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Ion-regulation, acid/base-balance, kidney function, and effects of hypoxia in coho salmon, *Oncorhynchus kisutch*, after long-term acclimation to different salinities

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ABSTRACT

Land-based salmon farming in closed containment recirculation aquaculture systems (RAS) is an emerging industry with the potential for reducing disease prevalence, improving production efficiency, and reducing environmental impacts compared with open net-pen aquaculture. Salinity and oxygen levels are environmental factors that have significant impacts on the physiology of fishes and possibly influence the production efficiency of fishes in aquaculture. These parameters are particularly relevant for fish production in RAS, where most water parameters can be manipulated and tightly controlled. While much is known about the specific mechanisms associated with the acute transfer between FW and SW and the reverse, little is known about the effects of long-term acclimation on ion- and acid/base-regulation, especially at intermediate salinities. We measured gas-exchange, ion-regulation, acid/base balance, and renal function in coho salmon (*Oncorhynchus kisutch* Walbaum, 1792) acclimated to four salinities (2.5, 5.0, 10, and 30 ppt) for over a year in RAS and investigated how animals prioritize these functions when faced with 24 h exposure to hypoxia (PO_2 = 63 mmHg = 8.4 kPa = 3.76–4.48 mg l^{-1}). We show that fish that were long-term acclimated to 30 ppt had substantially higher plasma osmolality and [Na^+] than fish acclimated to lower salinities. These changes were associated with a marked reduction in blood pH at 30 ppt relative to 2.5 ppt, and we discuss a possible thermodynamic link between salinity acclimation and acid/base regulation. Further, we show that hypoxia exposure results in changes in plasma osmolality by over 80 mOsm kg^{-1}, but only in 30 ppt water, demonstrating a salinity-dependent trade-off between gas exchange and osmoregulation. This study provides insight into the physiological state and hypoxia sensitivity of market sized salmon reared under industry-relevant conditions in RAS.

1. Introduction

Salmonids are economically important aquaculture species with an annual world-wide production of > 3.4 million metric tons (FAO, 2019). Conventionally, salmon are reared from hatch to smolts in FW systems until smoltification and are then transferred to SW in open water net-pens and grown to market size. Water conditions, such as temperature, salinity, oxygen, and pH in net-pens are not possible to regulate so that production efficiency may be low due to sub-optimal water-conditions for growth. Furthermore, open water net-pen production is associated with unpredictable changes to the environment, such as drastic reductions in oxygen levels associated with algal blooms. To increase production efficiency and to reduce environmental impact, there is an increasing interest in rearing salmon in recirculation aquaculture systems (RAS) over their full life-cycle (Fang et al., 2019; Hines et al., 2019; Thorarensen and Farrell, 2011), where most water parameters can be manipulated and controlled to optimize growth and control the treatment and elimination of waste products through filtration and deposition. Production in RAS is financially costly, thus it is important to rear fish under optimal conditions that improve production efficiency (optimal growth and feed conversion) which require assessments of physiology, welfare, and growth across a range of potential water conditions. Salinity is an important factor affecting the physiology of fishes, which in turn largely influences their metabolism and growth (Fang et al., 2019; Hines et al., 2019). Thus, understanding physiological responses to long-term salinity acclimation is essential for optimizing fish growth and profitability of this emerging industry.

Much is known regarding the specific mechanisms related to osmoregulation in salmonids at different salinities (Evans et al., 2005). In FW, where the blood of fishes is hyper-osmotic relative to the

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Glossary

αCO_2	Plasma solubility of carbon dioxide
$[CO_2]_p$	Concentration of plasma carbon dioxide
CR (X)	Clearance ratio of an ion X
DR	Drinking rate
ER (X)	Renal excretion rate of an ion X
FW	Freshwater
FR (X)	Filtration rate of an ion X
GFR	Glomerular filtration rate
[Hb]	Tetrameric hemoglobin concentration in the blood
$[HCO_3^-]_i$	Intracellular red blood cell concentration of bicarbonate
$[HCO_3^-]_p$	Concentration of bicarbonate in plasma
P_aCO_2	Arterial partial pressure of carbon dioxide
P_aO_2	Arterial partial pressure of oxygen
pH_a	Arterial pH
pH_i	Intracellular red blood cell pH
pK	Apparent dissociation constant for carbon dioxide hydration in plasma
RR (X)	Renal reabsorption rate of an ion X
SW	Seawater
UFR	Urinary flow rate

environment, fishes passively lose ions across the gills and gain water. Ion losses are actively compensated for by branchial ion-uptake and renal ion-reabsorption, while the kidney excretes the water load. In SW, where the blood in fishes is hypo-osmotic relative to the environment, fishes passively gain ions and lose water, which is compensated for by intestinal ion-uptake to drive water uptake and active branchial ion-excretion (Edwards and Marshall, 2012; Evans et al., 2005; Hwang et al., 2011). In isosmotic water (~10 ppt), the osmotic gradients and most ionic gradients are highly reduced, reducing the passive water- and ion-fluxes across the gills, and possibly the energetic requirements for osmoregulation.

In the studies conducted to date, FW salmon display lower plasma ion concentration and higher resting arterial pH (pH_a) compared to SW acclimated fish (*e.g.*, Eddy and Bath, 1979; Morgan and Iwama, 1998; Wood and Nawata, 2011). These differences have been documented between FW and SW stenohaline species, between pre-smolt in FW and post-smolt in SW, and in short term transfer studies in salmonids (hours to weeks) (Gilmour et al., 2012; Handeland et al., 1998; V. Maxime et al., 1991; V. R. Maxime et al., 1990). However, less is known about the degree to which these changes are transient and are compensated for during long-term acclimation (months to years) across a range of salinities within the same life stage. Furthermore, while the mechanisms for osmoregulation are well described in salmon acclimated to FW and SW (Evans et al., 2005; Hwang et al., 2011), the salinities to which these fish are evolutionarily adapted, much less is known about osmoregulation at intermediate salinities between FW and SW, an environment that is of increasing interest in RAS production (Fang et al., 2019; Hines et al., 2019; Thorarensen and Farrell, 2011).

