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Effects of elevated temperature on osmoregulation and stress responses in Atlantic salmon *Salmo salar* smolts in fresh water and seawater

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Smolting in Atlantic salmon *Salmo salar* is a critical life-history stage that is preparatory for downstream migration and entry to seawater that is regulated by abiotic variables including photoperiod and temperature. The present study was undertaken to determine the interaction of temperature and salinity on salinity tolerance, gill osmoregulatory proteins and cellular and endocrine stress in *S. salar* smolts. Fish were exposed to rapid changes in temperature (from 14 to 17, 20 and 24°C) in fresh water (FW) and seawater (SW), with and without prior acclimation and sampled after 2 and 8 days. Fish exposed simultaneously to SW and 24°C experienced 100% mortality, whereas no mortality occurred in any of the other groups. The highest temperature also resulted in poor ion regulation in SW with or without prior SW acclimation, whereas no substantial effect was observed in FW. Gill Na^+–K^+-ATPase (NKA) activity increased in SW fish compared to FW fish and decreased with high temperature in both FW and SW. Gill Nkaα1a abundance was high in FW and Nkaα1b and Na^+–K^+-2Cl- cotransporter high in SW, but all three were lower at the highest temperature. Gill Hsp70 levels were elevated in FW and SW at the highest temperature and increased with increasing temperature 2 days following direct transfer to SW. Plasma cortisol levels were elevated in SW at the highest temperature. Our results indicate that there is an important interaction of salinity and elevated temperature on osmoregulatory performance and the cellular stress response in *S. salar*, with an apparent threshold for osmoregulatory failure in SW above 20°C.

KEYWORDS
heat shock protein, ion transport, salinity, *Salmo salar*, smolts

1 | INTRODUCTION

As part of their anadromous life history, Atlantic salmon *Salmo salar* L. 1758 migrates from fresh water (FW) to seawater (SW) as juveniles. This normally occurs after undergoing preparatory changes in behaviour, morphology and physiology that are adaptive for downstream migration and seawater entry (McCormick, 2013). This parr–smolt transformation occurs in spring and is mediated primarily through photoperiod and temperature cues. One of the hallmarks of the parr–smolt transformation is a large increase in the capacity for ion regulation in seawater that is adaptive for rapid movements through estuaries and into the open ocean.

There has been substantial research on the ion-transport mechanisms and control of the ability of salmonids to move from fresh water to seawater. The gill plays a principal role in the maintenance of ion homeostasis in both FW and SW-acclimated fish (Evans *et al*., 2005). In order to maintain the internal ionic balance, the gills have specialized cells called ionocytes (also known as mitochondrion-rich or chloride cells) that are involved in chloride and sodium secretion in SW and uptake in FW (Hiroi & McCormick, 2012; Marshall & Grosell, 2006). There are three major ion-transport proteins involved in sodium and chloride secretion by the SW gill. Na^+–K^+-ATPase (NKA) pumps three sodium ions out of the cell while pumping in two potassium ions, making the inside of the chloride cell both low in sodium and negatively charged (Marshall & Grosell, 2006). The sodium gradient is then used by the Na^+–K^+-$2Cl^-$ co-transporter (NKCC) to bring chloride into the cell (Cutler & Cramb, 2002). Chloride subsequently leaves the cells on a favourable electrical gradient through an apical

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chloride channel, which has been shown to be a homologue of the cystic fibrosis transmembrane conductance regulator (CFTR) (Marshall, 2002).

NKA consists of three major subunits; the α-subunit is the major catalytic component, the β-subunit is important for structural maturation of the complex and the γ-subunit (also known as FXYD) performs a regulatory role. There are two major isoforms of the Nkaα-subunit in the salmon gill, the Nkaα1a and Nkaα1b, that are most abundant in FW and SW, respectively (McCormick *et al*., 2009; Nilsen *et al*., 2007; Richards *et al*., 2003). During smolting, gill NKA activity, the abundance of Nkaα1b and NKCC and the number of seawater type ionocytes increases, in tight association with increased salinity tolerance (McCormick *et al*., 2013; Pelis *et al*., 2001) and are thus useful metrics for smolt development and adaptive responses to SW.

Temperature has been described as the master abiotic factor for fish (Brett, 1971) that controls and limits virtually all physiological and behavioural parameters of ectotherms (Fry, 1947). In this sense, teleosts must react to significant changes in ambient temperature if they are going to survive. These reactions may be ethological (flight to tolerable thermal environments) or may involve physiological responses to the new thermal environment. Three main phases of physiological response can be classified: immediate and direct responses to changes in temperature; metabolic and behavioural compensation processes (acclimation); adaptive changes at the genetic level (Hazel & Prosser, 1974; Shaklee *et al*., 1977, Somero, 2004), the latter occurring over generations.

Increased temperature above a species-specific threshold can produce a stress response at the cellular level that is in part mediated by the actions of a family of proteins known as heat shock proteins (HSP). These proteins are highly conserved and have been measured in almost all organisms studied (Iwama *et al*., 1998). Studies in fish have demonstrated that in addition to temperature, several other stressors, including salinity, can induce HSP expression (Iwama *et al*., 2004). HSPs can act with other factors to repair damaged proteins and metabolize those that cannot be repaired, thus helping maintain cellular function following stressful events. The HSP response can vary as a function of species, development stage, tissue examined, stressor and concentration and duration of exposure (Fowler *et al*., 2009; Hori *et al*., 2010; LeBlanc *et al*., 2011, 2012; Niforou *et al*., 2014).

