Salinity effects on plasma ion levels, cortisol, and osmolality in Chinook salmon following lethal sampling

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ABSTRACT

Studies on hydromineral balance in fishes frequently employ measurements of electrolytes following euthanasia. We tested the effects of fresh- or salt-water euthanasia baths of tricaine mesylate (MS-222) on plasma magnesium (Mg^{2+}) and sodium (Na^+) ions, cortisol and osmolality in fish exposed to saltwater challenges, and the ion and steroid hormone fluctuations over time following euthanasia in juvenile spring Chinook salmon (*Oncorhynchus tshawytscha*). Salinity of the euthanasia bath affected plasma Mg^{2+} and Na^+ concentrations as well as osmolality, with higher concentrations in fish euthanized in saltwater. Time spent in the bath positively affected plasma Mg^{2+} and osmolality, negatively affected cortisol, and had no effect on Na^+ concentrations. The difference of temporal trends in plasma Mg^{2+} and Na^+ suggests that Mg^{2+} may be more sensitive to physiological changes and responds more rapidly than Na^+. When electrolytes and cortisol are measured as endpoints after euthanasia, care needs to be taken relative to time after death and the salinity of the euthanasia bath.

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1. Introduction

The study of hydromineral balance of fishes frequently necessitates the measurement of plasma electrolytes (Clarke and Blackburn, 1977; Congleton, 2006). Changes in blood chemistry such as spikes in glucose or cortisol, which can cause ion movement, occur in as little as 2 min following a stressor (Svobodová et al., 1999). To avoid progressive changes in ion concentrations, fish are typically captured and immediately euthanized with lethal doses of anesthetic (Wedemeyer et al., 1990; Congleton, 2006). Unlike lower immobilizing doses of anesthetic, the lethal dose is believed to prevent a stress response that would cause a cascade of physiological changes (Wedemeyer, 1970; Strange and Schreck, 1978; Barton and Peter, 1982; Congleton, 2006). Fish from freshwater or saltwater holding salinities are euthanized by anesthetic overdose (Strange and Schreck, 1978; Ewing and Birks, 1982; Carter et al., 2011). Acute physiological changes can indicate passive water movement or active ion regulation; these processes can be affected by stress associated with experiments and sampling protocols.

Blood ion levels, measured following saltwater challenges are used as an indicator of the osmoregulatory ability of fish and as a predictor of successful survival and growth in a saline environment (Clarke and Blackburn, 1977; Folmar and Dickhoff, 1980; Shrimpton et al., 1994; Zydlewski et al., 2010; Aykanat et al., 2011). When sampling fish following saltwater challenges, anesthetics such as tricaine mesylate (MS-222) are used to reduce potential confounding handling stress (Strange and Schreck, 1978; Iversen et al., 2003) as well as for animal welfare concerns. Studies employing MS-222 typically examine changes in plasma sodium (Na^+) ions, but little is known about circulating levels of magnesium (Mg^{2+}) ions. Magnesium is particularly important because it reportedly increases with stress when other ions do not (Redding and Schreck, 1983; Staurnes et al., 1994; Arends et al., 1999; Raggs and Watts, 2015), and it is known to play a role in Atlantic salmon (*Salmo salar*) osmoregulation (El-Mowafi et al., 1997). However, the mechanism of Mg^{2+} regulation is not well understood (McDonald and Milligan, 1992; Prodocimo and Freire, 2001; Al-Jandal and Wilson, 2011).

Saltwater challenges were developed to identify salmon that were prepared for seaward migration to accelerate or to correctly

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time entry of juvenile salmon into seawater net pens (Clarke and Blackburn, 1977). Clarke and Blackburn (1977) found that fully smolted salmon had plasma Na$^+$ concentrations less than 170 mM following a 24-hour exposure to saltwater after direct transfer from freshwater. Cortisol concentration positively correlates with smolting (Barton et al., 1985; Richman and Zaugg, 1987; Madsen, 1990), but can also be induced by stress. As such, the goal of our study was to assess the effects of salinity and time on plasma ion and cortisol concentrations following saltwater challenges in juvenile spring Chinook salmon (*Oncorhynchus tshawytscha*).

