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RESEARCH ARTICLE

Reduced thermal tolerance during salinity acclimation in brook trout (*Salvelinus fontinalis*) can be rescued by prior treatment with cortisol

Ciaran A. Shaughnessy[1,2,*] and Stephen D. McCormick[1,2]

ABSTRACT

The aims of this study were to assess whether thermal tolerance of brook trout (*Salvelinus fontinalis*) is affected during seawater (SW) acclimation and to investigate the role of cortisol in osmoregulation and thermal tolerance during SW acclimation. Freshwater (FW)-acclimated brook trout at 18°C (T_{acc}) were exposed to SW for 16 days, whilst maintaining a FW control. Fish were examined for critical thermal maximum (CT_{max}) 0 (before), 2, 5 and 16 days after SW exposure, and sampled at T_{acc} and CT_{max} for analysis of plasma cortisol, glucose and Cl^-, gill Na^+/K^+-ATPase (NKA) activity and heat shock protein 70 (HSP70) abundance, and white muscle water content. At 2 days in SW, CT_{max} was significantly reduced (from 31 to 26°C), and then recovered by 16 days. This transient decrease in thermal tolerance coincided with a transient increase in plasma Cl^- and decrease in muscle moisture content. Salinity itself had no effect on gill HSP70 abundance compared with the large and immediate effects of high temperature exposure during CT_{max} testing. To examine the role of cortisol in osmoregulation, brook trout were administered a cortisol implant (5 and 25 μg g^{-1} CORT) prior to SW exposure. Both CORT doses significantly increased their capacity to maintain plasma Cl^- during SW acclimation. Treatment with the 25 μg g^{-1} CORT dose was shown to significantly improve CT_{max} after 2 days in SW, and CT_{max} was associated with plasma Cl^- and muscle moisture content. These findings indicate that brook trout are sensitive to temperature during SW acclimation and that thermal tolerance is associated with ion and water balance during SW acclimation.

KEY WORDS: Osmoregulation, Temperature, Critical thermal maximum

INTRODUCTION

Migratory and estuarine fishes encounter a wide range of environmental conditions throughout their life, including changes in temperature, salinity and dissolved gases, and it is important to understand how changes in environmental conditions impact the physiology of these fishes. Although the physiological processes of thermal tolerance and salinity acclimation in fishes are relatively well understood, whether and how thermal tolerance is affected by salinity acclimation remains unclear.

In fishes and other ectotherms, temperature is known to have rapid effects on metabolism and behavior (Clarke and Johnston, 1999; Gillooly et al., 2001), and long-term effects on growth, fecundity and survival (Berrigan and Charnov, 1994; Jonsson and Jonsson, 2009). A widely used metric by ecologists and physiologists for quantifying acute thermal tolerance in a repeatable manner is critical thermal maximum (CT_{max}) (Becker and Genoway, 1979; Beitinger et al., 2000; Gunderson and Stillman, 2015; Lutterschmidt and Hutchison, 1997), which is determined experimentally by increasing the temperature of water in a test chamber at a constant rate – the temperature at which the subject loses equilibrium (T_{LOE}) is considered its CT_{max}. A widely used metric for evaluating the cellular response to thermal stress is the protein abundance of heat shock proteins (HSPs), a large family of highly conserved cellular proteins that are involved in various cellular processes including protein assembly, folding, translocation and disassembly, and the regulation and expression of which have been studied extensively in fishes (Deane and Woo, 2011). HSPs have a well-documented role in fishes in response in tolerating thermal stress (Basu et al., 2002; Iwama et al., 1999).

Studies assessing thermal tolerance in fishes and other aquatic ectotherms have been conducted using freshwater (FW)-acclimated (Barrionuevo and Fernandes, 1995; Currie et al., 1998; Elliott and Elliott, 1995; Zaragoza et al., 2008; Zhang and Kieffer, 2014) or SW-acclimated animals (Fangue et al., 2006; Healy and Schulte, 2012a,b; Madeira et al., 2013; Murchie et al., 2011; Tsuchida, 1995), or using comparisons between FW- and SW-acclimated animals (Metzger et al., 2016). However, whether salinity affects thermal tolerance remains inconclusive, varying between species and studies, with some studies reporting an effect of salinity on thermal tolerance (Chen and Chen, 1999; Cheng et al., 2013; Everatt et al., 2013; Jian et al., 2003; Metzger et al., 2016; Sardella et al., 2008a,b), and others reporting no effect at all (Re et al., 2006, 2012; Haney and Walsh, 2003). Even among studies reporting an effect of salinity on thermal tolerance, there appears to be no consistency in how thermal tolerance is associated with salinity.

Anadromous fishes make a deliberate FW-to-SW migration as juveniles then return to FW as mature adults. In FW, fishes passively gain water and lose ions to the dilute environment. To counter this, they must actively uptake ions (primarily Na^+ and Cl^-) across the gill epithelium by utilizing a suite of ion transporters, a process facilitated by the electrogenic gradients produced by the activity of the basolateral Na^+/K^+-ATPase (NKA) (Marshall and Grosell, 2006). Upon exposure to SW, fishes passively lose water and gain ions in the solute-concentrated environment. To counter this, the gill epithelium transforms to actively excrete ions. This is accomplished by the transcellular transport of Cl^- ions via a basolateral $Na^+/K^+/2Cl^-$-cotransporter (NKCC), and an apical Cl^- channel (CFTR) and the paracellular transport of Na^+ (via 'leaky' junctions in the gill

[1]Graduate Program in Organismic and Evolutionary Biology, University of Massachusetts, Amherst, MA 01003, USA. [2]US Geological Survey, Leetown Science Center, Conte Anadromous Fish Research Center, Turners Falls, MA 01376, USA.

*Author for correspondence (cshaughnessy@umass.edu)

C.A.S., 0000-0003-2146-9126

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epithelium) (Silva et al., 1977). The excretion of these ions in SW is also highly dependent on NKA activity. Thus, to survive a transition between FW and SW (which most freshwater fish species are incapable of), the gill of anadromous fishes must transform from a primary role of ion uptake to one of salt excretion (McCormick, 2013).

