Comparative Biochemistry and Physiology, Part A 193 (2016) 29–35

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Effects of short-term hyper- and hypo-osmotic exposure on the osmoregulatory strategy of unfed North Pacific spiny dogfish (*Squalus suckleyi*)

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ARTICLE INFO

Article history:
Received 2 August 2015
Received in revised form 8 December 2015
Accepted 9 December 2015
Available online 11 December 2015

Keywords:
Elasmobranchs
Osmoregulation
Rectal gland
Salinity
Nitrogen

ABSTRACT

The North Pacific spiny dogfish (*Squalus suckleyi*) is a partially euryhaline species of elasmobranch that often enter estuaries where they experience relatively large fluctuations in environmental salinity that can affect plasma osmolality. Previous studies have investigated the effects of altered salinity on elasmobranchs over the long term, but fewer studies have conducted time courses to investigate how rapidly they can adapt to such changes. In this study, we exposed unfed (no exogenous source of nitrogen or TMAO) spiny dogfish to hyper- and hypo-osmotic conditions and measured plasma and tissue osmolytes, nitrogen excretion, and changes in enzyme activity and mRNA levels in the rectal gland over 24 h. It was shown that plasma osmolality changes to approximately match the ambient seawater within 18–24 h. In the hypersaline environment, significant increases in urea, sodium, and chloride were observed, whereas in the hyposaline environment, only significant decreases in TMAO and sodium were observed. Both urea and ammonia excretion increased at low salinities suggesting a reduction in urea retention and possibly urea production. qPCR and enzyme activity data for Na^+/K^+-ATPase did not support the idea of rectal gland activation following exposure to increased salinities. Therefore, we suggest that the rectal gland may not be a quantitatively important aspect of the dogfish osmoregulatory strategy during changes in environmental salinity, or it may be active only in the very early stages (i.e., less than 6 h) of responses to altered salinity.

1. Introduction

A number of elasmobranch species are fully or partially euryhaline and thus, one area of research interest has been the effect of salinity, particularly low salinity, on plasma and tissue composition, and on nitrogen excretion. Elasmobranchs are ureosmotic; converting ammonia into urea and retaining this urea such that their plasma is isosmotic or slightly hyperosmotic to the ambient seawater (see review by Hazon et al., 2003). This strategy results in a slight influx of water and thus reduces the need to gain water via drinking (the strategy used by marine teleosts), which, in turn, reduces the intake of salts. Unfortunately, urea can destabilise proteins at these high concentrations (Rajagopalan et al., 1961) and thus must be counteracted; a feat elasmobranchs accomplish by maintaining relatively high plasma levels of trimethylamine oxide (TMAO; Yancey and Somero, 1979). TMAO is present in a wide variety of marine organisms (Seibel and Walsh, 2002), however not all species have the ability to synthesise TMAO from choline or trimethylamine precursors (Baker and Chaykin, 1962; Goldstein and Funkhouser, 1972; Goldstein and Dewitt-Harley, 1973) and thus they must obtain TMAO from their diet, a strategy believed to be used by the North Pacific spiny dogfish (*Squalus suckleyi*; Baker et al., 1963).

Although retaining urea reduces the salt intake that elasmobranchs would experience if they drank seawater, influxes of salt still occur due to the specific ion gradients that exist across the gills. Marine teleosts use their gills to eliminate excess sodium from the body but elasmobranchs have a specialised organ called the rectal gland that is responsible for excreting excess Na^+ and Cl^- (Burger and Hess, 1960). A number of studies have examined different aspects of the rectal gland and observed changes in response to the salt load experienced during feeding. For example, Walsh et al. (2006) observed large increases in the activity of a number of enzymes such as NKA and lactate dehydrogenase (LDH) following a meal. The latter was believed to be fueling anaerobic metabolism when gland activity outpaces oxygen delivery. A second recent study by Deck et al. (2013) used suppression subtractive hybridization to investigate changes in the mRNA expression of relevant genes and observed changes in the transcript levels of genes associated with metabolism and transport such as *Nka* and *Ldh*. It was also revealed that the gland may be storing RNA for rapid protein

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http://dx.doi.org/10.1016/j.cbpa.2015.12.004
1095-6433/

synthesis upon activation as a number of genes, most notably *Nka*, had higher mRNA levels in the fasted relative to the fed glands, a pattern that does not coincide with the increases seen in protein or enzyme activities, but would be consistent with a rapid subsequent increase in protein synthesis leading to increased enzyme activities. In regards to salinity, Piermarini and Evans (2000) observed that NKA activity and abundance was greater in rectal glands of seawater acclimated stingrays (*Dasyatis sabina*) relative to freshwater acclimated rays. However, Cramp et al. (2015) observed no such changes in rectal gland NKA activity in *Chiloscyllium punctatum* exposed to hypersaline conditions.