Our study had two objectives. First, we determined whether blood plasma Mg^{2+} and Na$^+$ ion concentrations and cortisol change when fish from a saltwater challenge are euthanized in a MS-222 euthanasia bath of a different salinity than the challenge tank. We predicted that the salinity of the anesthetic would affect osmoregulation, causing lower plasma Mg^{2+} and Na$^+$ concentrations in fish euthanized in freshwater compared to those euthanized in saltwater. We also predicted that plasma cortisol levels would be greater in treatments where fish were subjected to salinity changes and thus an osmoregulatory stress in addition to the confinement stress that is inherent to such tests. Second, we determined the magnitude of ion and cortisol changes over time following euthanasia. We predicted that over time there would be passive diffusion of water across the gill following death, when osmoregulation ceased, and this diffusion would alter plasma ion and cortisol concentrations, with decreases in freshwater and increases in saltwater.

While of interest from a biological perspective, our aim was also to help provide an understanding of possible sampling methodology. Because of constraints inherent in sampling under many field situations, it is often not safe or feasible to obtain blood samples immediately. Based on our experience we selected 30 min as an extreme time during which fish might sit in anesthetic before sampling. Also because of practical constraints in the field, we were interested in testing whether or not salinity of the anesthetic might have an effect.

2. Material and methods

2.1. Animals

We reared juvenile spring Chinook salmon originating from Oregon's McKenzie Fish Hatchery 2013 brood year at the Fish Performance and Genetics Laboratory (FPGL; Oregon State University, Corvallis, Oregon) under temperature and feeding regimes to achieve body size characteristics similar to those of sub-yearling or fall-migrating smolts. This included flow-through 11–12 °C, pathogen-free well water, exposure to natural daylight, low stocking density, and a low-lipid diet (fat content = 11.5% dry weight; manufactured and provided by W. Sealy, Bozeman Fish Technology Center of the US Fish and Wildlife Service). We fed fish this diet to emulate growth characteristics more like their wild counterparts compared to typical hatchery growth rates. We did not feed fish on the day they entered the 24-hour saltwater challenge. The fish ranged between 33 and 102 g in body weight and 109 and 216 mm in fork length at the time of the experiment. We conducted the 24-hour saltwater challenge in December 2014 when, based on our experience with these fish, they would presumably be undergoing or have completed smoltification.

2.2. Saltwater challenge and euthanasia

We used eight static 85 L opaque tanks (4 saltwater and 4 freshwater) with 20 fish each for a 24-hour saltwater challenge. We filled each tank with water from the stock tank from which the fish were reared. In half of the tanks, we added 3058 g of salt (Oceanic Natural Sea Salt Mix, Franklin, WI, USA) to produce a salinity of 34 ppt (comparable to seawater), once dissolved. We placed fish into each challenge tank at 1-hour

intervals to allow sufficient time for sampling at the end of the 24 hour challenge test. Each tank had an opaque cover to minimize outside light from entering, with aeration from compressed air through diffusing stones to maintain dissolved oxygen levels at atmospheric saturation. Tanks were immersed in a water bath to maintain stable temperature (11.3–11.6 °C). Following the 24-hour test exposure, we placed 10 fish from each tank in a freshwater euthanasia bath of 200 mgL^{-1} MS-222 buffered to pH 7.0 with 500 mgL^{-1} sodium bicarbonate (NaHCO$_3$), and placed the other 10 fish in a saltwater euthanasia bath with the same concentration of MS-222. We repeated this for each tank following its 24-hour exposure with a new euthanasia bath prepared for each tank. In total, we had four final treatments, each in quadruplicate: freshwater challenge to freshwater anesthetic, saltwater challenge to freshwater anesthetic, saltwater challenge to saltwater anesthetic, and freshwater challenge to saltwater anesthetic. All fish lost equilibrium in less than 20 s (stage 4 anesthesia) after being placed in the anesthetic and were unresponsive to external stimuli in less than 90 s (stage 5 anesthesia, as defined by Summerfelt and Smith, 1990). From each euthanasia bath, we sampled 5 fish immediately (1.5–2 min after being placed in the bath) and sampled the other 5 fish after they had remained in the euthanasia bath for 30 min. For each sampling, bleeding of all the fish took less than 5 ½ min. Due to mechanical failure, we omitted one of the saltwater challenge tanks from the study.