Most studies investigating the mechanisms of osmoregulation have been performed on fish in normoxia. However, fish in high-density aquaculture production may experience hypoxia that is compensated for by an increased blood perfusion and ventilation of the gills. This increased perfusion not only increases the oxygen uptake from the water, but it also increases the conductance for transbranchial ion-movements, causing increased ion loss during hypoxia in FW (Gilmour and Perry, 2018; Gonzalez and McDonald, 1992; Randall et al., 1972). This compromise between osmoregulation and gas-exchange (known as the osmo-respiratory compromise (Nilsson, 1986) has mainly been investigated during exposure to hypoxia and exercise in FW fishes (Fu et al., 2011; Robertson et al., 2015; Sardella and Brauner, 2011), while little is known concerning this compromise in fishes adapted to salinities greater than FW (such as brackish- and SW fishes), where the responses may differ markedly (Stevens, 1972).

This study was designed to test three hypotheses on the physiology of salmon reared across salinities in RAS for over a year. 1) We hypothesize that there is reduced demand for osmoregulation in fish in isosmotic water due to the reduced gradients for passive branchial water and ion fluxes. This hypothesis predicts low drinking rate (DR), low glomerular filtration rates (GFR), and low urinary flow rates (UFR) in fish acclimated to isosmotic water. 2) We hypothesize that salmon reared at the same salinity for over a year display similar ion- and acid/base-status, as they have had sufficient time to regulate these parameters. 3) We hypothesize that hypoxia exposure results in changes in plasma ion concentrations, for which the magnitude and direction of disturbance is a function of the external salinity. This hypothesis predicts that during hypoxia exposure, fish in FW lose ions, fish in SW gain ions, and that ionic concentration changes are minimal in fish in isosmotic water.

To test these hypotheses, we used coho salmon (*Oncorhynchus kisutch* Walbaum, 1792) that were acclimated to four salinities ranging from near FW to SW (2.5, 5.0, 10, and 30 ppt) for over a year in RAS during an on-going growth trial. This long-term acclimation protocol reflected realistic RAS production conditions and provided the opportunity to study the effects of external salinity on steady-state ion- and acid/base-balance. To our knowledge, this is the longest intermediate salinity acclimation protocol in a physiological study on salmonids. To shed light on the mechanisms and interactions between gas-exchange, acid/base-balance and ion-regulation across salinities, fully acclimated fish were instrumented with dorsal aorta and urinary bladder cannulae to assess the status of arterial blood gases and ions as well as acid/base-balance, drinking, and renal function. Acclimated animals were also challenged with moderate hypoxia (PO_2 = 40% of air saturation = 63 mmHg = 8.4 kPa, equivalent to an O_2 concentration of 3.76–4.48 $mgO_2\ l^{-1}$, depending on the salinity) for 24 h to determine how they prioritize these functions when faced with an oxygen stressor and the degree to which prioritizations are salinity-dependent. This information sheds important insight on the physiological implications of rearing fish in RAS at salinities to which they are evolutionarily adapted, and those that may be desirable from an industry perspective.

2. Materials and methods

2.1. Animal holding

Juvenile, female coho salmon (*Oncorhynchus kisutch* Walbaum, 1792) were purchased from Target Marine Hatcheries (Sechelt, BC, Canada) as smolts at one year of age and ~ 100 g. Six-thousand animals were randomly divided among four 5 m^3 tanks and held in flow-through dechlorinated Vancouver tap water for four months. The tanks were connected to individual recirculating aquaculture systems that allowed for water filtering, CO_2 stripping, oxygenation, protein skimming, and ozonation. Then water salinity was increased to 2.5, 5.0, 10, or 30 ppt over five days using Instant Ocean™ aquarium salt (Blacksburg, Virginia, USA) in the respective holding tanks (see Table S1 for calculated water ion compositions). The salinity of 2.5 ppt was chosen to represent a near-FW salinity that prevented fungal infections, 5.0 ppt was chosen as intermediate between FW and isosmotic, 10 ppt was chosen as an isosmotic salinity, and 30 ppt was chosen as a full SW condition. No incidence of fungal infections were observed in this study at any of these salinities.

For the first four months following the salinity change, the feeding rate was set to 0.5% body weight daily to minimize ammonia accumulation in the recirculation systems. After that, animals were fed at 1% body weight daily with commercial dry pellets using automated feeders for 12 h a day for over a year. Water conditions were $[NH_3] < 0.35$ μmol l^{-1}, $[NO_2^-] < 0.87$ μmol l^{-1}, $[NO_3^-] < 2.6$ μmol l^{-1}, temperature = 13 °C. Some of these fish were used in two other simultaneous studies (Fang et al., 2019; Hines et al., 2019), and the final growth parameters are reported in (Hines, 2019). Fish were randomly netted out of their tank, transferred to a 1 m^3 holding tank provided with water from the same recirculation system, where the fish were fasted for 48 h before experimentation. Individuals from different salinity acclimation groups did not differ in body mass or length (both $P > .05$, 1-way ANOVA).

2.2. Experimental series 1: drinking rate and renal function

The purpose of this experimental series was to measure renal function and drinking rate in animals acclimated for over a year to 2.5, 10, and 30 ppt. The 5.0 ppt acclimation group was omitted for Experimental series 1, as this group was terminally sampled for the concurrent growth trial at the time of experimentation. Twenty animals with a body mass of 1091 ± 293 g and a fork length of 38.2 ± 3.34 cm (mean ± standard deviation) were used in this experimental series, comprising 6, 7, and 7 animals from the 2.5, 10, and 30 ppt acclimation groups, respectively. Fish were anesthetized individually in normoxic water with 100 mg l^{-1} pH-neutralized 3-aminobenzoic acid ethyl ester (MS222 and $NaHCO_3$ in water) at the respective acclimation salinity, and when ventilation stopped, the fish were transferred to a surgical table where the gills were irrigated with aerated water containing 50 mg l^{-1} pH-neutralized MS-222 at the acclimation salinity.

