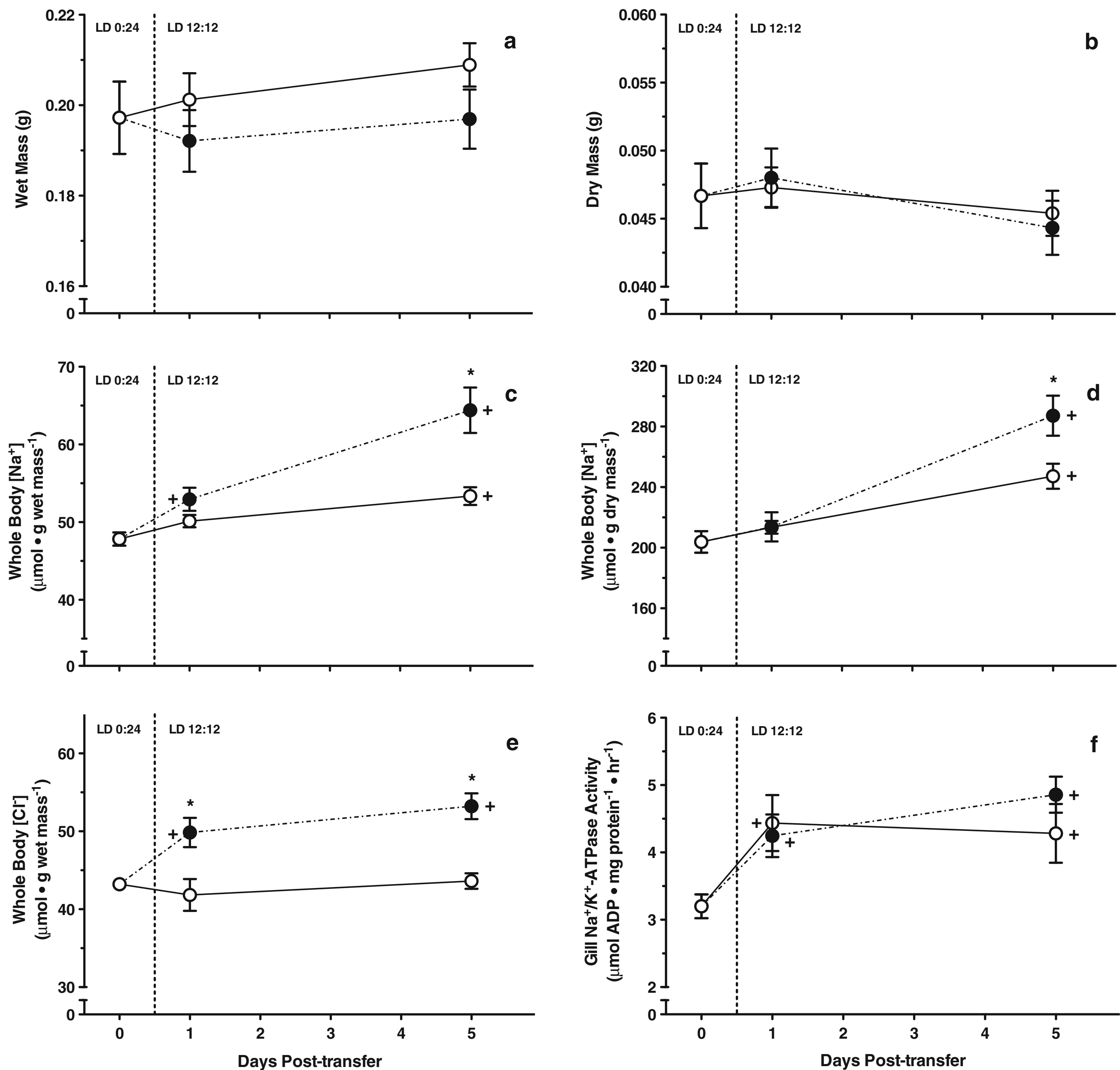


**Fig. 4** Wet mass (**a**), dry mass (**b**), whole body [$Na^+$] relative to wet and dry mass (**c**, **d**, respectively), body [$Cl^-$] relative to wet mass (**e**) and gill $Na^+/K^+$-ATPase activity (**f**) of pink salmon fry. *Open circles* at day "0" represent fry reared in freshwater under total darkness (pre-transfer FW), *open circles* at day "1" and "5" represent fry exposed to a simulated natural photoperiod while still in freshwater (FW + SNP), and *closed circles* represent fry simultaneously exposed to both seawater and a simulated natural photoperiod (SW + SNP). Values are means ± SEM ($n = 10$). $^+$indicates a significant difference from the pre-transfer FW value; * indicate significant differences between time-matched FW + SNP and SW + SNP values ($P < 0.05$)

fry for SW survival; yet, fry unexpectedly experience a greater whole-body ionic disturbance than alevins following SW transfer. Furthermore, the observed $Na^+$ loading also occurs in fry following FW + SNP exposure, albeit to a lesser degree (20 % in FW + SNP vs. 40 % in SW + SNP).