In teleost fishes, cortisol acts as both a glucocorticoid and a mineralocorticoid, involved in the regulation of many physiological functions including metabolism (Mommsen et al., 1999) and osmoregulation (McCormick, 2001). Many studies in fishes have shown that cortisol increases metabolic demand and stimulates gluconeogenesis and activity of gluconeogenic enzymes (see reviews by Faught and Vijayan, 2016; Mommsen et al., 1999). In addition to its more conserved role as a glucocorticoid in teleost fishes, cortisol, along with growth hormone and insulin-like growth factor 1, acts as a mineralocorticoid by improving osmoregulation in SW and promoting the differentiation of chloride cells in the gill (McCormick, 1996, 2001). Studies in Atlantic salmon (*Salmo salar*) have shown that cortisol treatment increases gill NKA activity and SW tolerance (measured as relatively lower plasma ion concentration after SW exposure) (Bisbal and Specker, 1991; McCormick et al., 2008; Specker et al., 1994; Veillette et al., 1995). The role of cortisol in promoting osmoregulation has never been demonstrated in brook trout.

It may be that thermal tolerance is limited during (although not after) SW acclimation, when osmoregulatory status (ion and water balance) is outside its normal homeostatic range. Until now, a time-course perspective of changes in thermal tolerance during SW acclimation has not been reported. Monitoring thermal tolerance throughout SW acclimation could provide important information on the thermal sensitivities of fishes with unique osmoregulatory life histories, such as anadromous and estuarine fishes (which experience daily fluctuations in salinity), and help determine potential impacts of future warming. The brook trout is a particularly useful and interesting species in which to study this question because of (1) its wide distribution across many different climates in the United States, including many small and shallow FW systems that can experience substantial warming during summer (Meisner, 1990), (2) its facultatively anadromous life history, including a southern distribution around Long Island Sound and Cape Cod, which can reach temperatures of 25°C (US Environmental Protection Agency, 1994) and has experienced substantial population declines in the last century (Limburg and Waldman, 2009), and (3) its amenability to laboratory studies. The aims of this study were to assess whether thermal tolerance of brook trout is affected during SW acclimation and to investigate the role of cortisol in osmoregulation and thermal tolerance during SW acclimation. It was hypothesized that thermal tolerance would be limited during SW acclimation, and that improving the osmoregulatory capacity of the fish using a cortisol treatment would help maintain thermal tolerance during SW exposure.

MATERIALS AND METHODS

Experimental procedure

All rearing and experiments were conducted at the US Geological Survey S. O. Conte Anadromous Fish Research Center in Turners Falls, MA, USA, and were carried out in accordance with USGS Animal Care Guidelines under IACUC 9070. Sandwich State Hatchery (Sandwich, MA, USA)-raised juvenile (2 years old) brook trout (16.0±0.1 cm, 44.7±0.9 g) were held under a natural photoperiod in 1.5 m diameter tanks supplied with Connecticut River water and fed to satiation daily. One month prior to experimentation, fish were randomly assigned to 1.5 m diameter experimental tanks containing recirculating (particle and chemical filtered), temperature-controlled (18.0±0.2°C; T_{acc}) dechlorinated municipal water under a natural photoperiod. Fifty-percent water changes were performed every third day. Food (BioTrout, Bio-Oregon, Westbrook, ME, USA) was administered using a feed-matched (between treatments) regimen throughout experimentation. Feeding was withheld for 48 h prior to any experimentation and sampling.

Experiment 1: CT_{max} during SW acclimation

Fish were exposed to either 25 ppt salinity (hereafter referred to as 'seawater' or 'SW') or kept in FW as a control for 16 days and monitored for changes in thermal tolerance using a CT_{max} test (described below). Target salinity in all experiments was achieved using artificial sea salt (Crystal Sea Salt, Baltimore, MD, USA) by first removing 50% of the acclimation water and replacing it with 50 ppt salt water over the course of 1 h. Before SW exposure (day 0) and 2, 5 and 16 days after SW exposure, eight fish from FW and SW were removed for sampling at T_{acc} and an additional eight fish from FW and SW were transferred to 0.6 m diameter flow-through tanks maintained at respective salinities for determination of CT_{max} followed by blood and tissue collection.

CT_{max} was determined by increasing the temperature of the water in each CT_{max} tank until a fish exhibited loss of equilibrium (LOE) (Becker and Genoway, 1979), at which point the fish was removed from the CT_{max} tank and immediately sampled. LOE was assessed using the most widely used LOE endpoint determination in the relevant literature, failure of righting response, which is indicative of the inability of a fish to escape conditions that would eventually lead to its death (Becker and Genoway, 1979; Beitinger et al., 2000; Lutterschmidt and Hutchison, 1997). As an objective measure in determining LOE, only a fish exhibiting LOE behavior for longer than 10 s without any effort to right itself was considered as having reached CT_{max}. Temperature in the CT_{max} tanks was increased at an initial rate of 4°C h^{-1} for the first hour (18 to 22°C), then at a test rate of 2°C h^{-1} thereafter until all fish had exhibited LOE. Temperature during the CT_{max} tests was controlled by allowing heated water to flow from header tanks through solenoid valves (Granzow, Inc., Charlotte, NC, USA) that were controlled by temperature controllers (Omega cn7500, Omega Engineering, Inc., Stamford, CT, USA) with a resistance thermometer input installed in each CT_{max} tank. The temperature controllers were optimized to the CT_{max} test conditions prior to experimentation. Each CT_{max} tank was provided with supplemental aeration for the duration of CT_{max} testing to ensure that oxygen levels remained at nearly saturating levels (90–100%), which is far above the levels of air saturation at which CT_{max} is reduced (Ern et al., 2016).

Experiment 2: Effect of cortisol treatment on osmoregulation

Fish were administered one of two doses of a cortisol implant (CORT) and analyzed for gill NKA activity and plasma Cl^- before and after SW exposure. Experimental water conditions were achieved as described for Experiment 1. Cortisol (Sigma-Aldrich, St Louis, MO, USA) was suspended in a 1:1 vegetable oil:shortening solution (as described in Specker et al., 1994) and warmed to ∼25°C prior to injection. Fish were anesthetized with MS-222 (50 mg l^{-1} buffered with $NaHCO_3$, pH 7.0; Argent Laboratories, Redmond, WA, USA), weighed and injected intraperitoneally with either a 5 or 25 µl g^{-1} body mass CORT dose or a vehicle control (VEH) using a 1 cc syringe and 25 gauge needle. Fish were sampled 6 (gill biopsy only), 12, 14 and 21 days after cortisol injection. After the sampling on day 12, all fish were transferred to SW.