2.3. Sampling and plasma analyses

We collected blood from each fish via caudal peduncle transection with ammonium heparinized blood collecting tubes (Natelson tubes, 250 μl), prior to collecting data on individual wet mass (g), fork length (mm), and sex. We separated the plasma from blood using centrifugation (Beckman Coulter Microfuge 16 Centrifuge) and froze samples at −20 °C until analysis. We measured plasma Na$^+$ and Mg^{2+} ion concentrations in an atomic absorption spectrophotometer (Perkin Elmer AAnalyst 100). Prior to measurement, we diluted plasma with deionized water by 1:1000 for Na$^+$ and by 1:200 for Mg^{2+}. We measured total cortisol using the radioimmunoassay procedure described by Redding et al. (1984), employing an antibody obtained from Fitzgerald (lot # P7071075), and validated by us for cortisol. Finally, we measured osmolality on duplicate 10 μl samples using vapor pressure osmometers (Wescor vapor 5100B and 5520, Wescor, Inc., Logan, Utah, USA).

3. Statistics

Analyses of plasma Na$^+$, Mg^{2+}, cortisol, and osmolality concentrations indicated the presence of a tank-level effect, requiring the use of a linear mixed model to evaluate the differences among treatments. We conducted statistical analyses using the lme4 (Bates et al., 2014) and lmerTest (Kuznetsova et al., 2014) packages in program R (R Core Team, 2014). We assessed goodness-of-fit for each model by plotting residuals vs. predicted values (Lindsay and Roeder, 1992) and examining normal Quantile–Quantile (Q–Q) plots (Ricci, 2005). These evaluations indicated that no transformations were needed for Na$^+$ or osmolality, while an inverse transformation of Mg^{2+}, and a square root transformation of cortisol data were needed to meet constancy of variance assumptions. We evaluated the effect of challenge salinity, euthanasia bath salinity, and time on Na$^+$, Mg^{2+}, cortisol, and osmolality using additive linear models controlling for tank effects with a random variable. The best approximating linear model was selected using Akaike's information criterion (AIC) (Burnham and Anderson, 2002). The significance of variables in the best approximating models was assessed using Satterthwaite's approximation for degrees of freedom (Gaylor and Hopper, 1969). We considered differences as significant using an α of 0.05.

4. Results

4.1. Plasma sodium concentrations

There was a significant effect of challenge salinity on plasma Na^+ concentration ($F = 48.78$, $P < 0.01$, $df = 6.96$, Fig. 1A), such that fish challenged in freshwater had significantly lower plasma Na^+ concentrations than those challenged in saltwater. There was also a significant effect of euthanasia bath salinity on plasma Na^+ concentration ($F = 13.11$, $P < 0.01$, $df = 129.05$, Fig. 1A). Fish placed into a saltwater euthanasia bath had higher plasma Na^+ concentrations than fish placed into freshwater euthanasia baths. Time in the euthanasia bath did not significantly affect plasma Na^+ concentration ($F = 1.36$, $P = 0.25$, $df = 129.00$, Fig. 1A), indicating that plasma Na^+ concentrations did not change significantly over time. Sex of fish did not have an effect on plasma $Na+$ concentration ($F = 0.06$, $P = 0.80$, $df = 133.09$) but there was a slight effect of fork length ($F = 3.78$, $P > 0.05$, $df = 129.60$).

4.2. Plasma magnesium concentrations

All three variables of challenge salinity ($F = 12.12$, $P = 0.01$, $df = 7.02$, Fig. 2), euthanasia bath salinity ($F = 17.31$, $P < 0.01$, $df = 131.01$ Fig. 2), and time in the euthanasia bath ($F = 192.25$, $P < 0.01$, $df = 130.95$, Fig. 2) significantly affected plasma Mg^{2+} concentration. Freshwater challenged fish had significantly lower plasma Mg^{2+} concentrations than saltwater challenged fish. Fish placed into a saltwater euthanasia bath had higher plasma Mg^{2+} concentrations than fish placed into freshwater euthanasia baths. Finally, fish held in the euthanasia bath for 30 min, regardless of salinity, had significantly higher plasma Mg^{2+} concentrations than fish bled immediately. Size of fish did not have an effect on plasma Mg^{2+} concentration ($F = 2.52$, $P = 0.11$, $df = 131.21$) but there was an effect of sex ($F = 4.49$, $P = 0.04$, $df = 133.23$) with males having, on average, slightly greater concentrations of Mg^{2+} after 30 min in the saltwater to freshwater and saltwater to saltwater treatments.