A PE50 catheter was inserted into the urinary papillae and fitted through a flanged PE160 sleeve, which was attached to the animal with silk sutures. Then, the gills were irrigated with water without MS-222, and the fish were allowed to recover overnight in individual flow-through black Perspex boxes (l × w × h = 100 × 7.0 × 15.7 cm) connected to a 500 − l water reservoir at 10 °C in normoxia at the respective acclimation salinity. Urinary flow was monitored continuously for catheter patency. After a minimum of 20 h of recovery, the chambers were isolated from the recirculating system, water volume was set to 7 l, and 1 μCi tritium-labelled polyethylene glycol 4000 ($[^3H]$PEG-4000; American radiolabeled chemicals, Saint Louis, MO, USA) was added to the water. During the following 8 h, water and urine were collected, and the fish was subsequently euthanized in 200 mg l^{-1} pH-neutralized MS-222 in its Perspex box. A 1 ml blood sample was taken from the caudal vein using a heparinized syringe fitted with a 23G hypodermic needle, blood was centrifuged (2 min 641 *g*), and plasma was removed and frozen for later measurement of ions and osmolality. The body cavity was opened, the gastrointestinal tract was ligated at both ends with silk sutures, removed, and digested for 48 h at 65 °C in sealed containers containing 250 ml of 2 mol l^{-1} nitric acid (Robertson and Wood, 2014).

2.3. Experimental series 2: ion- and acid/base status

The purpose of this experimental series was to assess baseline values for arterial blood gases, ions, and acid-base status among salinities and to determine how these parameters change during 1-h and 24-h hypoxia and subsequent exposure to normoxia for 1 h. A total of 32 animals with a body mass of 1025 ± 315 g and a fork length of 40.2 ± 3.76 cm (mean ± standard deviation) were used in this experimental series, where 5, 9, 10, and 8 individuals were used from the 2.5, 5.0, 10, and 30 ppt acclimation groups, respectively. Fish were anesthetized as in Experimental series 1, and a polyethylene catheter (PE50; inner- and outer diameter 0.6 and 1.0 mm, respectively) filled with heparinized (50 IU ml^{-1}) Cortland saline (Wolf, 1963) was implanted into the dorsal aorta between the first and second gill arch, extended through the rostrum through another catheter (PE160; inner- and outer diameter 1.2 and 1.7 mm, respectively), and secured by silk sutures in the dorsal part of the mouth and on the back of the animal (Soivio et al., 1975). The fish was placed in a flow-through black Perspex box (l × w × h = 51.5 × 7 × 11 cm) at the acclimation salinity for recovery. The catheter was subsequently flushed with heparinized saline every 12 h.

The fish were allowed to recover for at least 20 h permitting for a full recovery of ion- and acid/base-status, blood cortisol, and hematocrit, which has been shown to occur within 1 h post-surgery in SW Atlantic salmon (Eliason et al., 2007). During recovery and subsequent exposures, fish were held in individual boxes coupled to a recirculating system of 500 l aerated water at the respective water salinity in an environmental chamber set at 10 °C. The experiment was initiated by withdrawing a 0.6 ml blood sample from the dorsal aorta catheter into a heparinized, gas-tight syringe for measurement of blood gas- and ion-status (see below). Hypoxia was then induced by bubbling N_2 into the 500 l water reservoir at a fixed flow rate resulting in a water oxygen level of 40% air saturation within 15 min (*i.e.*, PO_2 = 63 mmHg = 8.4 kPa, equivalent to an O_2 concentration of 3.76–4.48 mgO_2 l^{-1}, depending on the salinity). Then, blood was sampled at 1 h and 24 h of hypoxia exposure, and subsequently 1 h after return to normoxia.

2.4. Analysis of radioactivity

To measure $[^3H]$PEG-4000 activity, 5 ml Ultima Gold AB scintillation fluid (Perkin-Elmer, Waltham, MA, USA) was mixed with either 1 ml tissue digest/plasma/urine or 5 ml water, and activity was measured on a scintillation counter (LS6500, Beckman Coulter, Fullerton, CA, USA) and quench-corrected to the same counting efficiency as water samples.

2.5. Analysis of blood gases and ions

Immediately after blood sampling in Experimental series 2, the arterial partial pressure of oxygen (P_aO_2) was measured using a thermostatted PO_2 electrode coupled to a PHM 71 Mk2 Acid-Base analyzer (both Radiometer, Copenhagen, Denmark), which had been two-point calibrated with pure nitrogen gas and water-saturated air before each measurement. Arterial pH was measured using a flow-through pH- and reference electrode (MI-16-705 and MI-16-705, respectively, Microelectrodes, Inc., Bedford, USA) coupled to an Orion Star A211 pH meter (Thermo Fisher, Waltham, MA, USA) and was calibrated daily. Blood tetrameric hemoglobin concentration ([Hb]) was measured spectrophotometrically after conversion to cyano-methemoglobin in Drabkin's reagent (Blaxhall and Daisley, 1973). The remaining blood was centrifuged (3 min 641 *g*), red blood cells and plasma were separated into individual tubes, flash-frozen in liquid nitrogen, and stored at −80 °C for subsequent analysis.

To measure intracellular red blood cell pH (pH_i), frozen red blood cells underwent two freeze/thaw-cycles using liquid nitrogen with subsequent thawing on ice, and the pH of the lysate was measured as described above for pH_a (Zeidler and Kim, 1977).