Although there are a large number of studies that have examined acclimation of fish to temperature or salinity (Bjornsson *et al*., 2011; Donaldson *et al*., 2008; McCormick & Bradshaw, 2006; Somero, 2004), only a few have examined simultaneous change of both salinity and temperature (Handeland *et al*., 1998, 2000). In nature, however, many anadromous and estuarine species can be faced with simultaneous change in both these factors as fish move rapidly between rivers and oceans that can differ substantially in temperature. Even greater changes in both salinity and temperature can occur in applied aquaculture settings when direct transfer from fresh water to seawater often occurs. The present study was undertaken to determine the interaction of temperature and salinity on salinity tolerance, gill osmoregulatory proteins and cellular stress pathways in the gill of *S. salar* smolts. Changes in gill NKA activity, Nkaα1a, Nkaα1b, NKCC and Hsp70 were used to monitor osmoregulatory responses and plasma glucose, Cl^- and cortisol were measured in order to examine the underlying endocrine signalling pathways.

2 | MATERIALS AND METHODS

2.1 | Animals and experimental protocols

Salmo salar juveniles were obtained from the Kensington State Hatchery (CT, USA) and brought to the Conte Anadromous Fish Research Center (Turners Falls, MA, USA) in autumn. Fish were reared in 1,000 l tanks supplied with ambient Connecticut River water at a flow rate of 4 l min^{-1} and provided with supplemental aeration from autumn to spring. Temperature in the winter reached a low of 1.8°C on 3 January and increased gradually in spring to 13.4°C on 2 April. The fish were maintained under natural photoperiod conditions throughout early rearing and the study utilizing both natural light and supplemental artificial light controlled by timer set for sunrise and sunset of the local latitude (42° 36′ N). Fish were fed a commercial salmon diet (Zeigler Bros; www.zeiglerfeed.com) and fed to satiation using automatic feeders.

On April 2, 2014, fish (n = 240; mean ± S.E. mass, M = 42 ± 7 g) were placed in two 1,000 l tanks at 14°C with particle, charcoal and biological filtration, ultraviolet light treatment and continuous aeration. This was just prior to peak smolt development and all fish were large enough to have undergone the parr–smolt transformation and had silvering and darkened fin margins characteristic of *S. salar* smolts. The fish were acclimated to either fresh water (no salinity change) or seawater (salinity 30; increased by 5 every second day) over a 2 week period. Fifty percent water changes occurred at least once per week. During the acclimation experiments, fish in FW and SW were fed ad libitum once daily.

For experimental conditions, 150 l tanks were prepared at 14, 17, 20 and 24°C in FW and SW (salinity 30). Fish were directly transferred from FW (14°C) or SW (14°C) to each of the temperature treatments. Thus, there were three major groups: FW–FW, FW–SW and SW–SW, at each of four temperatures (n = 20 fish per salinity and temperature condition). Each tank was held at its specific target temperature for the remainder of the study. Water temperature was maintained by use of stainless steel heat exchangers in the fish tanks that were connected with flexible tubing to a 100 l tank containing a bayonet heater. A temperature sensor controlling the heater was placed in the tank containing fish. The water temperature of each tank was measured and recorded every 15 min using Hobo pendent temperature loggers (Onset Computer Corporation; www.onsetcomp.com). Each tank had an independent recirculating pump system with particle, charcoal and biological filtration and UV sterilization, along with continuous supplemental aeration. Fish were fed ad libitum once daily. Dissolved oxygen levels were measured daily and were always above 90% saturation and ammonia was <1 mg l^{-1}, (KIT: ammonia NH3/NH4 liquid test API).

Fish were sampled after 2 and 8 days post transfer (dpt). Food was withheld for 24 hr prior to transfer and sampling of fish, which occurred between 1000 and 1200 hours Eastern Standard Time. Fish were anesthetized with MS-222 and blood was drawn from the caudal

vessels into a 1 ml ammonium heparinized syringe, spun at 3,200 *g* for 5 min at 4°C and plasma was aliquoted and stored at −80°C. Gill arches were removed, the gill filaments trimmed from the ceratobranchials and placed in a 1.5 ml microcentrifuge tube and frozen immediately at −80°C for Western blots. Four to six gill filaments were placed in 100 μl of ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and frozen at −80°C for measurement of NKA activity.

2.2 | Plasma variables

Plasma glucose levels were measured by enzymatic coupling with hexokinase and glucose 6-phosphate dehydrogenase (Stein, 1965) adapted to a 96-well microplate format (Glucose Assay Reagent, G3293, Sigma-Aldrich; www.sigma-aldrich.com). Plasma Cl^- was measured by silver titration using a digital chloridometer (Labconco; www.labconco.com). Plasma cortisol was measured by direct enzyme immunoassay (Carey & McCormick, 1998).

2.3 | Gill and kidney NKA activity

Gill and kidney NKA activities were determined using the micro-assay method of McCormick (1993). In this assay, ouabain-sensitive ATPase activity was measured by coupling the production of ADP to nicotinamide adenine dinucleotide (NADH). Samples (10 μl) were run in duplicate in 96-well microplates at 25°C and read at a wavelength of 340 nm for 10 min on a BioTek Synergy 2 spectrophotometer (BioTek; www.biotek.com). Protein concentration of the homogenate was determined using a BCA protein assay (Pierce #23225, Thermo Fisher Scientific; www.thermofishe.com).

