



Habitat Characteristics in Created Vernal Pools Impact Spotted Salamander Water-Borne Corticosterone Levels

Alice R. Millikin¹  · Sarah K. Woodley² · Drew R. Davis³ · James T. Anderson¹

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Abstract

Spotted salamanders (*Ambystoma maculatum*) require vernal pools for breeding habitat. Limited protection and preservation of vernal pools makes suitable habitat creation important. Differences in corticosterone levels, a hormone associated with growth, development, and stress in amphibians, could indicate population health and habitat quality. Our objective was to determine if habitat characteristics in created vernal pools influence corticosterone levels of spotted salamander larvae. In May and June of 2015 and 2016, we sampled water-borne corticosterone levels of larval spotted salamanders in 34 created vernal pools constructed 1–5 years earlier. Using multiple regression, we determined the best model predicting corticosterone levels included larval total length, pool-water temperature, year sampled, and pool diameter. Pool-water pH, depth, and age; percent cover; and predator presence were not significant predictors. Annual variation in corticosterone levels and habitat characteristics, and positive associations with water temperature and salamander body size highlighted the importance of controlling for external influences. The negative association between pool diameter and corticosterone indicated that larvae in larger pools (up to 12.75-m maximum diameter) were less stressed and potentially healthier. These results indicate that pool diameter contributes to habitat quality and may be important when constructing vernal pools for spotted salamanders.

Keywords *Ambystoma maculatum* · Caudata · Habitat creation · Hormones · Stress

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✉ Alice R. Millikin
alicemillikin@gmail.com

Sarah K. Woodley
woodleys@duq.edu

Drew R. Davis
drew.davis@utrgv.edu

James T. Anderson
jim.anderson@mail.wvu.edu

¹ School of Natural Resources, West Virginia University, PO Box 6125, Morgantown, WV 26506, USA

² Biological Sciences, Duquesne University, 600 Forbes Avenue, Pittsburgh, PA 15282, USA

³ School of Earth, Environmental, and Marine Sciences, University of Texas Rio Grande Valley, 100 Marine Lab Drive, South Padre Island, TX 78597, USA

Introduction

Vernal pools are seasonal palustrine wetlands that lack permanent, surface-water connections to other bodies of water. These pools provide critical, fishless, breeding habitat for amphibians like spotted salamanders (*Ambystoma maculatum*). Despite wetland mitigation efforts, vernal pools are susceptible to destruction and degradation. Vernal pools are difficult to detect and regulate due to the ephemeral nature of their pool water, and in some cases, small size (Dibello et al. 2016). Under current interpretation of the Clean Water Act, federal regulation and conservation only apply to larger wetlands, i.e., wetlands >0.04 ha, with permanent, surface-water flow to rivers. This leaves vernal pool habitat vulnerable to destruction and makes habitat creation an important conservation tool to reduce the impact of habitat losses.

Created vernal pools have varying levels of success, as measured by reproduction and metamorphosis of pool-breeding amphibians. Habitat characteristics incorporated during construction could determine the success of vernal pool creation. When creating pools, it is difficult to replicate

conditions of natural pools such as hydroperiod length, pool size, pool depth, canopy closure, and vegetation (Calhoun et al. 2014). Habitat quality in created pools is important for spotted salamander reproductive success and completion of metamorphosis for larvae. Permanent hydroperiods in created pools can support predators that would otherwise be excluded, such as eastern newts (*Notophthalmus viridescens*), which can be detrimental to vernal pool amphibians (Vasconcelos and Calhoun 2006; Denton and Richter 2013).

Several habitat characteristics have been associated with increased number of spotted salamander eggs present and their hatching success in lab, mesocosm, and field studies including both natural and created wetlands. These habitat characteristics include larger pool size, higher water pH, deeper water, and longer hydroperiod (Clark 1986; Rowe and Dunson 1993; Petranka et al. 2003a; Skidds et al. 2007). Low pH levels (< 5.5) in pools reduced hatching success, larval growth, development and survival, and a pH \leq 4.0 was lethal (Gosner and Black 1957; Brodman 1993). Egg mass density was positively correlated with forest cover within 1000 m of a pool, with spotted salamanders seemingly avoiding pools in clear-cut forests (Skidds et al. 2007; Felix et al. 2010; Scheffers et al. 2013). Vegetation or cover in a pool is important for oviposition, and also provides refuge for larvae from intra- and interspecific predators and a water temperature gradient (Formanowicz and Bobka 1989; Kern et al. 2013). Pools with more complex vegetation structure have been found to have more spotted salamander egg masses (Egan and Paton 2004). Time since pool construction is also an important factor when examining vernal pool success to determine if the colonizing population in a pool stabilizes or goes extinct over time (Petranka et al. 2003b; Vasconcelos and Calhoun 2006).

Physiological condition of amphibians inhabiting created pools may also be a valuable measure of habitat creation success. In particular, corticosterone (CORT) is a hormone that could be a useful indicator of sublethal impacts and habitat quality (Romero and Wikelski 2001; Homyack 2010; McCormick and Romero 2017). Corticosterone is a glucocorticoid hormone produced by the hypothalamus–pituitary–interrenal (HPI) axis and is related to stress, development, and growth in amphibians. Corticosterone causes behavioral and physiological changes such as increasing available energy to maintain allostasis (McEwin and Wingfield 2003). Chronic stressors can cause prolonged elevation of CORT with harmful consequences that include suppression of the immune system and growth (Wingfield et al. 1998; McEwin and Wingfield 2003; Moore and Jessop 2003; Romero 2004; Belden and Kiesecker 2005). The CORT-fitness hypothesis posits that baseline CORT levels are negatively related to fitness (Bonier et al. 2009).

