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Characterization of smoltification in the Tasmanian strain of Atlantic salmon (*Salmo salar*) in recirculation and flow-through systems

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ABSTRACT

This study examined morphological, physiological and molecular indicators of smoltification in Atlantic salmon (*Salmo salar*) juveniles in a flow-through (FT) and recirculating aquaculture system (RAS). Fish were exposed to 24-h light to initiate smoltification, for 5 (FT) and 7 (RAS) weeks prior to transfer from freshwater (FW) to seawater (SW) and were sampled weekly preceding and following SW transfer. Mass, length, condition factor, plasma chloride, gill Na^+/K^+-ATPase (NKA) activity and expression of salinity-specific isoforms of NKA mRNA transcripts were monitored. Fish raised in FT had significantly lower specific growth rate (SGR) in FW than in SW and showed a significant 5-6-fold increase in gill NKA activity, and high plasma chloride levels after transfer to SW. These fish also exhibited no significant reduction in relative mRNA expression of NKAα1a in FW but a sharp significant downregulation post-SW transfer. No significant increase in NKAα1b was seen until week 8 (3 weeks post-SW transfer). The $\log_2$ ratio of NKAα1b to NKAα1a showed a significant 8-fold increase throughout the study. Fish raised in the RAS had significantly higher SGR in FW than SW, and showed significantly higher plasma chloride in saltwater challenged fish compared to the freshwater control at all weeks during the FW phase. Fish had a 50% higher initial NKA activity than in FT, increasing significantly 2-3-fold and showed an immediate down-regulation of NKAα1a after exposure to 24-hr light in FW, and a further reduction after SW-transfer. There was no significant increase in NKAα1b in the RAS-raised fish for the duration of the study, and there was a significant 8-fold increase in $\log_2$ ratio of NKAα1b to NKAα1a. Whilst there were too many varying factors to statistically compare hatchery type in this study, it's evident that there are potentially system-related effects worthy of future investigation.

1. Introduction

Atlantic salmon (*Salmo salar*) is an anadromous teleost that is commonly cultured for food around the globe, with present production approximately 2.2 M tonnes (FAO, 2018). There are many challenges that face the commercial culture of anadromous species; perhaps the most crucial aspect is the process of transferring fish from their land-based freshwater (FW) hatcheries to ocean-based sea pens. Wild *S. salar,* along with other members of the *Salmonidae* family have developed several physiological mechanisms in order to facilitate their downstream migration that results in such a drastic change in environment. *S. salar* juveniles that are above a size/growth threshold are sensitive to a seasonal increase in photoperiod, water temperature and stream flow (McCormick, 2013; Stefansson and Björnsson, 2008). These along with several other parameters are thought to stimulate the migratory life phase and induce physiological, morphological and behavioural changes that allow for post-migration survival at sea (Hoar, 1988; McCormick et al., 2013; Stefansson and Björnsson, 2008). This developmental phenomenon is referred to as the parr-smolt transformation, or smoltification. In commercial hatcheries, these seasonal increases in photoperiod are often artificially manipulated in an attempt to induce the onset of smoltification-related changes before the fish are transferred to sea.

Perhaps the most important change during smoltification is the development of a higher tolerance to seawater (SW) (McCormick, 2001). Teleosts attempt to maintain a relatively constant osmolality (around 300–350 mOsmol L^{-1}), no matter the salinity of their surrounding environment (McCormick, 2013). FW and SW are

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approximately 0 mOsmol L^{-1} and 1000 mOsmol L^{-1} respectively. Consequently, during the transitional "migration" phase, the gill must switch from active ion uptake in FW to active ion secretion in SW, essentially reversing in function (Evans et al., 2005; Handeland et al., 2013; McCormick et al., 2009).

The gill is the primary osmoregulatory organ in all teleosts. Gill Na^+/K^+-ATPase (NKA) are transport proteins within the chloride cells (or ionocytes) found in the gill epithelium, and are the primary drivers of branchial ionic regulation in both FW and SW. Gill NKA activity is widely used as an indicator of advanced hypo-osmoregulatory ability in salmon smolts and has been correlated with development of SW tolerance (McCormick and Saunders, 1987). NKA provides an electrochemical gradient which allows the passive exit of Na^+ and Cl^- ions from the gill chloride cells (Hwang et al., 2011). Several isoforms of NKA proteins have been identified in Atlantic salmon and other salmonids (Blanco and Mercer, 1998; Madsen et al., 2009; Richards et al., 2003). Of particular interest are NKAα1a and NKAα1b, which are differentially regulated by salinity and have been linked with ion-uptake in FW and ion-secretion in SW respectively (Christensen et al., 2018; McCormick et al., 2009; Richards et al., 2003). NKAα1b is normally upregulated during smolt development in FW, and shows an increase in both the abundance of the protein and the number of ionocytes in which the protein is expressed (Christensen et al., 2018). The NKAα1b-expressing ionocytes are thought to be inactive in FW smolts, and become activated once fish are transferred to SW (McCormick et al., 2013). NKAα1a abundance remains relatively stable in FW, however significantly decreases post-SW transfer (Christensen et al., 2018; McCormick et al., 2013).

Globally, while the aquaculture sector continues to grow and become a more prominent contributor to human nutrition, there has been increased pressure on the industry to improve its sustainability credentials on multiple levels, including reduced carbon footprint and reduced nutrient-rich effluent from hatcheries and other operations. Recently there has been a shift from more traditional flow-through (FT) hatcheries, which use ambient water from a nearby water source (typically a river) that is crudely filtered before running through the hatchery systems and are then expelled back into the natural environment (Bergheim et al., 2009). There is often little or no treatment to reduce waste metabolites from the outflow of these systems. Developments in recirculating aquaculture system (RAS) technology means that hatcheries can reduce water requirements by 100-fold or more (Roque d'Orbcastel et al., 2009). This style of production is being promoted in the European aquaculture industry (Badiola et al., 2012), and make it possible to provide a highly controllable environment and water quality conditions that can result in year round production, as well as improved biosecurity and disease management (Summerfelt et al., 2009).

The effects of different FW rearing systems on production of *S. salar* smolts has not been well studied. One study by Kolarevic et al. (2014) compared growth performance, survival, physiological indicators and welfare of *S. salar* smolts raised in either a RAS or FT systems. They found that whilst there was no significant difference in growth performance or mortality between the two system types, there were differences at the physiological and molecular level. Fish from the FT system presented significantly higher mRNA expression of NKAα1b at the time of SW transfer compared to fish produced in the RAS, but also saw a higher prevalence of fin damage and short operculum deformity. This disparity in welfare parameters still showed significance for 4 months after SW transfer.

The Tasmanian salmon industry is following the global trend and moving the majority of its smolt production to RAS hatcheries (Tassal, pers. Comm.). It is crucial for industry to know precisely when smolts are ready to be transferred to sea in both FT and RAS hatcheries. Traditionally, morphological indicators such as silvering of scales, darkening of fin margins and body elongation were used to determine smoltification readiness. However, it has been shown that these morphological changes do not necessarily happen in conjunction with the physiological changes that are necessary for successful survival at sea (Folmar and Dickhoff, 1981). Commercial salmon hatcheries in Tasmania (Australia) currently use a 24-h SW challenge method similar to that outlined by Blackburn and Clarke (1987) to determine smolt transfer readiness, however hatcheries in the northern hemisphere commonly use a gill NKA activity assay (Christensen et al., 2018; McCormick et al., 2002; Zydlewski and Zydlewski, 2012).

