EVALUATION OF AMPHIBIAN COMMUNITIES OF PLAYA WETLANDS IN THE SOUTHERN HIGH PLAINS, TEXAS AND PHYSIOLOGICAL EFFECTS OF ENVIRONMENTAL STRESSORS ON THE NEW MEXICO SPADEFOOT TOAD, *SPEA MULTIPLICATA*

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CHAPTER I

EFFECTS OF LANDUSE ON AMPHIBIAN POPULATIONS OF THE SOUTHERN HIGH PLAINS, TEXAS, USA

Abstract

The Southern High Plains (SHP) contains approximately 25,000 playa wetlands, which provide the primary source of breeding habitat for resident amphibians. Intense row crop agriculture in the SHP has affected playas, primarily through increased sediment deposition and altered hydrology. In this study, amphibian community diversity was assessed in 94 playas over two years and among the three dominant land use types: cropland, native grassland, and land enrolled in the USDA Conservation Reserve Program (CRP). Overall, playas located within CRP watersheds had sediment depths, water loss rates, and starting water depths intermediate to cropland and grassland playas. However, hydroperiod, playa area, and amphibian richness did not differ among landuses. Although species richness did not differ among land use types, distribution of amphibian species among land use types did differ, particularly in the drier 2008 season. For example, in 2008, Great Plains toads (*Bufo cognatus*) were found almost half as frequently in CRP playas than in cropland or grassland playas, whereas plains leopard frogs (*Rana blairii*) were found twice as often in CRP playas. Further, CRP explained

52% of species distribution in canonical correspondence analysis, with plains leopard frogs strongly associating with CRP. In addition, sediment depth, hydroperiod, and percent native grass within the watershed accounted for the majority of variance within species distributions. This study is the first to definitively investigate CRP effects on playa amphibian communities.

Introduction

Amphibians are both predator and prey in aquatic and terrestrial systems, make up a large portion of the biomass in temperate and tropical regions (Stebbins and Cohen, 1995), and are considered keystone species in some habitats (Murphy et al., 2000). This certainly holds true for amphibians in the Southern High Plains (SHP) of Texas, a semi-arid environment, with 13 amphibian species present (including one salamander) (Smith 2003). Playa wetlands are the primary aquatic features in the SHP. Playas are shallow, temporary, recharge wetlands, contained within individual watersheds, and receive water exclusively through precipitation or runoff (Smith, 2003). The dynamic hydrological nature of playas exemplifies the need for many resident amphibians to exhibit strategies such as phenotypic plasticity and rapid development, which allow them to successfully inhabit and breed in playas. In addition, most playas are presently or historically situated in heavily cultivated landscapes.

Cultivation in the SHP has led to both direct and indirect effects on playa hydrology (Smith 2003). Sedimentation has become the greatest threats to playas, with most playas having lost over 100% of their total volume, by reducing hydroperiod and increasing water loss rate (Luo et al, 1997; Haukos and Smith, 2003; Tsai et al., 2007). Because of the potential impacts of cultivation on playa hydrology and the subsequent

effects on amphibian populations in the SHP, there have been numerous amphibian studies focusing on amphibians in playas surrounded by either native grassland or cropland (Gray et al., 2004a; Gray et al., 2004b; Gray and Smith, 2005; Venne, 2006). Gray et al. (2004b) observed a correlation between landscape characteristics surrounding playas and amphibian assemblages. Similarly, Houlahan and Findlay (2003) found that cultivated habitat, similar to that surrounding playas in cropland watershed, altered amphibian community composition in Ontario wetlands.

Abiotic and biotic stressors can alter the fitness and survival of amphibians, as well as altering population structure (Kiesecker et al., 2001; Pounds et al., 2006). Amphibian populations around playas could be threatened (e.g. altered population structure, decrease in size at metamorphosis, decreased survival probability) by decreased hydroperiod and increased sediment load (Gray et al., 2004a, 2004b; Smith, 2003), as well as agricultural contaminants due to landuse practices (Hayes et al., 2002; Smith, 2003; Hayes et al., 2006; Venne et al., 2008). Hydroperiods of playas are dependent on the amount and frequency of precipitation the watershed receives and the land use surrounding the wetland (i.e., sediments). For amphibians, shorter hydroperiods will increase desiccation stress and the amount of inter and intra-specific competition for food and space. Most species' development rate in this region is influenced by hydroperiod, where playas with longer hydroperiods should yield larger individuals. For example, Gray and Smith (2005) collected individuals of four amphibian species from eight crop and eight grass playas, over two years, and found that there can potentially be over 100% greater difference in body size in native grassland playas with longer hydroperiods than in cropland playas with shorter hydroperiods. The relative abundance of spadefoot toads

(*Spea bombifrons* and *S. multiplicata*) is influenced by landuse, with a greater abundance being found in cropland playas (Gray et al. 2004b) compared with native grass playas. This indicates that spadefoot toads may be more adapted to playas with more dynamic hydrology and lack of predators found in crop playas (due to shorter hydroperiods) (Ghioca-Robrecht et al. 2009). However, *Spea* spp. may also be more common in crop playas due to a lack of vagility when compared to other species, resulting in less emigration from the natal pond (Pearson, 1955).

Cultivation increases landscape complexity which can negatively affect amphibian dispersal (Wiens 1997). Gray et al. (2004a) investigated this concept in regard to landuse effects on amphibian community composition in the SHP. They found that some species (e.g. Great Plains toad and Texas barred tiger salamander) were not influenced by landscape structure, but spadefoot toads were found more often in cropland playas and negatively correlated with landscape complexity. In addition to landscape complexity, vegetative structure within playas affects amphibian habitat use (Anderson et al. 1999); with most species preferring areas with greater vegetation cover. However, although there was a distinct difference in habitat preference that can be influenced by landuse characteristics, landuse itself was not found to affect species distribution (Anderson et al. 1999).

Amphibian physiology has also been shown to be affected by the surrounding landuse of playa wetlands. Ghioca-Robrecht et al. (2010) assessed fatty acid composition in spadefoot toads from both cropland and native grassland playas and found lower polyunsaturated fatty acids in *Spea* spp. tadpoles and metamorphs in crop playas when compared with grass playas. Fatty acids play a key role in metabolism of estivating toads,

and a decrease in overall levels could potentially cause individuals to be in poorer body condition during the subsequent breeding season (Ghioca-Robrecht et al., 2010). Along with metabolic effects, immune function may also be influenced by land use. In a study investigating body size, spleen size, and spleen leukocyte cellularity, all measures were lower in spadefoot toads metamorphosing from crop playas than in native grassland (McMurry et al., 2009). Spadefoot toads have been shown to exhibit polyphenism, expressing both omnivore and carnivore morphotypes (Pfennig, 1992; Ghioca-Robrecht et al. 2009). Ghioca-Robrecht et al. (2009) found that landuse indirectly affects the presence of the carnivore morphotype by altering hydroperiod and water loss rate, which subsequently decrease the number of salamander predators in a playa. Carnivore morphotypes are positively correlated with the presence of the predator, the Texas barred tiger salamander (*Ambystoma tigrinum mavortium*), which is a species that requires long hydroperiods for larval development and tends to be found in grassland playas (Ghioca-Robrecht and Smith, 2008), and negatively correlated with faster water loss rates and short hydroperiods (Ghioca-Robrecht et al., 2009).