4.3. Plasma cortisol concentrations

There was no significant effect of challenge salinity ($F = 1.10$, $P = 0.32$, $df = 7.02$, Fig. 1B) or euthanasia bath salinity ($F = 0.80$, $P = 0.37$, $df = 129.05$, Fig. 1B) on cortisol concentrations. Time in the euthanasia bath ($F = 43.12$, $P < 0.01$, $df = 129.03$, Fig. 1B) affected cortisol concentrations with levels being lower in fish that remained in the bath for an additional 28 min following death. Neither sex of fish ($F = 0.72$, $P = 0.40$, $df = 130.71$) nor size ($F = 1.36$, $P = 0.25$, $df = 129.26$) affected cortisol concentrations.

4.4. Osmolality

All three variables of challenge salinity ($F = 32.29$, $P < 0.01$, $df = 7.05$, Fig. 1C), euthanasia bath salinity ($F = 18.21$, $P < 0.01$, $df = 126.17$, Fig. 1C), and time in the euthanasia bath ($F = 35.73$, $P < 0.01$, $df = 126.08$, Fig. 1C) significantly affected osmolality concentrations. Freshwater challenged fish had significantly lower osmolality than saltwater challenged fish. Fish placed into a saltwater euthanasia bath had higher plasma osmolality than fish placed into freshwater euthanasia baths. Fish held in the euthanasia bath for 30 min, regardless of salinity, had significantly higher osmolality than fish bled immediately. Neither sex of fish ($F = 0.01$, $P = 0.94$, $df = 128.92$) nor size ($F = 0.12$, $P = 0.73$, $df = 126.51$) affected osmolality concentrations.

5. Discussion

Based on their ability to regulate plasma Na^+ concentration below 170 mM as suggested by Clarke and Blackburn (1977), 93% of the fish
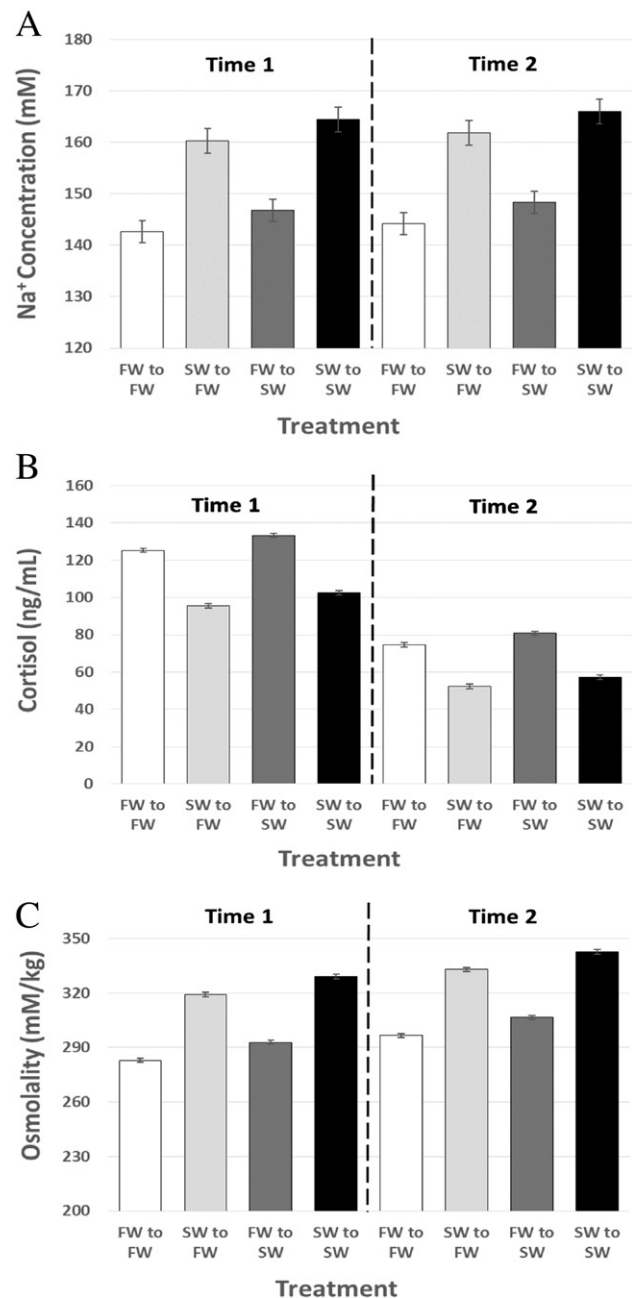