Our goal was to characterise smoltification and to collect baseline information on morphometric (growth parameters) and physiological indicators of smoltification (plasma chloride, gill NKA activity, gene expression) in the Tasmanian strain of Atlantic salmon *S. salar* reared in two different hatchery systems; FT and RAS respectively. This will ultimately result in a better understanding of smoltification in the Tasmanian stock of *S. salar,* and the long-term improvement of smolt quality and farm level practices that will lead to reduced mortality of smolts upon transfer to sea-pens.

2. Materials and methods

2.1. Fish rearing

Two studies to assess smoltification in juvenile Atlantic salmon were conducted in FW hatchery facilities (Tassal Operations, Hobart, TAS, Australia). The first study was conducted at SALTAS Hatchery (Tasmania) in which fish were kept in a flow-through system (FT), and then transferred to marine pens at Tassal's North West Bay lease. The second study was conducted at Rookwood Road Hatchery (Tasmania), where fish were maintained in RAS, before being transferred to marine pens at Tassal's Tasman lease. Juvenile Atlantic salmon used in these studies were reared from the egg stage at the above-mentioned hatcheries following the company's standard operational procedures. Feeding regime, sampling times, number of replicate tanks, number of fish, and transfer schedules were closely linked to farm operating procedures. Fish were fed commercial salmon diets (Skretting Australia) to satiation with automatic tank feeders. Transfer to SW occurred after fish were deemed to be capable of osmoregulation in SW, according to plasma chloride levels after a 24 h SW challenge (Blackburn and Clarke, 1987) (see details below).

2.2. Study 1 – Flow-through (FT) hatchery

Fish for this study were part of a commercial production run at the SALTAS Florentine hatchery Wayatinah, Tasmania. Fish were hatched in October 2014 and raised as per normal hatchery protocols. The hatchery operates a FT system, drawing water from the Florentine river. Water temperature was ambient, decreasing from 9 °C down to 4 °C during the study period, and the average temperature during the FW phase was 6.07 °C. There were four replicate tanks; 70,000 L concrete circular tanks, each containing approximately 30,000 fish (39 kg $m^{3\ -1}$ at time of transfer). In June 2015, tanks were exposed to a shortened photoperiod of 10L: 14D for 8 weeks using halogen lights suspended above the tanks. They were then exposed to constant 24:0 LD photoperiod for 5 weeks until SW transfer. This 24hr light period equated to 212degree days. Sampling commenced at week 1 of the 24-h light period.

During the 24-h light period, 10 fish per tank (40 fish in total) were sampled weekly for 5 weeks. At each sampling time fish were quickly captured and placed in a bucket containing a lethal dose (30 mg L^{-1}) of AQUI-S (AQUI-S New Zealand Ltd.). Blood samples were collected from the caudal vein immediately after fish were anaesthetized using a 3 mL pre-heparinised syringe. Plasma was separated by centrifugation at 3000 *G* for 15 min. Samples were stored at −80 °C until further analysis.

Approximately 6–8 gill filaments from the first 2 gill arches (left side of the fish) were collected and transferred to a microcentrifuge tube (1.5 mL) containing 0.5 mL of ice-cold sodium-EDTA-imidazole (SEI)

buffer (McCormick, 1993). These were then frozen immediately on dry ice, and subsequently stored at −80 °C until analysed. Another small section of gill was removed for molecular analysis, and was preserved in a 1.5 mL microcentrifuge tube containing RNA*later*® (Sigma Aldrich). These sections were left at room temperature overnight to allow time for the tissue to be saturated, and then frozen at −80 °C until analysed.

In addition, 10 more fish were taken from each tank and exposed to a 24 h SW challenge test (Blackburn and Clarke, 1987). Four 1000 L tanks containing 100 L of system water at were used for SW challenges. Water was made up to a salinity 35 g L^{-1} using FW system water aquarium-grade NaCl, and aerated using an air stone to strip CO_2 and maintain dissolved oxygen (DO). Water temperature remained at ambient system temperature of ~5 °C. After 24 h of SW exposure, fish were anesthetized and blood was collected following the protocol above, for plasma chloride analysis. These samples were frozen on dry ice and stored at −80 °C until analysis. Plasma samples were run in duplicate on a digital chloridometer (Labconco® cat. # 442–5000). This sampling procedure was repeated weekly for 5 weeks, at which point all SW challenged fish exhibited plasma chloride levels < 150 mEq L^{-1} after a 24 h 35 g L^{-1} SW challenge. As per normal industry protocol, fish were subsequently transported to a marine grow out site and transferred by tanker truck to a marine pen at North-West Bay, Tasmania. The truck was filled with FT system water (5 °C), supplied with liquid O_2 and DO was monitored throughout transfer (~3 h). Water temperature at the marine site was 9.3 °C, DO was 8.43 mg L^{-1} (93.25% sat.), and salinity was 35 g L^{-1}. As all 4 replicate tanks were transferred to a single marine pen, 40 fish were sampled weekly for 5 weeks (10 weeks in total). Sampling commenced on 22/06/2015 and concluded on the 24/08/2015.

2.3. Study 2 – Recirculating (RAS) hatchery

The second study followed a group of fish from the Rookwood Road Hatchery (Tassal Operations), south of Hobart Tasmania. Fish were hatched in October 2014 from the same cohort as the fish from the first study.This FW RAS hatchery is run at a constant water temperature of 14 °C, and CO_2 levels ranged from 7 to 19 mg L^{-1}. Three 400,000 L circular tanks containing approximately 100,000 fish (33.89 kg $m^{3\ -1}$ at transfer) in each were sampled weekly for 7 weeks (FW phase), and then for 5 more weeks after transfer to marine pens (a total of 12 weeks). Fish were transferred by tanker truck to a marine pen at Tasman, Tasmania. The truck was filled with RAS system water (14 °C), supplied with liquid O_2 and DO was monitored throughout transfer (~4 h). Water temperature at the marine site was 8.9 °C, DO was 9.80 mg L^{-1} (99.22% sat.) and salinity was 35 g L^{-1}. Tanks were initially exposed to a shortened “winter” photoperiod (10L: 14D) for 8 weeks prior to sampling, and then exposed to 24-h light. Halogen lamps were suspended above the tanks, as well as submerged beneath the surface. Sampling began at week one of the 24-h light regime, which lasted for 7 weeks and equated to 686 degree days.

The sampling protocol was similar to the FT study outlined above, except for a few minor differences. During the FW phase, 12 fish were taken from 3 tanks at each sampling time. Fish were euthanized and tissues collected as above. A further 12 fish were taken from each tank to conduct the 24 h SW challenge (Blackburn and Clarke, 1987), after which blood was taken and analysed for plasma chloride concentration (see above). For the SW challenge tanks, system water was made up to a salinity 35 g L^{-1} using aquarium-grade NaCl, and aerated using an air stone to strip CO_2 and maintain DO. Water temperature remained at system temperature of ~14 °C. During the SW phase, 12 fish were taken from 3 pens at each sampling time. Weekly sampling commenced on the 14/07/15 and concluded on the 29/08/15.