There have only been two studies investigating contaminant exposure in playa amphibians, one assessing heavy metals and one organochlorine (OC) exposure. In both studies, landuse did not play a key role in differences in contaminant residues in amphibian tissues and the levels found were below the reported level of toxicity (Venne et al., 2006; Venne et al., 2008). Venne et al. (2008) found that landuse also did not affect differences in OC residues in sediments collected from both cropland and grassland playas. Tissue metal residues did not differ among landuse in *Spea* spp. tadpoles; however, cadmium was two times higher in *Bufo cognatus* tadpoles in cropland playas

and nickel was higher in grassland playas (Venne et al., 2006).

As mentioned above, most of the SHP has been, or is currently cultivated, with only small amounts of remaining native grassland. Indeed, approximately 90% of natural vegetation in the SHP has been converted for agriculture (USGS, 2006); however, much of the previously cultivated uplands are currently enrolled in the United States Department of Agriculture (USDA) Conservation Reserve Program (CRP), and planted in exotic perennial grasses to decrease soil erosion (USDA, NRCS). In the Texas SHP, approximately 6800 km^2 of land were enrolled in CRP as of April 2011, one of the highest enrollments in the nation (USDA FSA, 2011). This program has been successful and soil erosion has been reduced significantly (USDA FSA, 2011) and although not designed to protect wetlands, CRP influences wetland characteristics and their function. For example, CRP effectively reduces erosion of top soil and thus protects playas from additional sediment input. However, many CRP plantings typically consist of non-native grasses (e.g. old world bluestem, weeping lovegrass), which although effective at decreasing sedimentation (Smith, 2003), these tall grass species also likely decrease the inundation frequency, amount of water entering the playa, and hydroperiod by intercepting runoff and increasing upland infiltration (Smith and Haukos, 2002, Cariveau et al., 2011). van der Kamp et al. (2003) showed that following replanting of grasses in previously cultivated fields, wetlands subsequently dried and remained dry due to a lack of runoff. The effects of CRP on amphibian assemblages are of interest as 5.4% of all playas throughout the Playa Lakes Region are located in CRP catchments (11.5% of all Texas playas) and the CRP is one of the most widely applied USDA conservation program in the U.S. (Smith et al., 2011).

The goal of this study was to assess amphibian communities among the dominant landuse types in the SHP; native grassland, cropland, and CRP. Although there have been numerous studies on amphibian populations in this region that have investigated effects of land use on amphibian diversity and development (Gray et al. 2004a, Gray et al. 2004b, Houlahan and Findlay 2003, Gray and Smith 2005, Venne 2006, Ghioca-Robrecht et al. 2009, McMurry et al. 2009), they have focused on currently cultivated cropland and native grassland. The CRP is a dominant feature of the SHP landscape, and current research suggests that playas surrounded by CRP receive less precipitation runoff and are inundated less frequently (Carivaeu et al. 2011) than those in cropland or native grass. Yet CRP also protects playas from additional sediment input, which has direct ramification on hydroperiod. Thus, it is unclear how amphibian communities might respond to the effects of CRP on playa hydrology. We predicted that sediment depth, hydroperiod, water loss rate, and amphibian diversity of CRP playas would be intermediary to that of cropland and grassland playas. Secondarily, we wanted to determine if wetland characteristics can be used to predict amphibian species occurrence and determine potential land use preferences of each species which can subsequently be used to aid in future management of amphibian populations located in these isolated wetlands.

Methods

Study Area

This study was conducted in the Texas SHP, a semi-arid region that receives 33-45 cm of precipitation yearly. Playas occupy approximately 2% of the total landscape (Haukos and Smith 1994), with an estimated 21, 800 playas in the SHP (Guthery and

Bryant 1982) providing over 160,000 ha of wetland habitat (Haukos and Smith 1992). The main crops of this region are cotton, wheat, corn, and grain sorghum (Colaizzi et al., 2008).

A total of 94 playas was selected after their initial inundation in 2008 (N=46) and 2009 (N=48). Inundation rate varies among landuse type, with CRP playas requiring more rain than either cropland or grassland playas, thus lending to mild site selection bias. Playas were distributed among nine counties and embedded within three major landuse types: cropland (2008 n = 18; 2009 n = 21), native grassland (2008 n = 14, 2009 n = 13), and CRP (2008 n = 14, 2009 n = 14) (Fig. 1). Playa landuse type was assigned if ≥75% of the immediate surrounding landscape was of a particular landuse type. All playas were selected and initially sampled within seven days of inundation. Geographic distribution of selected playas was constrained by precipitation patterns, particularly in 2009 when most spring and summer precipitation was localized in Castro and Swisher counties (West Texas Mesonet, www.mesonet.ttu.edu).

Water depth and amphibian surveys were conducted every two weeks after initial inundation until the playa dried, or until November 1 when most amphibians were no longer present. For all playas starting water depth (cm), hydroperiod (days), sediment depth (cm), rate of water loss (cm/day), and playa area (ha) were recorded. Water loss was measured by using a 2 m PVC pole placed at the center or near center of each playa. Overall water loss rate (cm/day) was determined by averaging each two week loss period (depth/days) for each playa, excluding occurrences when water was gained between surveys. Sediment depth was measured after playas dried. Six soil core samples were taken, one at the center of the basin and five points around the center and equidistant to

the wetland edge (Tsai et al, 1999). Cores were dug with a 76 mm auger to a depth where the soil consisted of ≥ 50% hydric soil (e.g. Randall clay).

Playa area, total watershed size, and percent landuse cover were delineated using ArcGIS 9.0 (ESRI®, Redlands, CA, USA). Watersheds were determined using 5 m topographic maps obtained from 2010 TerraServer data (Raleigh, NC, USA) and landuse data (digital orthophoto qudrangles) was acquired through Texas Natural Resources Information System (TNRIS) (Texas Water Development Board, Austin, TX, USA), using available 2008 and 2009 data. Contour lines and high points were used to create watershed boundary polygons (Ekanayake et al., 2009). Due to this region's lack of pronounced elevation changes, in cases where it was topographically indistinguishable to determine the true watershed boundary between two playas, the distance was split evenly.

Amphibian Surveys

Amphibian community composition and diversity was assessed every two weeks using a combination of netting animals in the water, walking transects along the wet edge, and nocturnal auditory surveys. Ten meter transects were randomly selected at four locations within the playa (1 per quadrant) and amphibian larvae captured using 40.6 x 40.6 cm square-frame large dip nets with 0.80 mm mesh. Dipnets were advantageous for increasing captures because many playas had dense vegetation that hindered the use of a large seine. Visual transects (250 m) were walked around the playa moist edge during day (0900-1900) and night (30 min after dark-0100) surveys. Moist edge was defined as the distinguishable boundary between dry and wet soil. A width 1 m (0.5 m either side of the transect) was accounted for during each transect walked. Additionally, nocturnal auditory surveys were conducted every two weeks at each playa for approximately 20

minutes, and all observers were proficient in identification. Surveys were postponed during heavy precipitation events as most amphibians were not detectable during those times.