Experiment 3: Effect of cortisol treatment on CT_{max}

Fish were administered a 25 µg g^{-1} CORT implant 12 days prior to SW exposure and tested for CT_{max} 2 days after SW exposure (14 days after CORT injection). Experimental water conditions, CORT treatment and CT_{max} testing was achieved as described for Experiments 1 and 2.

Sampling protocol

For terminal sampling, fish were euthanized using a lethal dose of MS-222 (100 mg l^{-1} buffered with $NaHCO_3$, pH 7.0), measured for length and body mass, and sampled for blood, gill and white muscle. Blood was collected from the caudal vessels using 1 ml ammonium heparinized syringes (25 gauge needle) and centrifuged (3000 ***g*** for 5 min at 4°C) for plasma extraction (aliquoted and stored at −80°C). Blood sampling was completed within 6 min of disturbing the tank, which is before any increase from basal cortisol levels can be detected (Wedemeyer, 1970). Additional gill tissue for immunoblotting was aliquoted and stored at −80°C. White muscle tissue (~2 g) was blotted dry, weighed (wet mass) and placed in a drying oven at 60°C until it reached a stable mass (dry mass) for determination of water content.

Non-lethal gill biopsies for enzyme activity analysis (taken on day 6 of Experiment 2 only) was achieved by anesthetizing fish with MS-222 (50 mg l^{-1} buffered with $NaHCO_3$, pH 7.0), and removing four to six filaments from above the septum on the first gill arch and storing them at −80°C in 100 µl of ice-cold SEI buffer (150 mmol l^{-1} sucrose, 10 mmol l^{-1} EDTA and 50 mmol l^{-1} imidazole, pH 7.3), following the protocol described by McCormick (1993).

Blood analyses

An aliquot of fresh blood was collected in a capillary tube for hematocrit (Hct) analysis, which was accomplished via centrifugation and is presented as percent packed red blood cell (RBC) volume. Plasma Cl^- was measured using a digital chloridometer (Haake Buchler Instruments Inc., Saddlebrook, NJ, USA). Plasma glucose was measured via enzymatic coupling of hexokinase and glucose-6-phosphate dehydrogenase (Stein, 1963). Plasma cortisol was measured using an enzyme immunoassay previously described (Carey and McCormick, 1998).

Gill Na^+/K^+-ATPase activity

Gill Na^+/K^+-ATPase (NKA) activity was analyzed using an NADH-linked kinetic assay in a 96-well microplate run at 25°C for 10 min, as described in McCormick (1993). Gill filaments were homogenized on ice in 150 µl of SEID (0.1% sodium deoxycholate in SEI buffer, pH 7.3) and centrifuged at 3200 ***g*** for 5 min at 4°C. The supernatant was assayed in duplicate for ATPase activity in the presence and absence of the NKA-specific inhibitor ouabain (0.5 mmol l^{-1}). NADH oxidation was determined spectrophotometrically at 340 nm, and the difference in absorbance between the inhibited and uninhibited assay mixtures was used to calculate NKA-specific activity and expressed as µmol ATP mg^{-1} protein h^{-1}. Protein concentration of homogenates was determined using the Pierce BCA Protein Assay (Thermo Scientific, Rockford, IL, USA). Both assays were run on a ThermoMax microplate reader using SoftMax software (Molecular Devices, San Jose, CA, USA).

Immunoblotting

Gill heat shock protein 70 (HSP70) protein abundance was determined using a western blot as previously described (Chadwick et al., 2015). Gill tissue homogenates were prepared and analyzed for protein content using the BCA protein assay described above. The supernatant was diluted in a 1:1 ratio with 2× Laemmli loading buffer, heated for 15 min at 60°C, and stored at −80°C. Samples were run at 10 µg protein per lane along with a 5 µg Precision Plus protein standard for reference on a 14% SDS-PAGE gel (Bio-Rad, Hercules, CA, USA) for electrophoretic separation, then transferred to Immobilon PVDF transfer membranes (Millipore, Bedford, MA, USA) in a transfer buffer (25 mmol l^{-1} Tris, 192 mmol l^{-1} glycine, pH 8.3) at 30 V overnight. The PVDF membranes were washed in phosphate-buffered saline with 0.05% Triton X-100 (PBST), blocked for 1 h at 23°C in 5% nonfat milk in PBST, washed in PBST again, then incubated for 1 h at 23°C in a 1:30,000 dilution of the HSP70 primary antibody in blocking buffer. The primary antibody is a rabbit polyclonal anti-HSP70 with no cross-reactivity with HSC70 produced from a synthetic peptide immunogen chosen from the C-terminus of salmonid HSP70 (AS05061; Agrisera, Vannas, Sweden), and has been validated and used in brook trout (Stitt et al., 2014). After primary antibody incubation, membranes were washed with PBST then incubated for 1 h at 23°C in a 1:1000 dilution of the secondary antibody (goat anti-rabbit) in blocking buffer. After secondary antibody incubation, membranes were washed with PBST, and labeling was detected via enhanced chemiluminescence (ECL) using a 1:1 mixture of ECL solution A (396 µmol l^{-1} coumaric acid, 2.5 mmol l^{-1} luminol, 100 mmol l^{-1} Tris-HCl, pH 8.5) and ECL solution B (0.018% H_2O_2, 100 mmol l^{-1} Tris-HCl, pH 8.5). Membranes were exposed to X-ray film (RPI, Mount Prospect, IL, USA) and digital photographs were taken of films. Band intensity was measured using ImageJ (National Institutes of Health, Bethesda, MD, USA), and protein abundance is expressed as relative band intensity. An identical pooled sample was run on each gel and used as a reference to correct for inter-blot differences.