Multiple amphibian species have shown associations between glucocorticoid hormones and environmental conditions. For example, spotted salamanders had elevated CORT

levels in habitat with anthropogenic disturbance (Homan et al. 2003). Larval spotted salamanders in high intraspecific density environments and juvenile salamanders in low moisture environments had higher levels of CORT (Charbonnier et al. 2018). Jefferson salamander (*Ambystoma jeffersonianum*) larvae in natural pools and mesocosms had higher CORT levels in more acidic environments (Chambers et al. 2013). Northern leopard frog (*Rana pipiens*) tadpoles had higher levels of CORT when there was limited food or high intraspecific density (Glennemeier and Denver 2002). Western spadefoot toad (*Spea hammondi*) tadpoles had increased CORT levels after food deprivation (Crespi and Denver 2005). Adult common toads (*Bufo bufo*) inhabiting plowed soil had higher CORT levels than toads using forest litter or meadows (Janin et al. 2012). Additionally, exposure to traffic noise increased CORT in European tree frogs (*Hyla arborea*; Troianowski et al. 2017).

In the eastern United States, created vernal pools should provide breeding habitat for spotted salamanders. This makes it crucial to understand how created habitats affect spotted salamander larvae and to determine which habitat characteristics should be incorporated during construction. Our objective was to determine if habitat characteristics of created vernal pools influence CORT levels in spotted salamander larvae. This information is needed to improve our understanding of the physiological condition of salamanders colonizing created vernal pools. We hypothesized that larvae would exhibit different levels of CORT in response to variation in pool age, pool-water pH, pool depth, pool diameter, refuge within the pool (pool cover), canopy cover, and predator presence. We chose these habitat characteristics because previous studies have shown that they contribute to habitat quality of spotted salamander breeding sites (Petranka et al. 2003a; Egan and Paton 2004; Skidds et al. 2007). We also examined the influence of larval total length, pool-water temperature, and year of sampling as competing null hypotheses to control for their influence and more accurately assess the effects of habitat quality. Water-borne hormone sampling is influenced by body size, so we had to account for differences in order to make comparisons among salamanders (Scott and Ellis 2007). We expected that larvae with larger bodies would produce higher levels of CORT. We expected that warmer temperatures would result in higher CORT levels because this species is ectothermic and subject to natural changes in CORT due to circadian variation (Cree et al. 2003; Novarro et al. 2018). We also expected that different sampling years would produce differences in CORT because these were different breeding years with different individual salamanders in environments subject to variation including different levels of precipitation and temperature. To quantify CORT, we measured the CORT levels of water that contained an individual larvae for 1 h. This method of measuring water-borne CORT has been used in many aquatic vertebrates as a less-invasive alternative to measuring

plasma CORT (Gabor et al. 2013; Baugh et al. 2018; Charbonnier et al. 2018).

Methods

Study Area

We sampled vernal pools on Cheat Mountain in Monongahela National Forest, WV from 19 May–13 June across two years ($n = 28$ in 2015, $n = 26$ in 2016, including 20 pools that were sampled both years). Only 20 pools sampled the first year had larvae present the second sampling season despite standing water and, in some cases, egg mass remains present. All vernal pools sampled were created by the U.S. Forest Service (USFS) between 2011 and 2014 as part of larger restoration projects. All sampled pools were located within 5 km of each other (Fig. 1). Pools were clustered together by year created: Barton Bench created in 2011, Lambert North created in 2013, and Lambert South created in 2014 (Fig. 1, Table S1). Vernal pool construction, including size and location, was determined by field conditions without predetermined designs or specifications (USFS 2014). During construction of vernal pools, branches and logs were placed in some of the vernal pools. Pools were constructed without liners so the pools would dry in mid to late summer most years. Presence of large green frog (*Rana clamitans*) tadpoles and crayfish (Decapoda) in 56% of pools indicated portions of our sample sites could have permanent hydroperiods, but we did not conduct surveys to confirm this. The monthly average air temperature and total precipitation varied between sample years with total precipitation from July 2014–June 2015 of 135.8 cm and from July 2015–June 2016 of 102.4 cm (Fig. S1 and S2 [The Weather Underground Elkins-Randolph County Station]).

Vernal pools were constructed in areas that had been strip-mined for coal in the 1970s before being acquired by the USFS in the 1980s. Restoration projects were initiated in 2009 and included ripping to mitigate soil compaction; removal of non-native Norway spruce (*Picea abies*) and red pine (*Pinus resinosa*) planted during mining reclamation; and planting seedlings, including aspens (*Populus spp.*), red spruce (*Picea rubens*), black cherry (*Prunus serotina*), wild raisin (*Viburnum nudum*), elderberry (*Sambucus nigra*), and service berry (*Amelanchier arborea*; Sandeno 2011, USFS 2014). The recent removal of non-native trees resulted in predominately open canopy for the vernal pools included in this study.

Field Sampling

Using dipnets and seines, we spent 6–82 min (average 35 min) catching 10 spotted salamander larvae per pool for water-borne hormone sampling (Table S1). Larvae capture typically occurred between 07:00–13:00 h. Upon capture, each larva

was placed in an individual high density polyethylene specimen cup (Dynarex Model 4254) in 20 ml of distilled water premixed with Kent R/O Right Water Conditioner (to prevent osmotic shock). Larvae were removed from specimen cups after 1 h. Larvae were then measured for total length to the nearest mm, processed for a separate genetics study (i.e., tail clipped), and released back to their original pool. The water-borne hormone samples in the specimen cups were frozen within 8 h of collection.