For ion-analysis, plasma and urine samples were thawed on ice. Total plasma CO_2 ($[CO_2]_p$) was measured on a Corning 965 Carbon Dioxide Analyzer in samples from Experimental series 2. Plasma and urine concentrations of Na^+ and Mg^{2+} were averages from triplicate measurements using atomic absorbance spectrometry (AA240 FS, Varian Inc., Mulgrave, Australia), and osmolality was measured using a vapor pressure osmometer (Model 3320, Advanced Instruments Inc., Norwood, MA).

2.6. Calculations

Urinary flow rate (UFR) was determined as the rate of urine accumulation over the 8 h sampling period. UFR was body mass corrected to a body mass of 1000 g using a 0.79 allometric scaling exponent

(Robertson and Wood, 2014) and is reported in ml kg^{-1} h^{-1}.

The excretion rate of an ion X [ER (X); in μmol kg^{-1} h^{-1}] was calculated by multiplying UFR in ml kg^{-1} h^{-1} and the urinary concentration of ion X {$[X]_u$; in μmol ml^{-1} (= mmol l^{-1})}:

$$ER(X) = UFR \bullet [X]_u$$

The glomerular filtration rate (GFR; in ml kg^{-1} h^{-1}) was calculated from the radioactivities of urine and plasma (A_u and A_p; in cpm ml^{-1}) and UFR, where A_p is multiplied by 0.5 to account for plasma radioactivity being zero at time zero:

$$GFR = \frac{A_u \bullet UFR}{0.5 \bullet A_p}$$

The filtration rate of an ion X [FR (X); in μmol kg^{-1} h^{-1}] was calculated by multiplying GFR and the plasma concentration of the ion ($[X]_p$; in μmol ml^{-1}).

$$FR(X) = GFR \bullet [X]_p$$

The renal reabsorption rate of water [RR (H_2O); in ml kg^{-1} h^{-1}] was calculated by subtracting UFR from GFR. Renal reabsorption rate of an ion X [RR (X); in μmol kg^{-1} h^{-1}] was calculated by subtracting ER (X) from FR (X):

$$RR(X) = FR(X) - ER(X)$$

Thus, a positive value of RR would represent the rate of net reabsorption, and a negative value would represent the rate of net secretion.

The clearance ratio of water [CR (H_2O)] was calculated by dividing UFR by GFR. Clearance ratio of an ion X [CR (X)] was calculated by dividing ER (X) by FR (X):

$$CR(X) = \frac{ER(X)}{FR(X)}$$

A clearance ratio of < 1.0 indicates net absorption, while a value > 1.0 indicates net secretion, though the index does not quantitatively separate the relative contributions of simultaneous secretory and reabsorptive processes. CR(X) is useful because it effectively distinguishes between changes in excretion rate due to variations in plasma concentration or GFR (CR(X) is constant) from those caused by alterations in tubular processes – *i.e.,* CR(X) changes when % net reabsorption or % net secretion vary.

Drinking rate (DR; in ml kg^{-1} h^{-1}) was calculated as:

$$DR = \frac{A_{\text{git digest}} \bullet V_{\text{git digest}}}{A_w \bullet t}$$

Here $A_{\text{git digest}}$ is the radioactivity of the gastrointestinal tract digest after 8 h of [^{3}H]-PEG exposure in cpm ml^{-1}, $V_{\text{git digest}}$ is the volume of the gastrointestinal tract digest in ml, A_w is the average water radioactivity in cpm ml^{-1}, and t is time in hours. Drinking rates were body mass corrected to a body mass of 1000 g using a 0.79 allometric scaling exponent (Robertson and Wood, 2014).

The arterial partial pressure of carbon dioxide (P_aCO_2) was calculated by rearranging the Henderson-Hasselbalch equation using the apparent dissociation constant for CO_2 hydration (pK' = 6.86–0.087 × pH) and the plasma solubility of carbon dioxide (αCO_2 = 0.063 mmol l^{-1} $mmHg^{-1}$) at 10 °C for rainbow trout, *Oncorhynchus mykiss*, from (Boutilier et al., 1984):

$$P_aCO_2 = \frac{[CO_2]_p}{\alpha_{CO_2} \bullet (1 + 10^{pH_a - pK'})}$$

Plasma concentration of bicarbonate ($[HCO_3^-]_p$) was calculated by subtracting physically dissolved CO_2 from $[CO_2]_p$.

$$[HCO_3^-]_p = [CO_2]_p - \alpha_{CO_2} \bullet P_aCO_2$$

Intracellular red blood cell [HCO_3^-] ($[HCO_3^-]_i$) was calculated from the Donnan distribution of protons across the RBC membrane (Jensen, 2004).

$$[HCO_3^-]_i = \frac{10^{-pH_a}}{10^{-pH_i}} \bullet [HCO_3^-]_p$$

2.7. Statistics

In Experimental series 1 and 2, a 1-way ANOVA was used to test for the significance of salinity effects on blood gas and ion parameters in normoxia, and a Tukey's honest significant difference test was used to test for significant pairwise differences. In Experimental series 2, the effect of 1-h hypoxia, 24-h hypoxia, and 1-h normoxia within each salinity was tested by mixed model analysis of variance using individual fish as a random effect, and pairwise differences were assessed with a Tukey's honest significant difference test with a Holm correction. A multivariate linear model was used to test the effects of P_aCO_2, $[HCO_3^-]_p$, and osmolality on pH_a.

The number of replicates in each acclimation group was not identical, as the catheters in some animals were dysfunctional after recovery. The significance level was set at 0.05. Values are reported as means ± standard error of the mean unless stated otherwise. All data, including mean, standard error, replicate numbers, degrees of freedom, and statistical output, are posted in Table S2. All data analysis was performed in R Studio v. 1.1.456.

2.8. Ethics

All experiments on animals were approved by The University of British Columbia Animal Use Protocol no. A15–0266.