Calculations and statistical analyses

Condition factor was calculated as: (body mass/length^3)×100. Percent water content was calculated as: [(wet mass−dry mass)×100]/wet mass. All values are presented as means±s.e.m. and n=8–11 for all group means except Experiment 1 SW day 16, where n=6. Normality and homogeneity of variance assumptions were tested using Shapiro–Wilk and Levene's tests, respectively. Student–Newman–Keuls *post hoc* analysis was used to identify significant differences between treatments after one-way (between treatment) or two-way (between treatment across time) ANOVA analyses were performed. An α-value of 0.05 was selected to denote statistical significance in all analyses and all P-values are presented in figures or figure captions. Statistics and figures were completed using R statistical software (version 3.2.2, https://www.r-project.org/) and GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA).

RESULTS

All fish remained active swimmers and in good condition throughout experimentation, except for in the final moments of CT_{max} testing, when the fish would lose equilibrium. Condition factor did not significantly differ between treatment or over time in any experiment.

Experiment 1: CT_{max} during SW acclimation

CT_{max} in FW remained stable at 30.4±0.1°C (pooled average; 0–16 days) throughout the 16-day experiment (Fig. 1A). CT_{max} in SW was reduced at 2 and 5 days after SW exposure, reaching a

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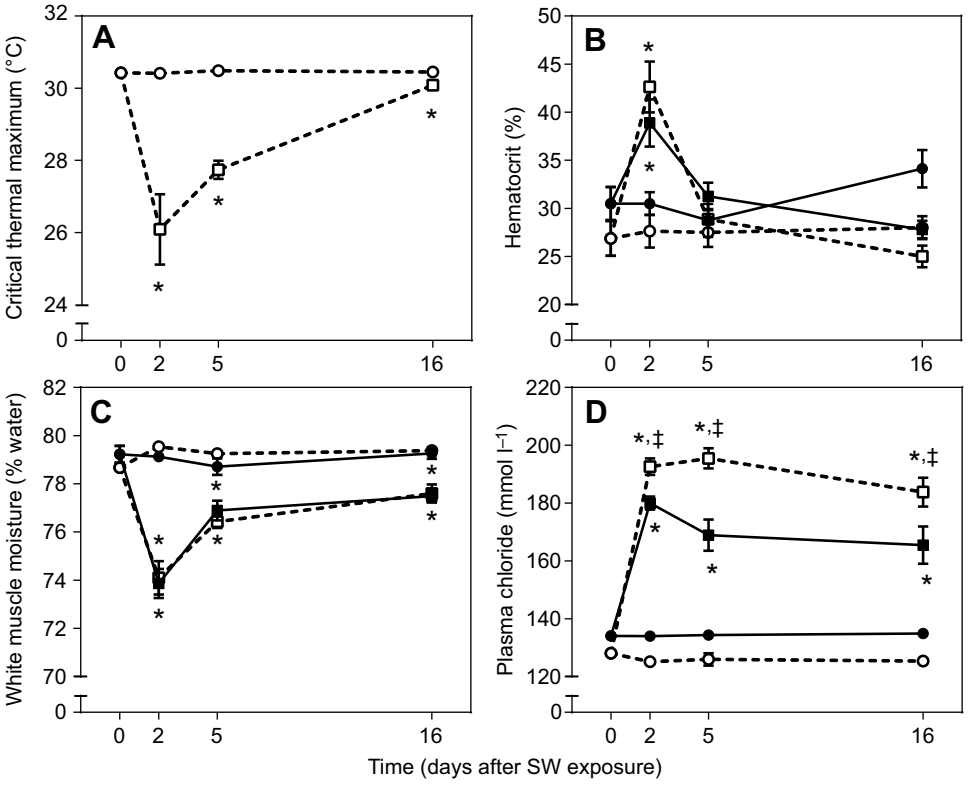

Fig. 1. Critical thermal maximum (CT_{max}) and ion and water balance in brook trout during seawater (SW) acclimation. (A) CT_{max}, (B) hematocrit, (C) white muscle moisture and (D) plasma Cl^- measured in brook trout for 16 days after SW exposure: freshwater (FW) acclimation temperature (T_{acc}; filled circles), SW T_{acc} (filled squares), FW CT_{max} (open circles) and SW CT_{max} (open squares). For A–D, $P<0.05$ for time, treatment and their interaction (two-way ANOVA; Student–Newman–Keuls *post hoc*). Data are presented as means±s.e.m.; $n=8$ for all groups except SW Day 16 $n=6$; *significant difference between salinity at respective time point; ‡significant difference within salinity at respective time point.

minimum of 26.1±1.0°C at 2 days, before returning to FW control levels by 16 days. At T_{acc}, Hct in FW remained stable at 31.0±0.7% (pooled average; 0–16 days), but was significantly elevated by 30% at 2 days in SW (Fig. 1B). Hct in fish at CT_{max} was similar to that from fish at T_{acc}, stable in FW and elevated at 2 days in SW. White muscle moisture at T_{acc} remained stable in FW at 79.1±0.14% water (pooled average; 0–16 days), but was significantly reduced to 73.9±0.6% water at 2 d in SW before partially recovering to a stable (although still significantly dehydrated) state at 5 and 16 days in SW (Fig. 1C). White muscle moisture data from fish at CT_{max} were nearly identical to those from fish at T_{acc}. Plasma Cl^- at T_{acc} was stable in FW at 134.3±0.5 mmol l^{-1} and significantly elevated to 180.0±2.3 mmol l^{-1} at 2 days in SW, then partially recovered to a stable (although still significantly elevated) state at 5 and 16 days (Fig. 1D). In FW, plasma Cl^- from fish at CT_{max} was 10% lower than from fish at T_{acc}, whereas in SW, plasma Cl^- from fish at CT_{max} was 10% higher than from fish at T_{acc}.

Plasma cortisol at T_{acc} remained stable in FW at 6.9±1.2 ng ml^{-1} (pooled average; 0–16 d) and was significantly elevated by 20-fold to 115.1±29.6 ng ml^{-1} in SW at 2 days (Fig. 2A). Exposure to high temperature during CT_{max} did appear to slightly elevate (threefold) plasma cortisol in both FW and SW fish. By day 5, plasma cortisol in fish in SW at T_{acc} had returned to FW T_{acc} levels, but plasma cortisol in fish in SW reaching CT_{max} still remained significantly elevated above fish in all other groups. By 16 days, plasma cortisol in all treatments had returned to control levels. At T_{acc}, plasma glucose in FW remained stable at 4.1±0.2 mmol l^{-1} (pooled average; 0–16 days) and was transiently elevated threefold to 13.2±2.2 mmol l^{-1} at 2 days in SW (Fig. 2B). Reaching CT_{max} elevated plasma glucose by twofold in FW fish. Gill NKA activity remained stable in FW at 1.81±0.17 (pooled average; 0–16 days), but steadily and significantly increased throughout SW acclimation by fourfold (Fig. 2C). In fish at CT_{max}, gill NKA activity did not differ from fish at T_{acc}. Gill HSP70 was not detected in any fish at T_{acc} and no significant differences were detected between FW and SW fish at CT_{max} over the 16 day experiment (Fig. 2D).