We also collected negative and positive hormone control samples to help assess assay quality (see below). Negative control samples consisted of 20 ml of distilled water premixed with Kent R/O Right Water Conditioner that was never exposed to salamander larvae. Positive control samples consisted of water-borne samples from 30 extra larvae per year. Within each year, the positive control samples were combined, aliquoted into vials of 20 ml and frozen.

We measured pool diameter as the average length of two transects, one extending across the longest distance of pool diameter and a second perpendicular to the first. Each transect measurement extended to the edge of the water on each side of the pool. We used the line intercept method to quantify cover along the same two transects used to measure the diameter of each pool (Barbour et al. 1999; Egan and Paton 2004). Pool cover included any form of refuge such as vegetation, rocks, and coarse woody debris. We measured the length of the transect covered by any cover type to the nearest cm and quantified pool cover as the total length of cover along the two transects in a pool divided by the cumulative length of both transects (Egan and Paton 2004). Canopy cover was measured using a spherical densiometer every 1.5 m along each transect, with 2–3 readings per transect based on pool size. Average pool depth was based on three equally spaced measurements across the longer transect of the pool.

Age of each pool was determined as the sampling year minus the year of pool creation. Water pH and temperature were measured with a PCTestr™ 35 Oakton® Waterproof Multi-Parameter Testr™. Predator presence was based on a rating of presence/absence (1/0) of eastern newt adults, diving beetle (Dytiscidae) larvae, and dragonfly (Odonata) larvae detected during dipnet and seine surveys for spotted salamander capture. A pool could have a score ranging from 0 when no predators were present, to 3 when all three were observed.

Water-Borne Hormone Extraction and Measurement

Samples collected in 2015, totaling 279 individual water-borne hormone samples from 28 pools, were processed in summer 2015 in 10 batches. Samples collected in 2016, totaling 277 individual water-borne hormone samples representing 26 pools, were processed in summer 2016 in 10 batches. Samples were evenly distributed into batches of 36 consisting

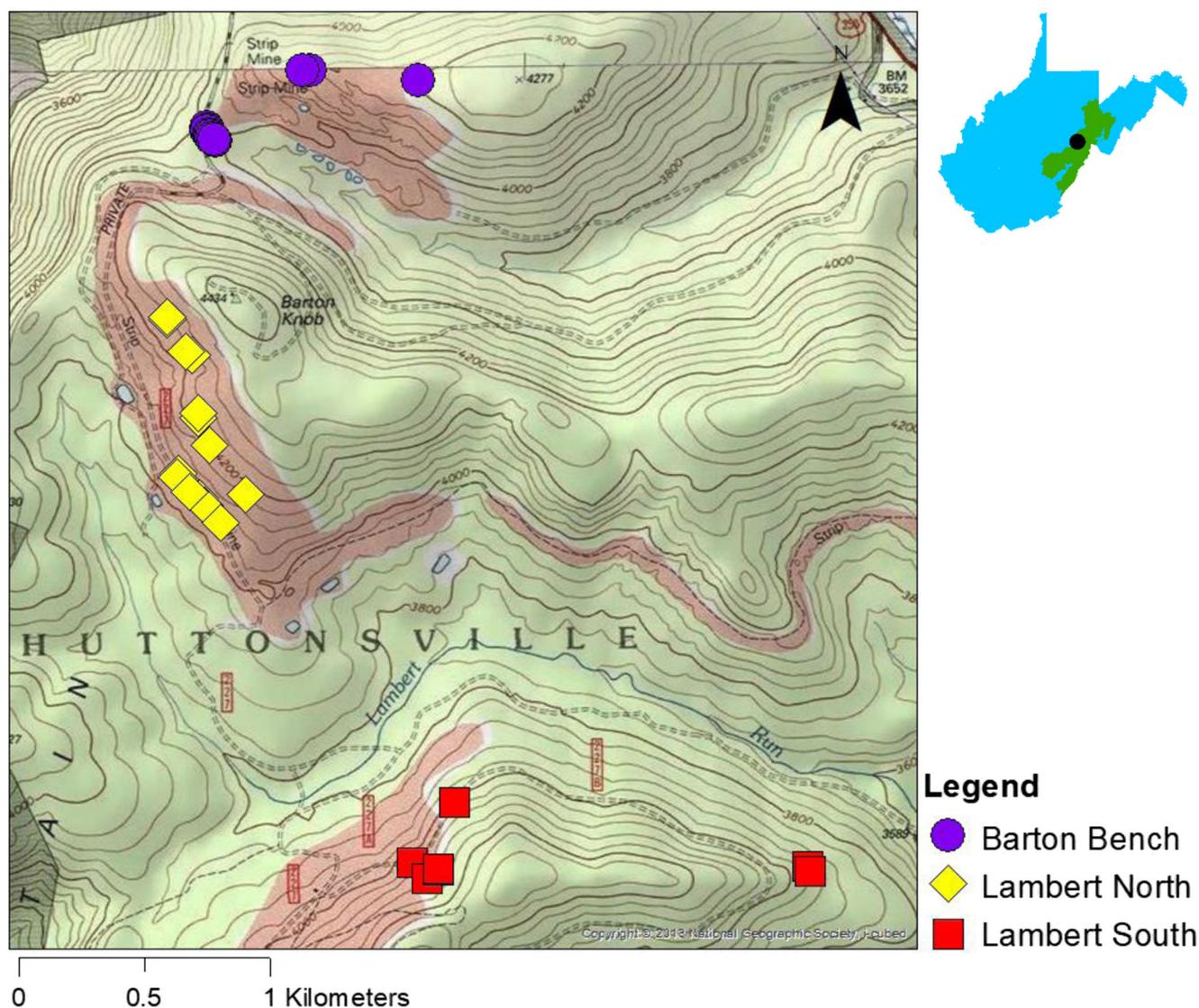


Fig. 1 Topographic map displaying the study area in Randolph County, WV in the Greenbrier District of Monongahela National Forest. Circles, diamonds, and squares represent the locations of created vernal pools that were sampled. West Virginia state map shows the outline of Monongahela

National Forest and general location of the study site marked with a black circle. USA Topo map accessed through ESRI © 2013 National Geographic Society, i-cubed

of 32 hormone samples, two positive controls, and two negative controls for processing.

