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Effects of salinity and a glucocorticoid antagonist, RU486, on waterborne aldosterone and corticosterone of northern leopard frog larvae

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ABSTRACT

Increased salinity is an emerging contaminant of concern for aquatic taxa. For amphibians exposed to salinity, there is scarce information about the physiological effects and changes in osmoregulatory hormones such as corticosterone (CORT) and aldosterone (ALDO). Recent studies have quantified effects of salinity on CORT physiology of amphibians based on waterborne hormone collection methods, but much less is known about ALDO in iono- and osmoregulation of amphibians. We re-assayed waterborne hormone samples from a previous study to investigate effects of salinity (sodium chloride, NaCl) and a glucocorticoid receptor antagonist (RU486) on ALDO of northern leopard frog (*Rana pipiens)* larvae. We also investigated relationships between ALDO and CORT. Waterborne ALDO marginally decreased with increasing salinity and was, unexpectedly, positively correlated with baseline and stress-induced waterborne CORT. Importantly, ALDO increased when larvae were exposed to RU486, suggesting that RU486 may also suppress mineralocorticoid receptors or that negative feedback of ALDO is mediated through glucocorticoid receptors. Alternatively, CORT increases with RU486 treatment and might be a substrate for ALDO synthesis, which could account for increases in ALDO with RU486 treatment and the correlation between CORT and ALDO. ALDO was negatively correlated with percent water, such that larvae secreting more ALDO retained less water. Although sample sizes were limited and further validation and studies are warranted, our findings expand our understanding of adrenal steroid responses to salinization in amphibians and proposes new hypotheses regarding the co-regulation of ALDO and CORT.

1. Introduction

Salts are emerging anthropogenic contaminants that can negatively affect the physiology, growth, and survival of freshwater organisms. Increased salinity in freshwater ecosystems has many causes, including the application of road salts as deicers, wastewaters from energy extraction, and encroachment from rising sea levels (Herbert et al., 2015). Sodium chloride (NaCl) is the most common anthropogenic salt, but other salts (e.g., magnesium chloride, $MgCl_2$) can also negatively affect ecosystems (Harless et al., 2011; Hopkins et al., 2013). Salinity can disrupt osmotic homeostasis of freshwater vertebrates and several physiological mechanisms mediate iono- and osmoregulation (e.g., McCormick, 2001; Uchiyama and Konno, 2006). Amphibians are particularly sensitive to salinity, yet some species can be tolerant (Hopkins and Brodie, 2015; Wu et al., 2012). However, investigations into the endocrinological changes involved in tolerance are still limited (reviewed in Hopkins and Brodie, 2015).

The adrenal steroid hormones corticosterone (CORT) and aldosterone (ALDO) are involved in amphibian iono- and osmoregulation in complementary ways (reviewed in Hillyard et al., 2008). For larval amphibians exposed to salinity, increases in CORT secretion are associated with a reduction in resource allocation towards growth and development and increases in water retention; these changes are thought to enhance survival (Bernabò et al., 2013; Hall et al., 2017; Lukens and Wilcoxen, 2020; Tornabene et al., 2021a). By contrast, ALDO stimulates sodium uptake in the kidneys and skin (Hadley and Levine, 2007). While ALDO is not studied as much as CORT in amphibians, ALDO of amphibians often decreases when exposed to saline conditions (e.g., Konno et al., 2005; Lukens and Wilcoxen, 2020), presumably to reduce sodium uptake. CORT actions on target tissues are mediated by mineralocorticoid receptors (MR) at low plasma concentrations but will activate glucocorticoid receptors (GR) at high concentrations during a stress response (Burraco and Gomez-Mestre, 2016; Hall et al., 2020; Hopkins et al., 2016; Tornabene et al., 2021a). While the

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actions of ALDO are thought to be mediated primarily through MR activation, studies have suggested that ALDO may have effects through GR (e.g., Rollins-Smith et al., 1997; Hillyard et al., 2008). However, in amphibians, the roles of CORT and ALDO and their interactions with MR or GR receptors in response to osmoregulatory challenges are still unclear (Hillyard et al., 2008; Tornabene et al., 2021a).