Experiment 2: CORT treatment on osmoregulation

By 12 d following CORT injection, plasma cortisol in the VEH group was 21.5±14.2 ng ml^{-1} and was elevated approximately fourfold in 5 µg g^{-1} CORT fish and ~10-fold in 25 µg g^{-1} CORT fish (Fig. 3A). Compared with VEH fish, gill NKA activity at 6 and 12 days after CORT injection was not elevated in the 5 µg g^{-1} CORT fish, but was significantly elevated 1.5-fold in 25 µg g^{-1} CORT fish after 12 d (Fig. 3B). After SW exposure, gill NKA activity increased in all fish, but the increase in activity was greatest in 25 µg g^{-1} CORT fish. At 2 d in SW, plasma Cl^- had increased by 40% in VEH fish, but only 15% in 5 µg g^{-1} CORT fish and 10% in 25 µg g^{-1} CORT fish (Fig. 3C).

Experiment 3: CORT treatment on CT_{max}

In FW, CT_{max} (~29°C) did not differ between VEH and CORT fish (Fig. 4A). At 2 days in SW, CT_{max} was significantly reduced in VEH fish to 27.1±0.6°C. However, CT_{max} in CORT fish in SW was 28.6±0.3°C and was not significantly reduced below FW controls. As plasma Cl^- is altered by reaching CT_{max} (Fig. 1D), the mean CT_{max} values for each experimental group were regressed with mean plasma Cl^- values of fish in T_{acc} under identical experimental treatment conditions as the CT_{max} fish. CT_{max} was significantly negatively correlated with plasma Cl^- [$n=4$, slope (m)=−0.034, $r^2=0.96$, $P=0.022$; Fig. 4B] and significantly positively associated with white muscle moisture ($n=41$, $m=0.212$, $r^2=0.60$, $P<0.001$; Fig. 4C).

DISCUSSION

Brook trout in this study experienced an elevation in plasma Cl^- and a dehydration of the white muscle immediately following SW

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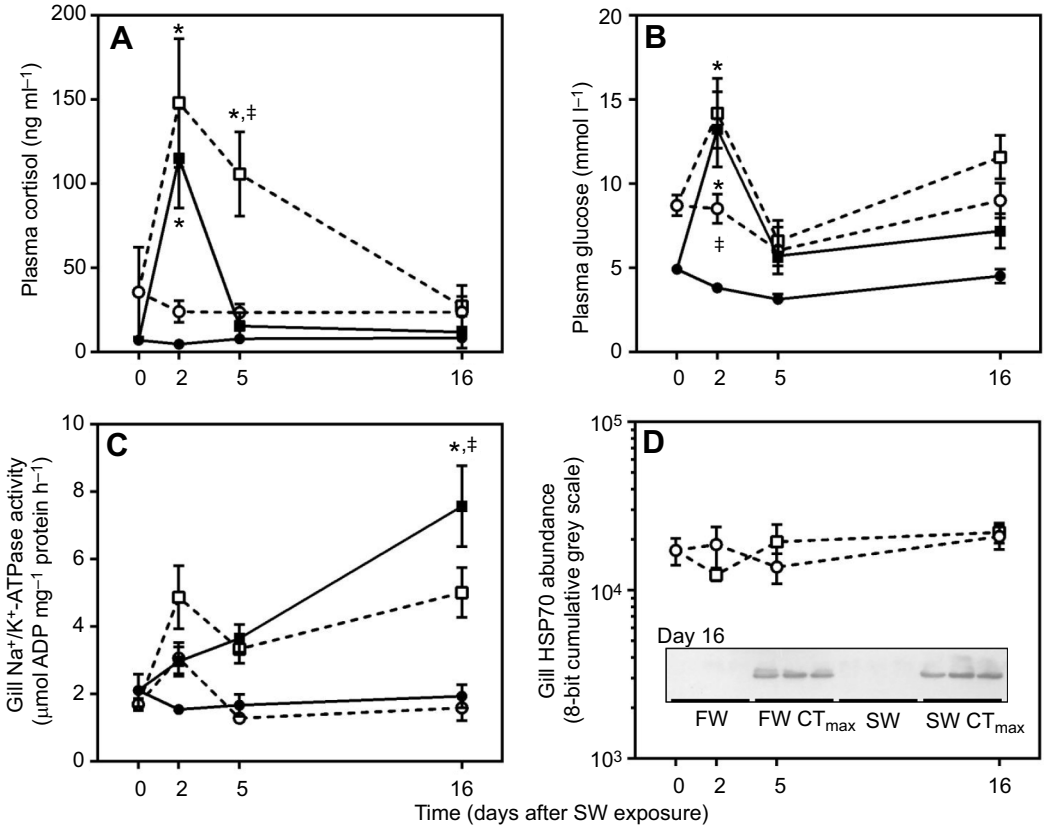

Fig. 2. Physiological responses of brook trout during SW acclimation and acute thermal tolerance testing. Plasma cortisol (A) and glucose (B), and gill Na^+/K^+-ATPase activity (C) and HSP70 abundance (D) measured in brook trout for 16 days after SW exposure: FW T_{acc} (filled circles), SW T_{acc} (filled squares), FW CT_{max} (open circles) and SW CT_{max} (open squares). For A–C, $P<0.05$ for time, treatment and interaction (two-way ANOVA; Student–Newman–Keuls *post hoc*); for D, no significant effects were detected. Data are presented as means±s.e.m.; *significant difference between salinities at respective time point; ‡significant difference within salinity at respective time point.

exposure, then recovered both of these indicators of osmoregulatory status back to stable states nearer to FW control values. This recovery of ion and water balance in SW followed a transient elevation in circulating cortisol concentration, the mobilization of glucose and an increase in gill NKA activity. Taken together, this is a well-documented pattern of SW acclimation by euryhaline fishes (Kultz, 2015; Marshall and Grosell, 2006; McCormick, 2001). Unique to this study are (1) a description of a transient reduction in thermal tolerance during SW acclimation, (2) a demonstration of the role of cortisol in osmoregulation in this species and (3) an association of osmoregulation with thermal tolerance.