After thawing, water-borne hormone samples were filtered with Q8 Whatman filter paper to remove suspended particles. Samples were purified and concentrated using solid phase extraction with C18 columns (SPE column, SepPak Vac 3 cc/200 mg; Waters, Inc.). Using vacuum pressure, columns were activated with 4 ml of HPLC-grade methanol and 4 ml of nanopure water. Next, samples and controls were loaded onto columns by pulling each into a 25-ml plastic serological pipette placed on the SPE column opening. Parafilm wax was applied around the pipette tip to provide a tight seal as a vacuum drew the sample into the column. CORT was eluted from the SPE column into glass test tubes using 4 ml of methanol. To evaporate off the methanol, test tubes were placed in

a 42 °C water bath, and a gentle stream of nitrogen gas was blown on the surface of the samples. Samples were checked every 15 min until all methanol had evaporated (about 1 h). Extracted CORT was resuspended in 400 ul of buffer (95% buffer from the ELISA kits [Cayman Chemicals Inc., #501320] and 5% ethanol). Each sample was vortexed for 10 s, covered, and refrigerated overnight or up to two nights until the ELISAs could be completed. If ELISAs could not be completed within 2 d of the solid phase extraction, samples were frozen at −20 °C in the interim.

Corticosterone concentrations were measured in 96-well plates using CORT ELISA kits following standard instructions provided with the kits. Samples were plated in duplicate, evenly distributed across plates, and randomly assigned to wells to control for intra- and inter-assay variation. Each

sample was measured with a plate reader set to 415 nm (Bio-Rad 3550).

Assay Quality Control

The positive control samples included in the plates were used to calculate inter- and intra-assay variation. Inter-assay variation was 30% in 2015 ($n = 10$ plates) and 19% in 2016 ($n = 10$ plates). Intra-assay variation was $14 \pm 11\%$ across 10 assays in 2015 and $4 \pm < 0.0001\%$ across 10 assays in 2016. This range of assay variation is normal for ELISA assays of water-borne hormone samples (Gabor et al. 2013).

Additional positive control samples were used to demonstrate parallelism between the standard curve of the assay and successive dilutions of the samples. The serial dilution curve (based on eight dilutions 1:1 to 1:128 run in duplicate) was not significantly different from the standard curve ($t_{(12)} = 1.26$, $p = 0.23$) demonstrating parallelism of standards and positive control samples.

To measure recovery, a positive control sample was diluted 1:2 and combined in equal parts with each of the eight standards of the ELISA standard curve. These hormone + standard samples were plated in duplicate along with one unaltered positive control sample diluted 1:2. Expected concentration was quantified as: (known CORT concentration in the unaltered pooled sample + concentration of the standard) / 2. We calculated recovery by dividing observed CORT concentration by expected CORT concentration. This measured recovery from the ELISA assay. Recovery averaged 111% and ranged from 64 to 181%. Observed and expected values were linearly related (slope = 1.40, $F_{(1,7)} = 3945$, $R^2 = 0.998$, $p < 0.0001$).

Calculating CORT Release

The amount of CORT measured by the ELISA in pg/ml was multiplied by 0.40 ml (the volume of ELISA buffer used to resuspend the sample). Due to matrix effects (Scott and Ellis 2007), negative control samples often have detectable levels of CORT. The standard procedure is to subtract the amount measured in negative controls from sample values. Average background CORT in negative control samples was calculated separately for plates run in 2015 and 2016. Annual average background CORT of the processing year was subtracted from salamander water-borne hormone samples. Finally, pg values were divided by 1 h.

To determine CORT pg/h/pool, we averaged CORT pg/h of salamanders caught per pool. We excluded individuals with CORT levels too high and too low on the standard curve and below background levels: 9.87% of data (Table S1). This resulted in 30 out of 56 pools with <10 larvae samples: 25 pools sampled in 2015 and 5 pools sampled in 2016. Larval sample

size at a pool ranged from 4 to 15 with an average of 9.11 (average 7.93 in 2015 and 10.38 in 2016, Table S1).

Statistical Analysis

All analyses were conducted in R (R Core Team 2017). Our sampling unit was pool, treating each pool as $n = 1$. We used multiple regression to determine significant linear relationships between environmental factors and water-borne CORT levels of spotted salamander larvae ($\alpha = 0.05$). Models with hypothesized environmental predictors including pool age, pool-water pH, pool depth, diameter, pool cover, canopy cover, and predator presence were compared using Akaike's Information Criterion (AICc, package 'AICcmodavg' [Mazerolle 2017, Burnham and Anderson 2002]). We also examined deviance explained by each model (package 'BiodiversityR' [Kindt and Coe 2005]). Predictors included in multiple regression models were not correlated (Pearson's correlation $r \leq 0.33$, $VIF \leq 1.14$; package 'car', Fox and Weisberg 2011). All variables met assumptions of normality and homoscedasticity. Those that did not (CORT, salamander total length, and total pool cover) were natural log transformed to meet assumptions of normality and homoscedasticity. Figures and graphs are based on non-transformed data (package 'ggplot2', Wickham 2009).