In a previous study focused on larval northern leopard frogs (*Rana pipiens*), sublethal salinity treatments reduced growth and development, but increased water retention (Tornabene et al., 2021a). When GR signaling was blocked using RU486 treatment (a selective GR antagonist), effects of salinity on growth were ameliorated (Tornabene et al., 2021a). CORT of salinity controls (< 32 mg/L Cl) increased or remained stable (stress-induced and baseline, respectively) as larvae grew and developed, but decreased for larvae exposed to 4000 mg/L Cl, revealing that salinity dysregulated CORT physiology (Tornabene et al., 2021a). CORT of larvae surviving salinity treatments was also higher than those that died (Tornabene et al., 2021a). With the aim of better understanding the role of ALDO and relationships between CORT and ALDO for larval amphibians exposed to salinity, we re-assayed waterborne hormone samples from the previous study with three main objectives: (1) To determine the influence of salinity on waterborne ALDO of larval amphibians; (2) To investigate relationships between waterborne CORT and ALDO; and (3) To determine whether blocking GR affected ALDO physiology. Because waterborne hormone sampling is noninvasive and has a longer temporal scope than instantaneous measures (Narayan et al., 2019), this method of hormone measurement is especially useful to understand effects of salinity and RU486 on hormones and receptors. Nevertheless, to our knowledge, no studies have determined whether ALDO can be detected in waterborne hormone samples from larval amphibians.

2. Methods

2.1. Experimental setup

Samples assayed in the present study were collected for a previous investigation of effects of salinity on CORT physiology (Tornabene et al. 2021a). Briefly, larval northern leopard frogs (hereafter, leopard frogs) were collected from wetlands uncontaminated by salinity (< 32 mg/L Cl; the lower limit of QuanTab chloride titration test strips; Hach Co., Loveland, CO). The experiment was a 4 × 2 factorial design and larvae were exposed to one of four levels of salinity (< 32, 200, 1000, and 4000 mg/L Cl) and to RU486 or not (96 μL of 1000 nM RU486; Ledón-Rettig et al., 2009; Tornabene et al., 2021a). Salinity treatments were made from tap water treated with Tetra AquaSafe Plus, to remove chloramines, and pure NaCl (ACS certified > 99% purity). RU486 (also known as mifepristone, SKU M8046, Sigma-Aldrich) is a receptor antagonist that suppresses actions of CORT and is thought to do so by specifically antagonizing GR in amphibian larvae (Crespi and Denver, 2004; Jung-Testas and Baulieu, 1983; Ledón-Rettig et al., 2009).

Twenty replicate larvae were included in each treatment combination and exposed for 24 d. We assayed four random samples from each treatment combination (salinity × RU486) from the last day of the experiment (day 24), when effects of salinity on larval physiology would be maximized. The previous study only collected and quantified CORT of four random samples from each treatment combination per sampling day (Tornabene et al., 2021a). Due to low survival in some groups, we only had two samples from the highest salinity group (4000 mg/L Cl) not exposed to RU486 and no samples from the highest salinity group exposed to RU486. Because of cost limitations and well limitations in EIA plates, we were only able to include 3 samples for < 32, 200, and 1000 mg/L groups for larvae exposed to RU486. Therefore, we included a total of 23 samples in the study. Larvae were euthanized with buffered MS-222 (tricaine methanesulfonate) at the conclusion of the experiment. Larvae were weighed at the end of the experiment to measure wet mass and then freeze dried to determine dry mass and percent water (calculated by dividing the remainder of wet mass minus dry mass by wet mass).

Protocols for animal collection, animal housing, and experiments were approved by the University of Montana Institutional Animal Care and Use Committee (UM IACUC Animal Use Proposal #024-18BHWB-050818 and #003-18BHWB-020618). We collected larvae under U.S. Fish and Wildlife special use permit #61530–18-003 and #62560–18-023; Montana Fish, Wildlife & Parks scientific collection permit #2019–100-W; and North Dakota Game and Fish collection license #GNF04882458.

2.2. Waterborne hormone collection, extraction, and quantification

Waterborne hormone samples were previously collected and concentrated, and CORT was quantified, by Tornabene et al. (2021a) following established methods (Earley and Hsu, 2008; Gabor et al., 2013; Gabor et al., 2016; Tornabene et al., 2021b). Briefly, to collect a baseline sample, larvae were placed in polypropylene containers with 200 mL of fresh respective treatment water for 60 min. To collect a stress-induced sample, larvae were immediately transferred to a new container with fresh respective treatment water, the container and larvae were shaken gently by hand for the first 5 min, and then larvae remained in containers for another 55 min (60 min total; modified from Gabor et al., 2013; Tornabene et al., 2021a). In the previous study, shaking elicited an elevation in CORT: stress-induced CORT was 1.2 × higher than baseline CORT (Tornabene et al., 2021a).