Statistical Analyses

Playa characteristic data were $\log_{10}$-transformed to satisfy normality assumptions and 2-way analyses of variance (ANOVA) were performed to assess the main and interactive effects of year and land use type on sediment depth, hydroperiod, water loss rate, starting water depth, percent native grass cover, and species richness. Landuse and year were independent variables and playa characteristics were dependent variables. When there was a significant interaction, one-way ANOVA was used to analyze differences among landuse types within year and year effects within land use. For post hoc comparisons we used Tukey's HSD. Chi-Square analysis was performed to determine differences in frequency of occurrence for individual species among landuse types. For the analysis, only the dominant species (*A. t. mavortium, B. cognatus, B. woodhousii, P. clarkii, R. blairii,* and both *Spea spp.* and tadpoles grouped together) were used to satisfy the assumption that the expected count was greater than 5. Because *Spea spp.* tadpoles cannot be distinguished between the two species at this stage (*Spea bombifrons* and *S. multiplicata*), and occasionally tadpoles but no adults were detected, we analyzed them as a separate group of amphibians to account for their presence for frequency distributions; they were not included in calculating species richness.

We also calculated Pearson correlation coefficients between biologically relevant variables (percent native grass cover, sediment depth, hydroperiod, water loss rate, and species richness) to determine the strength of relationships for potential use of wetland

characterization. For example, if wanting to make a generalization about a playa with a short hydroperiod, it would be advantageous to know if shorter hydroperiods are correlated with water loss rate or species richness. Percent native grass cover was used as a more quantitative variable to determine relationships between amphibian presence and playa characteristics to the amount of native grass present within the watershed. To include potentially relevant biological relationships among correlations, we increased alpha to 0.10 in assessing single species occurrence predictors. An alpha of 0.05 was used for significance level in all other analyses.

Binomial generalized linear models (GLMs) (R, Vienna, Austria 2010) were used to provide probability models for the presence of individual amphibian species given changes in hydroperiod, water loss rate, sediment depth, and percent native grass cover. The null hypothesis for these models was that the regression coefficient equaled zero. The z coefficient and p values reported for these results test this hypothesis.

We also performed canonical correspondence analysis (CCA) using CANOCO® to evaluate the combined influence of wetland characteristics (playa area, hydroperiod, water loss rate, sediment depth, and percent native grass cover) and landuse (crop, CRP, and native grassland) on species occurrence, with year as a covariable. For CCA we used combined data from both years to get a more robust distribution of playa characteristics, as well as analyzing 2008 and 2009 separately to account for the interaction between year and hydroperiod. The program square-root transformed the data and we chose to down-weight rare species to eliminate potential bias and influence of these species in the analysis (ter Braak and Šmilauer, 2002). Under the full model the program analyzed the data under 999 Monte Carlo permutations. Performing both GLMs and CCA allowed a

more robust determination of which playa characteristics may influence species occurrence, both singularly or with additive effect. CCA alone does not provide univariate measure of association, whereas GLM does, and therefore performing both analyses allowed us to identify specific relationships between environmental variables and individual species, calculate specific probabilities of species occurrence based on univariate relationships, and model species occurrence based on response to all variables combined.

Results

Average precipitation from April-July across weather stations closest to all sites was 38.0 cm in 2008 and 48.6 cm in 2009 (West Texas Mesonet, www.mesonet.ttu.edu), with 21% more precipitation in 2009 than in 2008. Precipitation in 2009 was localized within a four county radius, whereas precipitation was sporadic throughout the region in 2008. Subsequently, in 2008 playas were shallower and had much shorter hydroperiods (Table 1).

Hydroperiod was the only variable that had a significant year by land use interaction (Table 2), as hydroperiod length varied among landuse types in 2009 but not in 2008 (Tables 3 and 4). Hydroperiod of cropland playas was about 50 days longer on average than for grassland playas, but CRP playas did not differ from either cropland or grassland playas (Table 4). Hydroperiod also varied between years for all landuse types, with mean length in 2008 about 20% that of 2009. Water loss rate varied among landuse types and between years. Cropland playas lost an average rate of 1.25 cm per day, 30% faster than grassland playas (Table 5). Overall water loss rates were also about 33% faster in 2008 than in 2009 (Tables 3 and 4). Starting water depth varied among land use types

and between years. Cropland playas had the greatest initial water depth, averaging about 48 cm, which was 39 and 49% greater than for CRP or grassland playas, respectively (Table 5). However, starting water depth did not differ between CRP (34.27 ± 4.12) and grassland playas (24.57 ± 3.26). Starting water depth of playas in 2009 was twice that of playas in 2008 (Tables 3 and 4). Sediment depth in playas varied by land use, with 28% more sediment in cropland than CRP playas, which in turn were 88% deeper than for grassland playas (Table 5). Also, sediment depth was greater for playas in 2009 than 2008, a difference driven mostly by cropland and CRP playas, despite the lack of a year by landuse interaction (Tables 3 and 4). Playa area did not vary among landuse types or between years (Table 2). Overall playa size averaged 15.4 ha.

Mean number of species did not differ among landuse types (Table 2-4). Overall, we found 11 species total in crop playas (3.64 ± 0.26SE species per playa), 11 in CRP (3.43 ± 0.36SE species per playa), and 9 in grassland (3.11 ± 0.35SE species per playa). In 2009 we also saw a 50% increase in the number of species per playa than in 2008 (Tables 3 and 4). During both years, Texas barred tiger salamander (*A. t. mavortium*), Great Plains toad (*B. cognatus*), spotted chorus frog (*P. clarkii*), plains leopard frog (*R. blairii*), and spadefoot toad (*Spea* spp.) tadpoles were the dominant amphibians throughout all three land use types, being found in 55, 67, 73, 36, and 50% of all playas surveyed, respectively (Table 6). However, all five of these amphibians were found in greater frequency in 2009 than 2008. In 2008 there was greater variation in observed frequencies of species than in 2009, with more incongruity with expected values in crop and CRP playas (Table 6).

Some species tended to occur more often in certain landuses (2008 $X^2 = 39.10$,

$0.05 < p < 0.10$, 2009 $X^2 = 41.28$, $p < 0.05$) (Table 6). In 2008, *B. cognatus* were found 50% less in CRP playas than either crop or grass playas; however, in 2009 this did not occur and *B. cognatus* were seemingly ubiquitous throughout all landuse types and all playas in general. A similar species, *B. woodhousii* was found three times more often in 2009 than 2008, occurring mostly in crop playas. Although we cannot determine the ratio of *S. bombifrons* and *S. multiplicata* tadpoles found, *S. multiplicata* adults were seen twice as often as *S. bombifrons* in both years and across landuse types, with the exception of CRP playas in 2008 where *S. multiplicata* was found at the same frequency as *S. bombifrons*. In 2008, *Spea* tadpoles were identified in twice as many crop playas than CRP playas, which had the same frequency of occurrence as grassland playas, but in 2009 frequency among all landuse types was essentially the same. A similar trend also occurred with *R. blairii* in 2009, with a greater percentage being found in crop playas than either CRP or grass playas. However, in 2008 *R. blairii* were found more than twice as frequent in CRP playas than crop or grass playas. Another species that was found more often in CRP playas was *P. clarkii*, being found in 64 and 93% of all CRP playas in 2008 and 2009, respectively (Table 6). *B. speciosus* and *R. catesbeiana* were only seen in 2008, with *R. catesbeiana* being found in one CRP playa.