3. Results and discussion

3.1. Overview

This study was designed to test three hypotheses. The data support the first hypothesis that fish in isosmotic water have reduced demands for osmoregulation by showing reduced DR and UFR as well as a tendency for a reduced GFR. The data do not support the second hypothesis, as we show that salmon reared across salinities for over a year display pronounced differences in plasma ion and acid/base status. The data support the third hypothesis that the osmo-respiratory compromise is salinity-dependent by showing that the direction and magnitude of changes in plasma ion compositions during hypoxia are salinity-dependent, being most pronounced at 30 ppt.

3.2. Renal and intestinal osmoregulation

Drinking rate was higher in fish acclimated to 30 ppt than in fish acclimated to 10 ppt or 2.5 ppt (Fig. 1 A; see Table S2 for *P*-values), while UFR was higher in 2.5 ppt acclimated fish compared to fish in 10 ppt or 30 ppt (Fig. 1 B). Drinking rates in this data set exceed UFR, which could reflect a non-steady state condition, where fish accumulate water, but they are more likely a reflection of fluids passing through the gastrointestinal tract unabsorbed.

There was a tendency for a reduction in GFR with salinity (Fig. 1 C), but the relationship was not significant. The reduced UFR in 30 ppt is likely a reflection of a reduced GFR, as these parameters typically scale linearly in fishes (Miles, 1971). In fishes, GFR is primarily determined by 1) the rate of passive water gain, which increases blood pressure and hence GFR (Hickman Jr. and Trump, 1969), and 2) glomerular recruitment, where FW-acclimated salmon typically have higher glomerular number compared to SW-acclimated salmon (Ford, 1958). These are likely the same parameters that explain the variation in GFR and UFR among salinities in this data set (Wood, 1995).

Urine had higher concentrations of osmolytes, Na^+ and Mg^{2+}, at increasing salinity (Fig. S1 F,K,P), but since UFR decreased with

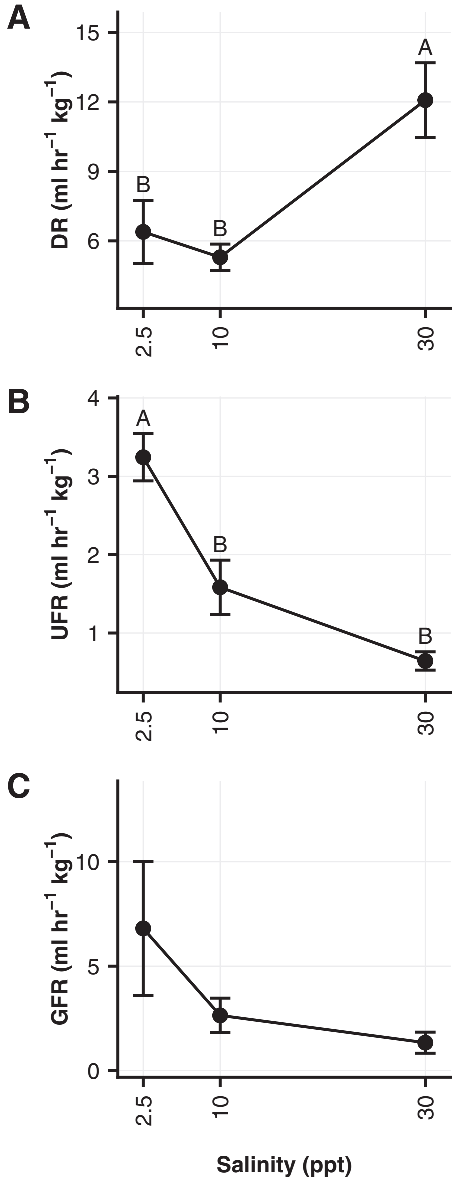

Fig. 1. Drinking rate and renal water handling in coho salmon acclimated for over a year to different salinities. Drinking rates (DR; A) and urinary flow rates (UFR; B), and glomerular filtration rate (GFR, C) measures in ^{3}H-PEG exposed animals fitted with a urinary bladder cannula. Differences in mean values among salinities were tested with a 1-way analysis of variance followed by a Tukey's honest significant difference test with a Holm correction. Significant pairwise differences among groups are indicated by letters that differ. Data are means ± s.e.m. (n = 20: 6 in 2.5 ppt, 7 in 10 ppt, and 7 in 30 ppt).

salinity, excretion rates were not significantly different among salinities for osmolytes (Fig. S1 G) and Na$^+$ (Fig. S1 L), but Mg^{2+} excretion rates were higher in 30 ppt compared to in 10 ppt (Fig. S1 Q). Osmolytes underwent renal reabsorption in 2.5 and 5.0 ppt indicated by a positive RR (osm) and a CR (osm) below unity. However, in 30 ppt, CR (osm) approached 1, and RR (Osm) was close to zero indicating reduced net renal reabsorption of osmolytes in 30 ppt. Na$^+$ was reabsorbed in all salinities indicated by a positive RR (Na$^+$) and a CR (Na$^+$) below unity. In contrast, Mg^{2+} was secreted across the kidneys in all three salinities indicated by a CR (Mg^{2+}) above unity. Further there was a trend (however, insignificant) towards increased Mg^{2+} secretion with increasing external salinity indicated by increasing CR (Mg^{2+}) (P = .051) and falling RR (Mg^{2+}) (P = .096).

These data show that demands for drinking, ultrafiltration, and urine production were reduced in 10 ppt compared to the relevant extreme salinity (*i.e.*, relative to 30 ppt for drinking, and relative to 2.5 ppt for GFR and UFR). Urine production, as well as intestinal water uptake, are energy-demanding processes (Grosell, 2010; Holmes and Donaldson, 1969; Takei and Balment, 2009), and potentially, these reductions may reduce metabolic demands in isosmotic environments compared to FW and SW. Hence, our physiological data are in support of the first hypothesis that there are reduced metabolic demands for osmoregulation in fish in isosmotic water which may be beneficial for growth of coho salmon in RAS. However, the investigated fish did not differ in size between salinities, which may have been a result of a marked size variation within acclimation groups, where a larger sample size of fish would have been required to detect a significant difference between acclimation groups within the first year of our growth trial. Hence, future studies should measure whole organism - as well as brachial-, renal-, and intestinal metabolic rates in Coho salmon long-term acclimated to different salinities to complement our data's support of this hypothesis.