Since their osmoregulatory strategy involves the synthesis of urea, elasmobranchs excrete the majority of their nitrogenous waste in this form; however this is primarily through diffusion at the gills rather than active excretion (Wood et al., 1995). In response to hypo-osmotic conditions, however, plasma osmolality would be expected to decrease in order to match the surrounding seawater. Indeed, Guffey and Goss (2014) showed decreases in plasma osmolality in the spiny dogfish (*S. suckleyi*) that could be accounted for by decreases in plasma sodium, chloride, and urea concentrations. The latter corresponded with a significant increase in urea excretion (Guffey and Goss, 2014), suggesting a reduction in the urea retention mechanisms. Steele et al. (2005) also observed an increase in urea excretion but no change in ammonia excretion when little skates (*Raja erinacea*) were exposed to 75% seawater. Two studies have exposed the small-spotted catshark (*Scyliorhinus canicula*) to both high and low salinities. The first by Hazon and Henderson (1984) showed that plasma osmolality, urea, sodium, and chloride all increased or decreased with salinity. This was also the case in a more recent study that exposed the brown-banded bamboo shark (*C. punctatum*) to both hypo- and hypersaline conditions (Cramp et al., 2015). In the second study on catsharks, fish that were fed a high protein diet and subsequently exposed to high salinities, had higher plasma concentrations of urea, sodium, and chloride than control fish (Armour et al., 1993). However, fish that were fed a low protein diet to hinder urea synthesis showed no ability to increase urea upon exposure to the increased salinity and thus, sodium and chloride were increased to a greater extent (Armour et al., 1993).

In the literature, there is a bias towards low salinity exposures for marine elasmobranchs because it is more common to encounter a hyposaline environment than a hypersaline one, although the latter can occur in estuaries during times of low water flow. In the three studies that included exposures to both hyper- and hypo-saline environments (Hazon and Henderson, 1984; Armour et al., 1993; Cramp et al., 2015), the salinity was altered gradually over days to weeks and the animals were held at the desired salinity for a number of days before measurements were taken. In addition, these studies did not measure TMAO or total nitrogen excretion, both of which are important aspects of the elasmobranch osmoregulatory strategy (albeit the Hazon and Henderson and Armour *et al.* studies were more focused on hormonal control of osmoregulation). One group did look at the short-term transfer of freshwater bull sharks (*Carcharhinus leucas*) to seawater but only one sample was taken within the first 48 h (Pillans et al., 2006). Thus, in this study, we used the North Pacific spiny dogfish (*S. suckleyi*), a species that has been shown to tolerate relatively large fluctuations in environmental salinity and can be found in estuaries (Burger, 1965; Guffey and Goss, 2014), to investigate the effects of short-term high and low salinity exposures on plasma and tissue osmolytes, nitrogen balance, and rectal gland activity. In contrast to the other studies, we used unfed fish because: 1) this approach would allow us to determine whether the dogfish are capable of increasing TMAO (which can only be synthesised *de novo* by select species) and urea as they were not receiving an exogenous source of nitrogen (an element many elasmobranchs appear to be limited in due to their sporadic feeding; Wood et al., 2005); and 2) the rectal gland appears to be inactive under these conditions (see Matey et al., 2009) so we believed that any changes in enzyme activity would be due solely to our salinity treatments. We measured plasma osmolality, sodium, chloride, urea, and TMAO as well as ammonia and urea excretion in order to determine how dogfish alter their osmoregulatory strategy to adapt to various salinities. All plasma parameters were expected to either increase or decrease with salinity as has been shown in the studies mentioned previously. In addition, we hypothesised that less urea would be produced at low salinities due to the predicted reduction in plasma osmolality and thus an increase in ammonia excretion was also predicted. Furthermore, we measured mRNA levels and enzyme activities of NKA and LDH in the rectal gland because we postulated that a hypersaline environment would activate the gland, mimicking the salt load experienced following a meal. Lastly, glucose (GLUT) and monocarboxylate (MCT) transporter mRNA levels were investigated since it is known that the gland requires glucose as a fuel and may be producing excess lactate during peak activity (Walsh et al., 2006). We expected that both types of transporters would be upregulated at high salinities as the rectal gland was hypothesised to be active under these conditions.

2. Materials and methods

2.1. Animals and salinity exposures

Male Pacific spiny dogfish (*S. suckleyi*; 1.5–2.5 kg) were caught by angling in Barkley Sound, British Columbia (Canada) in June 2014 and transported to Bamfield Marine Sciences Centre where they were held in a 155,000 L circular tank with running seawater and aeration at a maximum stocking density of less than 150 animals in the tank. Animals were allowed to acclimate to captivity for 7 days prior to experimentation during which time they were not fed. Following this period, the dogfish were anaesthetised using MS-222 (0.2 g L^{-1}) and fitted with a cannula of PE 50 tubing in the caudal artery (similar to methods published by De Boeck et al., 2001) before being placed in 40 L epoxy coated boxes with running seawater and aeration to recover for 24 h. The dogfish were then exposed to 70% (by addition of freshwater), 100%, or 130% (by addition of Instant Ocean) seawater ($N = 6$) for 24 h and blood (500 μL) and water samples were taken every 6 h. The blood was centrifuged at 13,000 ×*g* for 2 min to obtain the plasma which was then stored at −80 °C. During the experimental period, the boxes were not supplied with flow through seawater but the water was changed following each sample (every 6 h) by siphoning water from a header tank into the box as the old water was drained. This was done three times to ensure full replacement of the water. At the end of the 24 h period, dogfish were sacrificed by an overdose of MS-222 (1 g L^{-1}) and subsequent cervical dislocation. Muscle, liver, and rectal gland samples were taken and flash frozen in liquid nitrogen before being stored at −80 °C.