Fig. 1. Plasma ion concentrations for Chinook salmon (*Oncorhynchus tshawytscha*) by treatment for: (A) sodium, (B) cortisol, and (C) osmolality. Graphs show pooled replicate means (3–4 replicates) of each treatment type, coded on the X-axis, with standard error from the modeled data. The first letters in the treatment code correspond to the salinity of the challenge (FW for freshwater control and SW for saltwater challenge) and the second letters correspond to the salinity of the euthanasia (FW for freshwater and SW for saltwater). Time 1 represents fish sampled as soon as unresponsive to external stimuli/less than 90 s and time 2 represents fish sampled after 30 min of entering the euthanasia. Challenge salinity and euthanasia salinity were found to have a significant effect (α of 0.05) on sodium concentration. Only time in euthanasia had a significant effect on cortisol concentration. Challenge salinity, euthanasia salinity, and time in euthanasia had a significant effect on osmolality.

in our study were able to adapt to saltwater. Euthanasia bath salinity had an effect on the plasma ion concentration for both Mg^{2+} and Na^+; however, Mg^{2+} and Na^+ behave differently over time with regard to salinity after death by anesthetic overdose. This means that assessment of the osmoregulatory ability of fish following a saltwater challenge should use a euthanasia bath of the same salinity as the water used in the challenge to prevent post-morbidity ionic changes. When
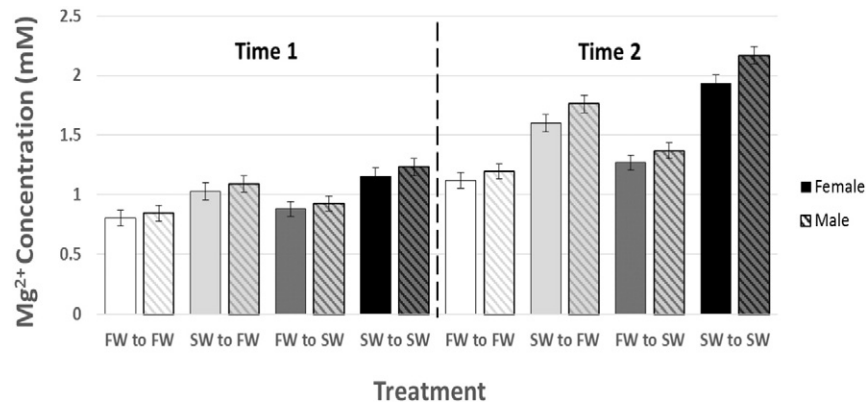
Fig. 2. Plasma magnesium concentrations for Chinook salmon (*Oncorhynchus tshawytscha*) by treatment for female (solid) and male (dashed). Graphs show pooled replicate means (3–4 replicates) of each treatment type, coded on the X-axis, with standard error from the modeled data. The first letters in the treatment code correspond to the salinity of the challenge (FW for freshwater control and SW for saltwater challenge) and the second letters correspond to the salinity of the euthanasia (FW for freshwater and SW for saltwater). Time 1 represents fish sampled as soon as unresponsive to external stimuli/less than 90 s and time 2 represents fish sampled after 30 min of entering the euthanasia. Challenge salinity, euthanasia salinity, time in euthanasia, and sex had a significant effect (α of 0.05) on magnesium concentration.