2.4 | Protein extraction and Western blot analysis

The gill protein ion transporter (Nkaα1a, Nkaα1b, Nkcc) and heat shock protein 70 (Hsp70) abundance were quantified by Western immunoblotting as outlined in McCormick *et al*. (2009) for Nkaα1a and Nkaα1b, Pelis and McCormick (2001) for NKCC and Chadwick *et al*. (2015) for Hsp70.

After protein extraction, all samples were thawed and run on a 7% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) gel. All gels included 10 μg Precision Plus protein standards in a single reference lane (Bio-Rad Laboratories; www.bio-rad.com). Following electrophoresis, proteins were transferred to Immobilon polyvinylidene difluoride (PVDF) transfer membranes (Millipore; www.emdmilliore.com) at 30 V overnight in 25 mM Tris, 192 mM glycine buffer at pH 8.3. PVDF membranes were blocked in phosphate-buffered saline with 0.05% Triton X-100 (PBST) and 5% non-fat dry milk for 1 h at room temperature, rinsed in PBST and exposed to primary antibody in PBST and 5% non-fat dry milk for 1 h at room temperature. After rinsing in PBST, blots were exposed to goat anti-rabbit, anti-mouse or anti chicken immunoglobulin (IgG) conjugated to horseradish peroxidase diluted 1:10,000 in antibody dilution buffer, for 1 h at room temperature. After rinsing in PBST, blots were incubated for 1 min in a 1:1 mixture of enhanced chemiluminescent solution A (ECL A; 396 μM coumaric acid, 2.5 mM luminol, 100 mM Tris-Cl pH 8.5) and ECL B (0.018% H_2O_2, 100 mM Tris-Cl pH 8.5), then exposed to X-ray film (RPI; www.rpicorp.com). Digital photographs were taken of individual gels and band-staining intensity measured using Image J (NIH; www.nih.gov); protein abundance has internal standard reference lanes for each protein for each gel to normalize across exposures and expressed as a cumulative 8 bit grey-scale value.

2.5 | Statistics

Individual *S. salar* smolts were our unit of replication and there were at least 20 fish in each salinity: temperature combination and 10 at each time point. Owing to limits on the number of temperature control systems, we were unable to test for uncontrolled tank effects, which are unlikely due to the short-term nature of our tests and the large physical changes that were induced in each tank. A mixed general linear model (MGLM) is the most appropriate test when tank replication is not feasible (Soane, 2014). Data were checked for normality, independence and homogeneity of variance before MGLM was conducted using salinity–time–temperature as factors of variance and fish as random effect, followed by a Tukey test to compare individual groups. Logarithmic transformations of the data were performed when necessary to fulfil the conditions of the parametric analysis of variance. The difference of experiment treatment was considered to be significantly different at a level of $p < 0.05$. All data are presented as mean ± S.E.

3 | RESULTS

3.1 | Mortality

Nineteen of 20 fish in the FW–SW group (seawater without acclimation) died within the first 48 h of transfer to 24°C. There were no mortalities in any of the other groups.

3.2 | Plasma ions and metabolites

Plasma chloride levels in the FW–FW group increased slightly (but not significantly) with increased temperature at 2 and 8 dpt (Figure 1(a), (b)). At 2 dpt plasma chloride levels were highest in the FW–SW group, although only at 14 and 24°C were there significant differences from the FW–FW group (Figure 1(a)). At 8 dpt plasma chloride levels of the 24°C SW–SW were substantially and significantly higher than FW–FW (Figure 1(b)). At 14, 17 and 20°C plasma chloride was similar in the FW–SW and SW–SW groups and only slightly higher than in the FW–FW group.

Plasma glucose levels showed two patterns depending on time and salinity. At 2 dpt plasma glucose was elevated in the SW–SW group at low temperature, but not at more elevated temperatures (Figure 1(c)). At 8 dpt there was a general increase in plasma glucose levels that occurred across all salinity groups compared to 2 dpt (Figure 1(d)).

Haematocrit levels were lowest in the SW–SW group in 14 and 20°C at 2 dpt (Table 1), but at 8 dpt there were no significant treatment effects (Tables 1 and 2).