2.2. Water analysis

The osmolarity of the water was measured using a Vapro vapour pressure osmometer. Ammonia was measured using the salicylate-hypochlorite method (Verdouw et al., 1978) and urea was measured using the diacetylmonoxime method (Rahmatullah and Boyde, 1980). For both assays, we used 100 μL of sample in each well and all samples were run in duplicate. Excretion rates were calculated as μmol of nitrogen per kilogramme body weight per hour for each 6 h sampling period.

2.3. Plasma and tissue analysis

Plasma osmolality was measured using a Vapro vapour pressure osmometer. Muscle and liver samples (~250 mg) were homogenised in 5 volumes of 5% trichloroacetic acid (TCA) using a glass homogeniser and mortar and pestle, respectively. They were then centrifuged at 13,000 ×*g* for 5 min to obtain the supernatant. Plasma samples (250 μL) were also combined with 5 volumes of TCA and centrifuged for 5 min. Urea was then measured using the diacetylmonoxime method (Rahmatullah and Boyde, 1980) while TMAO was measured

using the ferrous sulphate/EDTA method (Wekell and Barnett, 1991). An Atomic Absorption Spectrometer was used to measure plasma sodium and plasma chloride was measured using the mercuric thiocyanate method (Zall et al., 1956).

2.4. Enzyme activity

For measurements of lactate dehydrogenase activity, rectal glands were homogenised on ice in 20 mM Hepes, 1 mM EDTA, and 0.1% Triton X-100 at pH 7.0 and centrifuged at 8000 *g* for 5 min at 4 °C. The supernatant was then assayed spectrophotometrically in 50 mM imidazole (pH 7.0), 0.15 mM NADH, and 0.2 mM pyruvate, following the change in absorbance at 340 nm wavelength. Na^+/K^+-ATPase activity was measured using the methods of McCormick (1993) and a Bradford assay was used to determine protein content.

2.5. qPCR

Total RNA was extracted from each rectal gland using Trizol reagent (Invitrogen) according to the manufacturer's instructions and treated with DNase I (Invitrogen). The RNA (2 μg) was then combined with 375 ng random hexamers (Integrated DNA Technologies), 125 ng oligo dTs (IDT), and dNTPs (Invitrogen; 0.5 mM final concentration) and incubated for 5 min at 65 °C. The reaction was then chilled on ice for 5 min and first-strand reaction buffer (Invitrogen; 1x final concentration), dithiothreitol (5 mM final concentration), RNase Out (Invitrogen; 20 U), and nuclease-free water (0.5 μL) were added. The mixture was incubated at 42 °C for 2 min before Superscript II reverse transcriptase (Invitrogen; 1 μL) was added (total reaction volume of 20 μL). Lastly, the reaction was incubated at 42 °C for 50 min and then 72 °C for 15 min.

The cDNA template was diluted 5× and 1 μL was added to a 12.5 μL reaction containing 6.25 μL Rotor-Gene SYBR Green PCR master mix (Qiagen) and 200 nM of each gene specific sense and antisense primers for NKA (5′-TACTGCTCGAGGTGTTGTCG; 3′-GCTTCAAGCCAGCTGTATCC) and LDH (5′-AAAATGGTGGTGGAAAGTGC; 3′-TCAGACCATCAGCACT CAGG). Fluorescence was detected using a Rotor-Gene Q Real Time PCR cycler with the following cycling parameters: 95 °C for 5 min followed by 40 cycles of 95 °C for 5 s and 60 °C for 10 s. All reactions were run in duplicate. For the rectal gland, genes were normalised using the NORMA-Gene method (Heckmann et al., 2011). Relative mRNA levels were then calculated using the comparative Ct method (Schmittgen and Livak, 2008).

2.6. Statistics

Two-way repeated measure ANOVAs were used to analyse plasma osmolality, sodium, chloride, urea, and TMAO as well as ammonia and urea excretion while one-way ANOVAs were used to analyse tissue urea and TMAO as well as the qPCR data. The Holm–Sidak post-hoc test was used when significance was observed.

3. Results

3.1. Plasma

In all three treatment groups, mean plasma osmolality at the beginning of the experiment was between 950 and 960 mmol kg^{-1} and the seawater was approximately 910 mmol kg^{-1}. By 6 h, osmolality had decreased significantly to 923 mmol kg^{-1} ± 7 SEM in the low salinity group and it continued to decrease throughout the experiment, ending at around 831 mmol kg^{-1} ± 7 SEM (Two-way RM ANOVA $F_2 = 255.305$, $p < 0.001$; Fig. 1). The mean osmolality of the water for the low salinity group was 636 mmol kg^{-1} ± 25 SEM. In the high salinity group, osmolality increased significantly to 996 mmol kg^{-1} ± 8 SEM by 6 h and continued to increase to a value of 1083 mmol kg^{-1} ± 8 SEM at 24 h (Fig. 1). The mean osmolality of the water for this group was 1182 mmol kg^{-1} ± 25 SEM. Plasma osmolality in the control group remained constant throughout the experiment and the mean water osmolality was 933 mmol kg^{-1} ± 7 SEM.