Determination of CT_{max} as an indicator of thermal tolerance in ectotherms is a widely used method represented in an enormous body of literature (Beitinger et al., 2000; Lutterschmidt and Hutchison, 1997). This method estimates the maximum temperature an organism can tolerate by acutely increasing the temperature at a constant rate slow enough that a particular T_{LOE} can be identified but rapid enough to avoid acclimation by the test subject. The 2°C h^{-1} rate used in the present study was slower than the rates used in other CT_{max} tests, which range between 1 and 60°C h^{-1} (Becker and Genoway, 1979; Beitinger et al., 2000; Lutterschmidt and Hutchison, 1997). This slower rate was used for several reasons: (1) it was closer to ecologically relevant temperatures and fluctuations in temperature that a fish might experience in the wild (see Chadwick et al., 2015), (2) it was rapid enough to perform in a single day to resolve day-to-day changes in thermal tolerance during SW acclimation and (3) it resulted in consistency in control groups and repeatability between experiments.

In Experiment 1 (Figs 1 and 2), CT_{max} in FW remained stable at ∼30.5°C throughout the 16 day experiment. This result agrees with a previous estimate of thermal tolerance in brook trout of 29.8± 0.4°C reported by Lee and Rinne (1980) using a CT_{max} rate of 1.2°C h^{-1}, and falls within the range of estimates for members of the family Salmonidae of 26–33°C (Beitinger et al., 2000). Perhaps the most interesting finding of the present study is that upon SW exposure, thermal tolerance significantly declined to ∼26°C at 2 days before recovering to FW control levels by 16 days. This pattern of decline and recovery of thermal tolerance tracked changes in other parameters analyzed: plasma Cl^-, white muscle water content and Hct all remained stable in FW but significantly deviated from a stable state at 2 days in SW. This apparent synchrony in osmoregulatory status and thermal tolerance during SW acclimation suggests that these are associated in some way. It is particularly interesting that thermal tolerance is transiently affected by salinity because this may explain some of the inconsistencies in the literature regarding salinity effects on thermal tolerance (as described earlier, see Introduction). The timing of how salinity affects thermal tolerance clearly matters, but has not been addressed in studies investigating salinity effects on thermal tolerance.

An interesting finding of Experiment 1 was that, in contrast to the large increases in gill HSP70 abundance observed after the heat stress of the CT_{max} test, there was no detectable increase of gill HSP70 from osmotic stress over the 16 days following SW exposure

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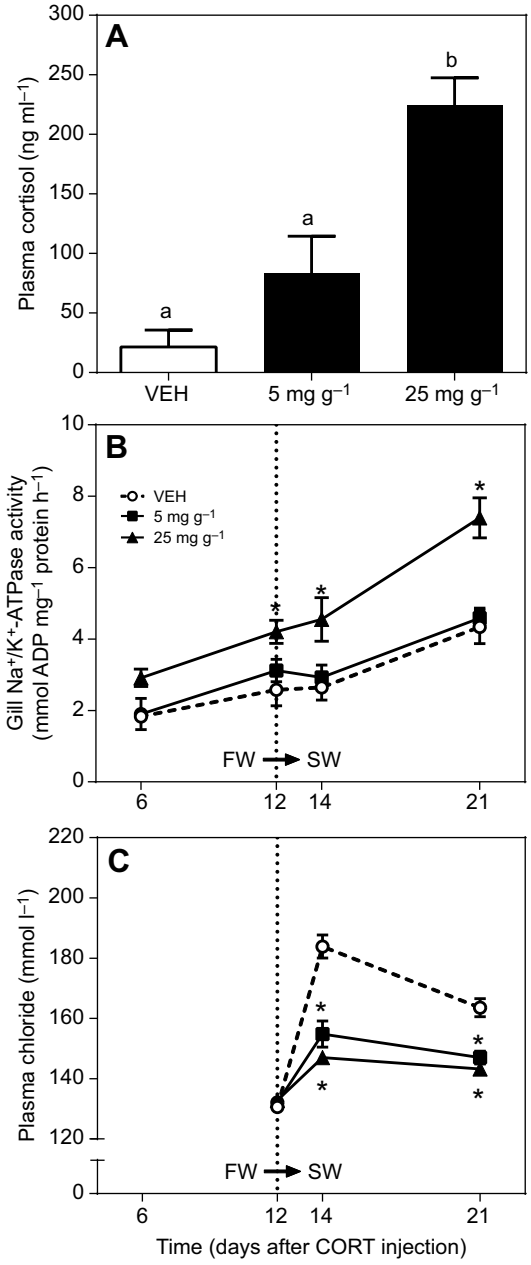

Fig. 3. Osmoregulation in brook trout during SW acclimation following cortisol treatment. (A) Plasma cortisol, (B) gill Na^+/K^+-ATPase activity and (C) plasma chloride measured at 6, 12, 14 and/or 21 days after a cortisol injection (CORT) of 5 µg g^{-1} (filled squares) or 25 µg g^{-1} (filled triangles), or injection with a vehicle control (VEH; open circles). Data are presented as means±s.e.m.; n=8–10 for all groups; letters indicate significance; *significant difference from VEH. Dotted line indicates FW-to-SW exposure.