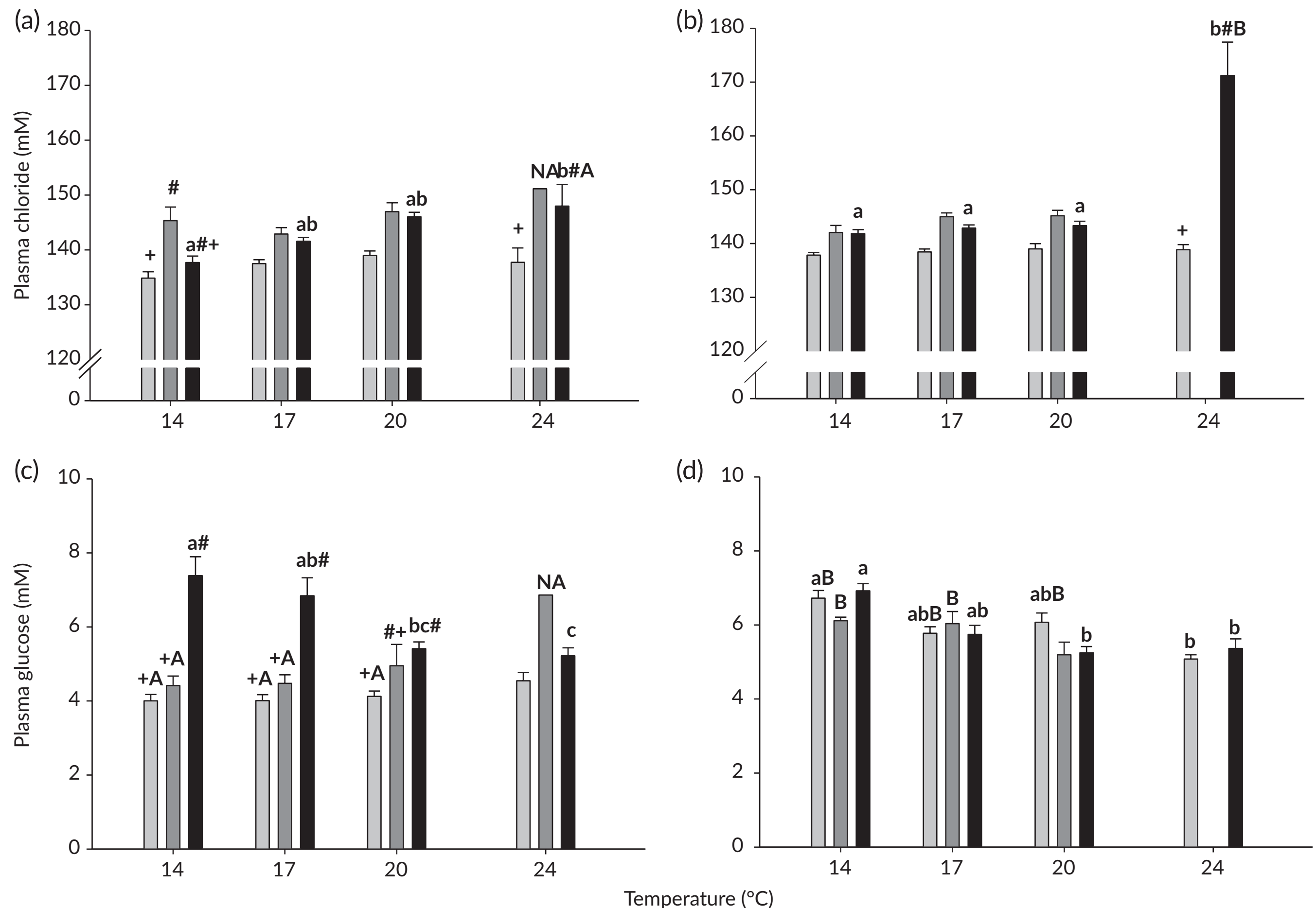

FIGURE 1 Plasma (a), (b) chloride and (c), (d) glucose levels (mean + S.E., n = 10) in *Salmo salar* smolts at (a), (c) 2 and (b), (d) 8 days post transfer (dpt) in fresh water (FW) to FW (FW–FW), FW to seawater (SW; FW–SW) and SW to SW (SW–SW) groups at different temperatures. Different lower case letters indicate significant differences between different temperatures with the same water conditions. Different capital letters indicate significant differences between different sampling times (2 *v.* 8 dpt) with the same temperature and water conditions. Different symbols (+, #, &) indicate significant differences between different water condition at same temperature (mixed general lineal model, Tukey–test, $p < .05$). NA: Not analysed for this treatment as only 1 fish survived (▭) FW–FW, (▭) FW–SW, and (▬) SW–SW

3.3 | Enzyme activity and protein abundance

At 2 dpt gill NKA activity in all three salinity treatments showed no apparent effect of temperature, while there was a clear effect of salinity, with the FW–FW having the lowest overall activity, FW–SW at intermediate levels and SW–SW at the highest levels (Figure 2(a)). By 8 dpt gill NKA activity in the FW–SW and SW–SW group were similar and both higher than the FW–FW group. There was a continuous decrease in gill NKA activity with increasing temperature in the FW–FW group and significantly lower values at 24°C than at other temperatures in the SW–SW group (Figure 2(b)). Gill Nkaα1a levels were highest in the FW–FW group, with strongly decreasing levels with time after SW exposure (Figure 2(c),(d)). The drop in gill Nkaα1a occurred more rapidly with increasing temperature in the FW–SW group. There was a general trend for decreasing Nkaα1a levels in FW–FW with increasing temperature at both 2 and 8 dpt, although the differences were not statistically significant ($p > 0.05$).

Gill Nkaα1b levels were intermediate in the FW–FW group and lowest in the FW–SW group at 2 dpt, but at 8 dpt the FW–FW group was lowest (Figure 3(a),(b)). Gill Nkaα1b levels were highest in the SW–SW fish at 2 dpt, although FW–SW group had increased to levels similar to the SW–SW group at 8 dpt. There was a general trend for lower gill Nkaα1b levels with increasing temperature. Gill NKCC levels were lowest in the FW–FW and decreased with temperature at 2 dpt, while the fish in seawater without acclimation (FW–SW) present highest levels at 12°C at 2 dpt, the highest levels were in the seawater-acclimated group (SW–SW) at 2 and 8 dpt, but at 8 dpt there was a four-fold increase in protein abundance in the FW–SW group, with the exception of the 24°C treatment (Figure 3(c),(d)).

