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GENERAL WETLAND SCIENCE

Effects of Agricultural Pollutants on Stress Hormones and Viral Infection in Larval Salamanders

Drew R. Davis[1,2] · Katie J. Ferguson[1] · Matthew S. Schwarz[3] · Jacob L. Kerby[1]

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Abstract

Declines in amphibians are a global problem, influenced by complex local factors. While many factors contribute to these declines, much attention has been focused on the roles of contaminants and pathogens. Throughout eastern South Dakota, row-crop farming has contributed to habitat degradation for many amphibians, often through increases in environmental contaminants. For two years we visited four wetlands (two reference wetlands, two agricultural wetlands) to measure water-borne corticosterone (CORT) release rates and ranavirus in larval Western Tiger Salamander (*Ambystoma mavortium*). We found that both water-borne CORT release rates and ranavirus infection load were greater in larval salamanders from agricultural wetlands compared to reference wetlands. We also found that water-borne CORT release rates were greater in ranavirus-infected individuals compared to uninfected individuals and that water-borne CORT is positively correlated with ranavirus infection load. Though the causal relationships among contaminants, CORT, and ranavirus infection are difficult to determine, chronically elevated CORT is known to be immunosuppressive and may result in high infection loads. This study further describes the negative effects of crop production on amphibian health, provides the first evidence of ranavirus in South Dakota, and supports the use of water-borne CORT as a biomarker of amphibian population health in row-crop landscapes.

Keywords Agriculture · Amphibian conservation · Contaminants · Corticosterone · Habitat degradation · Ranavirus

Introduction

Declines in biodiversity are a global problem due to numerous complex and interactive causes (Collins and Storfer 2003; Lawler et al. 2006; Wake and Vredenburg 2008). Observed declines in amphibian populations have been attributed to six major factors: habitat loss and degradation, global climate change, commercial exploitation, introduced species, environmental contaminants, and emerging infectious diseases (Collins and Storfer 2003). Both environmental contaminants (Carey and Bryant 1995) and emerging infectious diseases (Daszak et al. 2003) have been well documented to have strong individual and population level effects on amphibians, with some amphibian extinctions being attributed directly to pathogens (Skerratt et al. 2007; Scheele et al. 2019).

Most studies on amphibian declines examine the effects of a single factor at a time and their results can be difficult to apply in a field context because of interactions with other environmental factors (Blaustein et al. 2011; Cohen et al. 2019). While both environmental contaminants and diseases negatively affect amphibians, they are seldom studied in conjunction despite their potential for additive effects. Experimental studies have shown that environmental contaminants can increase the prevalence and virulence of diseases, resulting in higher morbidity and mortality of individuals than initially expected when examining diseases alone (Carey and Bryant 1995; Forson and Storfer 2006; Kerby and Storfer 2009). These studies stress the importance of examining whether environmental contaminants may be exacerbating the effects of diseases on amphibians under natural conditions. Unfortunately, few data exist on the interactive effects of contaminants and disease under field conditions compared to experimental studies.

✉ Drew R. Davis
drew.davis@utrgv.edu

[1] Department of Biology, University of South Dakota, 414 East Clark Street, Vermillion, SD 57069, USA

[2] School of Earth, Environmental, and Marine Sciences, University of Texas Rio Grande Valley, 100 Marine Lab Drive, South Padre Island, TX 78597, USA

[3] South Dakota Field Office, U.S. Fish and Wildlife Service, 420 South Garfield Avenue, #400, Pierre, SD 57501, USA

Agricultural practices such as row crop farming have strong negative effects on amphibians, primarily through the loss or degradation of habitat (Mann et al. 2009; Smith and Sutherland 2014). Throughout much of the Midwestern United States, many agricultural contaminants are present in wetlands and are likely responsible, in part, for regional amphibian declines (Lannoo 1998). Wetlands that directly receive surface or sub-surface water flows from nearby or adjacent row-crop fields, are especially at risk of environmental contaminants. A local factor that is contributing to increased contaminants in wetlands is the continued use and installation of surface drainage ditches and subsurface tile drains (Blann et al. 2009). These practices not only contribute to habitat loss by draining ephemeral wetlands (Wright and Wimberly 2013), but also serve as systems to transport pesticides, nutrients, and other contaminants into wetland habitats (Schwarz et al. 2018). Agricultural contaminants transported into these wetland habitats can consist of pesticides, nutrients, and heavy metals (Schwarz et al. 2018), many of which are known to contribute to amphibian mortality (Bridges and Semlitsch 2000; Davidson 2004) and interact with pathogens to have complex effects on individuals (Forson and Storfer 2006; Kerby and Storfer 2009).