assessing osmoregulatory ability and stress of fishes, it is insufficient to only examine Na^+ ion concentrations. Magnesium may be a better indicator of rapid physiological changes. Normal Mg^{2+} concentrations are less than 1 mM and typically do not exceed 2 mM (Bijvelds et al., 1998). At the first sampling time the fish that underwent the saltwater challenge were on average above 1 mM indicating difficulty maintaining homeostasis whereas Na^+ concentrations of these treatments were in a normal range. At the second sampling time, fish in all treatments had Mg^{2+} concentrations above 1 mM and fish in the saltwater to saltwater treatment had an average exceeding 2 mM. Again, Na^+ concentrations did not show such trends. Arends et al. (1999) found that the stressors of air exposure and confinement slightly increased plasma Na^+ concentrations of gilthead sea bream (*Sparus aurata*), whereas plasma Mg^{2+} concentrations doubled. Similar results have been shown in Atlantic salmon exposed to transport stressors with or without clove oil and Aqui-S sedation during truck transport prior to transfer to sea. The results from these studies showed that, while osmolality and plasma chloride temporally changed after transport and transfer to sea in stressed groups (unsedated), plasma magnesium became elevated and never returned to pre-stressed levels. The authors suspected that the prolonged elevated plasma magnesium (3 to 6 times) also were the reason for the elevated mortality experienced in the stressed groups (un-sedated) (Iversen et al., 2009; Iversen and Eliassen, 2009). There is some evidence to support the suggestion that Mg^{2+} may be a second messenger coordinating cellular responses to environmental changes (Flatman, 1984).

Leaving fish in the euthanasia bath for 30 min allowed us to detect passive movement of ions via gill permeability since all fish were past the point of osmoregulation. If the fish were osmoregulating then in the gill epithelium would be tight to limit passive ion loss in the fish in freshwater and more permeable in the fish in saltwater to facilitate Na^+ secretion (Sardet et al., 1979; Chasiotis et al., 2012). If the fish are unable to osmoregulate then the gills at both salinities would become permeable and ion secretion would stop causing internal ion concentrations to dilute. Thus we predicted that plasma Na^+ and Mg^{2+} concentrations would decrease in the fish challenged in saltwater and euthanized in freshwater over time. Congleton (2006) investigated the temporal changes (up to 35 min post euthanasia) in Na^+ and calcium (Ca^{2+}), as well as additional blood chemistry variables, when juvenile spring Chinook salmon from freshwater were euthanized in freshwater MS-222. Congleton (2006) found that plasma Na^+ concentration did not change, but total Ca^{2+} concentrations increased progressively over time. Although Congleton (2006) did not examine Mg^{2+}, our results were similar in that plasma Na^+ concentration remained constant over time while Ca^{2+} (Congleton, 2006) or Mg^{2+} (our study)

increased, regardless of salinity. Both Ca^{2+} (10 mM) and Mg^{2+} (50 mM) are divalent ions and abundant in saltwater, which may explain their similar patterns. Increased Mg^{2+} concentrations over time suggest changes were not related to passive movement.

Stress has been linked to increased plasma Mg^{2+} concentration over time in anesthetized rainbow trout (*Oncorhynchus mykiss*) when MS-222 was used following a stressor (being lifted out of the water for 20 s) (Soivio et al., 1977). Similar link between plasma cortisol and Mg^{2+} has also been shown in Atlantic salmon following daily crowding stress for 4 weeks with a subsequent vaccination (Iversen and Eliassen, 2014). Iversen and Eliassen concluded that fish who may have entered an allostatic overload type 2 became oversensitive to ACTH, reducing efficient negative feedback system and elevating base-line level of plasma cortisol. We tested cortisol concentrations to determine whether elevated Mg^{2+} concentrations were correlated. Contrary to our hypothesis, cortisol concentrations were the same between salinity treatments and there was no correlation with Mg^{2+} concentrations. Since it is insufficient to explain the difference of patterns we observed between Mg^{2+} and Na^+ based on stress alone, we propose that the cause of increased Mg^{2+} over time while Na^+ remained constant is due to a combination of factors: capacity to excrete, fluid shifts, and passive diffusion in concert.

Osmolality followed a similar pattern to Mg^{2+} increasing over time following euthanasia. Mean osmolality of all treatment groups fell in the normal range for euryhaline teleost fishes (Folmar and Dickhoff, 1980; McCormick and Saunders, 1987; Mortensen and Damsgård, 1998; Gallaugher et al, 2001; Velan et al., 2011). Osmolality was found to increase over time following death in another posthumous study on Na^+, chloride (Cl^-), and osmolality (Calhoun et al., 1964). In that study, Na^+ decreased in the first few minutes after death but returned to baseline at 30 min, prior to continuing to increase in concentration. Since our last sample point was at 30 min, this could be why it appeared that Na^+ concentration did not change. Without having sampled further out than 30 min, we cannot determine whether Na^+ concentration would have increased later.