Initial analysis indicated larval total length, pool-water temperature, and year of sampling were significant predictors of CORT. These were not predictors of interest, but were included in the models as the competing null hypotheses to control for their influence. Pools sampled both years were treated as two different sample units ($n = 2$) because CORT levels were significantly different (paired randomized t-test $p = 0.003$), making it inappropriate to average them. Additionally, pool habitat characteristics included in models were different from year to year at pools sampled both years (Hotelling t^2 test $p = 0.02$, Table 1). Although, when predictors were tested individually, only salamander total length and pool depth differed from year to year (paired t-test $p = 0.003$ and $p = 0.001$, respectively). We included both sample years to control for temporal and population level variation which is necessary because CORT can be influenced by environmental conditions and physiological state. It is critical to consider and control for outside influences on CORT to be able to accurately determine the impact of habitat characteristics of interest.

Other environmental traits had significant linear relationships with CORT, but were not included in the analysis because these predictors were either correlated with other predictors in the final model or the estimated coefficient was not significantly different from zero. These included: larval developmental stage, air temperature, day of the season sampled (mid-May vs mid-June), time of day sampled, and amount of time spent sampling in the pool. Larval stage was correlated with larval total length (Pearson's correlation $r = 0.65$). Air

Table 1 Summary data displaying ranges of all predictors of water-borne corticosterone levels (pg/h) for spotted salamanders (*Ambystoma maculatum*) included in analysis with means separated by sampling year

Predictor	Min	Max	2015 Mean \pm SE	2016 Mean \pm SE
Salamander Total Length (mm)	14.00	39.71	20.17 \pm 0.97	22.20 \pm 1.09
Water Temperature ($^{\circ}$ C)	10.07	28.03	18.79 \pm 0.78	17.57 \pm 0.77
Year Sampled	2015	2016	N/A	N/A
Pool Diameter (m)	2.08	12.75	6.57 \pm 0.44	6.86 \pm 0.44
Pool Age (years)	1.00	5.00	2.50 \pm 0.23	3.58 \pm 0.24
Water pH	5.37	7.47	6.70 \pm 0.10	6.93 \pm 0.08
Pool Depth (m)	0.13	0.66	0.27 \pm 0.02	0.37 \pm 0.02
Pool Cover (0–1)	0.00	0.70	0.19 \pm 0.03	0.26 \pm 0.02
Canopy Cover (0–1)	0.00	0.62	0.07 \pm 0.03	0.06 \pm 2.65
Predator Presence (0–3)	0.00	3.00	1.96 \pm 0.14	1.50 \pm 0.17

temperature was correlated with water temperature (Pearson's correlation $r = 0.77$) and time sampled (Pearson's correlation $r = 0.71$). Day of the season was correlated with larval total length (Pearson's correlation $r = 0.61$) and the coefficient was not different from zero. Time of day sampled was correlated with water temperature (Pearson's correlation $r = 0.67$) and the coefficient was not different from zero. Finally, the amount of time sampling in the pool (Table S1) had a coefficient not different from zero.

To control for differences in overall body size, we included total length in our models. Previous studies with amphibians corrected for body size by dividing water-borne hormone by salamander length (Gabor et al. 2013, 2016; Baugh et al. 2018). However, our preliminary analysis found that even after dividing by total length (TL), water-borne CORT pg/TL/h was still positively correlated with total length (mm) (Spearman's rank correlation: $r_s = 0.41$, $p = 0.002$). This indicates larger larvae released more CORT per mm of body length and dividing by total length would not standardize samples or correct for body size influence on CORT.

Results

We measured water-borne CORT levels of 10 salamander larvae per pool in 28 vernal pools in 2015 and 26 pools in 2016 (Table S1). This included 20 pools sampled both years. Pools varied in numerous environmental variables (Table 1). Pool diameter was a significant environmental predictor for CORT levels in spotted salamander larvae. The model including pool diameter was the top model: salamander total length + water temperature + year sampled + pool diameter (adj $R^2 = 0.73$, $F_{(4,49)} = 36.04$, $p < 0.01$; Tables 1, 2, 3). Pool diameter had a negative relationship with CORT (Table 3, Fig. 2c). Salamander total length and pool-water temperature were positively related to CORT levels (Table 3, Fig. 2). This top model outcompeted other predictors and all other null models

including total length, total length + water temperature, and total length + water temperature + year sampled (Tables 1 and 2). The top model explained 75% of the variance in our data and received 80% of the total weight of all models, compared to $\leq 6\%$ for other models. The model with pool cover had $\Delta AICc < 7$, indicating it could be a significant predictor but only received 3% of the weight (Tables 1 and 2). The estimate of pool cover in the model was not significantly different from zero. All other models had $\Delta AICc > 7$ and the other predictors expected to influence CORT had non-significant coefficients.

Discussion

We sampled created vernal pools to determine if environmental characteristics were impacting water-borne CORT levels of spotted salamander larvae. We found that the strongest model included larval total length, pool-water temperature, year of sampling, and pool diameter. Pool diameter had a negative relationship with CORT. We can hypothesize based on the CORT-fitness hypothesis that lower baseline CORT levels indicate higher fitness (Bonier et al. 2009), although it is difficult to visualize a simple linear relationship due to the other contributing predictors of larval size and water temperature (Fig. 2c). With this interpretation, larvae in larger pools would have lower CORT and higher fitness. Therefore, larger pools would be considered higher quality habitat for spotted salamanders that could produce larvae with better physiological health than larvae in smaller pools. Our pool sizes were based on average diameter, with a range of 2.08–12.75 m. Support for our interpretation can be found in previous studies showing a positive correlation between pool size and number of egg masses in spotted salamanders, and selection for larger pools (up to 0.08 ha in Petranka et al. 2003a, > 0.05 ha in Egan and Paton 2004). In Maine, larger beaver ponds had greater spotted salamander occupancy and breeding effort than smaller