Gill Hsp70 levels were low in the FW–FW and SW–SW groups at 14, 17 and 20°C, but increased 20-fold in these groups at 24°C. In the FW–SW group at 2 dpt there was elevated gill Hsp70 abundance with increasing temperature and higher levels compared to FW–FW and SW–SW groups at all temperatures (Figure 4(a),(b)).

3.4 | Plasma cortisol

Plasma cortisol concentration was highest in the one surviving fish in the FW–SW at 24°C at 2 dpt (Table 1), while at 8 dpt FW–FW and SW–SW groups at 17 and 24°C had the highest values (Table 2).

TABLE 1 Haematocrit and plasma cortisol levels (mean ± s.e., n = 10) in *Salmo salar* smolts 2 days post transfer (dpt) in fresh water (FW) to FW (FW–FW), FW to seawater (SW; FW–SW) and SW to SW (SW–SW) groups at different temperatures

Salinity	Temperature (°C)	Haematocrit (%)	Plasma cortisol (ng ml^{-1})
FW–FW	14	34.0 ± 1.4#+	19.7 ± 5.3
FW–SW	14	30.0 ± 0.9+	25.0 ± 5.2
SW–SW	14	37.0 ± 1.1#	64.6 ± 24.8
FW–FW	17	35.0 ± 0.8	9.4 ± 2.1
FW–SW	17	33.0 ± 1.2	36.8 ± 11.1A
SW–SW	17	33.0 ± 1.5	29.6 ± 6.3
FW–FW	20	36.0 ± 1.2#	18.2 ± 5.9
FW–SW	20	28.0 ± 1.6+	8.9 ± 2.6
SW–SW	20	34.0 ± 1.8#+	27.7 ± 10.7
FW–FW	24	38.0 ± 0.8	8.3 ± 3.8
FW–SW[a]	24	32.0	101.5
SW–SW	24	34.0 ± 1.3	17.7 ± 9.6

Note. Different lower case letters indicate significant differences between different temperatures with the same water conditions. Different capital letters indicate significant differences between different sampling times (2 *v.* 8 dpt), but with the same temperature and water conditions. Different symbols (+, #, &) indicate significant differences between different water condition at same temperature (mixed general lineal model, Tukey-Test, $p < 0.05$).
[a] Only 1 fish survived, consequently, this datum was not considered in statistical analyses.

4 | DISCUSSION

Rapid changes in temperature are a possible scenario in wild salmonids as they experience diel cycles in streams (Chadwick *et al*., 2015), move from one tributary to another or into cool-water refuges in streams (Breau *et al*., 2011) and migrate between FW and SW (McCormick *et al*., 1998). Juvenile *S. salar* prefer water temperatures between 16 and 18°C (Javaid & Anderson, 1967) and 28°C is lethal under controlled laboratory conditions (Garside, 1973), yet in the wild, *S. salar* rivers reach water temperatures in excess of 30°C (Breau *et al*., 2007; Cunjak *et al*., 2005). Hatchery-reared fish may also experience rapid temperature increase following release from the hatchery or when moved directly into seawater net pens. In the present study, immediate increases in temperature of 3, 6 and 10°C, in the presence and absence of salinity change, were used to assess the interaction of temperature and salinity on survival, cellular and physiological responses. Abrupt increases of 10°C are admittedly severe and unlikely to be encountered under most natural conditions, but we included them to establish a potential upper limit of abrupt temperature increase. Perhaps surprisingly, *S. salar* smolts in fresh water were able to survive an increase from 10 to 24°C with no apparent ionic perturbations and only moderate increases in Hsp70. Our results indicate that high temperature strongly interacts with salinity change in *S. salar* smolts. After a 14 to 24°C change in temperature, there was 100% mortality in fish that had not been previously acclimated to SW and large ionic perturbations were observed in fish that had previously been acclimated to SW. In addition, the simultaneous exposure to increased temperature and salinity in the FW–SW group resulted in elevated gill Hsp70 levels, further indicating an interaction between these two stressors.

The reduced capacity for ion regulation in seawater at 24°C may be related to the capacity to elevate or maintain high levels of ion

TABLE 2 Haematocrit and plasma hormones cortisol levels (mean ± s.e., n = 10) in *Salmo salar* smolts at 8 days post transfer (dpt) in fresh water (FW) to FW (FW–FW), FW to seawater (SW; FW–SW) and SW to SW (SW–SW) groups at different temperatures

Salinity	Temperature (°C)	Hematocrit (%)	Plasma cortisol (ng ml^{-1})
FW–FW	14	34 ± 1.8	14.2 ± 2.9ab
FW–SW	14	31 ± 1.1	19.1 ± 6.7
SW–SW	14	34 ± 1.0	12.1 ± 3.1
FW–FW	17	38 ± 11	57.0 ± 13.5b
FW–SW	17	33 ± 1.7	10.0 ± 7.1B
SW–SW	17	34 ± 1.0	12.6 ± 2.3
FW–FW	20	35 ± 1.1	15.9 ± 1.5ab
FW–SW	20	34 ± 1.2	8.4 ± 2.9
SW–SW	20	34 ± 1.0	15.7 ± 2.7
FW–FW	24	36 ± 1.0	5.9 ± 1.5a
FW–SW[a]	24		
SW–SW	24	34 ± 1.4	76.8 ± 29.2

Note. Different lower case letters indicate significant differences between different temperatures with the same water conditions. Different capital letters indicate significant differences between different sampling times (2 *v.* 8 dpt), but with the same temperature and water conditions. Mixed general lineal model, Tukey-Test, $p < 0.05$.
[a] Only 1 fish survived, consequently, this datum was not considered in statistical analyses.