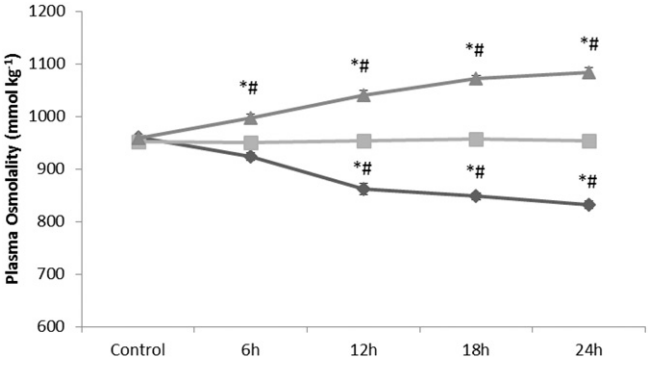

Fig. 1. Plasma osmolality (mmol kg^{-1}) for *Squalus suckleyi* in 70% seawater (♦), 100% seawater (■), and 130% seawater (▲). Data presented is means ± SEM. * indicates significant difference from the 100% seawater group and # indicates significant difference from the control value within a treatment (Two-way RM ANOVA; n = 6; $p < 0.001$).

Plasma urea did not differ significantly from the time zero value at any of the time points within each treatment group. However, there was a trend towards an increase in the 130% seawater group such that at 24 h, this group had significantly higher urea concentrations relative to the 100% and 70% seawater groups (two-way RM ANOVA $F_2 = 7.817$, $p = 0.004$; Fig. 2A). There were also no significant differences within treatment groups for plasma TMAO. However, plasma TMAO in the 70% seawater group decreased enough that at 24 h, it was significantly lower than in the other two treatment groups (two-way RM ANOVA $F_2 = 10.829$, $p = 0.002$; Fig. 2B). By 24 h, plasma sodium was significantly lower than the time zero value within the 70% seawater group and significantly higher than the time zero value within the 130% seawater group. However, only the 70% seawater group was significantly different from the 100% seawater group at this same time point (Two-way RM ANOVA $F_2 = 10.896$, $p < 0.001$; Fig. 2C). As with urea and TMAO, plasma chloride did not change within treatment groups. However, at 24 h, the 130% seawater group had significantly higher plasma chloride relative to the 70% and 100% seawater groups (two-way RM ANOVA $F_2 = 6.682$, $p = 0.006$; Fig. 2D). We also calculated the proportion of the osmolality that each osmolyte accounted for. In the 70% and 100% seawater groups, the percent composition for sodium, chloride, urea, and TMAO remained constant over the 24 h period. In the 130% seawater group, the proportions of urea and TMAO remained constant (~32% and 8% respectively) but those of sodium and chloride increased. At time zero, sodium composed 26% and chloride 19% of the osmolality whereas at 24 h, these increased to 31% and 22%, respectively.

3.2. Muscle and liver

In the liver, there were no significant differences in urea concentrations between the three groups (data not shown), but TMAO was significantly higher in the high salinity group (28 mmol L^{-1} ± 3 SEM) relative to the other two groups (both 19 mmol L^{-1}; One-way ANOVA $F_2 = 3.926$, $p < 0.05$). The high salinity group had significantly greater muscle urea concentrations (463 mmol L^{-1} ± 19 SEM) than both the control (404 mmol L^{-1} ± 14 SEM) and low salinity (361 mmol L^{-1} ± 15 SEM; one-way ANOVA $F_2 = 8.647$, $p = 0.003$) groups whereas muscle TMAO did not change.

3.3. Nitrogen excretion

Urea excretion rates for the low salinity group increased significantly from the control value of 327 μmol N kg^{-1} h^{-1} ± 40 SEM to