When comparing the relative frequency (frequency of finding a particular species based on the frequency of finding an amphibian in general) between years, there was little difference for most species (Table 7). However, in 2009 the chance of finding *B. cognatus* increased by over half in crop and grassland playas and four times greater in CRP playas than from 2008. Contrary to *B. cognatus*, the potential of finding *R. blairii* in a CRP or grass playa in 2009 decreased by over two thirds as it was in 2008 (Table 7),

and the potential for finding *P. clarkii* in a CRP playa also decreased by half in 2009.

Species richness was positively correlated with hydroperiod across 2008 and 2009 (Fig. 2). At hydroperiods of less than 50 days, species richness drops ($F = 25.06$, $p = <0.0001$). Past 50 days, species richness was variable, but relatively constant (Fig. 2). This relationship between richness and hydroperiod was most evident in 2008 (Fig. 3), particularly as related to short hydroperiods and low richness. Very few playas had hydroperiods of less than 50 days in 2009, and species richness was fairly constant with relation to hydroperiod (Fig. 4).

Hydroperiod and sediment depth were the only two playa characteristics found to be useful indicators of species occurrence probabilities. Although sediment depth and hydroperiod were correlated, they did not have the same overall effect on species occurrence. For pooled years, hydroperiod was a strong predictor of occurrence for *A. t. mavortium, B. cognatus, B. woodhousii, P. clarkii, R. blairii,* and *Spea* tadpoles (Table 8). Sediment depth was found to predict occurrence of *A. t. mavortium, B. cognatus, B. speciosus, B. woodhousii,* and *R. blairii* (Table 8). Additionally *B. speciosus* occurrence was further predicted with water loss and *B. woodhousii* with percent cover of native grass within the watershed (Table 8). However, when data were separated between years it was evident that these relationships were being driven by the distinctive difference in hydroperiods. Crop playas have greater sediment depths and in 2009 had longer hydroperiods, thus sediment was a strong predictor for some species that prefer long hydroperiods. When yearly data were separated, sediment was no longer a reliable predictor for *A. t. mavortium* or *B. cognatus*. Hydroperiod was still found to be a significant predictor in 2008 for *A. t. mavortium* (Fig. 5) but not in 2009, and similarly for

B. cognatus (Fig. 6) and *P. clarkii* (Table 8). The degree of importance for sediment, hydroperiod, and percent grass cover changes for *B. woodhousii* between years. In 2008, sediment was no longer a strong predictor; whereas in 2009 it remained highly important (Fig. 7) (Table 8). Without combined data, hydroperiod was no longer a predictor of *B. woodhousii* occurrence. Only in 2009 was percent grass cover a strong predictor of occurrence for *B. woodhousii*. In 2008 presence of *R. blairii* was not related to hydroperiod, but was in 2009 (Table 8). In *Spea spp.* tadpoles, hydroperiod is not necessarily a limiting factor to presence, but in 2008 it was a significant predictor due to the short lived nature of all playas that year (Table 8). For all other species, none of the environmental variables were found to be reliable for predicting species occurrence.

For combined years, landuse type did not explain overall variation within the full CCA model ($F = 0.990$, $p = 0.341$). In 2008, the full model was significant ($F = 1.50$, $p = 0.075$), with CRP explaining 52% of the variation in species distribution ($F = 1.95$, $p = 0.043$) (Fig. 8). *R. blairii* strongly associated with CRP playas and the majority of all other species were driven towards either crop or grass playas (Fig. 8). Axis 1, driven by CRP, accounted for 65% of the total variation, while Axis 2, driven by crop and grassland properties explained the remaining 35% of the distribution (total inertia = 1.116, $\lambda_1 = 0.054$, $\lambda_1 = 0.029$, $\lambda_1 = 0.229$, $\lambda_1 = 0.190$). In 2009, however, landuse did not explain the variation of species distribution ($F = 0.806$, $p = 0.677$) (Fig. 9).

Similar to the GLM univariate analyses, there is a strong relationship between hydroperiod and *R. blairii,* but not with *A. t. mavortium,* in the playa characteristics CCA (Fig. 10A, B). *Spea* tadpole occurrence was strongly related with playa area and increasing hydroperiod. *Bufo woodhousii* was strongly linked with increasing sediment

depth, whereas *S. multiplicata* and *P. clarkii* tended to be found in playas with a higher percentage of native grass within the watershed (Fig. 10A, B). Overall, sediment depth explained 34.8% ($F = 2.32$, $p = 0.014$) of the data, hydroperiod explained 28.1% ($F = 1.85$, $p = 0.058$), and percent grass cover 25.8% ($F = 1.72$, $p = 0.073$). With all variables in the analysis only *B. woodhousii*, *S. multiplicata*, *R. blairii*, and *Spea spp.* tadpoles showed distinct preferences (Fig 10). Although there was a significant landuse effect on amphibian distribution in 2008, overall wetland characteristics did not fully explain the variation within species distributions ($F = 0.986$, $p = 0.455$) (Fig. 11). In 2009, all five wetland characteristics contributed some explanation of species distribution ($F = 1.57$, $p = 0.025$) with hydroperiod explaining 37% ($F = 2.87$, $p = 0.01$) of species distribution, sediment depth 28.6% ($F = 2.14$, $p = 0.03$), and percent native grass in the watershed 24% ($F = 1.80$, $p = 0.11$) (Fig 12). *Bufo woodhousii* was again strongly associated with increasing sediment depth and *P. clarkii* was associated with percent native grass within the watershed, although to a lesser extent than with pooled data (Fig. 10). Both *S. bombifrons* and *S. multiplicata* were associated with playas that had short hydroperiods, whereas *R. blairii* preferred long hydroperiods (Fig. 10). The distribution of *A. t. mavortium, B. cognatus,* and *Spea* tadpoles were not explained by any of the five wetland characteristics assessed.

When considering all playa variables together, univariate relationships dissipate. Compared to other playa characteristics, water loss and playa area do not play a role in amphibian community characteristics (Fig 10 and 12). The pooled CCA (Fig. 10) also illustrates that playa area, hydroperiod, and water loss are correlated, but percent grass cover and sediment depth are not dependent of either hydroperiod or playa area.

Sediment depth and water loss rate were correlated and this further suggests and provides evidence that both are dependent on surrounding upland vegetation because crop playas tend to have both faster water loss rates and more sediment. Similar to the combined data, in 2009 hydroperiod, sediment depth, water loss rate, and percent native grass cover explained most of the species distribution variation (Fig. 12).

Discussion

We predicted that sediment depth, hydroperiod, water loss rate, and amphibian diversity of CRP playas would be intermediary to that of cropland and grassland playas. Our results show that with playa characteristics, this tends to be true; however species richness did not differ among land use types. Sediment depth was the only characteristic where CRP playas differed from both crop and grass playas; whereas with starting depth, water loss rate, and hydroperiod, CRP was similar to either crop or grass playas. These data are supported by the fact that CRP playas were previously cultivated and therefore will share some characteristics with crop playas, but are currently planted with grasses (although non-native) and will also be similar to native grassland playas.

The USDA CRP was initiated in 1985 to prevent soil erosion in highly erodible cropland and the replanting of grasses has been successful for this endeavor. However, introducing exotic grasses can alter ecosystem processes (Berthelsen et al., 1989; Ehrenfeld, 2003) and in this region it has affected playa wetland hydrology and wildlife and plant communities (2003 Smith et al., 2011). A recent survey of 261 playas from six states found that in an average rainfall season CRP playas were 45% less likely to be inundated than cropland or grassland playas, supporting the idea that although CRP plantings are efficient at mitigating erosion, they also likely inhibit water runoff into

wetlands. This occurs under ‘normal’ precipitation years as well as dryer and wetter years (Cariveau et al., 2011). Lack of inundation could be more prevalent in dryer years and potentially have a significant impact on vegetation and wildlife (Moon and Haukos, 2006), especially amphibian communities.