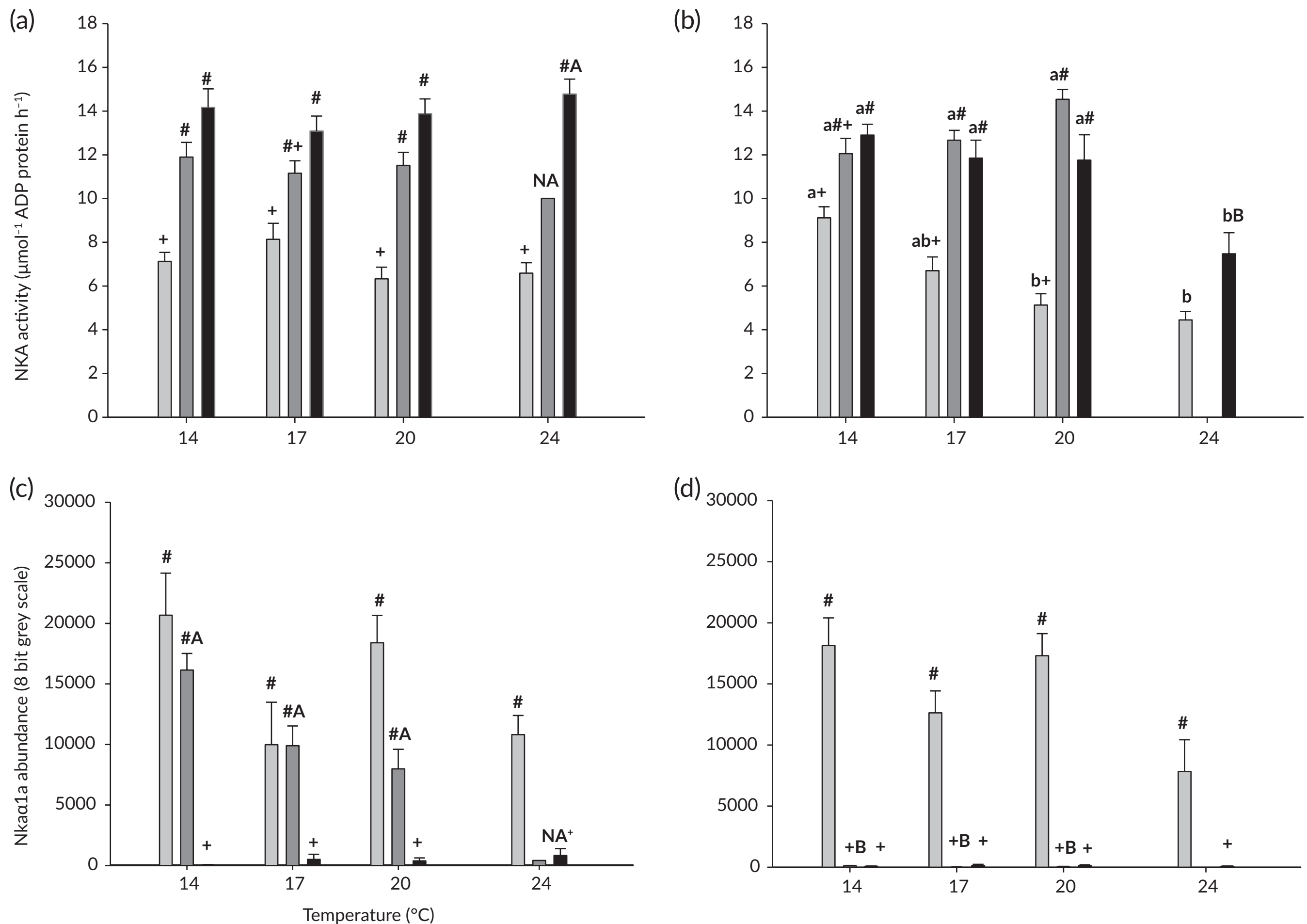

FIGURE 2 Gill (a), (b) Na^+–K^+-ATPase (NKA) activity and (c), (d) abundance of Nkaα1a (mean + s.e., *ssn* = 10) in *Salmo salar* smolts at (a), (c) 2 and (b), (d) 8 days post transfer (dpt)) in fresh water (FW) to FW (FW–FW), FW to seawater (SW; FW–SW) and SW to SW (SW–SW) groups at different temperatures. Different symbols (+, #, &) indicate significant differences between different water condition at same temperature (mixed general lineal model, Tukey–test, $p < 0.05$). NA: Not analysed for this treatment as only 1 fish survived (▭) FW–FW, (▭) FW–SW, and (■) SW–SW