Hormones were concentrated from waterborne samples using solid phase extraction and a solid phase extraction manifold, eluting hormones with methanol, and using an evaporating manifold to concentrate hormone pellets. Hormone pellets were then resuspended in enzyme immunoassay buffer (EIA) and stored at -80 °C for less than three months prior to quantification of ALDO. All samples were resuspended in 4 mL 95% EIA buffer/5% ethanol, which was optimized for CORT but not ALDO quantification in the previous study (Tornabene et al., 2021a). For each sample, we used EIAs to quantify ALDO from waterborne CORT samples (Cayman Chemicals Inc.). Cross reactivity of CORT in ALDO EIAs, and vice versa, are generally low (0.10 and 0.47%, respectively). We followed manufacturer instructions, ran samples in duplicate, and plates were read at 414 nm (5–7 nm bandwidth; Multiskan Ascent, Thermo Fisher Scientific). We multiplied waterborne ALDO values by resuspension volumes and divided by collection time (typically one hour) to yield release rates (pg/h).

2.3. Analytical validation of EIAs

We assessed parallelism, determined quantitative recovery, and documented intra- and inter-assay variation for ALDO EIAs. For parallelism and quantitative recovery, we used pooled samples from Tornabene et al. (2021a) where CORT was previously analytically validated. Pooled samples included 50 or 100 μL of 26 or 100 randomly selected samples for parallelism and recovery, respectively. For parallelism, we used a linear regression of percent ALDO bound against pg/mL concentrations for eight serial dilutions of the standard curve (~15–2000 pg/mL ALDO; 1:2–1:256) and five dilutions of the pooled sample (~100–300 pg/mL ALDO; 1:1.5–1:3). The dilution curve was parallel to the standard curve (comparison of slopes: $F = 0.480$, $p = 0.506$). For recovery, we spiked five aliquots of the pooled sample with one of five EIA standards and ran an unmanipulated pooled sample. Observed values compared to expected values were strongly correlated and linear (slope = 1.41; $F_{1,3} = 745$, $p < 0.001$, $R^2 = 0.995$). Average recovery, calculated as observed values divided by expected values, was 88% (Millikin et al., 2019). Mean intra-assay variation of 2 EIAs with positive controls was 8.22% and inter-assay variation was 11.82%.

Table 1
Summary statistics (β and standard error [SE] in natural-log scale) for linear models estimating effects of salinity (mg/L Cl) and RU486 treatment on waterborne aldosterone of larval northern leopard frogs. Mass was included to account for effects on waterborne aldosterone (pg/h). The reference level ('Intercept') is the salinity and RU486 control (<32 mg/L Cl and not exposed to RU486).

Variable	β	SE	t	p
Intercept	8.29	0.97	8.57	< 0.001
Salinity200	−0.03	0.34	−0.08	0.936
Salinity1000	−0.53	0.36	−1.45	0.166
Salinity4000	−1.23	0.63	−1.96	0.067
RU486	1.06	0.26	4.15	0.001
Mass	−0.45	0.47	−0.95	0.355

2.4. Statistical analyses

We used ordinary least-squares regression to estimate the influence of salinity and RU486 on waterborne ALDO of larvae. We used likelihood ratio tests (package 'lmtest'; Hothorn et al., 2019) to test between additive (salinity + RU486) and interactive models (salinity × RU486). Support for the interactive model would indicate that effects of salinity depended on whether larvae were exposed to RU486 or not. To account for effects of mass on hormone release rates (pg/h; Narayan et al., 2019; Scott et al., 2008), we included wet mass as a covariate in models and log transformed ALDO release rates (Archard et al., 2012; Tornabene et al., 2021a, b). Pooled across salinity treatments, we also separately tested for effects of RU486 treatment on waterborne ALDO using ordinary least squares regression and including mass as a covariate.