Amphibian population declines have been attributed to pathogens including ranavirus and the fungus *Batrachochytrium dendrobatidis* (*Bd*; Berger et al. 1998; Daszak et al. 1999; Lips et al. 2006), though *Bd* has received considerably more attention from the research community than ranaviruses (Duffus 2009). Ranaviruses (family *Iridoviridae*) are a group of viruses known to infect amphibians, reptiles, and fish (Gray and Chinchar 2015). Amphibians infected with ranaviruses can show both physical (skin sloughing, erythema, lesions, swollen limbs) and behavioral (lack of equilibrium, erratic movements, lethargy) symptoms of infection, often culminating in organ necrosis and massive hemorrhaging (Gray et al. 2009; Miller et al. 2011). Ranavirus infections have been linked to amphibian die-offs throughout North America, including states throughout the Great Plains (Green et al. 2002; Vandenlangenberg et al. 2003; Collins et al. 2004; Davis and Kerby 2016). Previous experimental studies have found that the presence of the herbicide atrazine and the insecticides chlorpyrifos and carbaryl each increased the susceptibility of salamanders to ranavirus infection (Forson and Storfer 2006; Kerby and Storfer 2009; Kerby et al. 2011). Therefore, these common and widely used environmental contaminants may be contributing to increased prevalence rates of ranaviruses and likely, increased infection intensity.

The amphibian immune response to infection is modulated by the hypothalamus–pituitary–interrenal (HPI) axis (Rollins-Smith et al. 2011). Glucocorticoid (GC) stress hormones are typically released within a few minutes of activation of the HPI axis after exposure to stressors (Moore and Ochinik 1994) and exert most of their effects on individuals through rapid secondary messenger cascades, providing fitness-enhancing effects to respond to immediate stressors. Initial releases of corticosterone (CORT), the main amphibian GC, may initially activate the immune system (Dhabhar 2002). However, over longer periods (hours or days) with chronic exposure to a stressor, CORT levels rise, resulting in inhibition of immune cell function and a negative feedback on the HPI, ultimately resulting in decreased growth and delayed development (Sapolsky et al. 2000). When confronted with pathogens, negative effects of chronic CORT can result in increased susceptibility to disease (Rollins-Smith et al. 2011).

In amphibians, elevated CORT can accelerate metamorphosis at the cost of additional immune responses (Denver 2009; Warne et al. 2011). Several studies have examined aspects of the immune and glucocorticoid responses in amphibians to infection with *Bd* and found higher circulating leukocytes (Davis et al. 2010) and elevated urinary CORT profiles in infected individuals (Kindermann et al. 2012). Additionally, tadpoles infected with ranavirus have elevated whole-body CORT, which drives faster metamorphosis at a lower body mass (Warne et al. 2011). Poor condition at metamorphosis and the additional costs of responding to a pathogen can decrease the probability of survival in amphibians (Garner et al. 2009, 2011). In sum, sustained high CORT levels during amphibian development may suppress immune responses to ranaviruses and may increase suboptimal timing of metamorphosis, which eventually contributes to greater likelihood of death.

Populations of amphibians vary in their susceptibility to infection by ranaviruses and this differential response owes to both host- and pathogen-dependent components (Blaustein et al. 2011). Knowledge of the physiological mechanisms that mediate susceptibility to infection may provide insights into why populations vary in infection levels and why some are extirpated and others persist. However, the link between physiology and ranavirus infection is difficult to establish, especially in free-living amphibians (Blaustein et al. 2011).

In this study, we measured the physiological stress and ranavirus infection levels of larval Western Tiger Salamander (*Ambystoma mavortium*) in the row-crop-dominated landscape of eastern South Dakota. Traditional methods of measuring circulating hormone concentrations in aquatic vertebrates involve assaying blood plasma. Here, however, we measured corticosterone (CORT) levels using a recently developed, non-invasive process of measuring water-borne hormone levels using enzyme-immunoassay (EIA) plates (Gabor et al. 2013a). We examined how water-borne CORT and ranavirus infection intensities (measured as viral gene copy number) differ between larval salamanders collected in agricultural wetlands and reference wetlands and investigated whether there is a relationship between water-borne CORT levels and ranavirus infection intensities. We predicted

that water-borne CORT levels and ranavirus infection intensities would be higher in agricultural wetlands compared to reference wetlands and that stress levels should correlate with infection levels. Additionally, we validated our methods by examining the relationship between plasma CORT and water-borne CORT in *A. mavortium*.