transporters at high temperatures. The levels of gill Nkaα1b after 2 days in SW (FW–SW group) were reduced compared to the FW–FW group, indicating increased turnover due to initial SW exposure. These levels were lowest in the 24°C group, albeit there was only one fish remaining at this temperature. In addition, the levels of gill Nkaα1b in the SW–SW group were reduced at 24°C at 8 dpt, corresponding to high levels of plasma chloride observed in this group. Thus, there is a strong correspondence between failure to maintain high levels of gill Nkaα1b and osmoregulatory failure at high temperature. While previous research has shown that gill Nkaα1b is positively correlated with salinity tolerance during smolt development, to the best of our knowledge this is the first study to demonstrate an effect of temperature on the salinity response of this ion-transport enzyme isoform that appears critical for salt secretion. The effect of temperature on gill NKA activity after SW exposure was reported by Handeland *et al.* (1998, 2000); after 30 days in SW, gill NKA activity was higher at 18°C compared with 4, 8 and 14°C. Our result indicated that NKA activity was highest in at 8 dpt in SW at 20°C, but significantly lower at 24°C. Thus, there is a strong correspondence between failure to maintain high levels of gill Nkaα1b and gill NKA activity and osmoregulatory failure observed at high temperature in SW.

The measurement of the freshwater specific NKA isoform (Nkaα1a) can also provide insight into the interaction of temperature and salinity. In the FW–SW group the abundance of gill Nkaα1a was relatively high at 2 dpt at 14°C, but decreased with increasing temperature. By day 8 gill Nkaα1a was largely absent in all SW groups. These results suggest that there is a temperature-dependent decrease in the abundance of Nkaα1a which may be related to the loss of FW ionocytes that has previously been observed after SW exposure of *S. salar* smolts (McCormick *et al.*, 2013). We hypothesize that the increased temperature promotes the loss (necrosis or apoptosis) of FW ionocytes or their transformation to seawater-type ionocytes. We also observed a general decline of Nkaα1a with increasing temperature in FW. Since there were no detectable perturbations in plasma chloride in this group, such a decline may be due to the loss of smolt characteristics that occurs at the end of smolt development that occurs more rapidly at elevated temperature (McCormick *et al.*, 2009). The differential regulation of Nkaα1a and Nkaα1b by salinity is consistent with previous reports at both the protein (McCormick *et al.*, 2009) and messenger (m)RNA levels (Nilsen *et al.*, 2007).

The abundance of gill Nkcc presented the highest levels in both fish groups in seawater, consistent with previous work in *S. salar*

FIGURE 3 Abundance of (a), (b) Nkaα1b and (c), (d) Nkcc (mean + s.e., n = 10) in *Salmo salar* smolts at (a), (c) 2 and (b), (d) 8 days post transfer (dpt) and in FW to FW (FW–FW), FW to SW (FW–SW) and SW to SW (SW–SW) groups at different temperatures. Different symbols (+, #, &) indicate significant differences between different water condition at same temperature (mixed general lineal model, Tukey–test, p < 0.05). NA: Not analysed for this treatment as only 1 fish survived (▭) FW–FW, (▭) FW–SW, and (▬) SW–SW

(Hiroi & McCormick, 2007; McCormick *et al*., 2009; Nilsen *et al*., 2007; Pelis *et al*., 2001). Although the lowest levels were those seen in FW, it should be noted that these are strongly elevated compared with *S. salar* parr (Pelis *et al*., 2001; A. M. Regish & S. D. McCormick, unpubl. data). Seawater exposure resulted in significant elevations in gill NKCC, as has been noted previously (Pelis *et al*., 2001). Even