We separately tested for influences of wet mass, dry mass, or percent water on ALDO using ordinary least squares regression. We also tested whether larvae exposed to RU486 had different responses than controls (i.e., not exposed to RU486) by including RU486 treatment as a response variable. We included salinity as a covariate to account for possible effects on relationships between ALDO and percent water and dry and wet mass. We used likelihood ratio tests to compare between additive (trait + RU486) and interactive models (trait × RU486) to test if effects were dependent on whether larvae were exposed to RU486.

Because of the complexity and number of interacting predictor variables, compared to the previous models that only compared between additive and interactive models, we used multimodel inference to estimate relationships between waterborne ALDO and waterborne CORT, salinity, and RU486 (package 'MuMIn'; Bartón, 2010). Multimodel inference and AIC allowed us to effectively consider and compare all combinations of, sometimes non-nested, predictor variables. We started with two global linear models (using ordinary least-squares regression) that included baseline or stress-induced waterborne CORT, salinity, and RU486 as interacting predictor variables (i.e., baseline CORT × RU486 × salinity or stress-induced CORT × RU486 × salinity). Mass was fixed and included in all models to account for effects of mass on hormone release rates. For models including baseline or stress-induced waterborne CORT, we separately created sets of all possible sub-models (19 models each for baseline or stress-induced). We used AIC corrected for small sample sizes (AICc) to select the top model or group of models. We included all models with delta AICc (ΔAICc) < 4 and averaged across these estimates weighted according to AIC weight (AIC_W). All analyses were conducted in program R (v4.1.0; R Core Team, 2021).

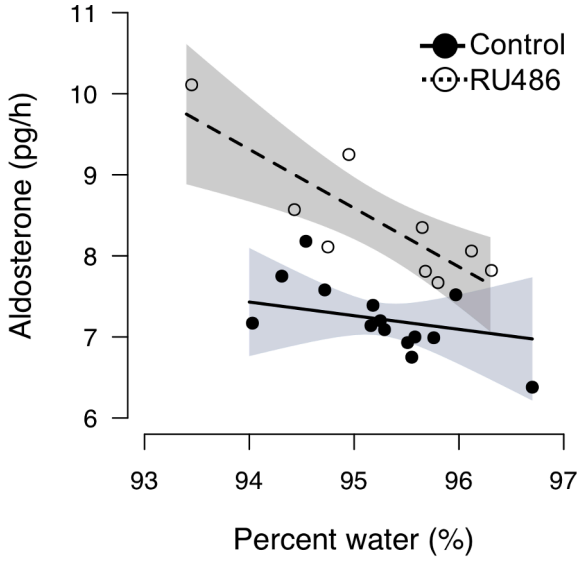

Fig. 2. Aldosterone (pg/h) decreased with increasing percent water for northern leopard frog larvae. Relationships were similar for larvae exposed to RU486 except that aldosterone was higher than for larvae that were not exposed to RU486. Solid points and lines are for larvae not exposed to RU486 (control) and open points and dashed lines are for larvae exposed to RU486. Means and 95% confidence intervals are presented.