Shorter hydroperiods and wetlands potentially with a limited amount of vegetative resources subsequently create an environment where fewer species will be able to occupy the wetland, decreasing breeding and developmental success (Alford, 1986; Pechmann et al., 1989; Anderson et al., 1999). In 2008, we surveyed and included in our analysis two CRP playas that never filled during the season, although they were located adjacent to grassland playas that were inundated and included in this study, therefore suggesting that this area received enough precipitation for inundation. This supports the idea that CRP playas fill less frequently, under the same conditions, as the other two catchment types. CRP playas require a larger amount of precipitation to inundate and sites selected for this study may reflect this discrepancy. Playas were selected within 7 days of initial inundation but there were many CRP playas in the same vicinity that never filled and this discrepancy of selecting playas after inundation may potentially have biased our results. CRP playas selected may have received more direct precipitation than others (or had a greater percentage of another landuse type within its watershed), allowing for an inaccurate depiction of CRP playa characteristics and amphibian communities. However, it is unlikely that any bias significantly impacted the results. Unfortunately, if projected climate change patterns occur, this region could see less precipitation over the next few decades, further decreasing playa hydroperiods and inundation rates (Seager et al., 2007), and subsequently impacting amphibian communities.

CRP playas were predicted to have intermediate or even lower species richness between crop and grass playas. This did not occur, with all three catchment types having the same mean number of species per playa. Previous studies investigating landuse effects on species richness found similar results (Anderson et al., 1999; Hall et al., 2004; Ghioca, 2005; Torrence, 2007), although each did find some differences between crop and grass playas in community composition. Hydroperiod was the main driver for species richness in 2008 when there was little precipitation, but not in 2009 when rainfall was locally abundant and hydroperiod was longer across all catchment types; thus allowing for more species to colonize the area and essentially doubling the number of species found in a small area (Duellman and Trueb, 1986; Pechmann et al., 1989; Snodgrass et al., 2000; Babbitt et al., 2003; Baber et al., 2004; Werner et al., 2007). Indeed, it is rare to find an inundated playa with no amphibians present, but in 2008, three playas (two crop and one CRP) did not have any amphibians present and all three had hydroperiods of 20 days or less. This evidence suggests that hydroperiod drives amphibian population diversity and species occurrence more so in drier years. It is also possible, that in playas that have remained dry for many years, resident amphibian communities were locally extirpated. Dry years, such that occurred in 2008 can affect species presence as well as potentially affecting breeding success (Donnelly and Crump, 1998), therefore during normal or wet years, more amphibian species are able to colonize playas with longer hydroperiods.

Although, cumulative species richness did not differ among land use types, community composition varied greatly both within and between years. Absolute frequency of species occurrence varied among years due to hydroperiod differences, but

the probability of finding most dominant species did not differ. This indicates that although environmental factors are driving amphibian presence and abundance, the proportion and probability of seeing dominant species is likely to stay the same if frequency of occurrence is already large. Our results show that the same four species (*A. t. mavortium, B. cognatus, P. clarkii*, and *R. blairii*) were dominant in both 2008 and 2009, suggesting that for at least these two years, these species' populations were in higher abundance.

Amphibian communities among all three landuse types were relatively similar in 2009, whereas in 2008 landuse, specifically CRP, played a greater role in community composition. This may have been driven by differences in landuse preference of *B. cognatus, P. clarkii,* and *R. blairii* as well as most species grouping towards either crop or grass playas. This implies that although CRP playas have the same number of species per playa as the other two land use types, community composition is different than in crop or grass playas. This disparity may be caused by two mechanisms: dispersal capability or landuse resource preference. Amphibians are capable of dispersing relatively long distances for their size (Gehlbach et al., 1969), but individuals will most likely stay in their natal ponds. Also, some species of amphibians have difficulty navigating a complex landscape or will not migrate to a different playa during dry years because it is too energetically costly. Spadefoot toads, for example, may travel shorter distances and have difficulties traversing heavily cultivated habitat (Gray et al., 2004a and 2004b), whereas spadefoot toads and other species such as the smaller-bodied spotted chorus frog (*P. clarkii*) may also have difficulties navigating the tall, perennial exotic grasses characteristic of most CRP. It is possible that the high abundance of *P. clarkii*

found in CRP playas throughout both years may be an artifact of difficulty traversing CRP grasses, causing populations to become isolated in their natal playas, similar to *Spea spp.* becoming isolated in crop playas due to habitat complexity (Wiens, 1997; Rothermel and Semlitsch, 2002; Gray et al., 2004a; 2004b; Venne, 2006). It is also possible that *P. clarkii* have thrived in CRP playas for other reasons. Desiccation stress increases in bare ground habitats (Rothermel and Luhring, 2005; Rittenhouse et al., 2008) and tall grasses may provide ample habitat for shelter.

Landuse and wetland characteristics had a major effect on species occurrence, indicating that some species have adapted and established themselves in areas thought to be less suitable for amphibians. This study as well as others (Gray et al. 2004a) found that cultivation may not have as large an impact on some species (with some more common in cultivated landscapes), however this does not indicate that there is no effect on species composition in wetlands highly impacted by cultivation. This study did not investigate potential effects of other anthropogenic alterations to wetland habitat (e.g., contaminants), but there is literature suggesting that contaminants play a large role in species and population survival in some amphibian species (Sparling et al., 2003; Hayes et al., 2006; Boone et al., 2007; Mann et al., 2009). Previous studies have shown that in playa wetlands, landuse has a significant effect on amphibian developmental rate, size, and immune development and function (Gray et al. 2004a, Venne 2006, McMurry et al. 2009). This may imply that although species are adapted to surviving in highly cultivated landscapes, there are potentially detrimental trade-offs.

Landuse affects the amount of surface runoff a wetland will receive (Euliss and Mushet, 1996; van der Kamp et al., 1999; Tsai et al. 2007). This study reflects this with

cropland playas having a deeper starting water depth than both CRP or grassland playas in 2008 and 2009, and percent native grass within the watershed and starting depth were negatively correlated ($r = -0.28$, $p = 0.01$). This accounts for cropland playas having longer hydroperiods even though they lose water more rapidly. Because crop playas have a larger proportion of bare ground in the surrounding upland, when precipitation events occur there is a greater chance of significantly more runoff into the playa, subsequently allowing for crop playas to have a greater starting depth. However, it is essentially hydroperiod and not starting depth of a playa that will affect amphibian communities. One exception may be spadefoot toads which prefer shallower water and tend to not breed in playas that remained inundated into the next breeding season (Bragg, 1944; Anderson et al., 1999).