Methods

Study Species and Sites

We sampled larval *Ambystoma mavortium* from wetlands in four Waterfowl Production Areas (WPAs), all part of the United States Fish and Wildlife Service's Madison Wetland Management District, in eastern South Dakota in early July 2013 and 2014 (Table 1). We chose *A. mavortium* as a focal species due to the abundance of larvae and known susceptibility to ranavirus infection from geographically proximate surveys (Davis and Kerby 2016). Further, *A. mavortium* is a widespread species, occurring throughout central and western North America, from northern Mexico to southern Canada. With such a widespread distribution, our findings may be applicable for better understanding this species in other localities where similar stressors are present on the landscape. The majority of *A. mavortium* from this region appear to metamorphose annually, with few multi-year larvae or paedomorphic individuals (< 5 individuals out of hundreds sampled) detected in over four years of fieldwork (DRD, unpubl. data). To minimize environmental differences among sites, we chose four spatially proximate (maximum straight-line distance among sites = 16 km) wetlands in northwestern Minnehaha County, South Dakota, USA (Table 1). Two wetland sites, Buffalo Lake WPA and Lost Lake WPA, were designated as reference wetlands as they are well buffered by surrounding native grasslands (>180 m buffer) and do not receive runoff from row-crop fields directly via surface ditches or tile drains. The two remaining wetland sites, Petri II WPA and Voelker II WPA, were designated as agricultural wetlands as they receive both surface runoff and tile drain effluent from adjacent row-crop fields (corn, soybean) with little (<50 m: Petri II WPA) to no (Voelker III WPA) surrounding grassland buffers. Previously collected data at these sites show that contaminant levels at agriculturally-influenced wetlands were significantly greater than those reference wetlands (Schwarz et al. 2018).

Table 1 Wetlands sites sampled for larval *Ambystoma mavortium* in northwestern Minnehaha County, South Dakota, USA in July 2013 and July 2014. All sites are Waterfowl Production Areas (WPAs) managed as part of the USFWS Madison Wetland Management District

Site	Wetland Type	Latitude	Longitude
Buffalo Lake WPA	Reference Wetland	43.82211°N	97.06056°W
Lost Lake WPA	Reference Wetland	43.67731°N	97.05740°W
Petri II WPA	Agricultural Wetland	43.67901°N	97.09344°W
Voelker II WPA	Agricultural Wetland	43.70787°N	97.11245°W

Sampling Protocol

We collected water-borne hormones and tissue samples from larval *A. mavortium* from 4–9 July 2013 and 8–13 July 2014 at each of the field sites mentioned above ($n = 20$ larvae/site/year). Larval salamanders were collected in small numbers with a seine and immediately (< 3 min) placed into individual glass containers ($n = 1$/container) containing 120 ml of reconstituted DI water (R/O Right™) and held for 1 h. This volume of water sufficiently covered the larvae and allowed individuals to rest at the bottom of the container without the need to swim in the water column. Reconstituted DI water was used instead of using water collected from the field site due to concern over high levels of background CORT (CORT present in the water; see Gabor et al. 2018). All hormone samples were collected between 1100 and 1300 h CDT to minimize any effect of circadian variation on CORT (Rovirosa et al. 2005). After the 1 h leaching period, salamanders were removed, and the water sample was poured into sterile plastic sample cups, labeled, and placed in a cooler on ice. Larval salamanders were then weighed, photographed against a standardized grid to obtain snout–vent length (SVL) measurements, and examined for clinical signs of ranavirus infection (e.g., erythema, sloughing skin, swollen limbs). Before each individual salamander was released, we collected a tail clip (ca. 1.5 cm) to screen for ranavirus infection. Tail tissue was stored in 95% ethanol in sterile 1.5-ml microcentrifuge tubes. Nitrile gloves were changed between handling individual salamanders and all equipment (e.g., forceps, scissors) was cleaned with a 10% bleach solution to prevent cross-contamination. Only a single site was sampled each day in order to quickly return water-borne hormone samples and tissue samples to the lab for storage in a freezer at −20 °C (within 2 h). All hormone-collection equipment was cleaned with 95% ethanol and rinsed with DI water between site visits and nitrile gloves were worn throughout the hormone collection period. At the conclusion of hormone sampling, we collected water temperature, pH, and conductivity measurements from wetlands using a YSI 6820 series water quality multimeter (YSI Inc., Yellow Springs, Ohio).

Relationship between Water-Borne and Plasma CORT

To validate water-borne hormone methods, we collected 10 larval *A. mavortium*, all of the approximate same size class (52.7–71.0 mm SVL), from a reference wetland site (Lost Lake WPA) on 22 July 2013. Salamanders were brought to

the University of South Dakota, placed in individual high-density polyethylene plastic containers, and allowed 48 h to acclimate. We then collected water-borne hormone samples and measurements from each individual salamander, similar to methods described above. Immediately after the 1 h leaching period, we then collected ca. 20–25 μl of blood from the caudal vein of each individual using pulled, heparinized capillary tubes. Blood samples were then centrifuged at 3000 × G for 10 min to separate plasma (5 μl) from blood. We stored plasma samples at −80 °C and water-borne hormone samples at −20 °C for less than 4 mo until they were extracted.

