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Physiological Adaptations of Gray Tiger Salamander Larvae (*Ambystoma tigrinum diaboli*) Inhabiting Saline Wetlands

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Abstract.—**Larval Gray Tiger Salamanders (*Ambystoma tigrinum diaboli*) that inhabit saline ponds in the Prairie Pothole Region of Canada provided us the opportunity to study the osmotic strategy used by amphibians to survive in elevated salinity environments. To better understand the osmotic and metabolic challenges these larvae face, we investigated the effect of environmental salinity on their nitrogenous waste production and metabolic function. In June 2018, we collected larvae from eight ponds with different specific conductivities. We measured and used specific conductivities as an indicator of environmental salinity. We measured nitrogenous waste (ammonia, urea, and total nitrogen) excretion rates, liver citrate synthase activity, and intestine Na^+/K^+ ATPase activity. Our results indicated larval *A. t. diaboli* were ammonotelic, with ammonia comprising 65.5% of nitrogenous waste excretion. We did not find a significant relationship between specific conductivity of the pond environment and ammonia production or liver citrate synthase activity. There was a relationship between pond-specific conductivity and larvae urea production, larvae total nitrogen production, and larvae intestine Na^+/K^+ ATPase activity. Based on the similarities in larval *A. t. diaboli* nitrogenous waste excretion and enzymatic activity across the variation in pond conductivity, these salamanders did not appear osmotically stressed and had acclimated to their saline environment.**

Key Words.—Ammonia, citrate synthase activity, Na^+/K^+ ATPase activity, Prairie Pothole Region, specific conductivity, urea

Introduction

Amphibians are typically associated with freshwater habitats and are generally considered saline intolerant due to their highly permeable skin and inability to maintain ion and water balance while in elevated salinity for prolonged periods of time (Gordon et al. 1961; Balinsky 1981). There are a few exceptional species of amphibian that can survive prolonged periods of time in elevated salinity (Gordon et al. 1961; Gordon 1962; Kirschner et al. 1971). Elevated saline habitats can be characterized as either slightly saline (1,000–3,000 ppm), moderately saline (3,000–10,000 ppm), very saline (10,000–35,000 ppm) or brine (> 35,000 ppm; Swenson and Baldwin 1965). Observations of saline-tolerant amphibians are mostly of adult stages (not juveniles) living in coastal areas influenced by oceanic salt, inland ditches exposed to road deicing salt or secondary salinization, or naturally saline inland lakes and ponds (see Hopkins and Brodie 2015). Saline tolerance appears to be advantageous in the ability to inhabit spaces that other amphibian species cannot, but comes with some limitations. Saline-tolerant amphibian species can withstand the short-term effects of elevated salinity, but the threshold between salinity tolerance and lethality varies depending on the species (Balinsky 1981). Furthermore, previous research has focused on the short-term impact of elevated salinity on amphibians, but the long-term effects have yet to be determined. Understanding long-term effects is necessary to better understand the potential impact of future increases in environmental salinity caused by climate change. Recent studies have focused on the morphological impacts of elevated salinity on amphibians (Alexander et al. 2012; Hua and Pierce 2013; Albecker and McCoy 2017), but the physiological mechanisms by which some amphibians withstand elevated salinity are still poorly understood.

To mitigate the effects of elevated salinity, saline-tolerant amphibians use osmoregulatory tissues such as the skin, gills, and kidneys to regulate ions and water (Gordon et al. 1961; Schmidt-Nielsen and Lee 1962; Uchiyama and Yoshizawa 1992), and forms of nitrogenous waste excretion (Wood et al. 1989). Depending on the species and environment, amphibians may excrete nitrogenous wastes in the form of ammonia, uric acid, or urea. The primary form of nitrogenous waste produced varies, and each waste product used presents different tradeoffs regarding energy and water allocation (Shoemaker et al. 1992; Loong et al. 2002;

Hillman et al. 2009). Ammonia is less costly to produce compared to other forms of nitrogenous waste, but it is toxic in high concentrations and needs to be rapidly diluted in large volumes of water to mitigate its harmful effects (Wright 1995; Moyes and Schulte 2008; Hillman et al. 2009); therefore, ammonotelism (excretion of ammonia and ammonium ions) is commonly seen in adult and larval stages of aquatic species of amphibians (Wood et al. 1989; Boutilier et al. 1992; Loong et al. 2002; Vanni et al. 2017). Uric acid is energetically costly to produce, but requires less water than either urea or ammonia production (Shoemaker et al. 1972; Wright 1995; Moyes and Schulte 2008; Hillman et al. 2009). Urea excretion is more common than either ammonia or uric acid production in terrestrially adapted adult amphibians (Hillman et al. 2009). Although urea also is energetically costly to produce, it requires less water compared to ammonia production (Gordon and Tucker 1965; Wright 1995; Moyes and Schulte 2008; Hillman et al. 2009). For some saline-tolerant species, urea production has an added benefit: the potential to store urea in their plasma and use it as an osmolyte to temporarily survive elevated salinity (McClanahan et al. 1994; Hillman et al. 2009). The use of urea can increase plasma osmolarity above osmolarity of the aquatic environment, thereby allowing external water to enter the skin, avoiding dehydration, and aiding in ion and water balance (Balinsky 1981; McClanahan et al. 1994).