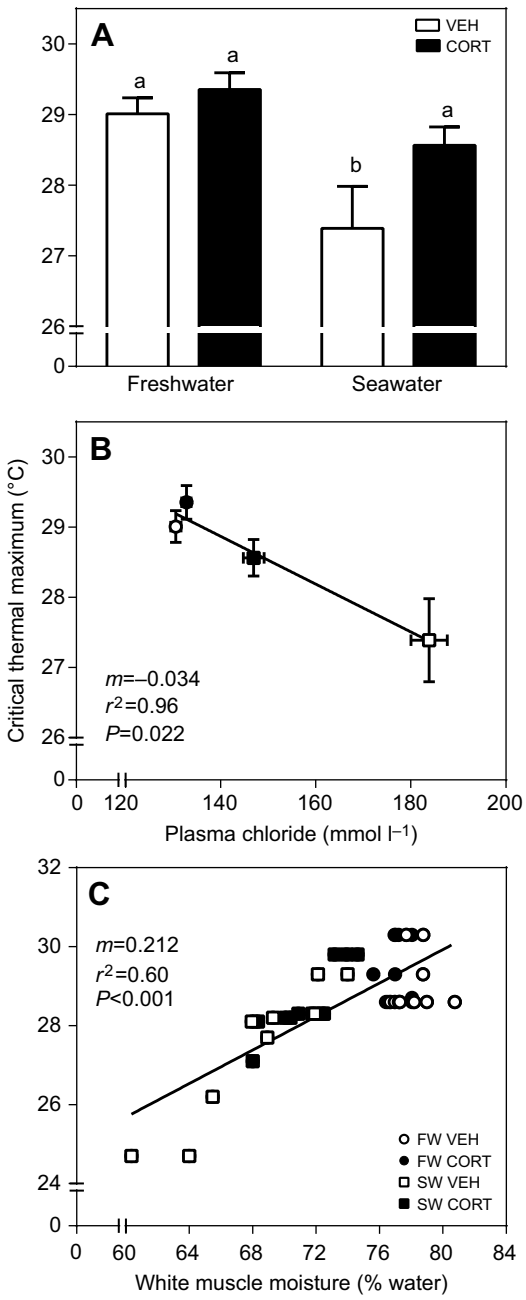

Fig. 4. CT_{max} in brook trout following cortisol treatment and SW exposure. (A) CT_{max}, (B) plasma Cl^- and (C) white muscle moisture measured 2 days after SW exposure (squares) or in a FW control (circles) in fish treated with either a 25 µg g^{-1} cortisol treatment (CORT; filled symbols) or a vehicle control (VEH; open symbols) 12 days prior to SW exposure. Data in A are presented as means±s.e.m. (n=10–11), with letters indicating significance (two-way ANOVA; Student–Newman–Keuls *post hoc*). In B, mean CT_{max} data are regressed with mean plasma Cl^- data of fish at T_{acc} (n=4). In C, individual CT_{max} and white muscle moisture data are presented (n=41).

(Fig. 2D). Many studies have investigated the response and regulation of HSP70 expression in response to heat stress in fishes (Basu et al., 2001; Deane and Woo, 2011; Iwama et al., 1999), but far fewer have seen an HSP70 response to osmotic challenges. Metzger et al. (2016) compared transcription of isoforms of *hsp70* in heat shocked marine and freshwater stickleback following salinity acclimation and acute salinity transfer. They observed a more rapid transcriptional response for *hsp70-2* than *hsp70-1* after heat shock from 18 to 28°C, and a general trend of higher *hsp70-1* transcription in fish acclimated to 2 ppt than fish in 20 ppt after heat shock. Smith et al. (1999) showed an increase in HSP70 abundance in isolated Atlantic salmon branchial lamellae after a hypersaline exposure *in vitro*, and Deane et al. (2002) showed an increase in hepatic HSP70 in black sea bream acclimated to elevated salinity. Additionally, heat shock treatments have been shown to improve survival of Atlantic salmon (DuBeau et al., 1998) and improve ion homeostasis of rainbow trout (Niu et al., 2008) after transfer to hypersaline water. The term ‘cross-tolerance’ refers to the phenomenon in which a physiologic response to one stressor

confers protection against an additional stressor. The mechanism of cross-tolerance between heat stress and osmotic challenge remains unclear, but may involve the moderation of effects of cellular dehydration by HSP-mediated changes to protein stability, folding or solubility (DuBeau et al., 1998). The HSP70 data in the present study show no evidence for an upregulation in gill HSP70 abundance from salinity exposure or cross-tolerance between osmotic and heat stresses, and thus explain neither the reduction nor recovery of thermal tolerance throughout SW acclimation. It is worth mentioning that in the present study we have only examined one member of the HSP family and only at one time point upon heat exposure (at CT_{max}), and because other induced or constitutively expressed HSPs such as HSP90 or HSC70 may be involved, we have a limited ability to make firm conclusions about cross-tolerance.

This is the first work to demonstrate the role of cortisol in osmoregulation in this species. The increased plasma cortisol from CORT treatment resulted in elevated gill NKA activity and improved osmoregulatory performance in SW (Fig. 3). Exogenous cortisol treatment has been shown to promote SW tolerance in Atlantic salmon and many other species (Bisbal and Specker, 1991; Foskett, 1977; McCormick et al., 2008; Singer et al., 2003; Specker et al., 1994; Veillette et al., 1995), as well as to promote many of the underlying mechanisms for salt excretion, including increasing the abundance of NKA, NKCC and CFTR (reviewed in Marshall and Grosell, 2006). In the present study, the dose of CORT treatment appeared to impact gill NKA activity and SW tolerance differently. Whereas the 5 µg g^{-1} CORT dose appeared to have no effect on gill NKA activity, gill NKA activity was significantly elevated in the 25 µg g^{-1} CORT dose prior to SW exposure and appeared to be more responsive than in VEH fish after SW exposure. It is interesting that, by contrast, SW tolerance was improved by a similar magnitude in both CORT doses. McCormick et al. (2008) made a similar finding when gill NKA activity was elevated by a 50 but not 10 µg g^{-1} CORT dose in Atlantic salmon, yet SW tolerance was improved by both doses. This may indicate that components of ion and water balance other than gill NKA activity may be more responsive to cortisol.

In addition to improving SW tolerance, CORT treatment improved thermal tolerance in SW-exposed fish and had no effect in FW-exposed fish. Plasma Cl^- and white muscle water content were associated with CT_{max} across salinity and hormone treatments (Fig. 4). Together, these findings support a hypothesis that decreased thermal tolerance after SW exposure is associated with higher plasma Cl^- and lower white muscle moisture. Determining the mechanism of the interaction between osmoregulatory status and thermal tolerance undoubtedly requires further investigation, but some possible explanations may be gleaned from this study.