Hormone Extraction, Validation, and Analyses

Hormone extraction, validation, and CORT analysis followed similar methods outlined by Gabor et al. (2013a). We primed C18 solid phase extraction columns (Sep-Pak, Waters Corporation, Milford, Massachusetts) by slowly passing 4 ml of HPLC-grade methanol and 4 ml of millipore ultrapure water through them. In order to collect total (free + conjugated) CORT, we then eluted water-borne hormone samples through these primed columns using a vacuum manifold, thereby extracting hormones from the water samples. Hormones were then eluted from these columns into borosilicate test tubes with 4 ml HPLC-grade methanol. Test tubes were then labeled, placed in a 37 °C water bath, and dried under a stream of low-flow nitrogen gas. Prior to hormone assays, we re-suspended dried samples in a 400 μl solution (380 μl EIA buffer +20 μl molecular-grade ethanol).

To obtain free, non-conjugated CORT from the water-borne hormone samples, we extracted hormones following the methods above, but re-suspended dried samples with 200 μl of millipore ultrapure water and 2 ml of diethyl ether. For our plasma samples, we re-suspended 5 μl plasma in a sterile borosilicate test tube with 200 μl of millipore ultrapure water and 2 ml of diethyl ether. All samples were then vortexed for 4 min. Once layers had separated for 2 min, a bath of dry-ice and methanol was used to freeze the water layer, allowing the diethyl ether containing the free CORT to be decanted into a separate vial. This was repeated an additional time to obtain a total volume of 4 ml for both the water-borne and plasma samples that were used in the correlational study. We then placed samples in a 37 °C water bath and dried them under a gentle stream of nitrogen gas. Dried samples of water-borne hormones were re-suspended similar to other water-borne samples from the field (380 μl EIA buffer +20 μl molecular-grade ethanol), and dried plasma samples were re-suspended in a 500-μl solution (475 μl EIA buffer +25 μl molecular-grade ethanol). We then used commercially available EIA plates (#500655, Cayman Chemicals Inc., Ann Arbor, Michigan) to measure CORT levels. All samples were run in duplicate on 96-well plates and read by a fluorescent plate reader set at 415 nm (Epoch Microplate Spectrophotometer, BioTek, Winooski, Vermont).

We also validated the use of CORT EIA kits for *A. mavortium* water-borne hormone samples. We created a pooled control by combining re-suspended samples of ten non-experimental *A. mavortium* (collected during sampling events in July 2013). Two pooled control samples run in duplicate were included on each EIA plate to measure both intra-assay (1.43–19.13%) and inter-assay variation (18.43%). We created a serial dilution of the pooled controls (run in duplicate and diluted 1:2) by constructing a log-logit transformed dilution curve using average percent maximum binding and picogram per milliliter (pg/ml) concentrations for serial dilution samples. The serial dilution curve was parallel to the standard curve for CORT (comparison of slopes: $t = 0.103$, $df = 10$, $p = 0.92$). We also determined the quantitative recovery of the water-extracted CORT by mixing equal volumes of the pooled control samples (diluted 1:4) with each of the eight CORT standards and an unmanipulated pooled control sample. Expected recovery concentrations were based on the known amount of CORT in both the standards and the pooled control sample. The minimum observed recovery was 71.6%. The regression coefficient of the observed vs. expected concentrations of CORT was 0.96, indicating a linear relationship ($F_{1,8} = 819.68$; $r^2 = 0.986$; $p < 0.0001$).

Ranavirus Detection and Quantification

Tissue samples were screened for ranavirus at the Disease Testing Center at the University of South Dakota following previously published methods (Kerby et al. 2011; Davis and Kerby 2016; Davis et al. 2019). DNA was extracted from tissue samples using DNeasy Blood and Tissue Kits (Qiagen, Hilden, Germany), following the provided protocol. All samples were run in triplicate (20 μl reaction volume) and ranavirus presence was determined via quantitative PCR (qPCR; StepOnePlus, Applied Biosystems, Foster City, California). Each plate contained a negative control (water) and a standardized dilution series of gBlocks (Integrated DNA Technologies, Inc., Coralville, Iowa) that contained the target sequence of DNA to use as a standard curve. This method provides an estimate of the number of gene copies present in a sample. Samples were only considered positive (RV+) if: 1) amplification occurred in at least two of three replicates, and 2) the quantity was above 100 (our minimum standard quantity). Samples were rerun if there were two wells with quantities near 100, or if sample values differed by an order of magnitude. Viral gene copies were determined by averaging values from each RV+ well.