One of the most thoroughly studied marine-tolerant amphibians is the adult Crab-eating Frog (*Fejervarya cancrivora*; Gordon et al. 1961). To survive in brackish Mangrove Swamps in southeastern Asia, adult *F. cancrivora* substitute urea in place of plasma sodium and chloride ions (Gordon et al. 1961). This allows adult *F. cancrivora* to switch from an osmoregulating strategy, used by most freshwater amphibians, to an osmoconforming strategy (Gordon et al. 1961; Gordon and Tucker, 1965), otherwise used only by marine elasmobranchs and coelacanths (Yancey and Somero 1980; Wright 1995), thereby minimizing the cost of water balance and water loss to their surrounding environment (Wright et al. 2004). Few amphibian species are known to use an osmoconforming strategy; however, additional investigations on saline-tolerant species could indicate this strategy is more common than known.

The Gray Tiger Salamander (*Ambystoma tigrinum diaboli*) primarily inhabits freshwater ponds, lakes, and other wetlands (Kirschner et al. 1971; Lannoo et al. 2005); however, laboratory investigations indicate their larvae can tolerate moderate saline environments (Kirschner et al. 1971; Romspert and McClanahan 1981; Gasser and Miller 1986). Larval Blotched Tiger Salamanders (*A. tigrinum melanostictum*) from eastern Washington, USA, produce urea while living in saline aquatic environments (Gasser and Miller 1986), but the association of urea production and development stage or onset of metamorphosis are unknown. Osmoregulatory tissues involved with salt and water balance strategies, the energetic cost of living in saline environments, and the mechanisms(s) larvae use to mitigate elevated salinity have not been examined. We studied the osmotic and metabolic challenges occurring in larval *A. t. diaboli* living in these unusual saline ponds in the Canadian Prairie Pothole Regions (CPPR). The CPPR is comprised of many small lakes ranging from freshwater to brine, and the salt associated with the increased salinity is sodium/magnesium sulfate rather than sodium chloride (Last and Ginn 2005; Wissel et al. 2011). Historically, amphibian saline tolerance is examined in either marine environments or systems impacted by road salts, both of which are predominantly comprised of sodium chloride (Karraker et al. 2008; Libes 2009; Petranka and Francis 2013).

This study has two objectives: to determine whether pond salinity affects the production of urea by larval *A. t. diaboli* as seen in adult *F. cancrivora*, and to determine if energetic costs differ between larvae of *A. t. diaboli* in elevated saline environments and those living in more typical freshwater environments. We hypothesize that environmental salinity would change nitrogenous waste production in larval *A. t. diaboli* living in freshwater versus saline ponds. We predicted that *A. t. diaboli* larvae living in ponds with higher salinity will produce more urea (and potentially less ammonia) than individuals living in ponds with lower salinity. To determine osmotic stress of larval *A. t. diaboli* living in elevated salinity, we measured intestinal Na^+/K^+ ATPase activity, which is the main ionoregulatory enzyme used in osmoregulatory tissues of most animals. To determine energetic costs of larval *A. t. diaboli* living in elevated salinity, we measured liver citrate synthase activity, which is a general indicator of metabolic rate. We also hypothesized that environmental salinity would change larval citrate synthase activity and Na^+/K^+ ATPase activity living in freshwater versus saline ponds; therefore, we predicted that *A. t. diaboli* larvae living in elevated salinity will have higher citrate synthase and Na^+/K^+ ATPase activity compared to freshwater individuals. To investigate this, we determined rates of nitrogenous waste (ammonia, urea, and total nitrogen) production, liver citrate synthase activity, and intestine Na^+/K^+ ATPase activity for individual larvae inhabiting higher salinity and freshwater ponds.

Materials and Methods

Field sampling.—During May and June 2018, we examined the water chemistry of 104 pothole ponds near Saskatoon, Saskatchewan, Canada, for specific conductivity (µS/cm; our proxy for salinity) and the

Table 1. Preliminary pond sampling in Saskatoon, Saskatchewan, Canada. Range and group means ± standard error for pond specific conductivity where larval or adult Gray Tiger Salamanders (*Ambystoma tigrinum diaboli*) were found. We sampled ponds for salamanders using minnow traps, seines and dip nets.

Pond Type	No. Ponds	Specific Conductivity Range (µS/cm)	Mean Specific Conductivity (µS/cm)
All Sampled Ponds	104	137–6,810	1,577.37 ± 127.71
Ponds with Larvae	17	462–4,045	1,554.29 ± 250.44
Ponds with Adults	13	462–3,025	1,506.46 ± 249.84
Ponds with Larvae and Adults	9	462–4,045	1,380.89 ± 308.95

presence of larval *A. t. diaboli* (Table 1). We found *A. t. diaboli* larvae in 17 ponds, and chose eight ponds for this study (ponds A through H; Table 2). From each pond, we used a YSI (ProPlus Multiparameter Meter, Yellow Springs, Ohio, USA) to measure specific conductivity; we collected all measurements at depths ranging from 0 to 1 m. In June 2018, we collected larvae from these eight ponds using a variety of methods, including minnow traps, dip-netting, and seining (Chalmers and Droege 2002; Werner et al. 2007; Nowakowski and Maerz 2009).