Our data support previous studies investigating water loss rates among crop and grassland playas (Gray et al. 2004a, Gray et al. 2004b, Venne 2006, Tsai et al. 2007). In 2008, CRP playas lost water 34% faster than grassland playas, and had water loss rate 15% slower than crop playas, indicating that CRP catchments trap sediment in the upland, preventing volume loss and water occupying a larger surface area, subsequently preventing excessive evaporation and slowing infiltration loss. Because of the significantly greater starting depth in cropland playas, hydroperiod was not negatively affected by high water loss rate in this study. Although species richness did not correlate with water loss rate, numerous laboratory studies link amphibian size at metamorphosis to water loss rate (Newman 1994, Denver et al. 1998, Gervasi and Foufopoulos 2008, Székely 2010). Species located in cropland or CRP playas may have a higher risk of being smaller at metamorphosis, because of faster water loss rates, an effect that can

continue into adulthood and influence survival and reproductive success (Woolbright, 1983; Semlitsch, et al., 1988). Gray and Smith (2005) found that landuse indirectly affects body size where individuals of *Spea multiplicata*, *S. bombifrons*, and *Ambystoma t. mavortium* had smaller body sizes in areas that were cultivated as opposed to the same species in native grassland playas.

Our CCA models indicate that sediment depth, hydroperiod, and percent cover of native grass in the watershed are key components to species occurrence. Therefore, when discussing management projects in playa wetlands, high priority should be considered to requiring vegetative buffers around the playa and restoring native landscape features (Melcher and Skagen, 2005). CRP enrollments were contracted for 10years each, over the past 25 years, but many contracts have expired or are currently up for renewal. With the potential for CRP enrolled lands to return to cultivation, there is the risk of increasing landscape heterogeneity, creating a more patchy landscape, further isolating some playas and subsequently isolating amphibian populations. Our data do not indicate overall abundance of species found in playa wetlands, but do identify dominant species based on frequency of occurrence. Species such as *B. speciosus* and *G. olivacea* were rarely observed, and some species such as *B. debilus* and *A. creptitans* were not observed despite their occurrence in the region. These rarer species may be most prone to land use changes and the contaminant effects on wetland hydrology (Gray et al., 1990; Purvis et al., 2000).

Amphibian communities in the SHP rely on playas as fundamental resources for breeding each year and the risk of losing playas from sedimentation, tilling, and land use effects is high. Extreme weather patterns are also becoming more frequent due to climate

change, and amphibians are highly susceptible (Beebee 1996, Pounds et al. 1999). Piha 2007 report that increased cultivation of the landscape has a detrimental effect on amphibian populations during extreme climatic occurrences (e.g. drought) and that heterogeneous landscapes (with less cultivation) may enhance population survival (Ehrlich & Murphy 1987, Piha 2007). Although CRP catchments decrease inundation of playas and alter amphibian community distribution, they do provide habitat that reduces desiccation probability and reduces sediment input into wetlands.

Table 1. Total number of sites in each of three hydroperiod categories for playa wetlands in Southern High Plains, TX, based on surrounding upland landuse. In 2008, almost all sites had hydroperiods below 50 days, whereas in 2009 hydroperiods tended to be greater than 50. The three hydroperiod categories were chosen to reflect short (≤ 50 Days) hydroperiods and the effects they can have on wildlife populations based on previous data and this study.

	Hydroperiod		
Catchment Type	**≤ 50 Days**	**51-100 Days**	**≥ 101 Days**
2008			
Cropland	18	0	0
CRP	11	1	0
Grassland	13	0	0
2009			
Cropland	0	4	17
CRP	1	5	9
Grassland	1	7	5

Table 2. Two-way ANOVA table showing F and p statistics for year, landuse, and the interaction term for all wetland characteristics. Hydroperiod was the only characteristic with a significant interaction and the results from the subsequent 1-way ANOVAs are shown.

Wetland Characteristics	F	p
Sediment Depth		
year	9.75	0.002
landuse	18.24	<0.001
year*landuse	2.85	0.064
Starting Water Depth		
year	43.13	<0.001
landuse	13.13	<0.001
year*landuse	1.55	0.217
Water Loss Rate		
year	8.57	0.004
landuse	3.93	0.023
year*landuse	0.75	0.475
Hydroperiod		
year*landuse	4.60	0.013
Year		
Crop	141.65	<0.001
CRP	45.59	<0.001
Grass	26.65	<0.001
Landuse		
2008	0.15	0.861
2009	5.84	0.006
Playa Area		
year	0.43	0.515
landuse	1.22	0.299
year*landuse	2.84	0.063
Species Richness		
year	47.21	<0.001
landuse	0.09	0.917
year*landuse	0.39	0.681

Table 3. Mean ± SE of wetland characteristics measured in playa wetlands across three landuse types in Southern High Plains, Texas during 2008. No differences among landuse type were observed.

2008	Cropland (n=18)	CRP (n=14)	Grassland (n=14)
Sediment depth (cm)	23.24 ± 4.61	16.67 ± 1.83	10.39 ± 0.88
Starting water depth (cm)	34.23 ± 5.7	19.58 ± 3.75	17.68 ± 3.69
Water loss rate (cm/day)	1.47 ± 0.19	1.47 ± 0.27	0.93 ± 0.08
Hydroperiod (days)	23.06 ± 2.48	22.83 ± 6.31	24.08 ± 5.49
Playa area (ha)	10.17 ± 1.16	13.33 ± 2.93	26.27 ± 10.37
Species richness	2.56 ± 0.35	2.5 ± 0.56	2.21 ± 0.50

Table 4. Mean ± SE of wetland characteristics measured in playa wetlands across three landuse types in Southern High Plains, Texas during 2009. Superscript letters denote differences among landuse type with Tukey's HSD, alpha < 0.05.

2009	Cropland (n=21)	CRP (n=14)	Grassland (n=13)
Sediment depth (cm)	39.57 ± 4.22	24.64 ± 1.77	11.66 ± 0.83
Starting water depth (cm)	59.43 ± 1.47	48.96 ± 4.81	32.00 ± 4.81
Water loss rate (cm/day)	1.07 ± 0.08	0.91 ± 0.16	0.86 ± 0.05
Hydroperiod (days)	130.52 ± 8.07[a]	109.96 ± 11.81[ab]	82.88 ± 10.65[b]
Playa area (ha)	16.04 ± 1.27	15.03 ± 2.05	12.66 ± 1.68
Species richness	4.57 ± 0.23	4.36 ± 0.29	4.08 ± 0.31

Table 5. Mean ± SE of wetland characteristics measured in playa wetlands across three landuse types in Southern High Plains, Texas with pooled data from 2008 and 2009. Superscript letters denote differences among landuse type with Tukey's HSD, alpha < 0.05.