3.3. Ion- and acid-base status in normoxia

Plasma ion concentrations differed among salinities with higher osmolality and [Na$^+$] in fish acclimated to higher salinity (Fig. 2 AB), while plasma [Mg^{2+}] did not differ between salinities (Fig. 2 C). Plasma and intracellular RBC [HCO$_3$$^-$] both fell with salinity (Fig. 3 DE), which was associated with reduced pH$_a$ and pH$_i$ (Fig. 3C). These data do not support the second hypothesis that the disturbance to ion- and acid/base status following exposure to a new salinity is transient, and instead, the present data set shows that coho salmon are not perfect iono-osmoregulators when exposed to higher salinities. These data suggest that the changes in ion-status observed during transfer between salinities in other studies are not necessarily transient but may persist over much longer time scales. Whether fishes change their osmoregulatory set-point during salinity transfers or are simply unable to fully ion-regulate across salinities is not known.

Acclimation to different salinities results in remodeling of the functional morphology of the gill, including changes in the ion-regulatory phenotypes and branchial anatomy (Giacomin et al., 2019; Henriksson et al., 2008). These changes can affect the gas-exchange capacity of the gill by affecting the conductance for transbranchial gas diffusion. Arterial PO$_2$ and PCO$_2$ in normoxia did not differ among salmon at different salinities (Fig. 3 AB). Hence, despite differences in the ion-regulatory function of the gill, branchial gas-exchange is matched to metabolic CO$_2$ production and O$_2$ consumption across salinities in coho salmon.

3.4. A putative mechanism linking pH-set point and osmolality

Arterial pH is typically tightly regulated (Brauner et al., 2019; Occhipinti and Boron, 2015; Shartau et al., 2019), so the 0.15 pH unit reduction in 30 ppt compared to fish in 2.5 and 5.0 ppt is striking (Fig. 3 CE). This reduced pH is associated with a reduction in $[HCO_3^-]_p$ rather than an increase in P_aCO_2 (Fig. 3). Similar quantitative changes in acid/base status have also been observed during acute transfers between FW and SW in salmonids (Gilmour et al., 2012; Maxime et al., 1991; Maxime et al., 1990), as well as in longer-term acclimations (Wood and Nawata, 2011). It is unlikely that the low $[HCO_3^-]_p$ represents a limitation to acid-base regulation as salmonids exposed to elevated environmental CO$_2$ in SW were able to rapidly elevate pH$_a$ which was associated with an elevation in $[HCO_3^-]_p$ to > 35 mmol^{-1} (Larsen and Jensen, 1997; Tovey and Brauner, 2018), well above that reported here. Thus, salmonids should not be limited in their ability to regulate pH$_a$ across these salinities. Hence, the reduced pH$_a$ at 10 and 30 ppt compared to 2.5 and 5.0 ppt suggests that pH$_a$ is indeed regulated at different set points at different salinities.

A multivariate linear model of pH$_a$ as a function of P_aCO_2,

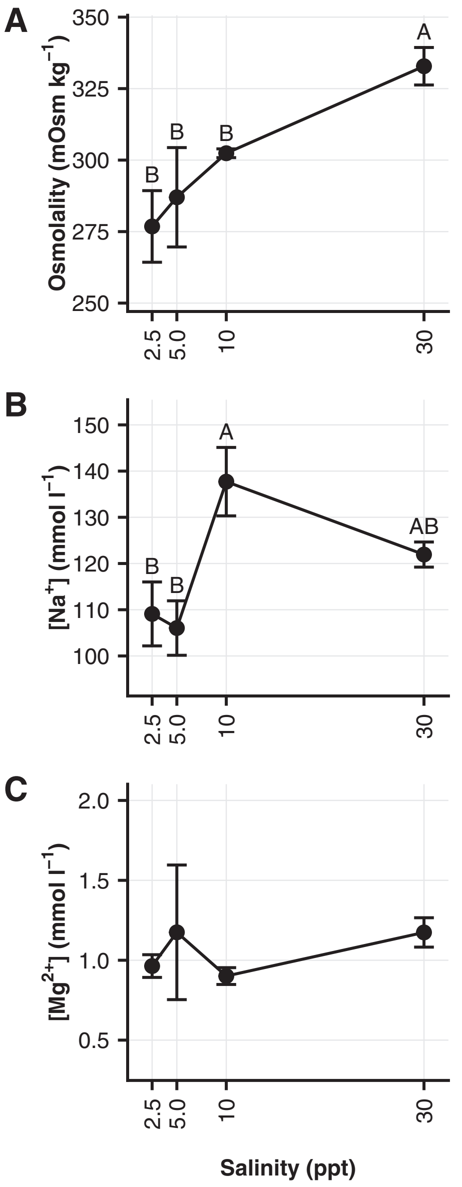

Fig. 2. Ion-status in coho salmon acclimated for over a year to different salinities. Blood was sampled from resting fish *via* indwelling catheters in the dorsal aorta in normoxia (Experimental series 2) for measurement of plasma osmolality (A), plasma sodium concentration ($[Na^+]$) (B), and plasma magnesium concentration ($[Mg^{2+}]$) (C). Differences in mean values among salinities were tested with a 1-way analysis of variance and a Tukey's honest significant difference test followed by a Holm correction to identify significant pairwise differences among groups, which are indicated by letters that differ. Data are means ± s.e.m. (N = 32: 5 in 2.5 ppt, 9 in 5.0 ppt, 10 in 10 ppt, and 8 in 30 ppt).