Excretion trials.—To determine nitrogenous waste excretion rates, we initiated excretion trials within 5 min of larvae collection from nets or traps (Burggren and Warburton 2007). We conducted excretion trials on 74 larvae: 10 larvae from most ponds, with the exceptions of ponds E (nine larvae used) and F (five larvae used). When collected, we placed larvae into an 18.9 L container filled with pond water (collected from their respective ponds) that had been filtered through a Whatman pre-combusted, 0.45 µm GF/F, filter (Cytiva, Marlborough, Massachusetts, USA). We then gently transferred larvae into individual 500 ml plastic excretion containers filled with 100–150 ml of filtered pond water. After transfer to excretion containers, we gave larvae 5 min to acclimate prior to initial water sample collection. We then collected two, 1 ml water samples from each container: an initial 1 ml water sample (time 0 min), and another after 30 min (Whiles et al. 2009). Following removal of each 1 ml sample, we added 1 ml of filtered pond water back to the excretion container to maintain the original volume of the water. We froze and stored water samples on dry ice for later analysis.

We used 0 min and 30 min water samples from excretion trials to determine larvae ammonia and urea excretion rates. We ran ammonia assays in duplicate using the Bower and Holm-Hansen (1980) salicylate/hypochlorite method. We processed microplates in the dark at 25° C for 1 h, then read at 595 nm on a microplate spectrophotometer (SPECTRAmax 384, Molecular Devices Corp., Sunnyvale, California, USA). Ammonia concentrations were calculated as mass-specific excretion rates and were expressed as N-µM g^{-1} h^{-1}. We also ran urea assays in duplicate, using the Rahmatullah and Boyde (1980) deproteinization method. We kept microplates in a 92° C bath for 30 min, then read at 540 nm on a microplate spectrophotometer. We also calculated urea concentrations as mass-specific excretion rates and were expressed as 2N-µM g^{-1} h^{-1}.

Tissue collection.—Following excretion trials, we euthanized all larvae using 2 mg/L of Orajel® (Church and Dwight Company, Inc., Ewing, New Jersey, USA; Crook and Whiteman 2006; Cecala et al. 2007) suspended into excretion containers. We weighed larvae, rapidly excised the liver and intestine, flash froze

Table 2. Group means for larval Gray Tiger Salamanders (*Ambystoma tigrinum diaboli*) from ponds in the Canadian Prairie Pothole Region of Canada (± standard error) for ammonia, urea, and total nitrogen excretion, liver citrate synthase and intestinal Na^+/K^+ ATPase activity. Ponds are listed in alphabetical order, and not ascending order from lowest specific conductivity to highest specific conductivity. Sample sizes are listed in parentheses. Abbreviations are SC = specific conductivity, AM = ammonia mean, TNM = total nitrogen mean, LCSAM = liver citrate synthase activity mean, INKAAM = intestine Na^+/K^+ ATPase activity mean.

Ponds	SC (µS/cm)	AM (N-µM g^{-1} h^{-1})	Urea Mean (2N-µM g^{-1} h^{-1})	TNM (3N-µM g^{-1} h^{-1})	LCSAM (nmol min^{-1} mg $prot^{-1}$)	INKAAM (µmol ATP mg $prot^{-1}$ $hour^{-1}$)
A	516 ± 3.90	1.31 ± 0.08 (10)	0.22 ± 0.06 (6)	1.42 ± 0.07 (6)	21 ± 5.45 (8)	2.28 ± 0.37 (10)
B	4335 ± 0.30	1.11 ± 0.09 (10)	0.32 ± 0.05 (10)	1.43 ± 0.11 (10)	21 ± 4.69 (7)	3.32 ± 0.33 (10)
C	1180 ± 1.00	1.50 ± 0.10 (10)	0.98 ± 0.13 (10)	2.47 ± 0.18 (10)	29 ± 6.90 (10)	2.32 ± 0.30 (10)
D	571 ± 12.10	0.95 ± 0.07 (10)	0.27 ± 0.07 (7)	1.19 ± 0.13 (7)	24 ± 4.96 (10)	3.11 ± 0.47 (10)
E	1010 ± 3.50	1.10 ± 0.14 (9)	0.32 ± 0.11 (4)	1.79 ± 0.11 (4)	22 ± 4.50 (8)	1.26 ± 0.32 (9)
F	967 ± 3.10	1.00 ± 0.38 (5)	1.70 ± 0.61 (4)	2.32 ± 0.68 (4)	26.40 ± 15.40 (3)	1.03 ± 0.49 (5)
G	3282 ± 38.00	1.11 ± 0.18 (10)	0.65 ± 0.13 (9)	1.77 ± 0.32 (9)	25 ± 7.17 (10)	2.46 ± 0.43 (10)
H	4020 ± 8.50	1.07 ± 0.16 (10)	0.67 ± 0.14 (9)	1.85 ± 0.22 (9)	25 ± 4.60 (10)	3.32 ± 0.51 (10)
All Ponds	1985 ± 12.41	1.15 ± 0.05 (74)	0.62 ± 0.06 (59)	1.78 ± 0.08 (59)	24 ± 2.08 (66)	2.49 ± 0.16 (74)

tissues in liquid nitrogen, and stored them at -80° C for future analyses.