Table 2 Hypothesized models explaining variation in corticosterone levels (pg/h) of spotted salamander (*Ambystoma maculatum*) larvae

Model	Δ AICc	w_i	Deviance Explained	All predictors significant $p < 0.05$
TL, C, Year, Pool Diameter	0	0.80	75%	*
TL, C, Year	5.31	0.06	71%	*
TL, C, Year, Pool Cover	6.68	0.03	71%	
TL, C, Pool Diameter	6.77	0.03	70%	*
TL, C, Year, Age	7.41	0.02	71%	
TL, C, Year, Predators Present	7.7	0.02	71%	
TL, C, Year, pH	7.71	0.02	71%	
TL, C, Year, Pool Depth	7.77	0.02	71%	
TL, C, Year, Canopy Cover	7.84	0.02	71%	
TL, C	10.83	0	66%	*
TL, C, Year, Pool Diameter, Pool Cover, Age, Predators, pH, Depth, Canopy Cover	15.83	0	76%	
TL	31.5	0	48%	*
~1	64.52	0	0%	

Δ AICc = change in AIC corrected for small sample size; w_i = weight of the model; TL = salamander total length; C = water temperature; Year = calendar year sampled; Pool Cover = any form of refuge such as vegetation, rocks, coarse woody debris; Age = time since pool creation

vernal pools (Groff et al. 2017). Our hormone data add to existing data indicating that pool size can be an important factor to consider when creating spotted salamander breeding habitat. Future studies should continue to monitor spotted salamander populations of the vernal pools we sampled to determine if reduced CORT and pool diameter are indeed predictors of pool creation success.

The top model also included larval total length, pool-water temperature, and year sampled. The model estimates indicated larval total length and water temperature were positively associated with CORT. This confirmed our expectations that larger animals would produce more CORT. It is also important to note that dividing by body size was not sufficient to control for its impact on CORT, making it necessary to include larval total length in the model. The top model also confirmed that warmer temperatures, which were correlated with time of day, would facilitate higher CORT production in ectotherms and reflect natural changes due to circadian variation (Dupont et al. 1979; Cree et al. 2003). Navarro et al. (2018) also found that warmer temperatures increased water-borne CORT levels in eastern red-backed salamanders (*Plethodon cinereus*). It is likely that, as was demonstrated in red-legged salamanders (*Plethodon shermani*), warmer temperatures were associated with increased metabolic rate, facilitating higher CORT levels (Wack et al. 2012). Since time of day and water temperature were correlated, it seems including water temperature in the

model also controlled for natural variations in CORT due to circadian rhythms (Dupont et al. 1979; Homan et al. 2003). The annual variation in CORT exemplifies the need for multi-year studies to control for temporal and population level variation. Corticosterone is highly variable and influenced by natural environmental conditions and physiological state. Here we showed CORT levels were influenced by body size and water temperature, and showed annual variation from year to year. Finding differences between years is representative of sampling unique individuals, but also environmental differences. Annual variation in salamander body size, pool-water temperature, pool diameter, pool-water pH, pool depth, pool cover, canopy cover, and predator presence likely contributed to differences in CORT levels between years (Table 3, Fig. S1, S2). Additionally, time since pool construction increased from year one to year two which could have influenced the habitat and salamanders. In order to assess the effects of habitat quality, it is imperative to account for all outside influential factors of hormone level by assessing their impact and, if found to be significant, including them in the model. By identifying that the model including pool diameter was the top model, we showed that in addition to all the natural factors influencing CORT, pool diameter was also influential. Including larval total length, water temperature, and year in the model controlled for the influence of these other factors on CORT levels, and facilitated a more accurate determination of habitat quality impacts.

We expected that vegetation would continue to grow and establish as more time passed after pool creation, and pool colonization would continue as an amphibian community was established. However, pool age was not found to be an influential factor for hormone levels of spotted salamander larvae. This could be because the sampled pool ages only varied from 1 to 5 years. We may have found more variation if our sites included pools that were > 5 years old. Alternatively, the initial available habitat may be sufficient. Mitigated wetlands in West Virginia were scored higher than reference wetlands in anuran, avian, invertebrate, and vegetation richness, abundance, diversity, density, and biomass (Balcombe et al. 2005). Created pools can be colonized quickly after creation and species richness can reach equilibrium within two years (Petranka et al. 2003a). Spotted salamanders used 90% of 10 created pools as early as the first year, the same occupation rate as 10 natural pools (Petranka et al. 2003a).

We did not find that pH was a significant predictor in our models. In Jefferson salamanders, CORT levels were negatively correlated with pH (5–7) when including ranges with the greatest hatching success (pH 5–6) for the species (Pough and Wilson 1977; Chambers et al. 2013). Allegheny Mountain dusky salamander (*Desmognathus ochrophaeus*) adults exposed to stream sites with pH values ranging from 4.1–8.1 did not exhibit correlated plasma CORT levels indicating that

Table 3 Coefficients for the top model explaining water-borne corticosterone levels (pg/h) for spotted salamanders (*Ambystoma maculatum*): $y =$ salamander total length (TL) + water temperature (C) + year sampled (Year) + pool diameter