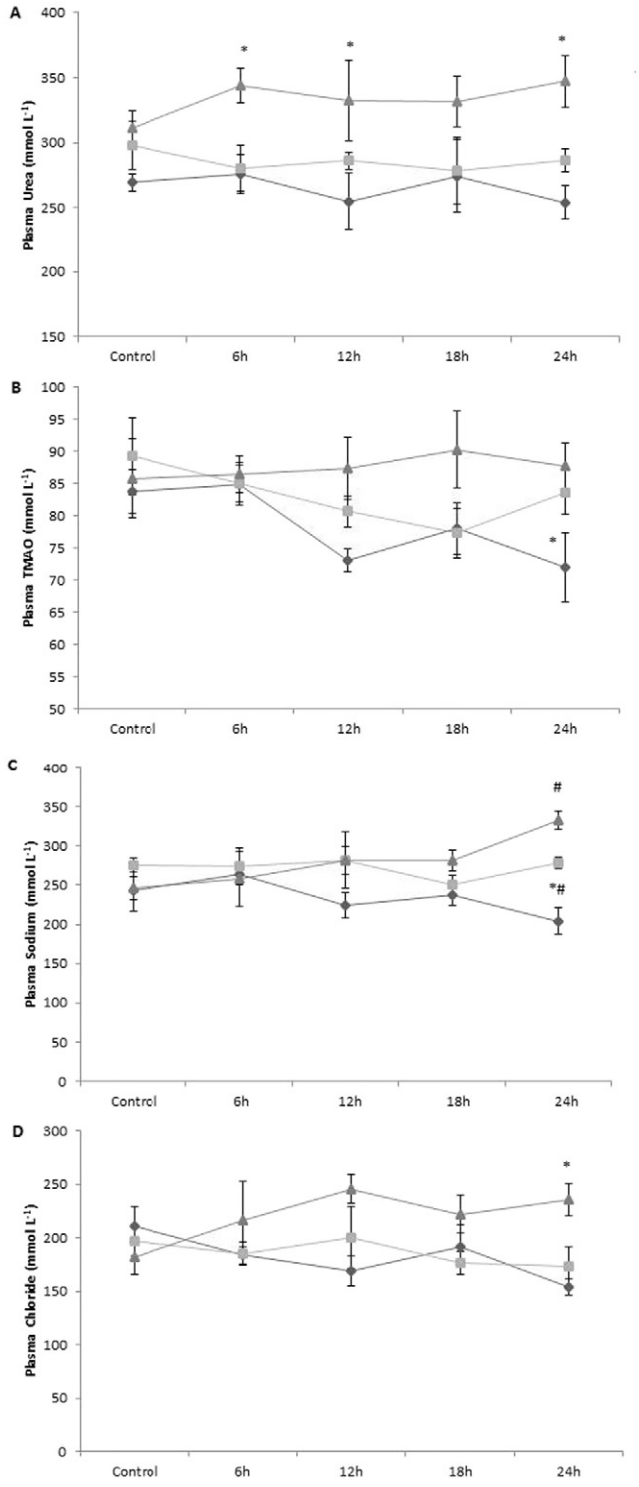

Fig. 2. Plasma urea (A), TMAO (B), sodium (C), and chloride (D) for *Squalus suckleyi* in 70% seawater (♦), 100% seawater (■), and 130% seawater (▲). Data presented is means ± SEM. * indicates significant difference within a time point and # indicates significant difference from the control value within a treatment (Two-way RM ANOVA; n = 6; p < 0.01).

612 μmol N kg^{-1} h^{-1} ± 53 SEM at the end of the experiment, but significant differences could be seen as early as 6 h (Fig. 3A). For the control and high salinity groups, urea excretion also increased gradually such that the rates were significantly higher than the time zero values at 18 h but by 24 h the rates had begun to decrease again, at which point the low salinity group had significantly higher excretion rates than the control and high salinity groups (Fig. 3A). Mean ammonia excretion rate increased

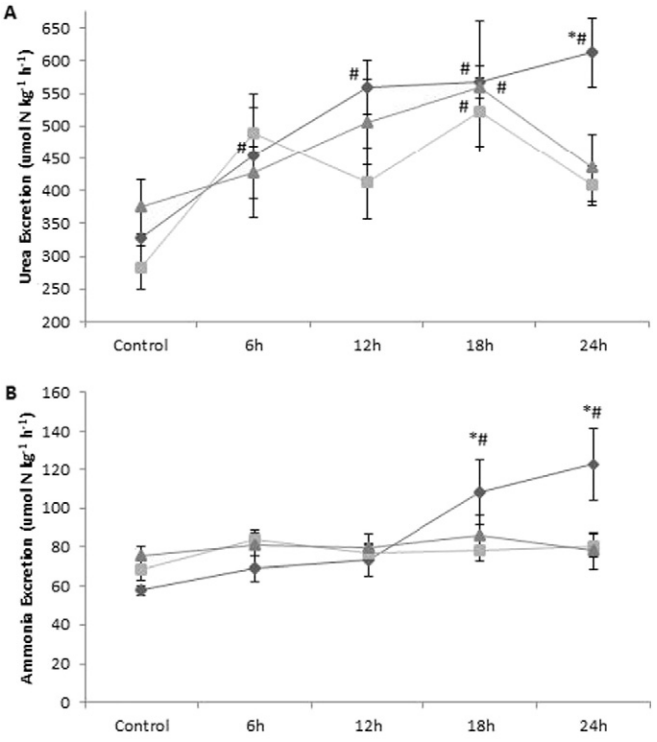

Fig. 3. Urea (A) and ammonia (B) excretion rates for *Squalus suckleyi* in 70% seawater (♦), 100% seawater (■), and 130% seawater (▲). Data are means ± SEM. * indicates significant difference within a time point and # indicates significant difference from the control value within a treatment (Two-way RM ANOVA; n = 6; p < 0.05).

significantly from 57 μmol N kg^{-1} h^{-1} ± 2 SEM to 123 μmol N kg^{-1} h^{-1} ± 18 SEM in the low salinity group and by 18 h was significantly higher than the ammonia excretion rate of the control and high salinity groups whose rates remained relatively constant throughout the experiment (Fig. 3B).

3.4. Enzyme activity and qPCR

In the rectal gland, NKA mRNA levels were significantly higher in the low salinity group compared to the other two groups but there was no change in activity levels (Fig. 4). In contrast, we observed no changes in LDH mRNA levels but LDH activity was significantly lower in the low salinity group (122 μmol min^{-1} g^{-1} ± 8.55 SEM) compared to the control (155 μmol min^{-1} g^{-1} ± 9.29 SEM) and high salinity (157 μmol min^{-1} g^{-1} ± 9.65 SEM) groups (Fig. 5). For GLUTs 1 and 4, there was a trend towards a decrease in mRNA levels with increasing salinity but these changes were not significant (Fig. S1 A,B). However, there were significantly higher levels of both MCTs 1 and 2 in the low salinity group relative to the control and high salinity groups (Fig. S1 C,D).