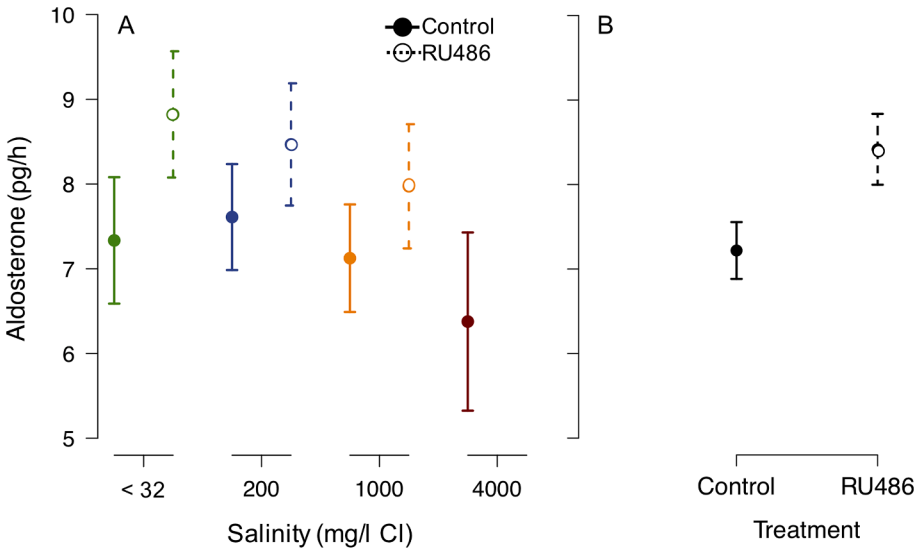

Fig. 1. (A) Interactive effects of salinity and RU486 treatment and (B) effect of RU486 treatment pooled across salinity treatments on waterborne aldosterone (pg/h) of northern leopard frog larvae. In both panels, solid points and lines are for larvae not exposed to RU486 (control) and open points and dashed lines are for larvae exposed to RU486. All larvae exposed to RU486 in the 4000 mg/L Cl treatment died before these waterborne hormone samples were collected. Means and 95% confidence intervals are presented.

Table 2
Summary statistics for likelihood ratio tests of model fit for multiple linear regression models investigating the influence of RU486 and one of three traits (wet mass, dry mass, and percent water) on aldosterone of northern leopard frog larvae comparing between additive (trait + RU486) and interactive models (trait × RU486).

Trait	χ^2	*p*
Wet mass	0.688	0.407
Dry mass	0.568	0.451
Percent water	0.106	0.745

Table 3
Summary statistics (β and standard error [SE] in natural-log scale) for linear models estimating effects of wet mass, dry mass, or percent water and RU486 on aldosterone (pg/h) of northern leopard frog larvae. The reference level ('Intercept') is the salinity and RU486 control (< 32 mg/L Cl and not exposed to RU486).

Trait	Variable	β	SE	*t*	*p*
Wet mass	Intercept	8.29	0.97	8.57	< 0.001
	Wet mass	−0.45	0.47	−0.95	0.355
	RU486	1.06	0.26	4.15	0.001
	Salinity200	−0.03	0.34	−0.08	0.936
	Salinity1000	−0.53	0.36	−1.45	0.166
	Salinity4000	−0.03	0.34	−0.08	0.936
Dry mass	Intercept	6.43	0.71	9.01	< 0.001
	Dry mass	10.54	7.35	1.44	0.169
	RU486	1.13	0.25	4.58	< 0.001
	Salinity200	0.17	0.30	0.57	0.576
	Salinity1000	−0.16	0.32	−0.51	0.619
	Salinity4000	−0.32	0.58	−0.54	0.596
Percent water	Intercept	61.63	11.68	5.28	< 0.001
	Percent water	−0.57	0.12	−4.65	< 0.001
	RU486	1.16	0.17	6.72	< 0.001
	Salinity200	−0.19	0.22	−0.87	0.398
	Salinity1000	−0.41	0.21	−1.94	0.070
	Salinity4000	−0.38	0.34	−1.14	0.270

Table 4
Top-ranked models for relationships between waterborne aldosterone and baseline corticosterone release rates (pg/h) of larval northern leopard frogs. Models are sorted by corrected Akaike information criterion (AIC_c) within each measure of CORT with log likelihood (Log lik.), difference in AICc from the best supported model (ΔAIC_c), and model weights (AIC_w). These models (i.e., those with $\Delta AIC > 4$) were included in the top model set and multimodel inference.

Model	*df*	Log lik.	AIC_c	ΔAIC_c	AIC_w
Baseline + Mass	4	−13.41	37.04	0.00	0.69
Baseline + RU486 + Mass	5	−12.57	38.67	1.62	0.31

3. Results

Larvae exposed to the highest salinity treatment had marginally lower waterborne ALDO (Table 1; $R^2_{adj} = 0.54$). For larvae not exposed to RU486, mean ALDO of larvae in the < 32 mg/L Cl treatment was 1.19 times higher than those in the 4000 mg/L Cl treatment (Fig. 1A). Larvae exposed to salinity and RU486 had 1.06 times higher waterborne ALDO than larvae only exposed to salinity (Fig. 1A). However, pooled across all salinity treatments, larvae exposed to RU486 had 1.16 times higher waterborne ALDO concentrations than those that were not exposed to RU486 ($F_{2,20} = 10.46$, $p < 0.001$, $R^2_{adj} = 0.46$; Fig. 1B). Waterborne ALDO was negatively correlated with percent water ($R^2_{adj} = 0.78$; Fig. 2 and Tables 2 and 3) and weakly, positively correlated with dry mass ($R^2_{adj} = 0.56$), but was not correlated with wet mass (Fig. 2 and Tables 2 and 3). Salinity had little effect on relationships between waterborne ALDO and percent water and dry and wet mass (Tables 2 and 3).