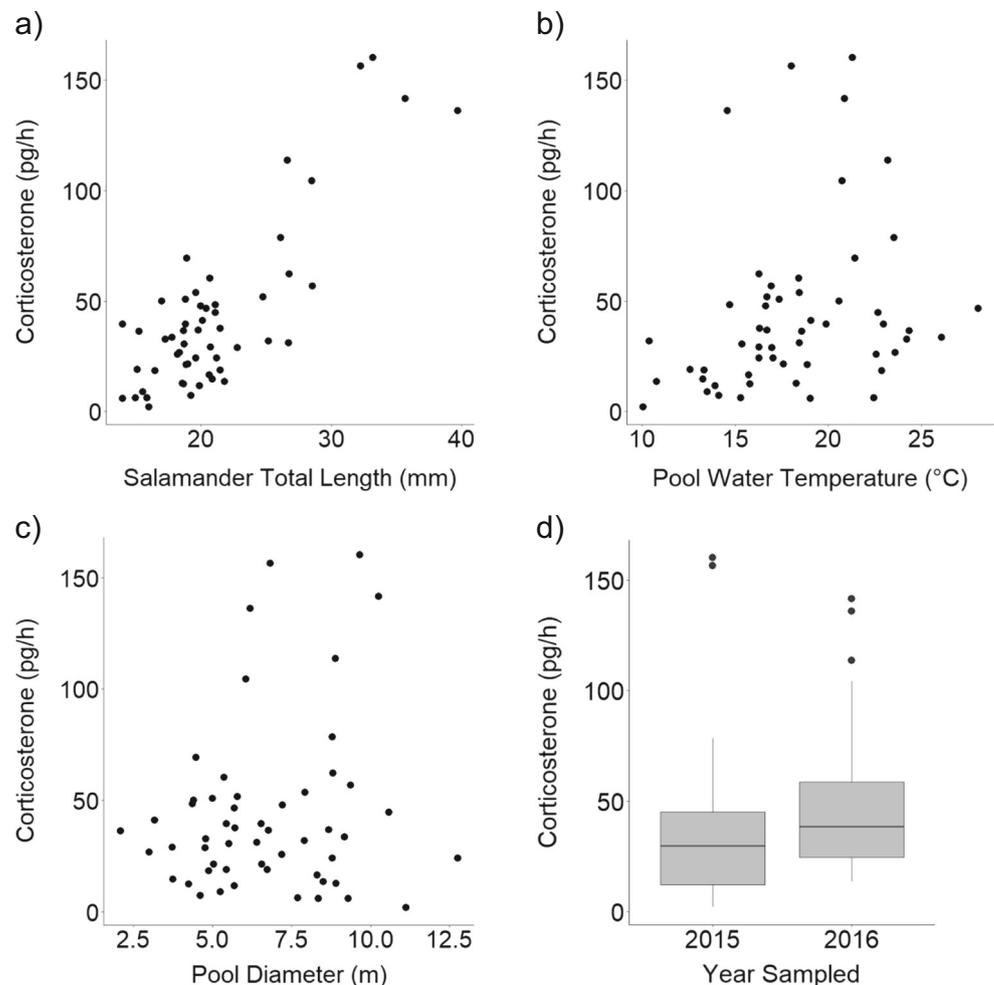
Coefficients	Estimate	Std Error	95% Confidence Interval		t	p
Intercept	-820.30	268.00	-1345.49	-295.09	-3.06	3.57E-03
TL	2.79	0.30	2.21	3.37	9.38	1.61E-12
C	0.10	0.02	0.07	0.13	6.26	9.39E-08
Year	0.40	0.13	0.14	0.66	3.04	3.83E-03
Pool Diameter	-8.12E-04	2.94E-04	-1.39E-03	-2.37E-04	-2.77	0.01

environmental pH levels may not activate the HPI axis to elicit a CORT response (Woodley et al. 2014). It is possible that impact of pH on salamanders may be based on a threshold. Spotted salamander embryos reared in water with a 4.5 pH had reduced mass compared to those in water with an 8.0 pH (Bianchini et al. 2012). Pool pH levels of 5–6 produced developmental abnormalities in spotted salamander larvae with more severe abnormalities occurring in pools with $\text{pH} < 5$ (Pough and Wilson 1977). The greatest hatching success for spotted salamanders was in pools with a pH between 7 and 9

(Pough and Wilson 1977). The limited range of pH in our created pools (5.37–7.47) may explain the lack of correlation, and it is possible that detection of pH effects on spotted salamander CORT levels requires a pH range of 4–9 to include both adverse and ideal pH levels. Future studies should examine a larger range of pH values in breeding pools to determine if spotted salamander larvae respond to suboptimal pH values with changes in CORT.

Pool depth, vegetation cover in the pool, and canopy cover have been shown to contribute to the number and density of

Fig. 2 Biplots displaying water-borne corticosterone levels (pg/h) and spotted salamander (*Ambystoma maculatum*) total length (mm) (a), pool water temperature ($^{\circ}\text{C}$) (b), and pool diameter (m) (c). Each dot represents average corticosterone (pg/h) levels for one pool in one sampling year ($n = 54$). Boxplot displaying corticosterone levels in each sampling year (d)



spotted salamander egg masses with clear positive correlations (Petranka et al. 2003a; Egan and Paton 2004; Skidds et al. 2007; Felix et al. 2010; Kern et al. 2013; Scheffers et al. 2013). However, we did not find that these were significant predictors in competitive models for CORT levels. We had expected to detect a threshold for depth at which a pool would be too shallow to provide suitable habitat. We predicted that pool cover would influence CORT levels because lack of cover within the pool exposes larvae to the sun, elevated temperatures, and predators. These influences may not have been identified as significant due to the limited ranges present at our study sites: pool depths ranged from 0.13–0.67 m and canopy cover varied from 0 to 62% (averaging 7%) with most pools under open canopy. Other studies have incorporated ranges in mean pool depths of 0.64–0.95 m up to 2.3 m and canopy cover from 0 to 100% (Petranka et al. 2003a; Skidds et al. 2007). Spotted salamander selection for oviposition sites in deeper water and denser submerged vegetation is evident with water depths up to 0.33 m and vegetation cover up to 100% (Kern et al. 2013). There may be an ideal depth as oviposition was documented most frequently in water 0.50 m deep rather than deeper areas up to 2.20 m (Egan and Paton 2004). While we expected these habitat traits to be important, it is possible that they are only critical as habitat for oviposition to attract breeding adults. Provided the pool does not dry before larvae complete metamorphosis, variation in these characteristics may not be critical for the physiological health of larvae or may not activate the HPI axis to cause changes in CORT.