4. Discussion

As previously mentioned, there has been a bias towards low salinity exposures for marine elasmobranchs because it is more common for them to experience such environments in the wild. Previous studies investigating the effects of hypersaline conditions used long-term exposures and thus were unable to show a time course for adapting to such environments. For our study, we investigated the effects of both elevated and reduced salinity on plasma and tissue osmolytes, nitrogen excretion, and the rectal gland in *S. suckleyi* over a 24 h period to assess the short-term response to osmotic challenges.

We first noted that plasma osmolality increased in the high salinity group and decreased in the low salinity group. It is well known that

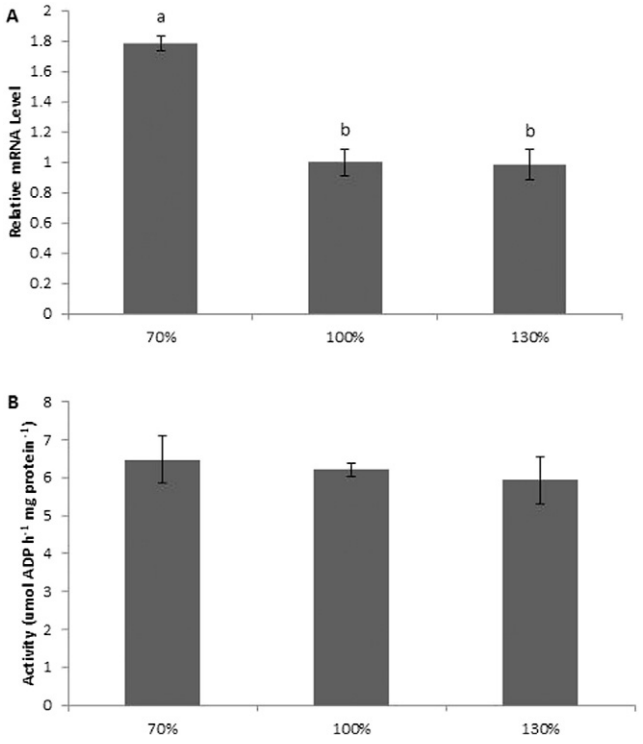

Fig. 4. Na^+/K^+-ATPase mRNA level (A) and enzyme activity (B) for rectal glands of *Squalus suckleyi* in 70%, 100%, and 130% seawater. Data are means ± SEM. Different letters indicate significant difference (one-way ANOVA; n = 6; p < 0.001).

the osmoregulatory strategy of marine elasmobranchs involves retaining high concentrations of plasma urea to maintain a slight hyper-osmolality relative to that of seawater. This causes a slight influx of water that reduces the need to drink. When the external salinity increases however, the plasma is no longer hyperosmotic to the environment, leading to larger salt influxes as well as water efflux. Thus, an increase in plasma osmolality must occur to match or exceed the ambient seawater so as to avoid these problems, which is what we have observed, and our findings concur with studies in *S. canicula* (Hazon and Henderson, 1984; Armour et al., 1993) *C. leucas* (Pillans et al., 2006), and *C. punctatum* (Cramp et al., 2015). In contrast, the dogfish lowered their plasma osmolality at low salinities to prevent the influx of water, something that has been observed previously in *S. suckleyi* (Guffey and Goss, 2014), *S. canicula* (Hazon and Henderson, 1984; Armour et al., 1993), *C. leucas* (Pillans et al., 2005), and *D. sabina* (De Vlaming and Sage, 1973; Piermarini and Evans, 1998; Janech et al., 2006). With the exception of Guffey and Goss (2014), samples were only taken after the 5–7 day acclimation period, not during, so no time course for the changes in osmolality was available. We have shown that the dogfish are able to significantly alter their plasma osmolality within 6 h of being exposed to a new salinity and that these changes begin to taper off by 18 h.

Altering plasma osmolality involves changing the concentrations of a number of solutes in the plasma. We observed a significant increase in plasma urea in the high salinity group, which has also been shown in *S. canicula* (Hazon and Henderson, 1984), but no changes in the low salinity group. Hazon and Henderson (1984) showed that at low salinities, sodium is preferentially retained over urea, which would suggest that a significant decrease in plasma urea would occur, as was the case in most of the studies mentioned previously. Janech et al. (2006), however, did not observe a decrease in plasma urea. In their study, *D. sabina* were only exposed to low salinity for 24 h rather than days or weeks, as was the case in our study. This, along with the lower control urea concentrations observed in our low salinity group (relative to the control and high salinity groups) could explain why we did not see a significant decrease in plasma urea. Another possibility is that urea is being released from the tissues into the plasma as muscle urea was lower in the low salinity group. Furthermore, urea is not the only solute that can change with salinity; plasma TMAO, sodium, and chloride are also of importance. Indeed, we observed a significant decrease in plasma sodium in the low salinity group.