2.4. Growth and condition

From each fish sampled, mass (g) and fork length (mm) were recorded. Fulton's condition factor (K) was calculated using formula $K = 10^n\ W/L^3$ (where: N = 5, W = mass in grams and L = length in mm). Specific growth rate was determined as percentage increase in total mass (g) per day, according to *SGR = [Ln (final mass) – Ln (initial mass)]/(number of days)* x *100*. Degree days were calculated according to Chezik et al. (2014): *DD = average daily temperature* x *number of days*.

2.5. Plasma chloride determination

Plasma chloride was measured using a Labconco® Digital Chloridometer. A standard of 100 mEq L^{-1} was used for calibration. 10 µl of sample was pipetted into the instrument receptacle along with the commercially produced reagent (Labconco® cat. # 442–5065). The instrument uses a coulometric assay technique, automatically titrating chloride ions (Cl^-) with silver ions (Ag^+) at a constant rate, forming silver chloride. The endpoint occurs when all chloride ions have bound to a silver ion, and the remaining (known) amount of free silver ions changes the conductivity of the solution. This instrument has previously been used to measure plasma chloride in studies investigating Atlantic salmon smoltification (Carey and McCormick, 1998; Shrimpton et al., 2000).

2.6. Na^+/K^+-ATPase activity assay

The gill NKA activity was determined following a similar procedure described by Brown et al. (2018) and McCormick (1993). Gill samples were taken from an −80 freezer and thawed on ice. 1 ml of ice-cold 0.15% SEID buffer (SEI buffer with 0.15% deoxycholate) solution was added, and the sample homogenised by hand using a glass tube and Teflon pestle. The supernatant was then centrifuged at 4 °C for 3 min at 5000 *G*. Samples were then run in a 96-well microplate in triplicate with two assay solutions: Solution 1 (ouabain negative) contained 4 U lactate dehydrogenase mL^{-1}; 5 U pyruvate kinase mL^{-1}; 2.8 mM phosphoenol pyruvate; 0.7 mM ATP; 0.22 mM NADH; 50 mM imidazole pH 7.5. Solution 2 (ouabain positive) is identical, but also contains 0.5 mM of the NKA inhibiting ouabain. Wells containing the ouabain negative (−) assay buffer indicated the total amount of ADP in the gill homogenate, and wells containing ouabain positive (+) buffer indicated ADP from other metabolic pathways. Therefore, ouabain (−) minus ouabain (+) = the amount of ADP generated specifically by gill NKA. The assay was run for 20 min at 26 °C, and the consumption of NADH as a function of ADP present was measured as a reduction in OD at 340 nm. The final calculation takes into account an ADP standard curve, and results are standardised by protein levels according to the Bradford method (Bradford, 1976). A final value of NKA activity is reported as µmol ADP · $(hr)^{-1}$ · $(mg\ protein)^{-1}$.

2.7. Real-time quantitative PCR

NKAα1a and NKAα1b mRNA transcripts were quantified using elongation factor 1-α (EF1-α) as a reference gene. Richards et al. (2003) has demonstrated that EF1-α does not vary when exposed to differing salinity treatments. It was also determined by Richards et al. (2003) that there was no significant variation in a selection of samples from varying time points and salinities, which lead to EF1-α being chosen as a reference gene for this study. Primers and assay protocols were designed based on previously published research (McCormick et al., 2008; Richards et al., 2003). Primers for each gene were manufactured by Integrated DNA Technologies (Melbourne, Australia) and were as follows: NKAα1a (forward: *GGC CGG CGA GTC CAA T*, reverse: *GAG CAG CTG TCC AGG ATC CT*), NKAα1b (forward: *CTG CTA CAT CTC AAC CAA CAA CAT T*, reverse: *CAC CAT CAC AGT GTT CAT TGG AT*) and EF1-α (forward: *GAG ACC CAT TGA AAA GTT CGA GAA G*, reverse: *GCA CCC AGG CAT ACT TGA AAG*).

Total RNA was extracted from approximately 10 mg of gill tissue stored in RNA*later*® using an Aurum™ Total RNA Mini Kit (Bio-Rad:

7326820). RNA quality of selected samples was visually assessed on a 1% agarose gel electrophoresis during initial testing stages, and then RNA quality and quantity of every sample was assessed by the ratio of absorbance at 260 nm and 280 nm on a benchtop NanoDrop. RNA was then normalised to 50 ng μl^{-1} using certified DNase/RNase-free H_2O and treated with DNase Turbo (Invitrogen) to eliminate any residual genomic DNA. This treatment consisted of 1 µl of DNase Turbo and 5 µl of 10× buffer being added to 44 µl of normalised RNA sample in a 1.5 mL microcentrifuge tube. Samples were incubated for 30 min at 37 °C, before 5 µl of inactivation reagent was added and the tube vortexed regularly for 5 min. Sample was then centrifuged at 10,000 *G* for 1.5 min to pellet reagent, and RNA supernatant was aliquoted into a fresh 1.5 mL tube.

cDNA was synthesised in 0.6 mL PCR tubes by adding 2 µl of RNA to 14 µl of iScript™ Reverse Transcription Super mix (Bio-Rad: 1708840) and 4 µl of RNase-free H_2O. The 20 µl reaction was then cycled in a thermocycler according to the manufacturer's instructions. No-reverse transcriptase (NRT) controls for each sample were also run under the sample conditions.

A qPCR Master Mix was prepared for each gene with 10 µl of SYBR green reagent (Bio-Rad, USA), 0.4 µl of 10 mM forward primer, 0.4 µl of 10 mM reverse primer and 7.2 µl of RNase-free H_2O per well. The total reaction volume of 20 µl results in a 200 nM:200 nM primer concentration ratio once 2 µl of cDNA template are added. Each plate contained duplicate templates for each sample and each gene. NRT controls from each plated sample were pooled and run in duplicate, along with a no-template-control (NTC) in duplicate for each primer set. PCR was started with an incubation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. To assure primer specificity, a melt curve analysis was then conducted with 0.5 °C increments from 65 °C to 95 °C for 5 s per temperature. Efficiency of PCR reaction was determined by running a pooled sample in a serial 10-fold dilution. Concentrations for the dilution curve were neat, 10×, 100×, 1000× and 10000×. Efficiency of each master mix were as follows: EF1α – 99.8%, NKAα1a – 100.2%, and NKAα1b: 99.0%. Fold-expression of target genes (NKAα1a and NKAα1b) was calculated relative to the reference gene (EL1-α) and relative to the initial time point (week 1) using the Delta-Delta-C_T ($\Delta\Delta C_T$) method (Livak and Schmittgen, 2001). For ΔΔCq calculations, an efficiency of 100% was assumed for each primer set.

2.8. Statistical analysis

Statistical analysis was conducted via a one-way repeated measures ANOVA using RStudio (R Core Team, 2018). All variables were analysed with time as the main factor and tank (replicate) number defined in the error term. For FT hatchery, tanks 1–4 were transferred into a single marine pen due to logistical constraints on farm. Therefore, for FT after transfer, fish was used as replicate unit ($N = 40$). All data were checked for equal variance and normal distributions both visually and using the Levene's test and the Kolmogorov-Smirnov test, respectively. Significant effects of time ($P < 0.05$) were investigated using Tukey post-hoc multiple pairwise comparisons. Graphic output was generated using ggplot2 for R. All graphic data is presented as mean ± SEM, where experimental units are replicate tanks.