Predator presence was expected to impact CORT levels but was not a strong predictor. During predatory interactions, prey can exhibit changes in CORT levels to facilitate escape behavior (Wingfield et al. 1998; Davis and Gabor 2015; Harris and Carr 2016). This may vary across species. Allegheny Mountain dusky salamanders exposed to predatory chemical cues did not exhibit a CORT response despite exhibiting reduced behavioral activity (Fonner and Woodley 2015). We are not aware of any research on spotted salamander CORT responses to predators specifically; however, spotted salamanders exposed to predators exhibit antipredator behavior by reducing their activity and feeding (Hossie et al. 2017). Our data suggests that eastern newt, diving beetle larvae, and dragonfly larvae presence alone may not influence CORT levels of spotted salamander larvae. Future research should examine if predator density or occurrence of larval predatory encounters impacts CORT levels in spotted salamander larvae.

Caveats to Interpretation of Water-Borne CORT Levels

There is debate regarding how to interpret CORT levels in terms of direct correlation to physiological health (Dickens and Romero 2013; Thomas et al. 2017). Previous studies that found associations between CORT levels and habitat variables

tend to assume that higher CORT reflects reduced fitness (Glennemeier and Denver 2002; Crespi and Denver 2005; Janin et al. 2012; Chambers et al. 2013; Troianowski et al. 2017; Charbonnier et al. 2018). This is reasonable considering that very high CORT inhibits reproduction, growth and immune function. Additionally, our data along with multiple other research projects have found a correlation between glucocorticoid hormones and habitat characteristics (Hopkins et al. 1999; Glennemeier and Denver 2002; Homan et al. 2003; Crespi and Denver 2005; Bonier et al. 2009; Janin et al. 2012; Chambers et al. 2013; Troianowski et al. 2017; Charbonnier et al. 2018). This indicates that both the impact of the environment on CORT and the mechanism behind it are research areas worth pursuing. However, CORT is a naturally occurring hormone that functions to facilitate growth and metamorphosis (Carr and Norris 1988; McEwin and Wingfield 2003; Moore and Jessop 2003; Romero 2004; Chambers et al. 2011). It also facilitates physiological changes in response to unexpected stimuli or stressors to make energy available. The biological meaning of differences in CORT levels is unsettled as it lacks a consistent profile in chronically stressed animals (Gormally et al. 2018). Thus, interpretation of CORT is still open, and more data from free-living animals in the natural environment is necessary.

Water-borne sampling is a relatively new method for sampling CORT levels in amphibians, and water-borne hormones are often assumed to reflect whole-body or plasma hormones. However, plasma CORT reflects a snapshot in time while water-borne CORT reflects cumulative release over a period of time and likely integrates changes to CORT due to handling, capture, or containment (Romero and Reed 2005). It has been argued that water-borne CORT may therefore provide a more accurate reflection of overall physiological condition due to its cumulative nature, compared to plasma CORT (Dantzer et al. 2014; Baugh et al. 2018). In support of this argument, associations between high water-borne CORT levels and physiologically relevant impacts have been demonstrated in several amphibian species. Common midwife toads (*Alytes obstetrician*) with more severe *Batrachochytrium dendrobatidis* infections had higher water-borne CORT levels and reduced righting response (Gabor et al. 2015). African clawed frogs (*Xenopus laevis*) had higher water-borne CORT in unnatural housing and greater body mass loss (Holmes et al. 2016). Spotted salamander larvae reared in high intraspecific density had higher water-borne CORT and reduced growth (Charbonnier et al. 2018).

Conclusions, Management Implications, and Future Research

We found that spotted salamander larvae inhabiting larger pools (diameters up to 12.75 m) have lower baseline CORT levels. This may indicate that pool diameter is an important

habitat characteristic contributing to the physiological health of salamander larvae. Research on selection for breeding sites supports the importance of larger pools, provided they do not contain or risk colonization by fish. Size of the pool should be carefully considered when creating breeding habitat for this species. To confirm our interpretation of CORT and its biological meaning, future studies should survey spotted salamander populations in these pools to determine if larger pools produced larger populations. Future research could also focus on determining an ideal pool size for spotted salamander larvae and assessing whether pool size contributes to other beneficial habitat characteristics. When using CORT as an indicator of habitat quality in field studies, it is critical to assess external influences for accurate habitat associations. One way to do this is to collect data over multiple years to account for annual variation in the natural environment. Other factors shown to impact CORT levels should also be controlled for or incorporated in analysis such as body size and water temperature for spotted salamanders. Additional studies could include more extensive ranges of age of the pool, pool-water pH, pool depth, canopy cover, specific types of vegetation cover and complexity, and predator density to determine their impacts on spotted salamander larvae. Laboratory studies would be beneficial to determine if pH influences CORT levels in spotted salamander larvae and at what extremes. Future research should examine how habitat characteristics in natural pools influence larval physiology and whether it differs from created pools.

Measuring physiological responses to habitat quality can improve understanding of population health and could be a useful tool to predict population declines and detect vulnerable populations. Physiological measurements, along with demographic studies, can better inform management decisions regarding habitat creation and restoration. Incorporating these tools will help determine ideal habitat characteristics and how they impact spotted salamander populations.

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