Seawater readiness of anadromous salmonids is typically measured by the degree of ionic disturbance sustained in the plasma following SW transfer (24 h seawater challenge, Clarke and Blackburn 1977; Clarke 1982; Blackburn and Clarke 1987). On a whole body level, water content may be a more suitable evaluation for pink salmon as "prepared" fry experience an ionic perturbation three-times greater than "unprepared" alevins. The exact origin and function of this ion load remains unknown, but its absence in alevins and presence in fry implies a connection to water balance. In SW, elevated ion levels could result from an inability to completely excrete excess ions absorbed at the gut during intestinal water uptake. As hypo-osmoregulatory

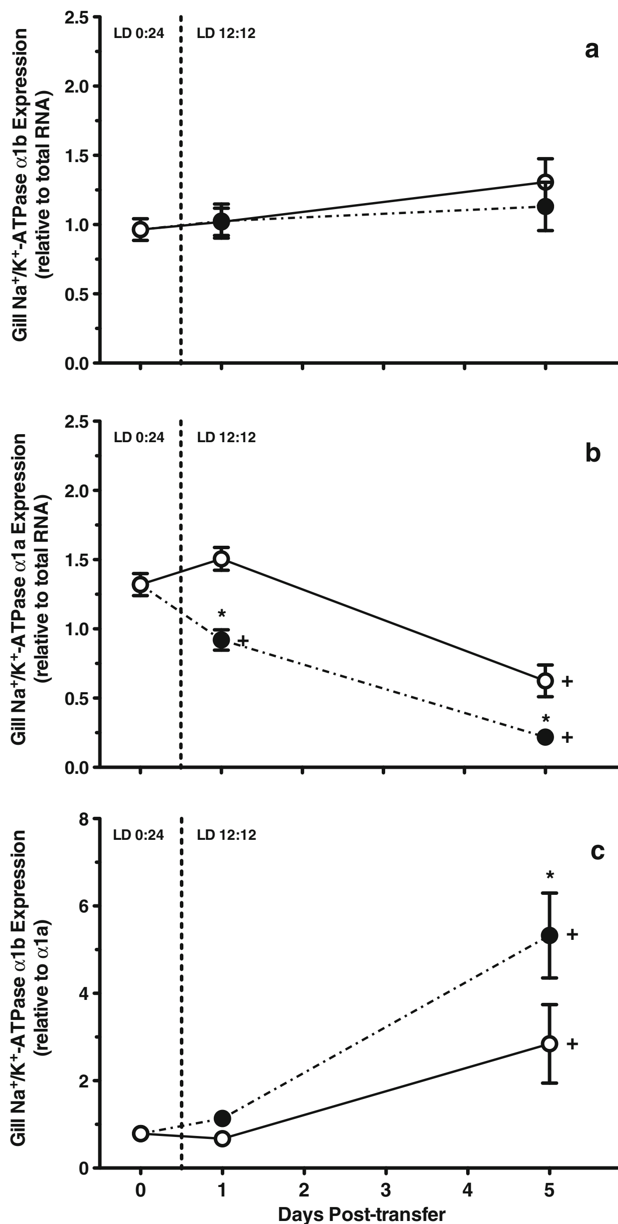


**Fig. 5** Gill $Na^+/K^+$-ATPase α1b mRNA expression relative to total RNA (**a**), α1a mRNA expression relative to total RNA (**b**), and α1b mRNA expression relative to α1a (**c**) of pink salmon fry. See legend of Fig. 4 for further details ($n = 6$–$10$). $^+$indicates a significant difference from the pre-transfer FW value; * indicate significant differences between time-matched FW + SNP and SW + SNP values ($P < 0.05$)

machinery develops, ion excretion rates would increase to match and exceed rates of uptake, ultimately restoring ionic balance as observed by Grant et al. (2009). This explanation, however, does not account for the $Na^+$ load incurred following FW + SNP exposure. FW ion loading could reduce the osmotic gradient between fry and the ocean, thereby facilitating water uptake and mitigating water loss upon SW entry. However, further investigation is required to determine the true nature of this phenomenon.