3. Results

3.1. Growth and condition

3.1.1. Flow-through hatchery

There was a significant effect of time on mass ($p < 0.001$) for the duration of the study, with significant increases between weeks 1 and 5, 6 and 8, and 9 and 10. Mean initial and final mass was 72.2 g (± 2.52) and 141.1 g (± 3.94) respectively (Table 1). Fork length also showed a significant effect of time ($P < 0.001$), with significant increases in length between weeks 1 and 5, 6 and 8, and 9 and 10 (Table 1). Initial mean fork length was 178 mm (± 2.09), and final mean fork length was 236 mm (± 2.34) (Table 1). Condition factor (CF) decreased significantly throughout the duration of the study ($P < 0.001$). The mean initial and final CF was 1.26 (± 0.04) (FW) and 1.07 (± 0.02) (SW). There was no significant difference between time-points during the FW phase. Fish in the SW phase had significantly lower CF than fish in FW at all time-points and showed a steep decline after transfer to SW (Table 1). SGR was significantly different between FW and SW phase of this study ($P = 0.03$). Mean specific growth rate was 0.72% (± 0.17) and 1.49% (± 0.20) mass gain per day in FW and SW respectively.

3.1.2. Recirculation (RAS) hatchery

There was a significant effect of time on mass ($P < 0.001$) for the duration of the study, although there was no significant increase from weeks 1–5 (FW). There was a significant increase in mass between weeks 4 and 6. Post-transfer, fish mass significantly declined between week 7 and week 8. Mass increased thereafter, although only significantly so at weeks 11 and 12. Mean initial mass was 78.2 g (± 3.92) and mean final mass was 162.1 g (± 7.92) (Table 1). There was a significant effect of time on fork length ($P < 0.001$), with significant increases between weeks 1 and 6 (FW), and no significant increase after this until week 11. Mean initial and final fork length was 178 mm (± 3.10) (FW) and 247 mm (± 3.64) (SW) (Table 1).

There was a significant effect of time on CF throughout the sampling period ($P < 0.001$). A significant decrease was evident between weeks 2 and 4 (FW), and after that remained steady until SW transfer. There was a significant reduction after SW transfer at week 8, after which CF of SW phase fish remained significantly lower than those of FW phase fish. Mean initial and final CF was 1.38 (± 0.02) (FW) and 1.05 (± 0.02) (SW) (Table 1). SGR was significantly higher in the FW phase of the study than the SW phase ($P = 0.02$). Mean SGR was 1.58% (± 0.08) and 1.26% (± 0.01) mass gain per day in FW and SW respectively.

3.2. Plasma chloride

3.2.1. Flow-through hatchery

There was a significant effect of time in SW challenged fish ($P = 0.013$), with significant decrease between week 2 and 3, and a significant increase between week 3 and 4. SW challenged fish showed significantly higher plasma chloride levels to the FW control at each week. Mean initial and final plasma chloride after a 24-hr SW challenge were 133.8 (± 1.46) and 132.7 (± 1.01) mEq L^{-1} respectively. Plasma chloride levels in the main population of fish showed a significant effect of time ($P < 0.001$), with significant differences in FW between week 2 and 3, but no other significant changes before transfer to SW. Plasma chloride increased significantly after transfer to SW (week 6), and between week 6 and 7. Plasma chloride levels then significantly decreased between week 7 and 8, with no significant change thereafter. Initial and final plasma chloride was 119.2 (± 1.01) mEq L^{-1} (FW) and 149.8 (± 1.71) (SW) (Fig. 1).

3.2.2. Recirculating (RAS) hatchery

There was a significant effect of time in SW challenged fish ($P < 0.001$), with significant differences between week 3 and 4, and 5 and 6. SW challenged fish showed significantly higher plasma chloride levels to the FW control at each week. Mean initial and final plasma chloride after a 24-hr SW challenge were 140.3 (± 2.55) and 133.6 (± 2.71) mEq L^{-1} respectively. There was a significant effect of time on plasma chloride levels in the main population of fish raised in the RAS ($P < 0.001$), however there was no significant change during the FW phase of the study. There was a significant increase in plasma chloride in the main population of fish after SW transfer. Subsequently there was significant reduction in plasma chloride between weeks 9 and 12. Levels plateaued at week 10. Mean initial and final plasma chloride

Table 1
Mean (± SE) values for total mass (g), fork length (mm) and condition factor (K). N = 40 replicate fish in FT, N = 3 replicate tanks, 12 fish/tank in RAS. Transfer to saltwater sea-cages was after week 5 in FT, and after week 7 in RAS. Significant differences ($p < 0.05$) between weeks are indicated by different letters.

System	Week	Salinity	Mass	Fork length	Condition Factor
FT	1	FW	72.16 ± 2.52^{a}	177.92 ± 2.09^{a}	1.26 ± 0.04^{c}
	2	FW	81.38 ± 2.79^{ab}	185.02 ± 1.87^{ab}	1.27 ± 0.03^{c}
	3	FW	83.3 ± 3.00^{ab}	188.43 ± 2.42^{abc}	1.23 ± 0.02^{c}
	4	FW	86.95 ± 2.98^{ab}	189.68 ± 2.07^{abc}	1.26 ± 0.03^{c}
	5	FW	93.01 ± 2.73^{b}	195.7 ± 1.66^{bc}	1.23 ± 0.03^{c}
	6	SW	83.75 ± 3.16^{ab}	201 ± 2.54^{cd}	1.02 ± 0.02^{ab}
	7	SW	95.75 ± 3.48^{bc}	212.35 ± 2.52^{de}	0.99 ± 0.03^{a}
	8	SW	110.95 ± 2.87c	218.32 ± 2.12e	1.06 ± 0.03b
	9	SW	112.55 ± 3.37^{c}	220.65 ± 2.32^{e}	1.04 ± 0.03^{ab}
	10	SW	141.12 ± 3.94^{d}	235.95 ± 2.34^{f}	1.07 ± 0.02^{b}
RAS	1	FW	78.22 ± 3.92^{a}	177.58 ± 3.1^{a}	1.38 ± 0.02^{c}
	2	FW	89.28 ± 5.03^{a}	184.86 ± 3.46^{ab}	1.39 ± 0.01^{c}
	3	FW	78.36 ± 8.78^{a}	180.19 ± 4.38^{a}	1.31 ± 0.05^{bc}
	4	FW	83.26 ± 5.4^{a}	185.56 ± 3.53^{ab}	1.26 ± 0.02^{b}
	5	FW	97.11 ± 4.2^{ab}	198.06 ± 2.78^{bc}	1.23 ± 0.01^{b}
	6	FW	120.44 ± 10.02^{bcd}	214.58 ± 6.51^{cd}	1.2 ± 0.02^{b}
	7	FW	135.56 ± 3.04^{de}	222.97 ± 3.41^{d}	1.21 ± 0.02^{b}
	8	SW	104.14 ± 5.37^{abc}	216 ± 3.67^{d}	1.03 ± 0^{a}
	9	SW	117.08 ± 7.35^{bcd}	223.81 ± 6.15^{d}	1.03 ± 0.02^{a}
	10	SW	125.22 ± 7.63^{cd}	226.78 ± 4.28^{de}	1.05 ± 0.02^{a}
	11	SW	154.28 ± 8.32^{e}	243.47 ± 5.02^{ef}	1.06 ± 0.01^{a}
	12	SW	162.14 ± 7.92^{e}	247 ± 3.64^{f}	1.06 ± 0.01^{a}

levels in the main population were 109.0 (± 0.23) (FW) and 145.8 (± 1.92) mEq L^{-1} (SW) (Fig. 1).