It is interesting that we observed an almost two-fold increase in urea excretion in the low salinity group because we did not observe a large reduction in plasma urea levels. Furthermore, we saw a significant increase in ammonia excretion in the low salinity group, which leads us to believe that urea production had been reduced. In elasmobranchs, most of the ammonia produced from protein catabolism is converted to glutamine and used to produce urea. Therefore, only a very small proportion of nitrogenous wastes are excreted in the form of ammonia (Wood et al., 1995). A two-fold increase in ammonia excretion then, suggests that less ammonia is being shuttled into the urea cycle and since large concentrations of ammonia are toxic, it is being excreted. Thus, although the increase in urea excretion was expected and it agrees with previous studies conducted on *S. suckleyi* (Guffey and Goss, 2014) and *R. erinacea* (Steele et al., 2005), it was not associated with a decrease in plasma urea as predicted.

Gradual but significant increases in urea excretion were also observed in the high salinity group, which was unexpected. Elasmobranchs that are exposed to higher salinities must increase their plasma osmolality as was discussed previously and part of this increase comes from urea. Therefore, it would be logical to reduce urea excretion in order to maintain a higher plasma osmolality. The gills, however, have an outwardly directed gradient by which urea passively flows and it is possible that the increase in plasma urea has led to greater outward diffusion simply based on the increased size of the gradient from the

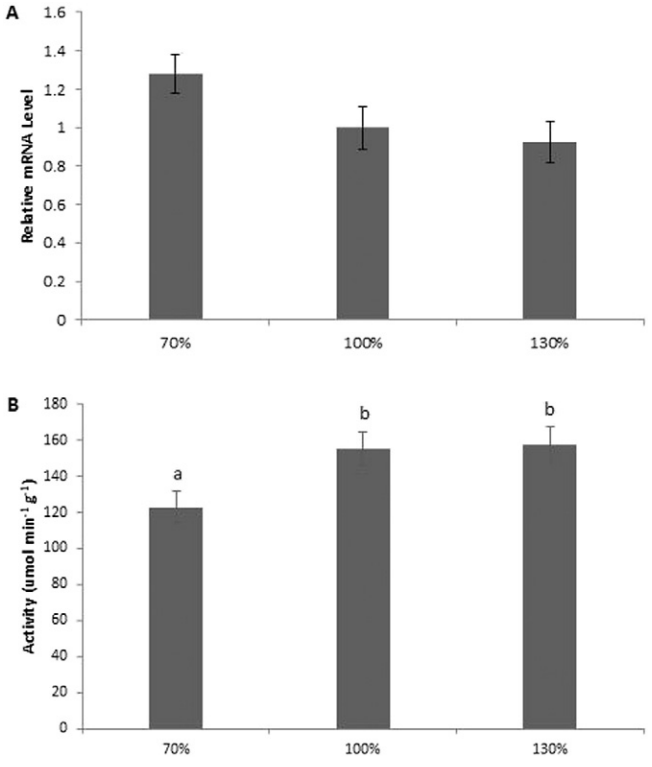

Fig. 5. Lactate dehydrogenase mRNA level (A) and enzyme activity (B) for rectal glands of *Squalus suckleyi* in 70%, 100%, and 130% seawater. Data are means ± SEM. Different letters indicate significant difference (one-way ANOVA; n = 6; p < 0.05).

plasma to the ambient seawater. Furthermore, the greater the urea concentration in the plasma, the more inefficient the renal urea transporter is (Kempton, 1953), which may help to account for the increases in urea excretion observed. In addition, elasmobranchs have been shown to excrete large amounts of urea in response to stress (Evans and Kormanik, 1985), and as increases in urea excretion were also observed in the control group, it is possible that the stress imposed by the water changes or being confined in a box may have been a factor. Elasmobranchs have a unique corticosteroid, 1α-hydroxycorticosterone, which is believed to be their primary stress hormone and has been shown to increase in the plasma in response to salinity challenges (Hazon and Henderson, 1984; Armour et al., 1993) so if this hormone were to increase in response to secondary stressors, it could affect our results. However, there is no direct evidence showing that the hormone is actually released in response to stress (see review by Anderson, 2012) and we observed no changes in plasma glucose throughout our experiment (data not shown). Regardless, it is still interesting that the dogfish were able to significantly increase plasma urea concentrations under hypersaline conditions despite these increases in excretion, as they were not provided with an external source of nitrogen.

A noteworthy point, however, is the difference in the proportion of the osmolality that is comprised by each osmolyte between the different salinities. Hazon and Henderson (1984) showed that at low salinities the percent osmolality that is accounted for by both sodium and chloride increases compared to higher salinities and vice versa for urea and this seems to hold true for the Cramp et al. (2015) study as well, although the urea levels in the latter study were exceptionally high. In contrast, we observed no changes in percent composition of any of the osmolytes measured between the 0 and 24 h time points of the 70% and 100% seawater groups. We also did not observe any changes in urea or TMAO for the 130% seawater group. However, the proportions of sodium and chloride (particularly sodium) did increase in the 130% seawater group relative to both the time zero value and the 24 h values for the 70% and 100% seawater groups. This, along with the lack of increase in the proportion of urea, may indicate that the dogfish were limited in their ability to increase their plasma urea to the levels required at the salinity tested, possibly due to the lack of a dietary nitrogen source, and thus sodium and chloride had to be increased to a greater extent in order to generate plasma that is at least iso-osmotic to the ambient water. This concurs with the study conducted by Armour et al. (1993) in which catsharks (*S. canicula*) fed a low protein diet to hinder urea synthesis showed no ability to increase plasma urea in response to increases in salinity. The percent composition of TMAO did not differ widely between treatments, and no increase in plasma TMAO levels was observed at the high salinity. It is believed that the spiny dogfish lacks the enzyme required to synthesise TMAO and must obtain it from their diet (Baker et al., 1963). Since they had been held without food for some time prior to experimentation, this finding may provide additional evidence for the inability of *S. suckleyi* to synthesise TMAO.