Enzymatic assays.—We analyzed liver samples in larvae to determine citrate synthase activity, which is an indicator of metabolic rate. We homogenized tissue on ice in imidazole buffer (50mml L-1; pH 7.4) by hand using a ground glass homogenizer. We centrifuged samples for 2 min at 4000× g at 4° C and used the supernatant for enzymatic assays. We measured citrate synthase in 50mmol-1 imidazole buffer, pH 8.0, 0.1 mmol·L-1 DTNB (5,5'dithiobis 2-nitrobenzoic acid), 0.3 mmol·L-1 acetyl CoA, 0.5 mmol·L-1 oxaloacetate (omitted for control). We determined activity of citrate synthase at $\lambda = 412$ nm using DTNB ($\varepsilon_{412} = 13.6$; Singer et al. 1990). We ran samples in duplicate and analyzed them using a microplate spectrophotometer with mean activities expressed per mg of protein. We determined tissue homogenate protein concentration using the Bradford (1976) method. If the gallbladder was pierced during removal of the liver, we did not determine citrate synthase activity.

To analyze Na^+/K^+ ATPase activity in larvae intestines, we homogenized tissue on ice in SEID buffer (150mM sucrose, 10mM EDTA, 50mM imidazole, and 0.1% sodium deoxycholate; pH 7.5) by hand using a ground glass homogenizer. We centrifuged samples for 2 min at 4000× g at 4° C and used the supernatant in a Na^+/K^+ ATPase enzyme assay. We determined Na^+/K^+ ATPase activity at 25° C using a NADH-linked assay (Gibbs and Somero 1989; McCormick 1993). During this process ADP (formed during hydrolysis of ATP by ATPases) is linked to NADH using commercially prepared pyruvate kinase and lactate dehydrogenase. With this method, we monitored the disappearance of NADH (ε_{340}=6.22) in the presence or absence of the Na^+/K^+ ATPase inhibitor ouabain, using a microplate spectrophotometer (Agilent Cary 60 UV-Vis, Agilent Technologies, Inc., Santa Clara, California, USA) at 340 nm. We analyzed samples in duplicate and mean Na^+/K^+ ATPase activity was expressed per mg of homogenate protein. We determined tissue homogenate protein concentration using the Bradford (1976) method.

Statistical analysis.—We conducted Linear Regressions to examine the relationship between pond specific conductivities and each of larvae ammonia, urea and total nitrogen excretion, liver citrate synthase activity, and intestine Na^+/K^+ ATPase activity. Pond-specific conductivity and urea excretion, total nitrogen excretion, and liver citrate synthase activity did not meet normality assumptions (Shapiro Wilks specific conductivity and urea: $W = 0.801$, $P < 0.0001$; specific conductivity and total nitrogen: $W = 0.898$, $P = 0.0001$; specific conductivity and citrate synthase activity: $W = 0.951$, $P = 0.011$). To meet normality assumptions, we log-transformed pond-specific conductivities, urea excretion, and total nitrogen excretion. Log pond-specific conductivity and log liver citrate synthase activity did not meet normality assumptions ($W = 0.752$, $P < 0.0001$), but did meet normality assumptions when citrate synthase activity was square root transformed. We recognized that all relationships may not be linear, and therefore we compared the fit of linear and polynomial relationships using a Likelihood-ratio Test and Akaike Information Criterion (AIC) model selection. We used a linear fit for log pond-specific conductivity and ammonia excretion as well as log pond-specific conductivity and square root liver citrate synthase activity (Likelihood-ratio Test result for log pond-specific conductivity and ammonia ($P = 0.305$) and square root citrate synthase activity ($P = 0.611$); AIC model selection for best ammonia model (linear: AIC weight of 0.52) and best square root citrate synthase activity model (linear: AIC weight of 0.63)). Based on the results of the Likelihood-ratio Test and AIC model selection, we used a quadratic fit for log pond-specific conductivity and log-urea excretion, log-total nitrogen excretion and Na^+/K^+ ATPase activity data (Likelihood-ratio Test result for log pond-specific conductivity and log-urea ($P < 0.001$), log-total nitrogen ($P < 0.001$), and Na^+/K^+ ATPase activity ($P = 0.001$); AIC model selection for best log-urea model (quadratic: AIC weight of 0.72), best log-total nitrogen model (quadratic: AIC weight of 0.72) and best Na^+/K^+ ATPase activity model (quadratic: AIC weight of 0.7228)). We determined significance using $\alpha = 0.05$. We used R version 3.6.0 to conduct all statistical analyses (R Developmental Core Team 2013, Vienna, Austria).