Statistical Analyses

Following Gabor et al. (2013a), we multiplied CORT release rates (pg/ml) by the volume of the re-suspension solution (0.4 ml for water-borne CORT, 0.5 ml for plasma CORT) and standardized values by dividing by the SVL of each individual, resulting in CORT release rates units being pg/SVL/h. Even though we corrected for body size (SVL) in CORT release rate values, Millikin et al. (2019) suggested that size still correlated with CORT measures. Therefore, we also directly examined the differences in the SVL of larval *A. mavortium* between years and wetland types using t-tests ($\alpha = 0.05$). A linear regression was used to examine the relationship between free water-borne CORT and free plasma CORT. Both water-borne CORT release rates and ranavirus gene copy number data failed to meet the assumptions of parametric analyses (even after LN-transformation) and were analyzed with non-parametric analyses ($\alpha = 0.05$). We compared water-borne CORT release rates between years (2013, 2014), wetland type (agricultural, reference), and ranavirus infection status (RV+, RV-). We compared ranavirus gene copy number between years and wetland type. Additionally, because values for water-borne CORT release rates and ranavirus gene copy number exist for each sampled individual, we examined the relationship between CORT release rates and ranavirus gene copy number using a linear regression to determine if these two variables are correlated. All analyses were conducted on LN-transformed data in JMP v13.0.0 (SAS Institute Inc., Cary, North Carolina); however, to aid in visualizing the data, we present non-transformed data in the figures.

Results

We found a significant positive relationship between free water-borne CORT and free plasma CORT in larval *Ambystoma mavortium* ($F_{1,9} = 11.84$, $r^2 = 0.60$, $p = 0.009$; Fig. 1), thus validating our methods of water-borne hormone collection. Both water-borne CORT release rates (Mann–Whitney U test: U = 2.54, DF = 1, $p = 0.11$) and ranavirus gene copy number (U = 3.17, DF = 1, $p = 0.08$) were not significantly different between years (2013, 2014) and as such, each was combined across years for further analyses. Water-borne CORT release rates were significantly higher from salamanders collected in agricultural wetlands compared to those from reference wetlands (U = 9.27, DF = 1, $p = 0.002$; Fig. 2a) and were significantly higher in infected (RV+) compared to uninfected (RV-) larval salamanders (U = 10.66, DF = 1, $p = 0.001$; Fig. 2b). Ranavirus infection prevalence was greater than 50% in all sampled wetlands across years (Table 2), and no individuals collected as part of this study showed visible signs of disease. Ranavirus gene copy number was significantly higher in tissue samples from larval salamanders collected from agricultural wetlands compared to those from reference wetlands (U = 25.44, DF = 1, $p < 0.001$; Fig. 3). Additionally, there was a significant positive relationship between water-borne CORT release rates and ranavirus gene copy number ($F_{1,158} = 98.38$, $r^2 = 0.38$, $p < 0.0001$; Fig. 4). We found no difference in the SVL of larval salamanders between year (t-test: t = −0.18, DF = 78, $p = 0.85$; mean ± SE: 2013 = 56.01 mm ± 2.80; 2014 = 58.17 mm ± 1.79) or wetland type (t = 0.26; DF = 78; $p = 0.79$; reference wetlands = 57.45 mm ± 1.30; agricultural wetlands = 58.11 mm ± 2.18).

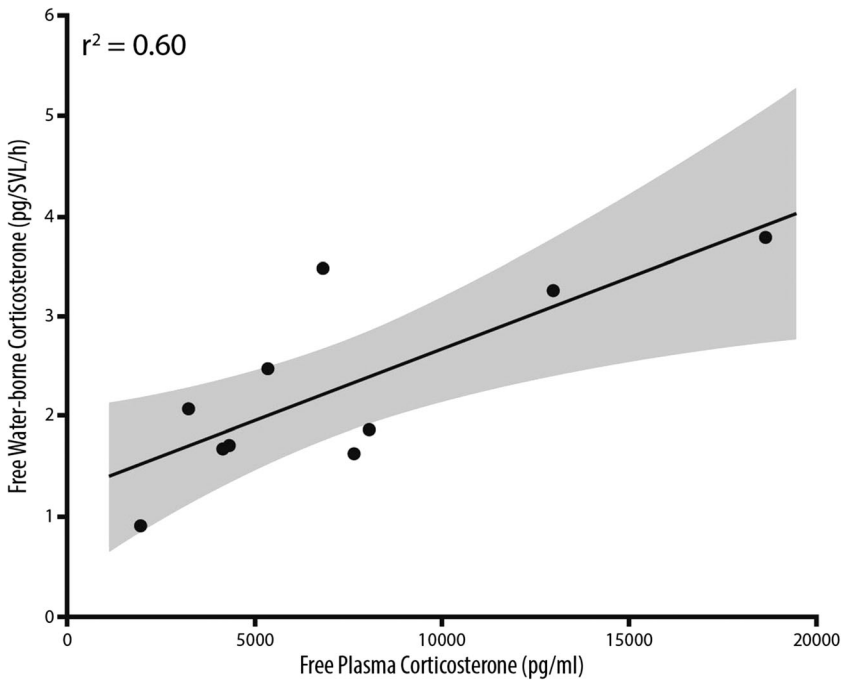

Fig. 1 Significant positive relationship (with 95% confidence interval) between free plasma corticosterone (pg/ml) and free water-borne corticosterone (pg/SVL/h) in larval *Ambystoma mavortium*