While the Na^+ pumps at the gills help to quickly regulate Na^+, Mg^{2+} enters the gut through drinking and has a more complex excretion process through intestinal uptake mechanisms and renal excretion. The difference of trends in ion concentration could have been due to different regulatory time-courses in response to the saltwater challenge. As Mg^{2+} is primarily excreted through the renal system (Oikari and Rankin, 1985) whereas Na^+ is excreted branchially, differential ion changes during the euthanasia could also be due to these excretion systems shutting down at different times. Thus, if the renal system shuts down first then more Mg^{2+} would accumulate.

Magnesium is maintained through active transport in live fish (Quamme et al., 1993). In the euthanasia bath, the fish become oxygen limited and stop respiring. Decreasing blood oxygen levels cause a decrease in blood pH and an increase in plasma catecholamine levels (Fiévet et al., 1990; Caldwell et al., 2006). If blood pH concentrations get sufficiently low, then acidosis occurs which causes a release of Ca^{2+} from bone (Ruben and Bennett, 1981) and presumably Mg^{2+}. Given that no hemolysis was visible in our samples, it is likely that Mg^{2+} elevations came from outside the blood. Bony tissues and scales are Mg^{2+} reservoirs which maintain ionic concentration in soft tissues when Mg^{2+} intake is low (Reigh et al., 1991; Bijvelds et al., 1996, 1998). These reservoirs account for 50–70% of the total Mg^{2+} in the fish's body (Van der Velden et al., 1989; Bijvelds et al., 1996, 1998) whereas extracellular magnesium makes up around 1% (Weisinger and Bellorín-Font, 1998). Studies in mammalian cells show that hormonal stimulation, such as increased catecholamine levels, can cause up to 15% of total intracellular Mg^{2+} to move out of the tissue within 15–30 min (Keenan et al., 1995; Romani and Maguire, 2002). Without active transport of Mg^{2+}, we propose that passive diffusion is occurring, moving pools of Mg^{2+} from bone or intercellularly to reach homeostasis. Fish remaining in the euthanasia bath for 30 min would have developed extreme respiratory acidosis compared to fish who have only been in the bath for 90 s. This respiratory acidosis corresponds with observed elevated Mg^{2+} concentrations. The fish remaining in the euthanasia baths would also have the greatest depletion of ATP, of which over 90% is bound to Mg^{2+} (Scarpa and Brinley, 1981). We propose that this depletion would cause large amounts of Mg^{2+} to become free in the cytosol and ready to be transported out. Additionally, stress affects muscle ATP content at the time of death (Berg et al., 1997; Thomas et al., 1999; Ribas et al., 2007) and concentrations of Mg^{2+} (Khalil et al., 2012).

The lower plasma cortisol levels in fish that remained in the euthanasia bath for 30 min compared to the fish that were only in the euthanasia bath for 90 s was unexpected. As the liver will unlikely continue to clear the cortisol from the blood after death, and typically in living fish, cortisol levels do not decrease for hours following stress (Redding et al., 1984; Barton et al., 1986; Shrimpton et al., 1994), we speculate that post-mortem, cortisol is diffusing out of the circulatory system and being bound to binding sites on various tissues. During and following morbidity, vascular tonus of capillaries would have been lost, thereby exposing cells to the circulation (the heart continues to beat for a considerable while after death and the blood flowed freely into collection tubes 30 min after euthanasia) that had been restricted and hence perhaps making glucocorticoid binding sites available, effectively removing the steroid from the circulation.

6. Conclusions

Our research is a unique study of the interactive effects of salinity and MS-222 over time on blood plasma. Although anesthetics are designed to decrease stress and lethal doses should not stimulate cortisol responses (Strange and Schreck, 1978; Wedemeyer et al., 1990; Carter et al., 2011), our study shows that there can still be plasma ionic and steroidal changes taking place during euthanasia. Furthermore, these fluctuations are not consistent across ions. Care needs to be taken to incorporate necessary controls and be aware of variables that could alter the correct interpretation of the obtained data. If only measuring plasma Na^+ concentrations, time is not as much of a concern as with Mg^{2+}, cortisol, and osmolality. When using plasma Mg^{2+}, cortisol, or osmolality concentration as a dependent variable, blood collection should be performed immediately after operculum movements cease. Published seawater challenge studies should include description of the salinities used, both in the challenge as well as in the anesthetic/euthanasia baths, together with the time fish are left in anesthetic before being sampled.

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