Results

Nitrogenous waste excretion.—The eight ponds we sampled ranged in specific conductivity from 516 to 4,335 µS/cm (about 0 to 2.2 ppt salinity). We did not find salamander larvae in any pond where salinity was > 4,335 µS/cm. All larvae excreted measurable amounts of ammonia over the 30-min sampling period. The mean ± (standard error) larva ammonia production for 74 larvae was 1.15 ± 0.05 N-µM g^{-1} h^{-1} (range, 0.07-2.53 N-µM g^{-1} h^{-1}) across all ponds (Table 2). There was no significant relationship between log pond-specific conductivity and larval *A. t. diaboli* ammonia excretion ($F_{1,72} = 0.267$, $P = 0.607$; Fig. 1). Although all larvae excreted ammonia, 79.7% of individuals excreted both ammonia and urea. Urea excretion was found in individuals of all sizes, with the two highest measured urea excretions produced by smaller individuals from intermediate salinity (967 µS/cm). Mean larva urea excretion rate for 59 larvae was 0.62 ± 0.06 2N-µM g^{-1} h^{-1} (range, 0.03-3.33 2N-

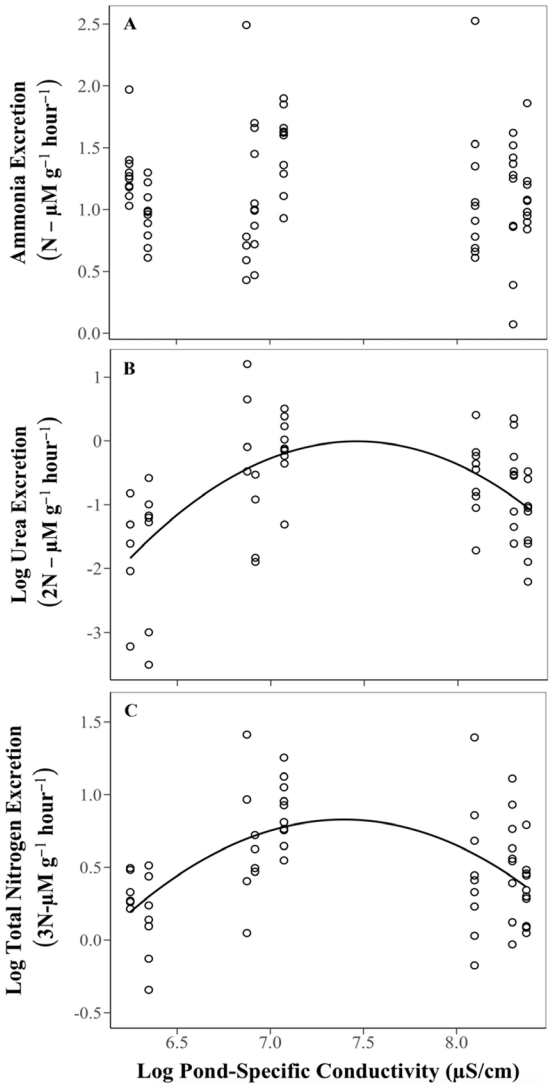

Figure 1. The relationship between salinity, measured as log pond-specific conductivity, and nitrogen waste production in larval Gray Tiger Salamanders (*Ambystoma tigrinum diaboli*) from Canada. (A) ammonia excretion, (B) urea excretion, (C) total nitrogen excretion. Log pond-specific conductivity ($P > 0.05$; Linear Regression) did not predict larvae ammonia. Log pond-specific conductivity did predict larvae log urea excretion ($P < 0.05$; Quadratic Regression) and larvae log total nitrogen excretion ($P < 0.05$; Quadratic Regression), as indicated by the solid line.