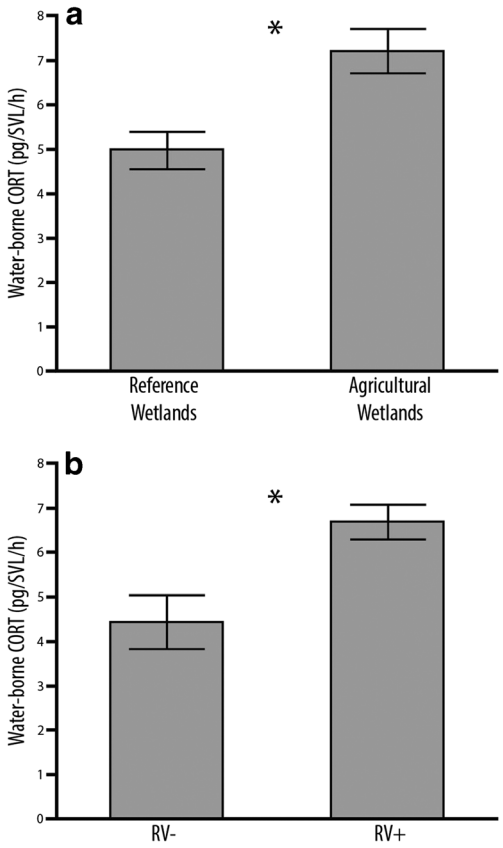

Fig. 2 Mean (± 1 SE) water-borne CORT release rates (pg/SVL/h) of larval *Ambystoma mavortium* between (**a**) individuals from reference and agricultural wetlands and **b** uninfected (RV-) and ranavirus-infected (RV+) individuals. Asterisk indicates significant difference ($\alpha = 0.05$)

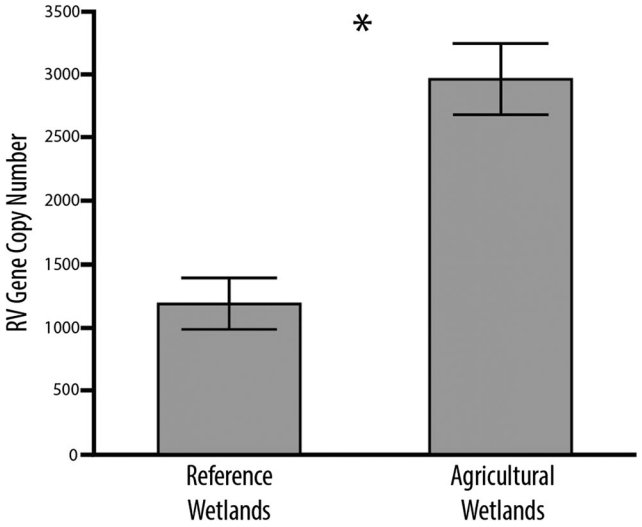

Fig. 3 Mean (± 1 SE) ranavirus gene copy number from tail tissue collected from larval *Ambystoma mavortium* from reference and agricultural wetlands. Asterisk indicates significant difference ($\alpha = 0.05$)

There was no significant difference in water temperature (mean ± SE: reference wetlands = 25.3 °C ± 0.9; agricultural wetlands = 25.4 °C ± 1.3), pH (reference wetlands = 7.61 ± 0.26; agricultural wetlands = 7.95 ± 0.22), and conductivity (reference wetlands = 235.9 μS/cm ± 5.7; agricultural wetlands = 231.6 μS/cm ± 3.9) between wetland types.

Discussion

We measured significantly higher water-borne CORT release rates in *A. mavortium* from agricultural wetlands compared to those in reference wetlands and higher water-borne CORT release rates in ranavirus-infected (RV+) salamanders compared to uninfected (RV-) salamanders. Further, we found a significant positive relationship between water-borne CORT release rates and ranavirus infection intensity (expressed as ranavirus gene copy number). These results matched our initial predictions and follow previously published studies. Additionally, our use of water-borne hormone methods was successfully validated, and water-borne CORT values correlated well with plasma CORT levels, following similar validations from other studies.