$[HCO_3^-]_p$, and osmolality showed a strong negative correlation between pH_a and osmolality ($P < .001$). While the molecular mechanism for pH_a-detection is unknown in fishes, it is most likely sensed *via* a histidine or cysteine side chain that exhibits pK_as within 2 pH units of physiological pH_a (Reeves, 1977; Reeves, 1972). Possibly, pH_a is regulated such that the ratio of populations of conjugate acid-base pairs of the amino acid side chains in the pH_a sensor is maintained constant. Since the pK_a of such side chains changes with ionic strength *via* the Debye-Hückel relationship, any changes in plasma ionic strength are expected to result in different steady-state pH_a values. Body fluid pH values are believed to be regulated in order to maintain constant ionization of protein surfaces to maintain functional integrity (Hazel et al., 1978; Reeves, 1972; Wang and Jackson, 2016), and hence, salinity-induced changes in plasma ionic strength may potentially introduce trade-offs to protein function. While detailed measurements of all plasma ions were not performed in our experiments, future studies should measure steady-state ionic composition and acid-base status in animals that are not perfect osmo-regulators, such as coho salmon, to provide deeper insight into putative molecular mechanisms for pH_a-sensors in fishes.

3.5. *Salinity dependent compromise between ionoregulation and gas-exchange*

Hypoxia exposure leads to hyperventilation in fishes, which is typically matched by concomitant increases in branchial perfusion (Petterson and Johansen, 1982), which in turn results in an increased conductance for oxygen to enhance oxygen uptake, but also in increased conductance for passive transbranchial ion/water fluxes (Gonzalez and McDonald, 1992; Nilsson, 1986; Randall et al., 1972). The rates and directions of passive ion/water fluxes during hypoxia are therefore expected to be a function of both branchial conductance (*i.e.*, morphology) and the electrochemical and osmotic gradients for ion/water fluxes across the gill. Our data show that hypoxia exposure did not affect plasma concentrations of osmolytes and Na^+in 2.5, 5.0, and 10 ppt (Figs. 4, S2–4), but hypoxia was associated with pronounced increases in these parameters in 30 ppt (Figs. 4, S2–4). These changes in plasma composition likely reflect the net effects of these fluxes. These findings demonstrate that the osmo-respiratory compromise is salinity-dependent, as the net changes are much more pronounced in 30 ppt compared with the other salinities. The electrochemical and osmotic gradients driving ion and water fluxes across the gills are greatest at 30 ppt; this, combined with unfavorable changes in transepithelial potential during hypoxia, likely explain the greatest changes in plasma composition at this salinity (Wood and Grosell, 2015). The direction of the osmolality changes during hypoxia acclimation was similar to those found during exhaustive exercise, where plasma chloride concentration increased after exercise in coho salmon in SW but decreased after exercise at 2.5 ppt (Fang et al., 2019). These findings suggest that the salinity-dependent compromise between passive water/ion-fluxes and gas-exchange at the gill is found under multiple physiological stressors, such as exercise and hypoxia. These data suggest beneficial physiological effects of rearing salmon at salinities below SW.

4. Perspectives and conclusions

In conclusion, our study shows that differences in ion-balance between fish in different salinities are not transient effects; rather they persist after long-term acclimation. We show that these iono-regulatory differences translate into different regulated resting pH_a values between salinities, which may potentially affect protein function. Lastly, our data demonstrate that the osmo-respiratory compromise at the gill is salinity-dependent, where osmotic disturbances are highly pronounced in 30 ppt only. These data provide insight into the interactions, prioritization, and trade-offs between physiological functions in multi-functional organs in response to multi-stressor environments. They further shed light on the optimal conditions for rearing salmon in aquaculture where there is an interest in rearing animals in RAS at intermediate salinities (Fang et al., 2019), as the fish are expected to have the lowest cost for osmoregulation that may potentially translate into improved growth. Further, our data may indicate an underlying physiological challenge of living in SW, where hypoxia exposure impairs ion/water homeostasis. Thus, the multiple stressors that can occur in an aquaculture environment may have a greater effects on fish in SW, relative to those reared at lower salinities, an area clearly worthy of further investigation. If supported, this may further justify the use of salinities below SW values in RAS.