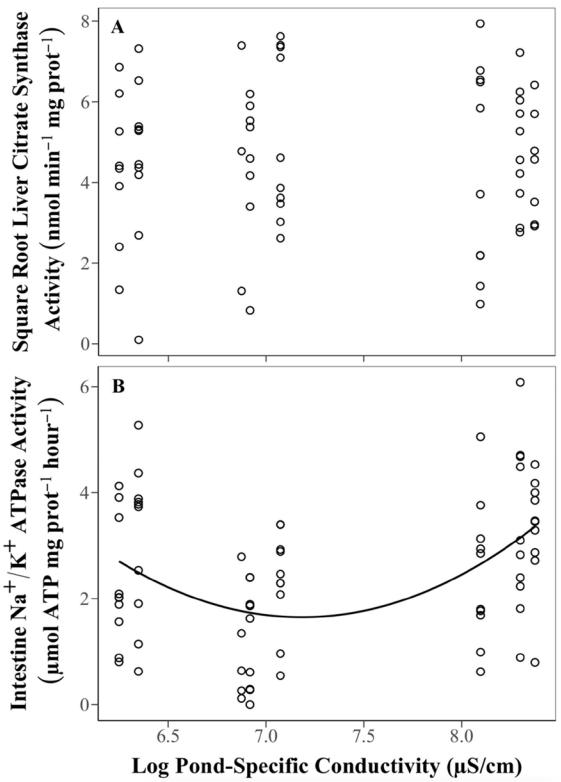

Figure 2. The relationship between salinity, measured as log pond-specific conductivity, and enzymatic activity of larval Gray Tiger Salamanders (*Ambystoma tigrinum diaboli*) from Canada. (A) square root liver citrate synthase activity, (B) intestinal Na^+/K^+ ATPase activity. Log pond-specific conductivity ($P > 0.05$; Linear Regression) did not predict larvae square root liver citrate synthase activity. Log pond-specific conductivity ($P < 0.05$; Quadratic Regression) did predict larvae intestine Na^+/K^+ ATPase activity, as indicated by the solid line ($P < 0.05$).

µM g^{-1} h^{-1}) across all ponds (Table 2). There was a significant quadratic relationship between log pond-specific conductivity and *A. t. diaboli* larva log-urea excretion ($F_{2,56} = 14.22$, $P < 0.0001$; Fig. 1). Of the total nitrogenous waste excretion (sum of ammonia and urea excretion) for all individuals sampled, ammonia represented 65.5% and urea represented 34.5%. Mean larva total nitrogen excreted (59 larvae) was 1.78 ± 0.08 3N-µM g^{-1} h^{-1} (range, 0.71-4.11 3N-µM g^{-1} h^{-1}) across all ponds (Table 2). There was a significant quadratic relationship between log pond-specific conductivity and *A. t. diaboli* larva log total nitrogen excretion ($F_{2,56} = 10.19$, $P < 0.001$; Fig. 1). The highest calculated larvae total nitrogen waste excretions were from individuals in pond F (967 µS/cm) and C (1,180 µS/cm). Pond C had the highest producer of ammonia, whereas Pond F had the highest producer of urea (Table 2).

Enzymatic assay activity.—Larvae from different ponds had similar mean citrate synthase activities. Mean larva citrate synthase activity (66 larvae) was 24.78 ± 2.08 nmol min^{-1} mg $prot^{-1}$ (range, 0.01-63.01 nmol min^{-1} mg $prot^{-1}$) across all ponds (Table 2). There was no significant relationship between log pond-specific conductivity and square root citrate synthase activity in livers of larval *A. t. diaboli* ($F_{1,64} = 0.015$, $P = 0.902$; Fig. 2). Although citrate synthase activity was > 50 nmol min^{-1} mg $prot^{-1}$ (1,180 µS/cm) in several larvae from pond C (Table 2), the highest recorded citrate synthase activity came from a larva in pond G (3,282 µS/cm).

Mean larva intestinal Na^+/K^+ ATPase activity (74 larvae) was 2.49 ± 0.16 µmol ATP mg $prot^{-1}$ $hour^{-1}$ (range, 0-6.08 µmol ATP mg $prot^{-1}$ $hour^{-1}$) across all ponds (Table 2). We detected high intestine Na^+/K^+ ATPase activity in larvae from several ponds. The highest activity (> 5 µmol ATP mg $prot^{-1}$ $hour^{-1}$) was from individuals in ponds D (571 µS/cm), G (3,282 µS/cm) and H (4,021 µS/cm). There was a significant quadratic relationship between log pond-specific conductivity and *A. t. diaboli* larva intestine Na^+/K^+ ATPase activity ($F_{2,71}$ = 8.194, P = 0.001; Fig. 2).

Discussion

Although larval *Ambystoma tigrinum* typically inhabit freshwater wetlands throughout most of the distribution of the species complex, some populations are known to inhabit saline environments, including those in saline ponds the Canadian Prairie Pothole Region, which range from 516 to 4,335 µS/cm. Larvae are consistently exposed to elevated salinity in some ponds in the CPPR, and our data indicates a relationship between urea and total nitrogenous waste excretion of larvae and environmental salinity. Mean urine urea concentrations in metamorphosed adult *A. tigrinum* exhibit a linear increase after a 10-d acclimation to hypersaline solutions (200 mOsm/l to 450 mOsm/l NaCl; Romspert and McClanahan 1981). Unlike Romspert and McClanahan (1981), we observed a quadratic relationship between urea and total nitrogen and environmental salinity. We predicted that larvae urea synthesis would increase with elevated pond salinity, which occurred at low salinities, but this trend did not continue as environmental salinity continued to rise. Although we expected larvae urea levels to continuously increase with salinity, they may not have due to differences in larvae diet or reduced feeding rates between ponds. If larvae feeding rates or protein content of larvae diet were lower in higher salinity ponds, we would expect to see lower urea production. Urea production increases in adult African Clawed Frogs (*Xenopus laevis*) acclimated to elevated salinity, and adult *X. laevis* can switch from an ammonotelic to a ureotelic strategy when salt-stressed (Wood et al. 1989). Unlike Wood et al. (1989), we did not see a switch in nitrogenous waste strategy, from ammonotelism to ureotelism, in larvae *A. t. diaboli.* A possible explanation is that the salinity in these naturally saline ponds is well below the hypersaline solutions (400 mOsmol) experimentally produced by Romspert and McClanahan (1981) and Wood et al. (1989). Although *A. t. melanostictum* can tolerate hypersaline environments (Gasser and Miller 1986), we did not find *A. t. diaboli* in any CPPR ponds with salinity > 4,335 µS/cm. Some ponds used in this study (3,282 µS/cm to 4,335 µS/cm) represent aquatic habitats that are only slightly saline, but they are elevated compared to the traditional freshwater habitats that *A. t. diaboli* larvae and other amphibian species typically inhabit. Further examination of nitrogenous waste in *A. t. diaboli* larvae found in low saline and hypersaline ponds are necessary to observe amphibian osmotic responses occurring at these extremes, which provides a better overall depiction of physiological response to salinity at all levels.