The factors limiting thermal tolerance in fishes have been widely discussed (Clark et al., 2013a,b; Farrell, 2009, 2013; Jutfelt et al., 2014; Pörtner, 2001, 2014; Pörtner and Giomi, 2013; Pörtner and Knust, 2007; Schulte, 2015). A leading hypothesis that has emerged states that at elevated temperatures, along with increases in standard metabolic rate and O_2 consumption, O_2 uptake and delivery is limited, resulting in a decrease in oxygen availability ('aerobic scope') for other metabolically demanding physiological processes, such as locomotion, growth, reproduction and osmoregulation (discussed in detail below). Although this hypothesis has been criticized for failing to explain thermal tolerance in many species (Clark et al., 2013a; Gräns et al., 2014; Norin et al., 2014), it appears to at least partially explain acute thermal tolerance in trout (Keen and Gamperl, 2012).

The transient reduction in thermal tolerance during SW acclimation observed in the present study may be the result of reduced O_2 availability. In rainbow trout, acclimation to elevated salinity increases O_2 consumption, decreases arterial and venous blood O_2 tension, and increases blood lactate levels within 24 h (Maxime et al., 1991). If the brook trout in the present study experienced a similar decrease in oxygen availability owing to an increase in metabolic demand whilst acclimating to SW, this may have limited the ability of these fish to tolerate the increasing metabolic load from the rising temperature during the CT_{max} test, leading to a lower observed CT_{max} at 2 days in SW. If basal metabolic rate and arterial and venous O_2 availability return to homeostatic levels once the fish fully acclimate to SW, this may explain the recovery of thermal tolerance at 16 days in SW.

Another possible explanation for the reduction in thermal tolerance at 2 days in SW may be related to rheological properties of the blood affecting O_2 transport. SW exposure transiently elevated Hct in the present study (Fig. 1B), but no hematological analyses were performed to determine whether this increase in Hct was the result of an increase in the number or cell volume of erythrocytes. Dehydration associated with SW exposure would likely reduce RBC size, so the increase in Hct at 2 days in SW may be due to an increase in RBC production to counter any reduced O_2 transport capacity. Blood viscosity is known to increase with Hct (Fletcher and Haedrich, 1987) and increase the work needed to be performed by the heart, reducing cardiac output and O_2 transport (Wells and Weber, 1991). If thermal tolerance is related to O_2 delivery, as has been suggested, then a potential decrease in cardiac output owing to elevated Hct may result in the reduced thermal tolerance observed in the present study. In rainbow trout, optimum blood O_2 transport capacity occurs at a Hct of 30% packed cell volume, and is reduced by one-fifth when Hct is 40% (Wells and Weber, 1991). In the present study, FW brook trout had a Hct around 30%, but by day 2 in SW, Hct had transiently increased to nearly 40% (38.9±2.4%). Additionally, it has been suggested that cardiac arrhythmia during warming occurs because of a lack of O_2 supply to the highly metabolically active myocardium (Clark et al., 2008) and that this coincides with upper thermal limit in salmon (Casselman et al., 2012; Clark et al., 2008) and rainbow trout (Verhille et al., 2013). It could be that if the heart was working harder during a state of elevated Hct at 2 days in SW, the switch to anaerobic metabolism (and onset of arrhythmias) in the myocardium during warming would have occurred at lower temperatures, leading to the reduced observed CT_{max}. These speculations about Hct effecting thermal tolerance, however, cannot explain why thermal tolerance was still reduced at 5 days in SW, when Hct had already fully recovered.

A third possible explanation for the transient reduction in thermal tolerance during SW exposure may be that either plasma ion concentration or a transient metabolic acidosis after SW exposure affects O_2 binding kinetics of hemoglobin. In an early study in trout on the effects of anion concentrations on oxygen affinity, both hemoglobin types HbI and HbII appeared to have reduced affinity for O_2 under increased Cl^- concentrations (Brunori et al., 1975). Maxime et al. (1991) observed a decrease in Hb–O_2 binding affinity (increase in P_{50}) in rainbow trout exposed to SW for 24 h and suggested that it could be the result of a metabolic acidosis (decrease in arterial pH) that was also observed. In more recent studies, exposure of rainbow trout to hyperosmotic conditions resulted in a similar metabolic acidosis and reduction in Hb–O_2 binding affinity (Brauner et al., 2002; Jensen et al., 2002), and the decrease in RBC intracellular pH was highly correlated with an increase in P_{50} (Brauner et al., 2002). Although blood pH was not measured in the

present study, it can be surmised that a transient metabolic acidosis after SW exposure may have reduced O_2 binding affinity in the blood of the brook trout and led to the reduced CT_{max} at 2 and 5 days after SW exposure. More hematological work producing O_2 equilibrium curves using salmonid RBCs under a variety of osmolality conditions is needed to address this question.

A comparative approach may be useful in learning more about this unique sensitivity to temperature during SW acclimation, and clarifying some of the questions which arise from this finding. For instance, does thermal sensitivity vary with SW tolerance among anadromous fishes, i.e. would an anadromous fish that does not experience large increases in SW tolerance during development (such as Arctic charr) experience a greater reduction in thermal tolerance during SW acclimation than an anadromous fish that does increase SW tolerance during development (such as Atlantic salmon do during smolting)? From the data presented in this study, it appears that a critical consideration in addressing questions such as these is observing transient changes in thermal tolerance during SW exposure in addition to comparing FW- and SW-acclimated fishes.

Conclusions

Unique to this study are the description of a transient reduction in thermal tolerance during SW acclimation, the demonstration of the role of cortisol in improving osmoregulation in SW in this species, and the association of elevated plasma Cl^- and reduced white muscle water content with reduced thermal tolerance during SW acclimation. This paper highlights the importance of understanding the thermal experience of anadromous fishes considering their relatively unique life history of moving between FW and SW. This paper also reveals an opportunity to better understand salinity effects on thermal tolerance in general, which is important considering anticipated future water warming and variability across freshwater and marine environments.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C.A.S., S.D.M.; Methodology: C.A.S., S.D.M.; Validation: C.A.S.; Formal analysis: C.A.S.; Investigation: C.A.S., S.D.M.; Resources: S.D.M.; Data curation: C.A.S.; Writing - original draft: C.A.S.; Writing - review & editing: C.A.S., S.D.M.; Supervision: S.D.M.; Project administration: S.D.M.; Funding acquisition: S.D.M.

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