2008-2009	Cropland (n=39)	CRP (n=28)	Grassland (n=27)
Sediment depth (cm)	26.42 ± 3.35^{a}	20.66 ± 1.47^{b}	11.00 ± 0.61^{c}
Starting water depth (cm)	47.8 ± 3.39^{a}	34.27 ± 4.12^{b}	24.57 ± 3.26^{b}
Water loss rate (cm/day)	1.25 ± 0.10^{a}	1.17 ± 0.16^{ab}	0.86 ± 0.05^{b}
Hydroperiod (days)*	80.92 ± 9.76	69.75 ± 10.90	53.48 ± 8.26
Playa area (ha)	13.33 ± 0.98	14.18 ± 1.76	19.72 ± 5.5
Species richness	3.64 ± 0.26	3.43 ± 0.36	3.11 ± 0.35

* Values are reported although there is a significant interaction between landuse type and year

Table 6. Absolute percent frequency of amphibian species in Southern High Plains, Texas playa wetlands surveyed embedded in three landuse types (cropland, CRP, and native grassland). This table illustrates the total percent of each landuse, and total number of playas, that a particular species was found in during our survey. The table is separated by year and with combined total survey data. Chi square analyses were performed using a reduced model including *A. t. mavortium, B. cognatus, B. woodhousii, P. clarkii, R. blairii,* and all three *Spea spp.* grouped as one category. 2008 $X^2 = 39.10$, $0.05 < p < 0.10$; 2009 $X^2 = 41.28$, $p < 0.05$; 2008-2009 $X^2 = 63.75$, $p < 0.05$

2008	**Crop (n=18)**	**CRP (n=14)**	**Grass (n=14)**	**Total (n=46)**
A. t. mavortium	38.90	35.70	21.40	32.60
B. cognatus	50.00	28.60	57.10	45.70
B. speciosus	16.70	7.10	0.00	8.70
B. woodhousii	16.70	7.10	14.30	13.00
G. olivacea	0.00	14.30	0.00	4.40
P. clarkii	44.40	64.30	57.10	54.40
R. blairii	16.70	35.70	14.30	21.70
R. catesbeiana	0.00	7.10	0.00	2.20
S. bombifrons	5.6	7.1	14.3	8.7
S. multiplicata	22.2	7.1	35.7	23.9
S. couchii	11.10	0.00	0.00	4.40
***Spea* tadpoles**	44.40	21.40	28.60	32.60
2009	**Crop (n=21)**	**CRP (n=14)**	**Grass (n=13)**	**Total (n=48)**
A.t. mavortium	81.00	78.60	76.90	79.20
B. cognatus	95.20	92.90	84.60	91.70
B. speciosus	0.00	0.00	0.00	0.00
B. woodhousii	66.70	42.90	30.80	50.00
G. olivacea	4.80	0.00	7.70	4.20
P. clarkii	76.20	92.90	84.60	83.30
R. blairii	61.90	42.90	46.20	52.10
R. catesbeiana	0.00	0.00	0.00	0.00
S. bombifrons	14.30	21.4	7.70	14.60
S. multiplicata	33.30	50.00	53.80	43.80
S. couchii	4.80	0.00	0.00	2.10
***Spea* tadpoles**	66.70	64.30	53.80	62.50
2008-2009	**Crop (n=40)**	**CRP (n=28)**	**Grass (n=26)**	**Total (n=94)**
A.t. mavortium	62.50	53.60	44.40	55.30
B. cognatus	72.50	53.60	70.40	67.00
B. speciosus	7.50	3.60	0.00	4.30
B. woodhousii	42.50	25.00	22.20	31.90
G. olivacea	2.50	3.60	3.70	3.20

Table 6 continued				
P. clarkii	65.00	82.10	74.10	73.40
R. blairii	37.50	39.30	29.60	36.20
R. catesbeiana	0.00	3.60	0.00	1.10
S. bombifrons	12.50	14.30	7.40	11.70
S. multiplicata	30.00	32.10	40.70	34.00
S. couchii	7.50	0.00	0.00	3.20
***Spea* tadpoles**	60.00	42.90	40.70	50.00

Table 7. Percent relative frequency of amphibian species (individual species frequency/total species frequency) in Texas playa wetlands embedded in three landuse types (crop, CRP, and grassland). Data was collected in 2008 and 2009. The total species frequency was calculated as the number of times an amphibian was found during each survey performed. Total number of observations for 2008-2009 combined Crop (426), CRP (264), Grass (235). Total species frequency of Crop 0.541, CRP 0.743, Grass 0.765. Individual species frequency was calculated by determining the percent occurrence out of the total number of observations per landuse. For example, in 2009 we saw *Ambystoma t. mavortium* in 81% of surveyed crop playas (Table 5); however, based on all data recorded and all individual visits to crop playas throughout the year, if we went to a crop playa and found an amphibian there would be a 13.5% chance that it would be *A. t. mavortium*.

	Crop	**CRP**	**Grass**
2008			
A.t. mavortium	9.61	12.80	6.54
B. cognatus	13.90	9.02	18.17
B. speciosus	7.39	1.28	0.00
B. woodhousii	5.36	1.28	2.61
G. olivacea	0.00	2.56	0.00
P. clarkii	17.00	42.30	31.10
R. blairii	7.39	16.70	14.25
R. catesbeiana	0.00	1.28	0.00
S. bombifrons	1.07	1.28	3.92
S. multiplicata	7.39	2.56	6.54
S. couchii	3.14	0.00	0.00
***Spea* tadpoles**	27.73	9.02	16.86
2009			
A.t. mavortium	13.50	11.90	15.70
B. cognatus	34.70	38.70	37.60
B. speciosus	0.00	0.00	0.00
B. woodhousii	12.20	7.70	4.37
G. olivacea	0.33	0.00	0.63
P. clarkii	12.80	21.60	20.70
R. blairii	9.67	4.67	5.04
R. catesbeiana	0.00	0.00	0.00
S. bombifrons	0.98	2.00	0.63
S. multiplicata	2.60	5.66	7.56
S. couchii	0.64	0.00	0.00
***Spea* tadpoles**	12.80	7.70	8.15

Table 7 continued			
2008-2009			
A.t. mavortium	12.60	12.10	12.60
B. cognatus	29.70	30.20	31.20
B. speciosus	1.73	0.37	0.00
B. woodhousii	10.60	5.88	3.79
G. olivacea	0.25	0.74	0.42
P. clarkii	13.90	27.20	24.40
R. blairii	9.17	8.09	8.01
R. catesbeiana	0.00	0.37	0.00
S. bombifrons	0.99	1.83	1.68
S. multiplicata	3.71	4.78	7.16
S. couchii	1.23	0.00	0.00
***Spea* tadpoles**	16.10	8.09	11.00

Table 8. Results for binomial GLMs showing species with significant relationships between probability of occurrence and select environmental variables. Data from 2008, 2009 and pooled years are shown. The null hypothesis was that the regression coefficient equaled zero, and the z coefficient a p values reflect this hypothesis.

	2008		**2009**		**2008-2009**	
	z	**p**	**z**	**p**	**z**	**p**
A. t. mavortium						
Hydroperiod	2.22	0.02	0.80	0.43	4.17	<0.001
Sediment depth	0.55	0.58	0.28	0.78	1.81	0.07
B. cognatus						
Hydroperiod	1.93	0.05	1.53	0.13	3.89	<0.001
Sediment depth	0.41	0.68	1.17	0.24	2.08	0.04
B. speciosus						
Sediment depth	2.05	0.04	-	-	2.31	0.04
Water loss rate	1.73	0.08	-	-	1.73	0.08
B. woodhousii						
Hydroperiod	0.59	0.56	0.51	0.61	3.34	<0.001
Sediment depth	1.79	0.07	2.68	0.01	3.50	<0.001
Percent native grass	-0.44	0.66	-2.60	0.01	2.29	0.06
P. clarkii						
Hydroperiod	1.69	0.09	1.07	0.28	2.61	0.01
R. blairii						
Hydroperiod	1.45	0.15	2.40	0.02	4.08	<0.001
Sediment depth	-0.80	0.43	0.81	0.42	1.81	0.07
Spea tadpoles						
Hydroperiod	2.46	0.01	0.68	0.50	2.93	0.003

Fig. 1. Distribution of study sites across the Texas region of the Southern High Plains. Black triangles denote sites in 2008 and gray triangles denote sites in 2009.