The observed ion loading does not imply that ion excretion is unimportant to fry SW survival. Whole-body ion levels still remain well below those of ambient SW, and the upregulation of gill NKA activity and NKCC IR cells indicates an increased capacity for ion excretion. Although ion balance is not fully restored until gill NKA activity peaks 8 weeks later (Grant et al. 2009), the degree of ion excretion afforded prior to peak levels may be just as important to performance and survival if ion levels are kept below a certain critical threshold. This may explain the observed similarity between whole body [$Cl^-$] of alevins and fry despite the presumed increase in intestinal uptake by the latter. The differences between [$Na^+$] and [$Cl^-$] following SW transfer are also of interest, and although the possible explanations for these observations remain too numerous for discussion here, they should be targeted by future study.

Another point of interest is that the increase in the gill NKA α1b/a mRNA expression ratio following SW transfer is driven entirely by a downregulation of α1a. For virtually all salmonids in which the α1 isoforms have been examined, SW transfer also elicits an increase in α1b expression. The exceptions are landlocked Arctic char (*Salvelinus alpinus*; Bystriansky et al. 2007), which fail to survive SW, and the pink salmon of this study, which do survive SW. This unique result further supports the idea of a novel strategy for SW entry in pink salmon. What remains unclear is if α1b is already upregulated maximally in preparation for SW entry, or if its role in SW acclimation is reduced relative to other salmonids. Future studies examining pink salmon development in FW over a longer period of time (both earlier in development and well beyond natural SW migration) should determine α1a/b expression dynamics and help clarify their relative importance to SW entry and survival.

## Photoperiod may trigger smoltification in pink salmon

Upon controlled emergence from darkness to a natural photoperiod, changes in gill NKA activity and α1 isoform mRNA expression in FW fry paralleled those of fry transferred directly to SW. This result provides the first experimental evidence that photoperiod may trigger preparatory changes in pink salmon typical of smoltification. This paradigm differs from the current belief that ontogeny largely dictates the acquisition of salinity tolerance in pink salmon, which is supported by previously reported increases in gill NKA activity of FW fry following a surge in plasma thyroxine and yolk absorption (Sullivan et al. 1983). Although the present study lacks a suitable control (FW dark) to conclusively dissociate developmentally timed change from that triggered by environmental cues, the likelihood of coincidence between controlled emergence and ontogenetic change is low enough to merit speculation.

**Table 2** Expression of gill $Na^+/K^+$-ATPase α1a and α1b mRNA relative to elongation factor 1α (EF-1α) and ubiquitin (Ub) in pink salmon fry

| Treatment | α1a/EF-1α | α1b/EF-1α | EF-1α/total RNA | α1a/Ub | α1b/Ub | Ub/total RNA |
|---|---|---|---|---|---|---|
| Pre-transfer FW | $1.36 \pm 0.06$ | $1.04 \pm 0.13$ | $0.97 \pm 0.04$ | $1.59 \pm 0.14$ | $1.13 \pm 0.08$ | $0.86 \pm 0.04$ |
| FW + SNP day 1 | $1.35 \pm 0.08$ | $0.91 \pm 0.07$ | $1.17 \pm 0.14$ | $1.56 \pm 0.12$ | $1.07 \pm 0.14$ | $1.00 \pm 0.08$ |
| SW + SNP day 1 | $1.02 \pm 0.05^{t,*}$ | $1.14 \pm 0.11$ | $0.94 \pm 0.12$ | $1.21 \pm 0.10$ | $1.35 \pm 0.16$ | $0.86 \pm 0.16$ |
| FW + SNP day 5 | $1.34 \pm 0.25$ | $3.08 \pm 0.54^{t}$ | $0.49 \pm 0.10^{t}$ | $2.27 \pm 0.55^{t}$ | $4.79 \pm 0.72^{t}$ | $0.29 \pm 0.03^{t}$ |
| SW + SNP day 5 | $0.72 \pm 0.07^{t,*}$ | $3.53 \pm 0.33^{t}$ | $0.31 \pm 0.02^{t}$ | $0.83 \pm 0.13^{t,*}$ | $4.40 \pm 0.75^{t}$ | $0.34 \pm 0.10^{t}$ |

Fish were held in freshwater in darkness (pre-transfer FW) and then exposed to a simulated natural photoperiod in either freshwater (FW + SNP) or seawater (SW + SNP). Data are means ± SEM; *t* indicates statistically significant differences from pre-transfer FW values; * indicate statistically significant differences between time-matched FW + SNP and SW + SNP values