Table 5
Summary statistics and model-averaged coefficients (β and standard error [SE] in natural-log scale) for linear models estimating relationships between waterborne aldosterone and baseline or stress-induced corticosterone and RU486 of larval northern leopard frogs pooled across salinity treatments. Mass was included to account for effects on aldosterone and corticosterone.

Measure	Variable	β	Adj. SE	*z*	*p*
Baseline	Intercept	5.56	0.62	9.01	< 0.001
	Baseline	0.53	0.14	3.89	< 0.001
	RU486	−0.17	0.38	0.46	0.649
	Mass	0.33	0.29	1.11	0.269
Stress-induced	Intercept	5.95	0.70	8.47	< 0.001
	Stress-induced	0.40	0.20	1.99	0.047
	RU486	−1.10	1.64	0.67	0.502
	Stress-induced × RU486	0.64	0.37	1.75	0.081
	Mass	0.16	0.36	0.45	0.651

Waterborne ALDO was positively correlated with waterborne baseline CORT and the association was not dependent on RU486 treatment ($R^2_{adj} = 0.70$; Tables 4 and 5 and Fig. 3A). Stress-induced CORT was also positively correlated with waterborne ALDO ($R^2_{adj} = 0.62$; Tables 5 and 6 and Fig. 3B). Based on model support (log likelihood and AIC; Table 6) and p-values (Table 5), there was weak evidence that the relationship between ALDO and stress-induced CORT was dependent on whether RU486 was applied.

4. Discussion

We quantified ALDO from waterborne samples from larval leopard frogs exposed to increased salinity and a GR antagonist (RU486). ALDO marginally decreased for larvae exposed to the highest salinity treatment. We provide evidence that ALDO production and secretion is affected by GR signaling given that ALDO increased when GR was blocked using RU486. We also found that ALDO was negatively correlated with percent water and positively correlated with CORT. Although sample sizes were limited and further validation and studies are warranted, our findings expand our understanding of the regulation of adrenal steroid responses to salinization in amphibians.

ALDO decreased weakly with increasing salinity in our study, particularly for the highest salinity treatment; this result is similar to previous studies that assayed ALDO from amphibian plasma (e.g., Konno et al., 2005; Lukens and Wilcoxen, 2020). Downregulation of ALDO may occur to reduce retention of sodium when larvae are exposed to salinity and passively take up sodium and chloride (Fournier et al., 2012; Hopkins and Brodie, 2015). Although statistical significance was marginal in our study, the effect size was large based on R^2; this suggests the relationship could be meaningful and further investigation is warranted. Compared to plasma collection methods, waterborne methods may also be less sensitive to small changes in ALDO concentrations. Changes in ALDO may also not be as responsive to salinity as they are to dehydration because of, for example, other changes in ion balance (reviewed in Hillyard et al., 2008). Additionally, receptors could be upregulated in specific tissues compared to upregulating circulating ALDO. Nevertheless, ALDO plays a role in iono- and osmoregulation for larvae exposed to increased salinity based on our finding that larvae excreting more ALDO retained less water. ALDO was also weakly, positively correlated with dry mass and this may be due to larger larvae secreting more ALDO, similar to CORT (Tornabene et al., 2021b).

In our study, we surprisingly found that ALDO increased strongly and consistently with exposure to RU486. This result is similar to effects of MR antagonists (Chong et al., 2017), even though RU486 was previously thought to only antagonize GR in freshwater vertebrates (Jung-Testas and Baulieu, 1983; Garty et al., 1994). There are several potential explanations for this unexpected result (Katsu and Baker, 2018; Sturm et al., 2005). First, *in vitro* studies previously suggested that RU486 could