In regards to the rectal gland, we found that mRNA levels of NKA were significantly higher in the low salinity group compared to the other two groups. This could be similar to the findings of Deck et al. (2013) where starved fish had higher levels of NKA than fed fish even though gland activation occurs with feeding (e.g. Walsh et al., 2006). Deck et al. (2013) put forth the notion that the rectal gland stores mRNA for proteins that would be required immediately upon activation. In the wild, exposure to hyposaline conditions is relatively short-lived as behavioural modifications allow a return to full strength seawater and thus the higher levels of mRNA in the low salinity group could signify the rectal gland preparing for this return. We did not, however, see an even greater reduction in NKA mRNA levels in the high salinity group relative to the controls and there were no differences in NKA activity (which is much more indicative of activation) across the three groups, suggesting that the rectal gland may not have been further activated during our experiment. Notably, the rectal gland NKA activity in our control group was approximately as high as the highest NKA activity observed by Walsh et al. (2006) at 6 h post-feeding, and higher than their unfed group (which displayed NKA activities of about 4 μmol ADP h^{-1} mg $protein^{-1}$). If NKA activity can be used as a proxy for gland activity, it is clear that the gland is 'active' in our experimental treatments. It is possible that the short time frame of our experiment, however, did not necessitate an increase in enzyme activity as only modest (although significant) increases in plasma sodium and chloride were observed. On the other hand, since our results suggest that the dogfish are capable of rapidly adapting to a change in salinity, the relevant time frame for increases in NKA activity could have occurred earlier on in the experiment, leading to reduced increases in plasma sodium and chloride. In a study by Piermarini and Evans (2000), higher NKA activity in the rectal glands of seawater acclimated *D. sabina* relative to freshwater rays was reported. This is a fully euryhaline species however, compared to the partially euryhaline dogfish so the osmoregulatory strategies likely vary. In contrast to NKA, we did observe higher activity levels of LDH in the rectal glands of control and high salinity dogfish suggesting that either the gland is more active at these salinities, or that blood or oxygen supply to the gland has diminished. However, since the activity level did not increase between the control and high salinity groups, the salinity may not have been high enough to require rectal gland activation or the relevant time point occurs prior to our first measurement.

We also measured mRNA levels of glucose and monocarboxylate transporters (which transport lactate and ketone bodies) in the rectal gland. Walsh et al. (2006) determined that the gland was glucose-dependent, but could use ketone bodies to augment secretion rates, and that it may be producing excess lactate since LDH activity increased with feeding but there was an inability for the gland to use lactate as a fuel. Since we believed that hypersaline conditions would activate the gland, we hypothesised that both types of transporters would be upregulated at this time. We observed no significant differences in mRNA levels of either GLUTs 1 or 4 although there was a trend towards a decrease with increasing salinity. On the other hand, mRNA levels of both MCTs 1 and 2 were significantly higher in the low salinity group. These findings oppose our predictions but as was previously mentioned for NKA in both this study and the Deck et al. (2013) study, the higher mRNA levels that appear when the gland is expected to be inactive likely indicate mRNA storage in preparation for reactivation of the gland. Indeed, MCT1 is responsible for transporting ketone bodies into cells and higher protein levels are associated with greater oxidative capacity of the tissue, while MCT2 is responsible for transporting lactate out of cells. Decreases in both of these at high salinities suggest that the gland has been activated and requires higher levels of these proteins.

Overall, this study has shown that *S. suckleyi* are capable of rapidly adapting to changes in salinity (within 24 h) as indicated by the positive relationships between salinity and plasma osmolality. They can also increase plasma urea in response to a high salinity exposure, but the increase appears to be limited, possibly due to the lack of dietary nitrogen. On the other hand, the dogfish did not elevate plasma TMAO in response to elevated salinity, suggesting that they lack the enzyme for TMAO synthesis. Furthermore, mRNA data for the rectal gland reiterates the idea of mRNA storage in order to prepare for reactivation of the gland and LDH activity suggests that the gland is less active at low salinities. However, the lack of difference between the control and high salinity groups for both LDH and NKA may indicate that activation of the gland is just not necessary at the salinities tested. Another possibility is that due to the apparent limited ability to increase plasma urea, the fish were retaining any salt that entered in order to help match their plasma osmolality to the ambient seawater. It would be interesting to conduct a similar study using recently fed dogfish to identify whether rectal gland activation is required under those circumstances.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpa.2015.12.004.

Acknowledgements

The authors would like to acknowledge Eric Clelland, the research coordinator at Bamfield Marine Sciences Centre for helping to ensure our experiments ran smoothly, as well as our field assistant Rachel Munger. This research was funded by a NSERC Discovery Grant to PJW who is also supported by the Canada Research Chair Program, a NSF grant (NSF EF-1316113) to BAS, and a JEB Travel Grant to ABB.

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