Most larval ammonotelic amphibians excrete more ammonia than urea (Nash and Fankhauser 1959; Moyes and Schulte 2008). Our data on larvae inhabiting natural saline ponds support observations from laboratory studies that larval *A. t. diaboli* are capable of producing both ammonia and urea, and are not exclusively ammonotelic (Kirschner et al. 1971; Stiffler et al. 1980; Gasser and Miller 1986). Ammonia comprises most of the nitrogenous excretion (65%), and urea represents about 35% of the total nitrogenous waste in larval *A. t. diaboli* in the Prairie Pothole Region of Canada. Also, some individuals in our study did not excrete urea during the 30-min trial, which is likely due to a bolus of urine released during the holding period just prior to the initial collection. Because urea excretion rates are needed to calculate total nitrogen excretion and % ammonia/urea-N, we could not determine these values for those individuals. Wood et al. (1989) show that urea comprises most of the nitrogenous waste in larvae *A. tigrinum;* however, larvae used by Wood et al. (1989) were larger (95 g) than individuals used in this study (3.1 ± 0.2 g). Milanovich and Hopton (2016) found that unlike most ammonotelic larvae, stream-dwelling Southern Two-lined Salamander (*Eurycea cirrigera*) larvae were ureotelic, in which urea comprised over 75% of their nitrogenous waste. The presence of urea in larval urine indicates that these individuals are synthesizing urea either through ornithine-urea cycle enzymes or through the degradation of uric acid or arginine (Nash and Fankhauser 1959; Wright 1995). Most adult amphibians produce urea through the ornithine-urea cycle (Balinsky et al. 1972; Wright 1995; Loong et al. 2002; Wright et al. 2004). Although these physiological mechanisms have yet to be examined exclusively in larvae, larval *A. t. diaboli* in the CPPR could possess enzymes needed to synthesize urea through the ornithine-urea cycle. Possessing urea synthesizing enzymes prior to metamorphosis and throughout development would be advantageous, because it would enable larvae to survive pond hypersalinity by osmoconforming. Additional sampling of larval *A. t. diaboli* from the CPPR is needed to confirm the presence of ornithine-urea cycle enzymes.

Based on our observations, pond specific conductivity does not appear to have a significant effect on liver citrate synthase activity. In contrast, Peña-Villalobos et al. (2016) report a significant increase in adult female *X. laevis* liver citrate synthase activity

when acclimated to isotonic vs. hypertonic solutions. Larval *A. t. diaboli* inhabiting saline ponds in the CPPR express liver citrate synthase activity similar to that of larvae inhabiting freshwater wetlands. We are uncertain why the citrate synthase activity is not elevated in larval *A. t. diaboli* inhabiting slightly saline ponds in the Prairie Pothole but suggest that the saline ponds we examined are less saline than the hypertonic solutions produced by Peña-Villalobos et al. (2016). It is possible that the larvae living in CPPR slightly saline ponds do not require the same metabolic demand to maintain osmoregulation compared to larvae living in moderate or very saline environments. Furthermore, the range of citrate synthase activity in Prairie Pothole *A. t. diaboli* larvae is large (0.01-63.01 nmol min^{-1} mg $prot^{-1}$). Juvenile Axolotls (*Ambystoma mexicanum*) exhibit citrate synthase activity that varies with temperature and fasting (Irwin et al. 1999); therefore, some of the citrate synthase activity variation could be associated with variation in fasting among larvae: the time since a last meal cannot be determine in larvae collected from ponds. Citrate synthase values in this study are similar to those measured in other fasted ectotherms, Arctic Char (*Salvelinus alpinus*; Bystriansky et al. 2007) and Spotted Lungfish (*Protopterus dolloi*; Frick et al. 2008). Adult anurans are typically used to examine citrate synthase activity in amphibians (Putnam and Bennett 1983; Walsberg et al. 1986), rather than examining and comparing activity between adults and larvae. More data are needed, especially from other saline tolerant amphibian species, to help us understand how amphibians respond metabolically to elevated salinity.