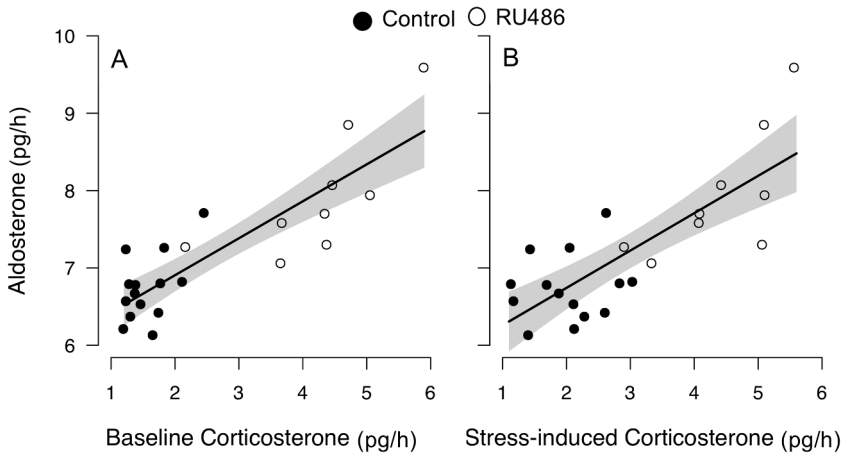

Fig. 3. Waterborne aldosterone (pg/h) was positively correlated with baseline (A) and stress-induced (B) corticosterone (pg/h) of larval northern leopard frogs. Data are pooled across salinity treatments for larvae exposed to one of four salinity treatments (< 32, 200, 1000, or 4000 mg/L Cl). Means and 95% confidence intervals are presented.

Table 6
Top-ranked models for relationships between waterborne aldosterone and stress-induced corticosterone (pg/h) of larval northern leopard frogs. Models are sorted by corrected Akaike information criterion (AIC_c) within each measure of CORT with log likelihood (Log lik.), difference in AICc from the best supported model (ΔAIC_c), and model weights (AIC_w). These models (i.e., those with $\Delta AIC > 4$) were included in the top model set and multimodel inference.

Model	*df*	Log lik.	AIC_c	ΔAIC_c	AIC_w
Stress-induced + Mass	4	−16.89	43.99	0.00	0.68
Stress-induced × RU486 + Mass	6	−14.73	46.70	2.71	0.18
Stress-induced + RU486 + Mass	5	−16.77	47.07	3.08	0.15

antagonize MR receptors in amphibians or that CORT and ALDO could both mediate their effects through GR (Garty et al., 1994; Rollins-Smith et al., 1997; Hillyard et al., 2008). Second, elevations in ALDO could be an indirect effect of the removal of GR-mediated negative feedback on CORT. RU486 treatment also elevated CORT in these larvae (Tornabene et al., 2021a), suggesting that negative feedback on the hypothalamic-pituitary-interrenal (HPI) axis was disrupted. Third, both ALDO and CORT are produced in the adrenal cortex, and ALDO is synthesized from CORT substrate in the presence of aldosterone synthase (CYP11B2; Bassett et al., 2004). Therefore, if more CORT is being synthesized with RU486 treatment, more ALDO synthesis could follow. This mechanism could also explain the positive correlation between CORT and ALDO in our study. Alternatively, although ALDO is primarily regulated by the renin-angiotensin system (Chong et al., 2017), it is possible that the HPI axis could play a direct role in the regulation of ALDO in larval amphibians, as it does in some mammalian genotypes (El Ghorayeb et al., 2016).

The regulation of hormones such as ALDO may allow amphibians to adaptively tolerate saline conditions by mediating effects of salinity, but other interacting hormones are likely involved (e.g., prolactin, arginine vasotocin, and atrial natriuretic peptide; Hopkins et al., 2016; Warburg, 1995). For example, amphibians exposed to increased salinity may also upregulate arginine vasotocin to reabsorb and conserve water (Warburg, 1995; Hillyard et al., 2008). Given the small sample sizes included in this study, studies replicating our experiment with larger sample sizes and quantifying multiple hormones would be beneficial to test our findings. Future studies using transcriptomics to better understand MR and GR receptor expression, investigating steroidogenic enzymes regulating ALDO and CORT synthesis, and analyzing hormonal and interrenal responses to salinity would also be useful to better understand iono- and osmotic regulation in amphibians. Future studies and physiological validation are also warranted to verify that waterborne ALDO reflects endogenous ALDO (e.g., comparing with plasma or whole-body samples). Our findings add to our understanding of relationships between ALDO and CORT across a gradient of salinities and proposes new hypotheses regarding the co-regulation of ALDO and CORT.

CRediT authorship contribution statement

Brian J. Tornabene: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition. **Creagh W. Breuner:** Conceptualization, Methodology, Resources, Supervision, Project administration, Funding acquisition. **Blake R. Hossack:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Erica J. Crespi:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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