3.3. Gill Na^{+}/K^{+}-ATPase activity

3.3.1. Flow-through hatchery

Fish from the FT hatchery showed a significant increase in Gill NKA activity through time in both fresh and salt water ($P < 0.001$). Mean initial activity was 2.2 (± 0.18) µmol ADP mg protein^{-1} hr^{-1}, and significantly increased to 6.6 (± 0.36) µmol ADP mg protein^{-1} hr^{-1} before fish before being transferred to salt water (after week 5). Post-transfer, NKA activity levels continued to significantly increase between week 6 and 7, with a peak value of 13.7 (± 0.56) at week 9. NKA levels then plateaued, showing mean a value of 12.5 µmol ADP mg protein^{-1} hr^{-1} between weeks 7–10 (Fig. 2).

3.3.2. Recirculating hatchery

Gill NKA activity in the RAS hatchery showed a significant effect of time ($P < 0.001$), with a mean value of 3.7 (± 0.34) µmol ADP mg protein^{-1} hr^{-1} at week 1, and no significant difference seen in the first four weeks of the study. There was a significant increase in NKA between week 3 and week 5, with no significant change until week 9. Following transfer, there was a significant increase between week 8 and week 9, where levels appear to plateau thereafter. Final mean level was 9.8 (± 0.39) µmol ADP mg protein^{-1} hr^{-1}. Mean value for weeks 9–12 was 10.8 µmol ADP mg protein^{-1} hr^{-1} (Fig. 2).

3.4. NKA - gene expression

3.4.1. Flow-through hatchery

There was a significant effect of time ($P < 0.001$) on gene

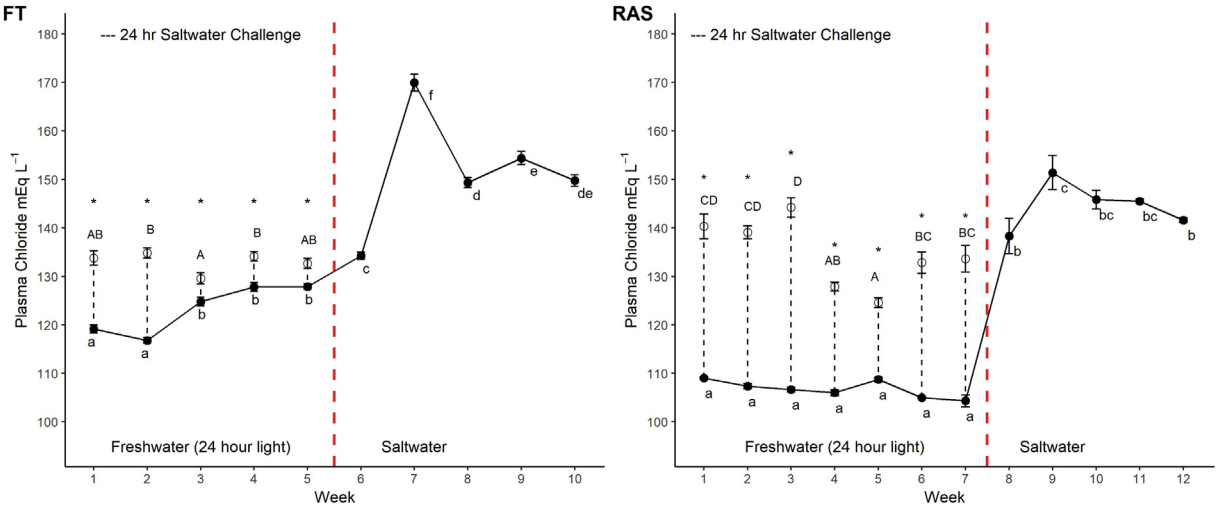

Fig. 1. Mean plasma chloride levels (± S.E.) (mEq L^{-1}). Fish from flow-through (FT) system were sampled weekly for 10 weeks (5 in freshwater and 5 in seawater, n = 40). Fish from recirculation aquaculture system (RAS) were sampled weekly for 12 weeks (7 in freshwater and 5 in seawater, n = 3). Transfer to seawater marine pens represented by red dashed line. Solid line refers to main population in freshwater/seawater. Dotted line represents plasma chloride in fish subjected to a 24 hr-seawater challenge. Significant differences ($p < 0.05$) among weeks are indicated by different upper-case (seawater challenged group) and lower-case (freshwater control group) lettering. Significant differences between salinities are indicated by asterisk (*). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

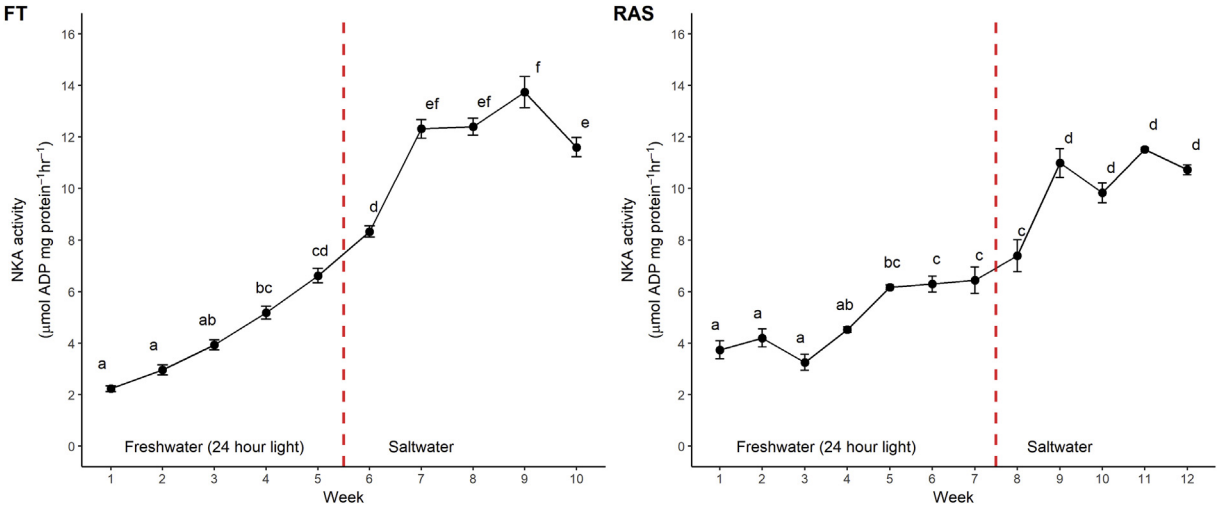

Fig. 2. Mean gill Na^+/K^+-ATPase activity (± S.E.) in μmol ADP mg protein^{-1} hr^{-1}. Fish from flow-through (FT) system were sampled weekly for 10 weeks (5 in freshwater and 5 in seawater, n = 40). Fish from recirculation aquaculture system (RAS) were sampled weekly for 12 weeks (7 in freshwater and 5 in seawater, n = 3). Transfer to seawater marine pens represented by red dashed line. Significant differences ($p < 0.05$) between weeks are indicated by different letters. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