Traditionally, intestinal Na^+/K^+ ATPase studies focus on changes in ion transport in euryhaline fish as they move between freshwater and saltwater environments (Jampol and Epstein 1970; Pillans et al. 2005; Chourasia et al. 2018; Vargas-Lagos et al. 2018), while saline tolerant amphibian species are largely ignored. Although similar studies have yet to be conducted on saline tolerant amphibians, non-amphibian studies provide potential insight into the changes that could occur in amphibian intestinal tissues, if they were to inhabit saline environments. Jampol and Epstein (1970) report an increase in intestinal Na^+/K^+ ATPase activity in adult freshwater American Eels (*Anguilla rostrata*) acclimated to seawater; whereas, intestine Na^+/K^+ ATPase activity in juvenile Bull Sharks (*Carcharhinus leucas*) does not change between freshwater and marine acclimated individuals (Pillans et al. 2005). Additionally, studies by Chourasia et al. (2018) indicate that intestinal Na^+/K^+ ATPase activity increases in adult Mozambique Tilapia (*Oreochromis mossambicus*), but activity does not change in adult Nile Tilapia (*O. niloticus*) when acclimated to seawater. In freshwater juvenile Atlantic Salmon (*Salmo salar*), Na^+/K^+ ATPase activity is not significantly different between parr and smolt groups or in the hindgut and foregut of the intestines, but is significantly different in the midgut (Vargas-Lagos et al. 2018. Based on these studies, intestinal Na^+/K^+ ATPase activity can vary among environments and species. In this study, we show that log pond-specific conductivity has a significant quadratic effect on intestinal Na^+/K^+ ATPase activity of *A. t. diaboli*. Intestinal Na^+/K^+ ATPase activity is responsible for driving active ion uptake in the intestines, which leads to the passive uptake of water (Chourasia et al. 2018). At low salinities, these amphibians possibly maximize mechanisms for uptaking salts, which would explain the higher larval intestine Na^+/K^+ ATPase activity. At higher salinities, larvae in the CPPR may begin losing water to the environment, so intestine Na^+/K^+ ATPase activity could elevate to maintain ion and water balance. Unlike low and high salinity ponds, intermediate salinity ponds used in our study could represent an ideal salt and water balance environment that allows larvae intestine Na^+/K^+ ATPase activity to subside. The average intestinal Na^+/K^+ ATPase activity of 2.51 ± 0.2 µmol ATP mg $prot^{-1}$ $hour^{-1}$ for larval *A. t. diaboli* in the CPPR is similar to that of terrestrial, adult, aestivating Green Striped Burrowing Frogs (*Cyclorana alboguttata*; Cramp et al. 2009). Future investigations of amphibian saline tolerance should examine Na^+/K^+ ATPase activity. Also, more data regarding larvae enzyme activity throughout development would be beneficial to determine, if and when, activity changes as larvae metamorphose into adult-form individuals (Burggren and Just 1992).

We collected larvae from ponds ranging in specific conductivity from 516 to 4,335 µS/cm. Our pond salinities were similar to the saline lakes in North Dakota and Montana of the U.S. (3,344–4,462 µS/cm), which contain larval tiger salamanders (Held and Peterka 1974; Hossack et al. 2018). We did not find any evidence that *A. t. diaboli* larvae in the Prairie Potholes are stressed osmotically at any of the salinities tested; rather, they appeared normal and healthy. Although we did not find *A. t. diaboli* in ponds with salinity over 4,500 µS/cm, larvae could inhabit hypersaline Prairie Pothole ponds. Therefore, to determine if *A. t. diaboli* can change their osmoconforming/osmoregulating capabilities, future studies should include hypersaline Prairie Pothole ponds and measure additional physiological indicators of osmotic stress (plasma ions). Future studies should also consider using long term data collection (i.e., throughout development) to determine at what environmental osmolarity larvae switch strategies.

Ambystoma tigrinum and other amphibian species can survive elevated saline environments, but the longevity of this tolerance in naturally occurring habitats is still poorly understood. Although this study focuses on the osmotic and metabolic effects of elevated salinity

in naturally saline inland ponds on *A. t. diaboli* larvae, more studies are needed to understand the physiological effects of elevated salinity on lesser-known saline tolerant amphibians. Furthermore, these studies could aid in furthering our understanding of amphibian salinity tolerance, especially as environmental salinities increase due to the effects of climate change. For example, amphibians living in inland saline ponds could be exposed to elevated salinity as the climate warms leading to increased water evaporation rates (Johnson et al. 2005; Werner et al. 2013); whereas amphibians living in coastal areas can be exposed to elevated salinity as sea-levels rise and frequency of storm surges and flooding occurs (Albecker and McCoy 2017). Therefore, the threat of increasing salinity due to climate change likely poses a challenge to all amphibians living in or near saline habitats (Hopkins and Brodie 2015), which may be more saline tolerant than we expect, and possibly better prepared for the uncertain future than what we predict.

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