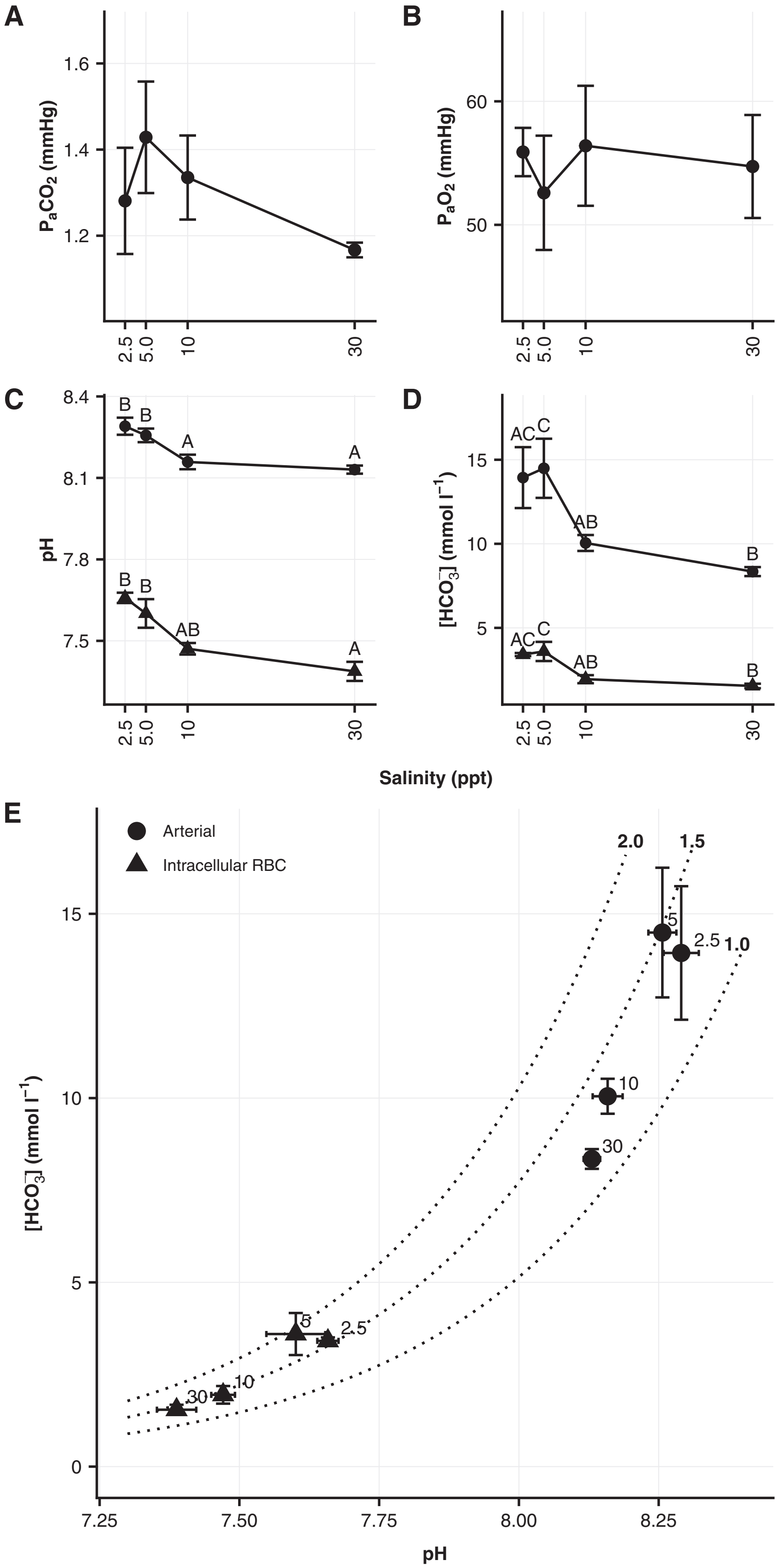

Fig. 3. Acid/base-status in coho salmon acclimated for over a year to different salinities. Blood was sampled from resting fish *via* indwelling catheters in the dorsal aorta in normoxia (Experimental series 2) for measurement of arterial partial pressure of carbon dioxide (P_aCO_2) (A), arterial partial pressure of oxygen (P_aO_2) (B), pH (C), and bicarbonate concentration ($[HCO_3^-]$) (D). Panel E shows the results in pH-$[HCO_3^-]$-plot, where dotted lines represent PCO_2 isopleths at 1, 1.5, and 2 mmHg calculated using the Henderson-Hasselbalch equation and pK' and αCO_2 from Boutilier et al. (1984). In panels C-E arterial and intracellular red blood cell values are depicted by circles and triangles, respectively. Differences in mean values among salinities were tested with a 1-way analysis of variance and a Tukey's honest significant difference test followed by a Holm correction to identify significant pairwise differences among groups, which are indicated by letters that differ in A-D. Data are means ± s.e.m. (N = 32: 5 in 2.5 ppt, 9 in 5.0 ppt, 10 in 10 ppt, and 8 in 30 ppt).

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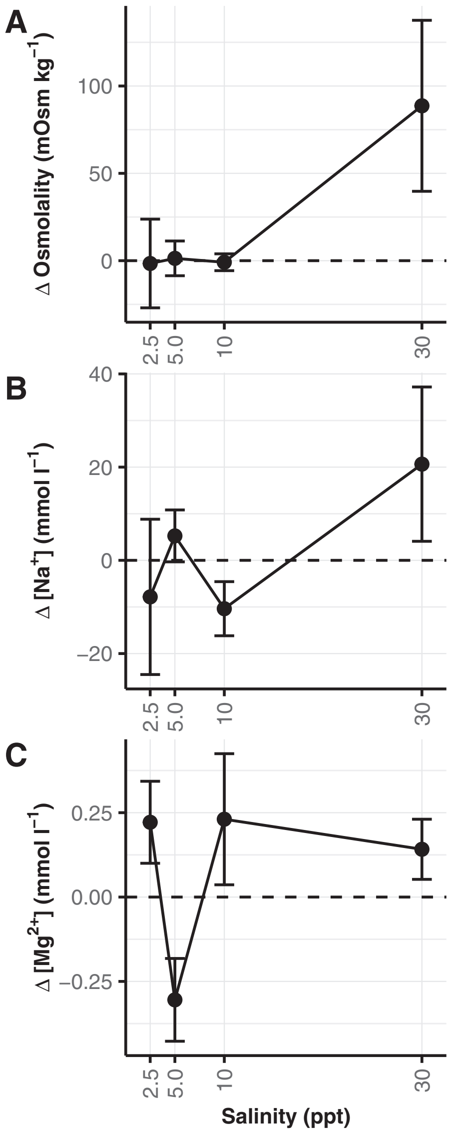

Fig. 4. Hypoxia-induced net-changes in plasma ion concentrations in coho salmon at 4 salinities. Salmon were instrumented with an indwelling dorsal aorta catheter, and blood was collected *via* the catheter in normoxia, before and after 24 h hypoxia (P_wO_2 = 63 mmHg) for measurement of net changes in plasma osmolality (Δ osmolality) (A), plasma sodium concentration (Δ [Na^+]) (B), and plasma magnesium concentration (Δ [Mg^{2+}]) (C). These measurements were made on salmon reared at four salinities for over a year. The horizontal dashed line shows zero net change. Data are means ± s.e.m. (*N* = 32: 5 in 2.5 ppt, 3 in 5.0 ppt, 9 in 10 ppt, and 3 in 30 ppt).

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Author contributions

C.D., C.M.W., J.G.R., and C.J.B. conceptualized the study, C.D. and M.M. performed the experiments, C.D. analyzed the data, prepared the figures and drafted the manuscript, all authors edited and revised the manuscript.

Disclosures

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2020.735571.

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