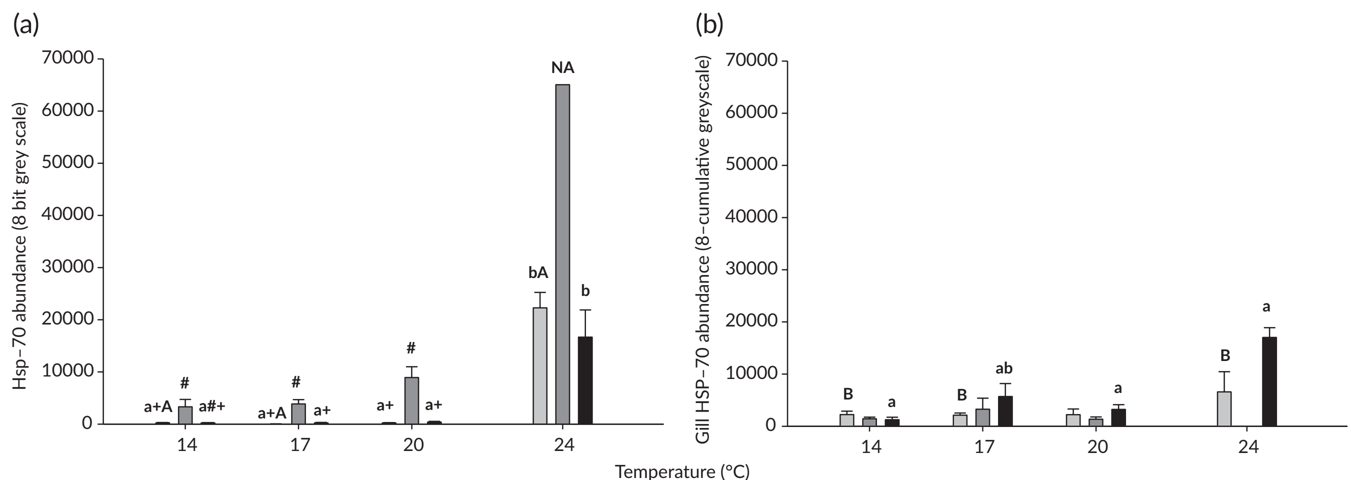

FIGURE 4 Abundance (mean + s.e., n = 10) in *Salmo salar* smolts of Hsp70 at (a) 2 and (b) 8 days post transfer (dpt) in fresh water (FW) to FW (FW–FW), FW to seawater (SW; FW–SW) and SW to SW (SW–SW) groups at different temperatures. Values are mean ± SEM. (n = 10 for each experimental group). Different symbols (+, #, &) indicate significant differences between different water condition at same temperature (mixed general lineal model, Tukey–test, p < 0.05) (▭) FW–FW, (▭) FW–SW, and (▬) SW–SW

8 days after seawater exposure the FW–SW group had not reached the levels seen in the SW-acclimated group, indicating that it takes weeks to reach the new set point for SW levels of gill NKCC. It should be noted, however, that gill Nkaα1b were similar in the FW–SW and SW–SW groups by 8 dpt, indicating a more rapid response of this ion-transport protein compared with Nkcc. In SW, we observed no effect of temperature on gill Nkcc between 14 and 20°C, but as with gill Nkaα1b levels were lower at 24°C. The fact that abundance of both of these proteins are decreased at high temperature suggests that the number of gill ionocytes or the density of ion transporters within ionocytes are reduced at elevated temperature in SW.

HSPs are upregulated in the presence of denatured proteins, which can result from a variety of environmental stressors, including elevated temperature (Deane & Woo, 2011; Tomanek, 2010). Many experimental studies have identified elevated HSP expression in response to temperature increases or decreases in salmonid species (Chadwick *et al*., 2015; DuBeau *et al*., 1998; Fowler *et al*., 2009; Smith *et al*., 1999) and silver sea bream *Chrysophrys auratus* (Forster 1801) (Deane & Woo, 2005). Salinity has also been shown to increase HSPs in some species including *S. salar* (Smith *et al*., 1999) and Mozambique tilapia *Oreochromis mossambicus* (Peters 1852) (Tang & Lee, 2013), though usually to a lesser magnitude than temperature. Dietz and Somero (1993) established that there are threshold temperatures for HSP concentrations in teleosts and perhaps for other indicators of cellular stress such as adenosine monophosphate-activated protein (AMP) activated kinase activity (Anttila *et al*., 2013). In the present study, there were minimal differences in Hsp70 between 14 and 20°C, but significant increases in all salinity groups at 24°C. This is consistent with sharp temperature thresholds for Hsp70 up-regulation in salmonids including *S. salar* between 22 and 25°C (Lund *et al*., 2002) and brook trout *Salvelinus fontinalis* (Mitchell 1814) up to 22°C (Chadwick *et al*., 2015). There was also a clear interaction with salinity, as the combination of simultaneous salinity and temperature exposure in the FW–SW group at 2 dpt resulted in elevated Hsp70 even at low temperature. In addition, Hsp70 was higher in the SW–SW group at 8 dpt at 24°C. If this elevated Hsp70 is indeed due to damaged proteins, it would help to explain the lower levels of gill Nkaα1b and Nkcc observed in this group.

Our results do not show a clear relationship of plasma cortisol with either temperature or salinity. The inherently variable levels in plasma cortisol, particularly during smolt development when basal levels are elevated (McCormick, 2013), may have prevented us from detecting statistically significant responses. Previous work has shown that basal plasma cortisol levels increase with increasing temperature in some teleost species (Arends *et al*., 1998; Costas *et al*., 2012), but that this is not a universal finding. Arjona *et al*. (2010) found no relationship between plasma cortisol levels and increasing temperature in Senegalese sole *Solea senegalensis* Kaup 1858. In trout *S. fontinalis*, exposure to high temperature for several hours, sufficient to induce Hsp70, did not result in elevated plasma cortisol (Chadwick *et al*., 2015), but the same temperatures did result in elevated plasma cortisol after 8 and 24 days of exposure (Chadwick & McCormick, 2017). It should be noted that the highest values of plasma cortisol observed in the present study were in the one surviving fish in the FW–SW group at 24°C after 2 days in SW and in the SW–SW group at 24°C at 8 dpt. These are the two groups that showed the greatest disturbance in plasma chloride and may indicate that elevated temperature and salinity that results in osmotic disturbance also induces an endocrine stress response.

In conclusion, we demonstrate that there is an important interaction between temperature and salinity on survival and physiological responses of *S. salar* smolts. Freshwater smolts were able to tolerate a rapid exposure to an increase in temperature from 14 to 24°C, whereas those exposed to simultaneous exposure to 24°C and a salinity of 30 had high mortality and even smolts that had previously been acclimated to SW experienced ionic perturbations at 24°C. Our studies were limited to only 8 days and it would be of value to examine longer-term effects of elevated seawater temperatures on survival, growth and physiological responses to seawater. Our results also indicate that Hsp70 can be a useful indicator of the effect of combined stressors on survival and physiological responses of fish and should be explored further for its potential to detect stressors in wild *S. salar*.

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