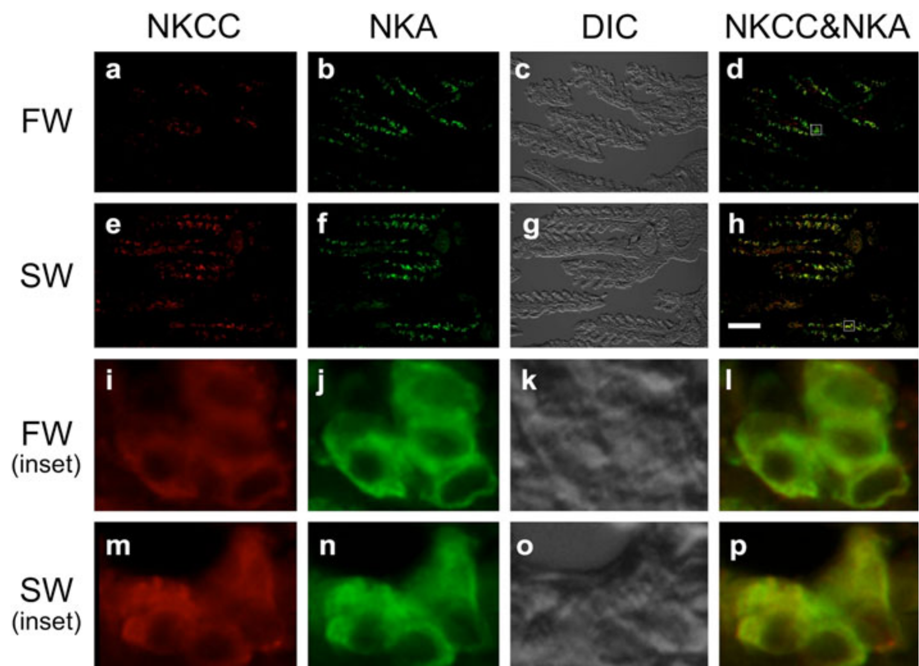


**Fig. 6** Immunofluorescent localization of $Na^+$:$K^+$:$2Cl^-$ cotransporter (NKCC; **a**, **e**, **i**, **m**), $Na^+/K^+$-ATPase (NKA; **b**, **f**, **j**, **n**), co-localization of these transporters (**d**, **h**, **l**, **p**), and differential interference contrast images (DIC; **c**, **g**, **k**, **o**) of gill sections of pink salmon fry kept in freshwater in darkness (**a**–**d**, **i**–**l**) or acclimated to seawater for 5 days under a simulated natural photoperiod (**e**–**h**, **m**–**p**). The high magnification insets (**i**–**l**, **m**–**p**) show similar cellular distributions of NKA and NKCC. NKCC immunoreactive cell frequency is greater in seawater than freshwater ($P < 0.05$; see "Results"). *Scale bar* **a**–**h** 1 µm; **i**–**p** 50 mm

The observed drinking rates and changes in α1 mRNA expression reported for alevins following SW + SNP exposure also support the notion of smolt-like environmental cue sensitivity, as they matched those observed in FW/SW + SNP fry and occurred well before the proposed ontogenetic switch at yolk absorption. Pink salmon are thus clearly capable of responding to environmental cues at transcriptional and behavioural levels prior to yolk absorption, but fail to mount an effective change in whole animal performance. Instead of an ontogenetic switch for hypo-osmoregulatory development, we propose that yolk absorption marks a point in pink salmon development where fry become capable of fully translating environmental cues into a preparatory smolt response. This proposed smolt competence could be linked to endocrine development and certainly warrants further investigation. Future work should also definitively determine if pink salmon do respond to photoperiod, and if so, whether specific periodicity or simply light exposure is required to cue the appropriate changes.

## Summary

This study sheds new light on the acquisition of SW tolerance in pink salmon. Contrary to long-held pre-conceptions, it was shown that pink salmon do not possess adult-like hypo-osmoregulatory abilities prior to yolk absorption and that post-absorption preparatory change might be triggered by photoperiod rather than ontogeny. Furthermore, maintaining water balance appears to be the key difference between alevins and fry critical to SW survival. Fry appear to incur large ion loads to maintain hydration in SW prior to the completion of hypo-osmoregulatory development, which may be a novel strategy among salmonids to facilitate early ocean entry.

Two major osmoregulatory hurdles accompany increasingly early ocean entry: (1) an accelerated transition from FW to SW; and (2) an elevated body surface area to volume ratio (SA:V). Predictive anadromy meets the first challenge by significantly reducing the lag-time between downstream migration and ocean entry common to facultative anadromy (McCormick 1994, 2009). Likewise, increased hypo-osmoregulatory ability can overcome the greater osmoregulatory challenge associated with a higher SA:V in smaller fish (McCormick and Naiman 1984). However, the ion loading observed in pink salmon may further combat both constraints by masking hypo-osmoregulatory deficiency and thus further reducing the need for post-emergent FW preparatory time. Entering seawater with underdeveloped ion excretion machinery distinguishes pink salmon anadromy from the more ancestral facultative and predictive forms. Therefore, we propose that this unique life history be termed "precocious anadromy." The ability to incur and tolerate large, sustained ion loads at the whole body level may be a key adaptation underlying this most extreme and perhaps derived form of salmonid anadromy.

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