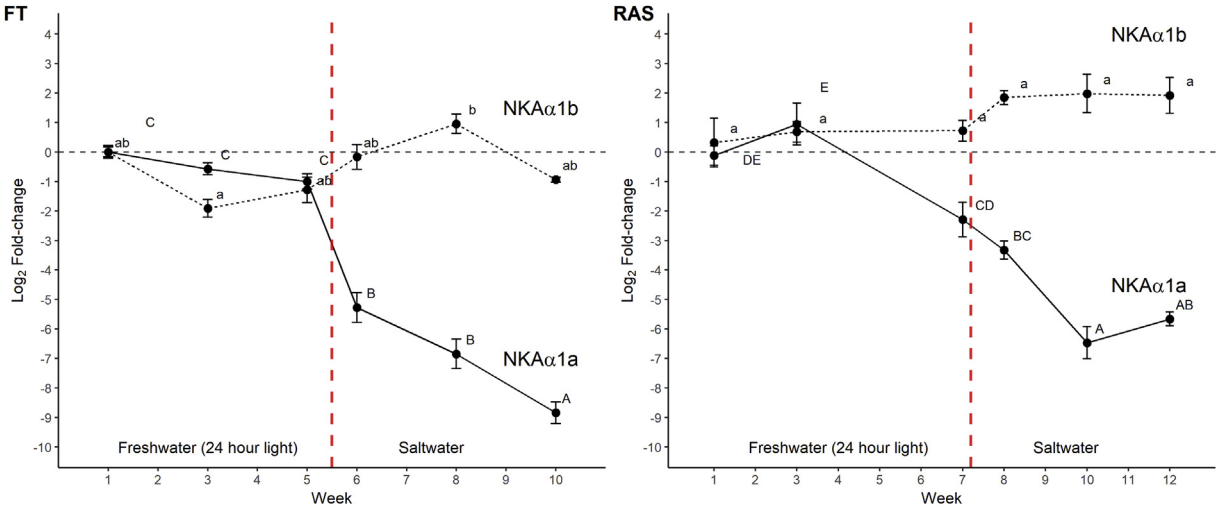

Fig. 3. Mean Log_2 fold-change in expression of branchial Na^+/K^+-ATPase alpha-1 subunits a & b (± S.E.). Fish from flow-through (FT) system were sampled weekly for 10 weeks (5 in freshwater and 5 in seawater, n = 40). Fish from recirculation aquaculture system (RAS) were sampled weekly for 12 weeks (7 in freshwater and 5 in seawater, n = 3). Transfer to seawater marine pens represented by red dashed line. Each gene was analysed separately, with significant differences ($p < 0.05$) between weeks indicated by different letters. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

expression of NKAα1a relative to EF1α, in fish raised in the FT hatchery. Relative expression of NKAα1a did not significantly change during the FW phase of the study, but decreased significantly after transfer to SW. There was a significant (5.5-fold) reduction between week 5 and 6, with a further significant (9-fold) reduction seen by week 10 (Fig. 3).

Relative expression of NKAα1b mRNA showed a significant effect of time relative to EF1α ($P = 0.019$), with the only significant difference between weeks 3 and 8. No other weeks were significantly different to the control group (week 1) (Fig. 3).

3.4.2. Recirculating hatchery

Fish raised in the RAS hatchery showed a significant effect of time ($P < 0.001$) on gene expression of NKAα1a relative to EF1α. Gill NKAα1a mRNA expression was significantly lower at week 7 (FW) when compared to weeks 1 and 3. After SW-transfer, NKAα1a mRNA expression levels continued to significantly decline. By week 10, expression had decreased by approximately 6.5-fold, with no significant change thereafter (Fig. 3).

Gill NKAα1b expression in fish from the RAS hatchery did not significantly change throughout the study ($P = 0.242$), with no significant effect of time on gene expression of NKAα1b relative to elongation factor 1-alpha EF1α. Expression stayed constant during the FW phase of the study, and although expression levels were consistently higher in the SW phase, there was no statistically significant difference detected (Fig. 3).

3.5. Log_2 ratio of NKAα1b to NKAα1a

3.5.1. Flow-through hatchery

There was a significant effect of time on Log_2 ratio of NKAα1b/NKAα1a ($P < 0.001$), with no significant effect throughout the FW phase. After transfer to SW, this ratio significantly increased approximately 5-fold, and then increased significantly to 5.11 (± 0.75) at week 8, where the ratio between these two genes appears to plateau at approximately Log_2 8-fold. Mean initial and final Log_2 ratio was −1.33

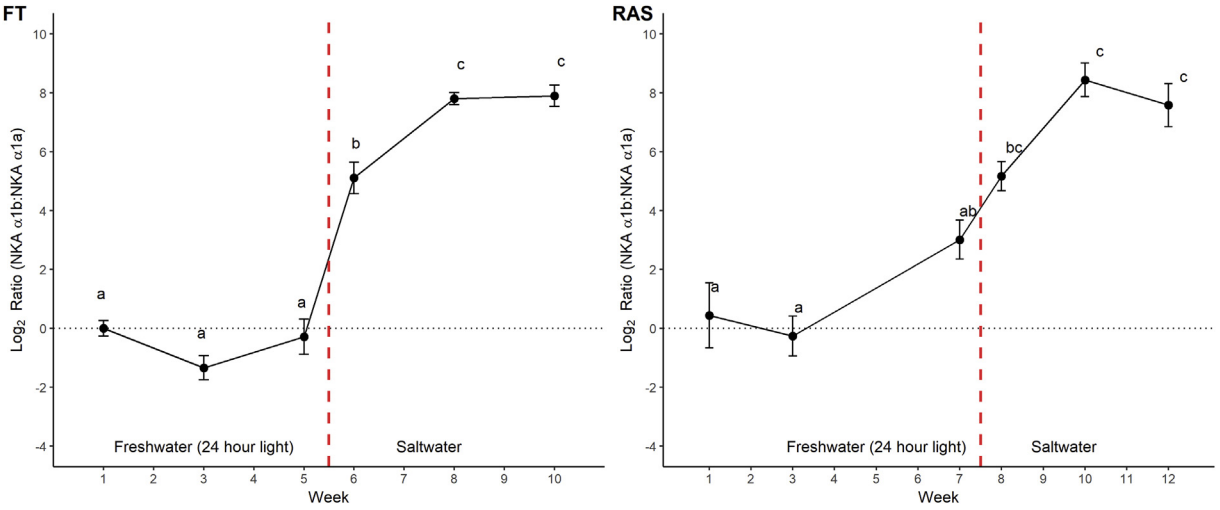

Fig. 4. Mean Log_2 ratio of branchial Na^+/K^+-ATPase alpha-1 subunit b to Na^+/K^+-ATPase alpha-1 subunit a (± S.E.). Fish from flow-through (FT) system were sampled weekly for 10 weeks (5 in freshwater and 5 in seawater, n = 40). Fish from recirculation aquaculture system (RAS) were sampled weekly for 12 weeks (7 in freshwater and 5 in seawater, n = 3). Transfer to seawater marine pens represented by red dashed line. Significant differences ($p < 0.05$) between weeks are indicated by different letters. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(± 0.33) and 7.90 (± 0.71) respectively (Fig. 4).