Though the exact reason for increased CORT at agricultural wetlands is uncertain, agricultural wetlands are known to have significantly higher levels of pesticides, nutrients, and metals due to surface or subsurface drainage from nearby row-crops compared to reference wetlands (Wauchope 1978; Donald et al. 1999; Blann et al. 2009; Mann et al. 2009; Main et al. 2014). Further, previously collected data on agricultural contaminants from all four sampled wetland sites (Schwarz et al. 2018), indicated elevated levels of nitrates, selenium, and neonicotinoid insecticides in these agricultural wetlands. Several previous studies have also found that environmental contaminants have effects on CORT levels in amphibians (Gendron et al. 1997; Hopkins et al. 1997,

Table 2 Ranavirus prevalence in larval *Ambystoma mavortium* across sites and between years. Ratios are provided as the number of individuals testing positive for ranavirus (RV+) over the total number of individuals examined

Site	Wetland Type	RV+ Prevalence 2013	RV+ Prevalence 2014	Total
Buffalo Lake	Reference Wetland	13/20 (65%)	11/20 (55%)	24/40 (60%)
Lost Lake	Reference Wetland	18/20 (90%)	12/20 (60%)	30/40 (75%)
Petri II	Agricultural Wetland	19/20 (95%)	16/20 (80%)	35/40 (87.5%)
Voelker II	Agricultural Wetland	16/20 (80%)	14/20 (70%)	30/40 (75%)

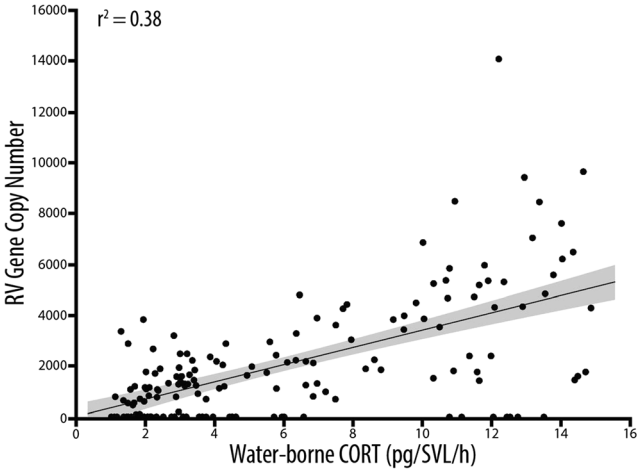

Fig. 4 Significant positive relationship (with 95% confidence interval) between water-borne CORT (pg/SVL/h) and ranavirus gene copy number in larval *Ambystoma mavortium* sampled in 2013 and 2014

1999; Glennemeier and Denver 2001; Hayes et al. 2006; Peterson et al. 2009; McMahon et al. 2011, 2017), though the directionality of response is difficult to determine due to potential for environmental contaminants to influence the HPI responsiveness (the ability for the individual to mount a CORT response; Dickens and Romero 2013).

Other factors such as climate (Narayan et al. 2012; Narayan and Hero 2014), water quality (Barrett et al. 2010; Chambers 2011; Chambers et al. 2013; Gabor et al. 2018), habitat characteristics (Millikin et al. 2019), predators (Narayan et al. 2013; Davis and Gabor 2015), reproduction (Reedy et al. 2014; Aspbury et al. 2017), and prey availability (Reeve et al. 2013) are known to influence CORT levels in amphibians, but none of these factors appear to be a major influence in our system. The proximity of these four sites to one another limits the potential for wetland sites to experience drastically different climate factors from one another and no noticeable differences in water quality parameters were observed between wetland types. Large predatory fish were absent from all four wetland sites; however, Fathead Minnows (*Pimephales promelas*) were found in one of the agricultural wetlands (Petri II WPA) and Brook Sticklebacks (*Culaea inconstans*) were found in one of the reference wetlands (Buffalo Lake WPA). These two fish species may prey upon salamander eggs or small larvae, but larval salamanders quickly grow to sizes where these fish become gape-limited predators. Larval salamanders of the sizes we sampled at these two sites (34.0–68.1 mm SVL) are more likely to function as predators of, rather than prey to, these two fish species (Larson et al. 1999). Further, no larval salamanders that we sampled were sexually mature paedomorphic individuals, and therefore, CORT levels were likely unaffected by reproductive behaviors. Reeve et al. (2013) observed elevated CORT levels in response to food limitation in Wood Frog (*Rana sylvatica*) tadpoles. Agricultural pollution in wetlands is associated with decreased aquatic invertebrate abundance for some species (Euliss and Mushet 1999; Riens et al. 2013; Van Dijk et al. 2013; Schwarz et al. 2018), and elevated CORT from salamanders in agricultural wetlands may be partially due to decreases in prey abundance.