3.5.2. Recirculating hatchery

There was a significant effect of time on Log_2 ratio of NKAα1b/NKAα1a ($P < 0.001$) in fish raised in the RAS system. There was no significant increase during the FW phase of the study, although an increasing trend was evident between weeks 3 and 7. Post SW-transfer, the Log_2 ratio between these two genes of interest was significantly higher at week 8 than weeks 1 and 3. Although no significant difference was seen thereafter, the increasing trend continued until week 10, where the ratio of NKAα1b/NKAα1a appears to plateau at approximately Log_2 8-fold higher. Mean initial and final Log_2 ratio was 0.44 (± 1.10) and 7.58 (± 0.73) respectively (Fig. 4).

3.6. Hatchery comparison: FT x RAS

Plasma chloride levels in 24-h SW challenged fish after 5 weeks of increased day length were 132.7 (± 1.00) mEq L^{-1} in FT and 124.6 (± 0.99) mEq L^{-1} in the RAS, suggesting similar levels of salinity tolerance, although a much smaller difference between the FW control and SW challenged levels was evident in the FT group. Fish from the FT hatchery showed a 3× increase in gill NKA activity during the FW phase, whereas fish from the RAS showed a 0.5× increase. After 5 weeks of increased day length, NKA activity increased to 6.6 (± 0.28) μmol ADP mg $protein^{-1}$ hr^{-1} in FT and 6.2 (± 0.08) μmol ADP mg $protein^{-1}$ hr^{-1}in RAS. Gill NKA values increased in both groups after SW exposure. NKAα1b expression was not altered substantially in either rearing condition, but NKAα1a expression decreased substantially after SW exposure in both systems. Specific growth rate after 5 weeks in SW was 1.50 (± 0.17) % day^{-1} in the FT group and 1.27 (± 0.01) % day^{-1} in the RAS group. Fish from both systems increased significantly in mass throughout the study. Fish from the RAS decreased significantly one week post-transfer, and whilst they increased significantly thereafter, they were not significantly different at week 12 when compared to their final week in FW (week 7). In the FT group, there was no significant change between FW and SW transfer, but after 3 weeks showed a significant increase in mass. The FT group showed a significant increase in fork length two weeks post SW-transfer, whereas the RAS group didn't show a significant increase until 4 weeks post SW-transfer. Overall, the results indicate that smolt development and salinity tolerance increased in the FW phase in both FL and RAS and that further development occurred after SW exposure, although there were some differences in performance evident between systems.

4. Discussion

The smoltification process in salmonids is characterised by morphological, behavioural and physiological changes that equip juvenile fish with the necessary adaptations to migrate to and survive in full strength SW (Hoar, 1988; McCormick, 2013; Stefansson and Björnsson, 2008). Increased osmoregulatory function and the development of a higher tolerance to SW is perhaps the most important change during the smoltification phase. Anadromous fish in the wild tend to migrate to seawater at their own pace after these changes have been completed. However, fish in aquaculture systems, such as Atlantic salmon, are transferred from their land-based freshwater hatcheries to ocean-based sea pens immediately after some morphological (colour, decrease in condition factor) and physiological (plasma chloride or gill NKA activity) indicators, measured in a sample of the population, have reached a threshold. This study examined some of the major physiological and molecular indicators of smoltification in Atlantic salmon reared in two commercial FW hatcheries in Tasmania (Australia). To our knowledge, this has never before been studied in Tasmanian Atlantic salmon smolts.

It's widely understood that photoperiod is the primary external factor in initiating this physiological transformation (Duston and Saunders, 1990; Hoar, 1988; McCormick et al., 2007), and it's thought that a thermal sum of approximately 300–400 degree-days is necessary for Atlantic salmon to complete smolt-related changes in physiology and morphology (Sigholt et al., 1998; Stefansson et al., 1998). The window for optimum transfer lasts approximately 300–350 degree-days (Sigholt et al., 1998; Stefansson et al., 1998; Stefansson and Björnsson, 2008). In this study, fish from the FT and RAS groups were exposed to 24 h light for 212 degree-days and 686 degree-days respectively. In spite of the fact that fish from the FT system may have been transferred earlier than their optimal smolt window and fish from RAS system nearing the end of this optimal window, both groups showed smolt-related changes in physiology (increased gill NKA activity, reduction in condition factor, increased salinity tolerance etc.) before and after transfer to SW.

Independent of system type, fish showed a significant increase in mass during the study. However, fish in the FT system did not increase in mass during the initial 2 weeks after transfer, and didn't significantly increase in mass until week 8 (3 weeks after SW transfer). Fish from the RAS system were significantly lower in mass at week 8 (SW) when

compared to week 7 (FW), and didn't show a significant increase in mass in SW.

It is possible that feed intake was affected after transfer to SW in fish coming from both systems. Whilst feed consumption data was not recorded in this study, it has been demonstrated that feed intake and growth were inhibited (Bendiksen et al., 2003; Usher et al., 1991) and SGR decreased (Handeland et al., 2003) after transfer to SW.

To our knowledge, this is the first study to measure gill NKA activity and gene expression in the Tasmanian stock of Atlantic salmon. In addition, little work has been done characterising gill NKA activity in different hatchery systems (one exception: Kolarevic et al., 2014). Gill NKA activity reported in the present study were in accordance with values previously recorded for this species in populations of Atlantic salmon from North America (Christensen et al., 2018; McCormick, 1993; McCormick et al., 2007; Shrimpton et al., 2000), Norway (Handeland et al., 2013; Solbakken et al., 1994), and Denmark (Kiilerich et al., 2011; Madsen et al., 2009). The present study revealed that gill NKA activity in Atlantic salmon smolts in FW (approximately 6.0 μmol ADP mg protein^{-1} hr^{-1}), independent of system type, were lower than values typically reported in smolts close to SW transfer/migration (8.0–25 μmol ADP mg protein^{-1} hr^{-1}) (McCormick et al., 2007; McCormick, 2013; Stefansson et al., 2007). It's worth noting that while it is likely that population-based differences in gill NKA activity exist, direct comparisons between studies on enzyme activity must be conducted with caution, due to potential technical variation between different laboratories.

Although gill NKA activity in fish maintained in the RAS was initially 2-fold higher than in the FT system, their gill NKA activity did not significantly increase in FW until week 5. At this point (week 5), fish from both systems displayed similar NKA activity levels. While the RAS fish were held for two more weeks in FW than fish from the FT group, they did not show any subsequent increase in NKA activity until after SW transfer. Fish used in this study were transferred according to the aforementioned plasma chloride SW challenge test, therefore one could question whether these fish would have shown an increase in gill NKA activity if they were maintained for longer in FW on 24-h light. Fish from both rearing systems were exposed to SW with similar gill NKA activity, and both groups showed a substantial increase in NKA activity after SW transfer. This suggests that salinity plays a role in driving gill NKA activity to the appropriate levels needed for long-term survival in SW. In fact, large juvenile Atlantic salmon have shown the ability to develop smolt-related characteristics (including increase in gill NKA activity) after SW transfer outside of the typical smolt window (Brown et al., 2018). There have been other studies in salmonids that have also demonstrated increases in gill NKA activity after exposure to SW (Bjerknes et al., 1992; Taylor et al., 2007).

It's been reported that smolts can be considered fully developed when plasma chloride levels are maintained below 150 mEq L^{-1} in SW (Arnesen et al., 2003). Plasma chloride typically increases initially after exposure to SW, and stays elevated for several days before reducing to a new basal level (Madsen and Naamansen, 1989). It's thought that smolts go through a “crisis” phase after SW transfer, where increased salt load and water loss to the external environment is at a higher rate than can be compensated for by ionic regulatory mechanisms (Madsen and Naamansen, 1989). In the present study, fish raised in the FT system showed a smaller degree of change in plasma chloride levels following a 24-h SW challenge. These fish also maintained plasma chloride levels at 134 mEq L^{-1} one week after transfer to SW (week 6). However, a significant increase in plasma chloride (169 mEq L^{-1}) occurred between week 6 and 7. At week 8, plasma chloride levels reduced to 149 mEq L^{-1} and there was no significant change thereafter.

Fish from the RAS system presented a significant increase in plasma chloride levels in all sampling times in FW following a 24-h SW challenge. These fish showed a peak plasma chloride level (151 mEq L^{-1}) at 2 weeks after transfer to SW, and then maintained plasma chloride levels constant thereafter. Fish maintained in the FT system showed less variation in plasma chloride levels after SW challenges during the FW phase than fish in the RAS system suggests that fish from the FT system were better prepared to cope with changes in salinity over a 24-h (SW challenge) period. It is possible that fish raised in this type of system may be more resilient to changes in their external environment than fish raised in RAS, where the environment is consistently maintained. In SW however, fish from both groups increased plasma chloride levels after transfer. The fish from the FT system were able to maintain their plasma chloride below 150 mEq L^{-1} (an industry-used threshold) during the first week of SW exposure, but by the second week in SW, plasma chloride levels peaked to ~170 mEq L^{-1}. The high peaks in plasma chloride in fish from both hatchery systems post-transfer, taken together with the lack of growth in the initial weeks of SW, suggests that fish may not have been sufficiently adapted to deal with their new high-saline environment. Plasma chloride levels after 3 weeks in SW suggest that fish from both hatcheries were able to adapt to their new environment. This profile is typical of what has been shown in previous studies that transferred smolts to SW (Handeland et al., 2003; Madsen and Naamansen, 1989; McCormick et al., 2013). Mechanisms such as increased drinking of SW allow fish to counter the net-loss of water and survive a sudden change in salinity, however water is desalinated in the oesophagus and Na^+ and Cl^- need to be excreted by the chloride cells located in the gills (Fuentes and Eddy, 1997). Ensuring that these mechanisms are sufficiently developed in the majority of the population to be transferred to SW is extremely important to reduce transfer-related stress that may lead to delayed mortality or increased disease susceptibility (Barton and Iwama, 1991; Berge et al., 2011; Björnsson et al., 1988).

Several important ion-transporting proteins have been identified in the gill epithelium of teleosts, including salinity-specific isoforms of the alpha-subunit of NKA (Richards et al., 2003). By measuring the gene expression of these ion regulators, it's possible to monitor their profiles during the 24:0 LD preparatory photoperiod regime and subsequent SW transfer. The timing of their up or down-regulation can be associated with salinity tolerance, total gill NKA activity and plasma chloride in fish prior to and after transfer to SW (Christensen et al., 2018). In the present study, fish from the FT system showed no significant response of the NKAα1a subtype during the FW 24:0 LD photoperiod, but did present a significant decrease after exposure to SW. The NKAα1b subtype also showed no increase during the FW (24-hr light) phase of the study, but was significantly elevated at week 8 (SW) when compared to week 3 (FW). This is contrary to what has been reported in the literature (Christensen et al., 2018; Kolarevic et al., 2014; Stefansson et al., 2007). Fish exposed to an increasing photoperiod typically show an increase in NKAα1b in FW during smolt development. Fish from the RAS system showed the expected significant reduction in NKAα1a in the FW phase, and then a further significant reduction post-transfer to SW. NKAα1b showed a slight but non-significant increase post-transfer, but overall no significant change was detected during the study. It is possible that a more frequent sampling interval would have revealed the significant changes reported in previous studies (Christensen et al., 2018; Madsen et al., 2009; Nilsen et al., 2007). As well as a significant increase in the abundance of NKAα1b both during smolt development and then again post SW-transfer, ionocytes containing both NKAα1a and NKAα1b (labelled by immunohistochemistry) were present in FW (McCormick et al., 2013). These co-labelled ionocytes were not present after SW transfer, suggesting their involvement in ion-uptake (McCormick et al., 2013), and might explain the lack of increase in upregulated NKAα1b mRNA transcript in the present study. Comparatively small increases (50%) in gill NKAα1b mRNA lead to large increases in NKAα1b protein abundance (Christensen et al., 2018). This is thought to be due to a slow yet steady build-up of protein over several weeks which is either inactive itself, or present in predominantly inactive ionocytes (Christensen et al., 2018). This hypothesis is supported by previous studies in Atlantic salmon (Madsen et al., 2009; McCormick et al., 2009; Nilsen et al., 2007; Pelis and McCormick, 2001).

Strong correlations have been shown to exist between salinity tolerance and the abundance of NKAα1a mRNA, ratio of gill NKAα1b to NKAα1a mRNA, and the ratio of NKAα1b to NKAα1a protein (Christensen et al., 2018). The present study explored the ratio of gill NKAα1b to NKAα1a mRNA. Fish from both the FT and RAS hatchery systems exhibited an 8-fold increase in Log_2 ratio, although only after exposure to SW. Although there was an increase in NKA activity in both hatcheries prior to SW transfer, this was not reflected in the NKAα1b: α1a mRNA ratio in FW. It's been suggested that the ratio between these salinity-specific isoforms of NKA mRNA or their relative proteins could serve as a suitable indicator of smolt-development (Christensen et al., 2018). Further work needs to be done investigating the appropriate ratio required for smolts to achieve optimum salinity tolerance resulting in successful smoltification.

From the present study, it appears that the FT system better prepared fish for transfer to the marine sites than the RAS system. Growth and condition factor profiles of the Tasmanian strain of *S. salar* were reflective of those previously reported for the species, however better post-transfer growth performance in terms of mass and length gain, and the ability to better regulate plasma chloride suggest that fish raised in the FT system may have been better adapted to cope with the changed conditions. Although gill Na^+/K^+-ATPase activity showed similar absolute values at time of transfer, the fold-change during the 24-hr light regime was far greater in fish raised in the FT than the RAS. Gene expression of the salinity specific isoforms of Na^+/K^+-ATPase showed similar profiles to those previously reported, however there seemed to be a lack of increase in NKAα1b in both the FT and RAS system, although variation between the two hatchery types was evident. It cannot be discounted that environmental factors specific to the different hatcheries may have had different effects on the smoltification parameters investigated. Further work under laboratory conditions is required to determine how variables that differ between these systems such as water quality, light quality/regime and water temperature interact to facilitate smolt development.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2019.734603.

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