In addition to examining CORT release rates at reference and agricultural wetlands, we also examined whether ranavirus infection status influenced water-borne CORT release rates. We found that RV+ larval salamanders had significantly higher water-borne CORT release rates than RV- salamanders. Our results are in agreement with other studies that found similar relationships between disease and stress-hormone levels (Dhabhar 2002; Davis et al. 2010; Warne et al. 2011; Kindermann et al. 2012; Gabor et al. 2013b). Overall, prevalence of ranavirus among sampled individuals was high across sites and between years, and ranavirus prevalence observed each year in this study (2013: 82.5%; 2014: 66.3%) was higher than that observed in nearby *A. mavortium* populations from Nebraska (37%; Davis and Kerby 2016). No year effect was detected in either water-borne CORT release rates or ranavirus gene copy number. Although ranaviruses are believed to be widespread across both geographic areas and amphibian host species, there are many regions where we know little about ranaviruses, including the midwestern United States (Duffus et al. 2015). The detection of ranavirus in larval *A. mavortium* is the first published report of ranaviruses in South Dakota and helps fill in a region where little is known about these pathogens (Duffus et al. 2015).

High ranavirus prevalence such as what was observed during this study may provide a scenario for which mass-mortality events occur, despite us not observing individuals showing clinical signs of disease. Mass-mortality events of *A. mavortium* have been reported from Colorado, Idaho, North Dakota, Utah, and Wyoming, all of which were attributed to ranavirus (Green et al. 2002). Though the exact triggers for mass-mortality events are poorly understood and difficult to predict, they are believed to be the result of complex interactions between biotic and abiotic factors (Collins and Storfer 2003; North et al. 2015). With such high ranavirus prevalence, if additional stressors are introduced to this system, it may create a scenario where immune function decreases to a point where ranavirus infection results in widespread mortality. Further, with such high ranavirus prevalence, there is concern over human-mediated movement of amphibians and ranavirus (e.g., pathogen pollution; Picco and Collins 2008). The commercial trade and use of bait amphibians, particularly *A. mavortium*, has been identified as a major source of pathogen pollution that may spread ranaviruses around North America, introducing novel strains to regions where virulence and resulting mortality is expected to be greater (Storfer et al. 2007; Picco and Collins 2008).

Given that both water-borne CORT release rates and ranavirus gene copy number were collected from each

sampled individual, we were able to examine the relationship between CORT and infection intensity. We observed a significant positive relationship between water-borne CORT release rates and ranavirus gene copy number. This relationship is the first to be reported for ranavirus and is similar to that reported for *Bd* (Gabor et al. 2013b). Despite the observed positive relationship between CORT and ranavirus infection intensity, it remains unclear whether: 1) the HPI axis is activated and increases CORT in response to ranavirus infection, 2) chronic stressors in this system such as environmental contaminants activate the HPI axis and increase production or CORT, resulting in immunosuppression, which allows ranavirus infection intensity to increase in individuals, or 3) it is some combination of the prior two scenarios. Future experimental studies should seek to address these scenarios.

Regardless of whether elevated CORT drives increasing ranavirus infection intensity or vice-versa, it is clear that these two variables are correlated in field settings. Chronic increases in CORT are known to decrease larval-amphibian growth and accelerate metamorphosis (Hayes et al. 1993; Glennemeier and Denver 2002; Belden et al. 2005), which is especially important given the influence of size at metamorphosis on post-metamorphosis fitness (Earl and Whiteman 2015). Further, increases in CORT are known to delay wound-healing abilities (Thomas and Woodley 2015), affect immunity (reviewed by Rollins-Smith 2017), and decrease survival (Murone et al. 2016). Agricultural wetlands, with elevated levels of contaminants, may be causing chronic stress in larval *A. mavortium*, contributing to higher ranavirus infection intensities that can result in mortality or other negative fitness effects on individuals post-metamorphosis. Further, other amphibians found in these agricultural wetlands (e.g., American Toad [*Anaxyrus americanus*], Boreal Chorus Frog [*Pseudacris maculata*], Northern Leopard Frog [*Rana pipiens*]) may be experiencing similar increases in CORT and ranavirus infection, and their populations may be at risk of regional declines, similar to *A. mavortium*.

The validation of using water-borne methods to measure CORT in *A. mavortium* builds upon information gained from other studies of a growing number of amphibian species in which water-borne hormone techniques have been used with success (Gabor et al. 2013a, b; Reedy et al. 2014; Gabor et al. 2016, 2017; Millikin et al. 2019). We found a significant positive relationship between free plasma CORT and free water-borne CORT ($r^2 = 0.60$), and similar r^2 values have been reported by Gabor et al. (2013a, b). Water-borne hormone methods provide a non-invasive, repeatable method, which can be especially useful when working with small or imperiled taxa or time-series experiments. Further, this study continues to build upon the use of water-borne CORT measurements as a biomarker for population health in field-based studies (Gabor et al. 2018) and helps fill a critically important gap on our understanding of how pathogens and contaminants may interact under field conditions. Given that amphibians rarely face stressors in isolation, it remains important to continue to explore the complexities of multiple stressors, especially in the field, to better understand global declines.

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