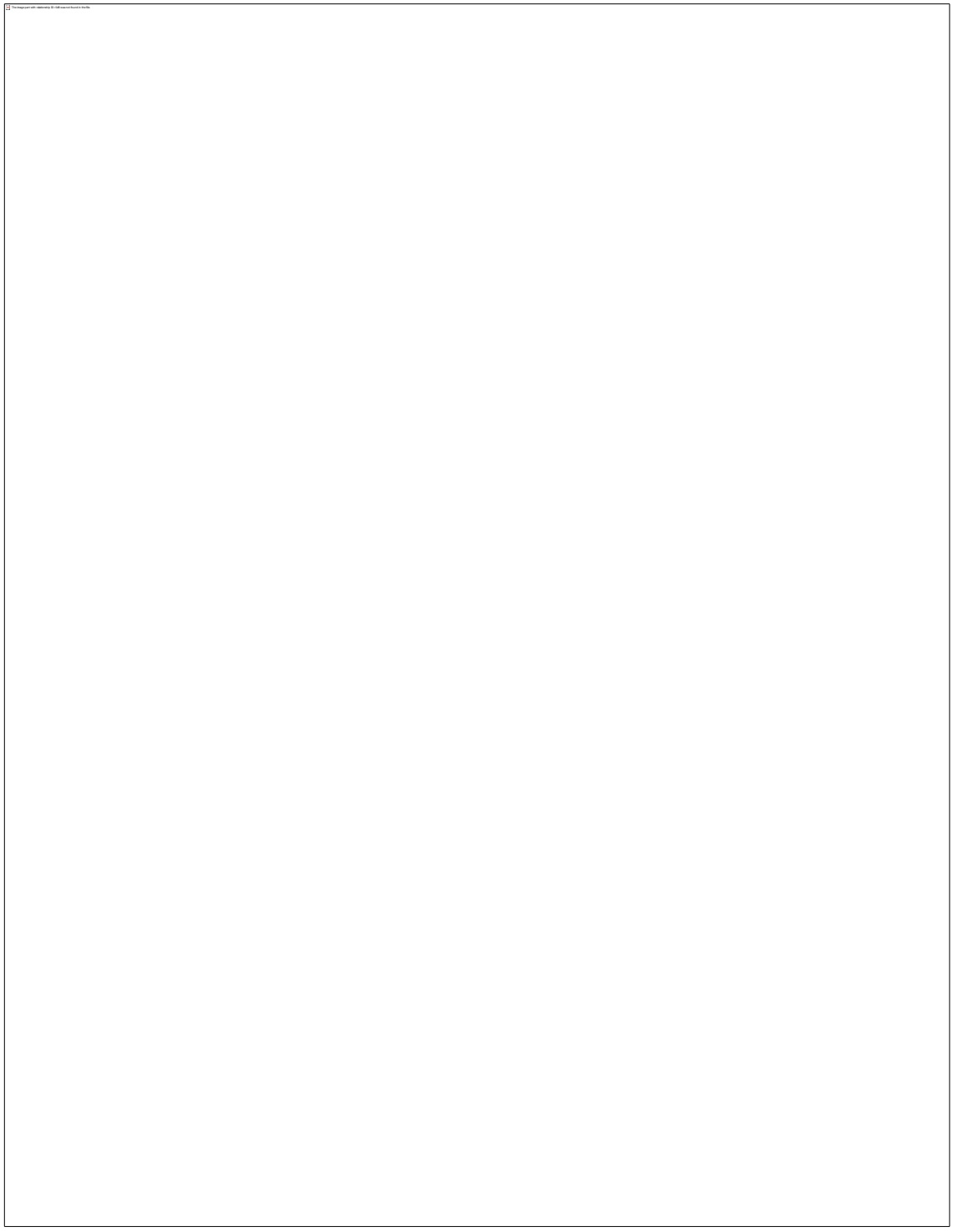


Fig 2. Correlation between species richness and hydroperiod for combined 2008-2009 data. The dotted line, at 50 days, is where a loss of species richness occurs (F = 25.06, p = <0.0001). At 100 days, there is a maximum in species richness that is maintained at longer hydroperiods. Solid circles represent playas from 2008 and open circles represent playas from 2009. The data are skewed by yearly differences where all of the shorter hydroperiods (< 50 days were surveyed in 2008 whereas all of the longer hydroperiods were from 2009. Pearson r = 0.584, p = <0.0001, R^2 = 0.42.

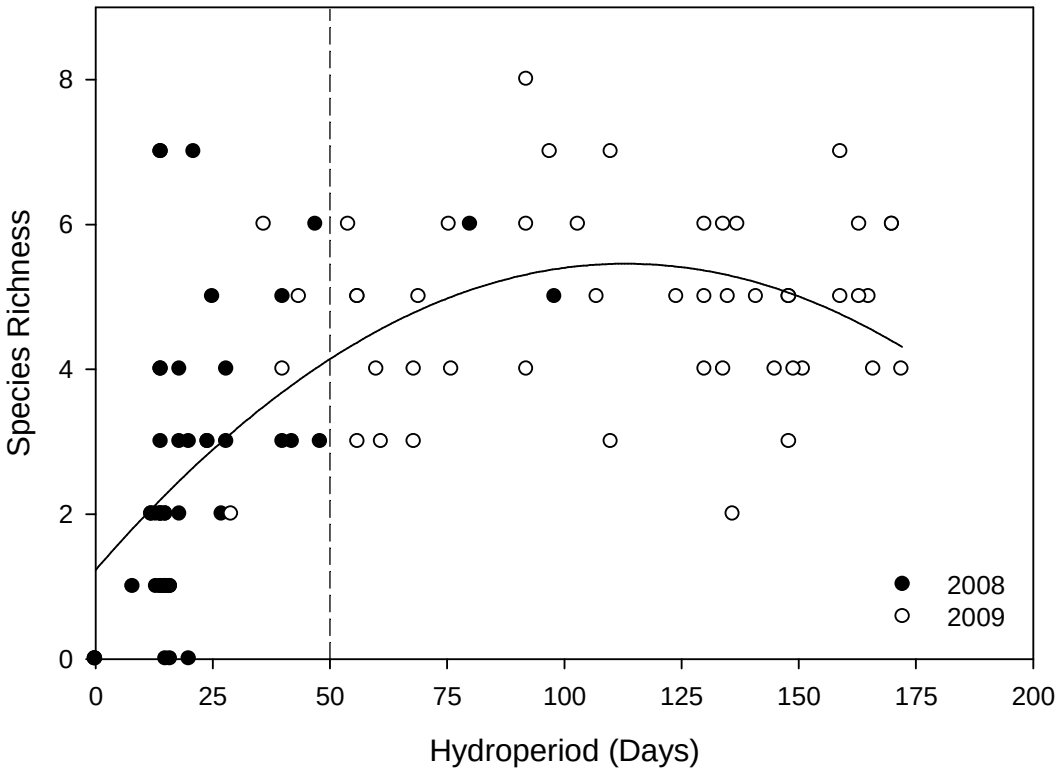


Fig. 3. Correlation between hydroperiod and species richness from playas surveyed in Southern High Plains, TX in 2008. There is a positive correlation between hydroperiod and species richness. Black circles represent individual playas. Person r = 0.505, p <0.0001, R^2 = 0.308.

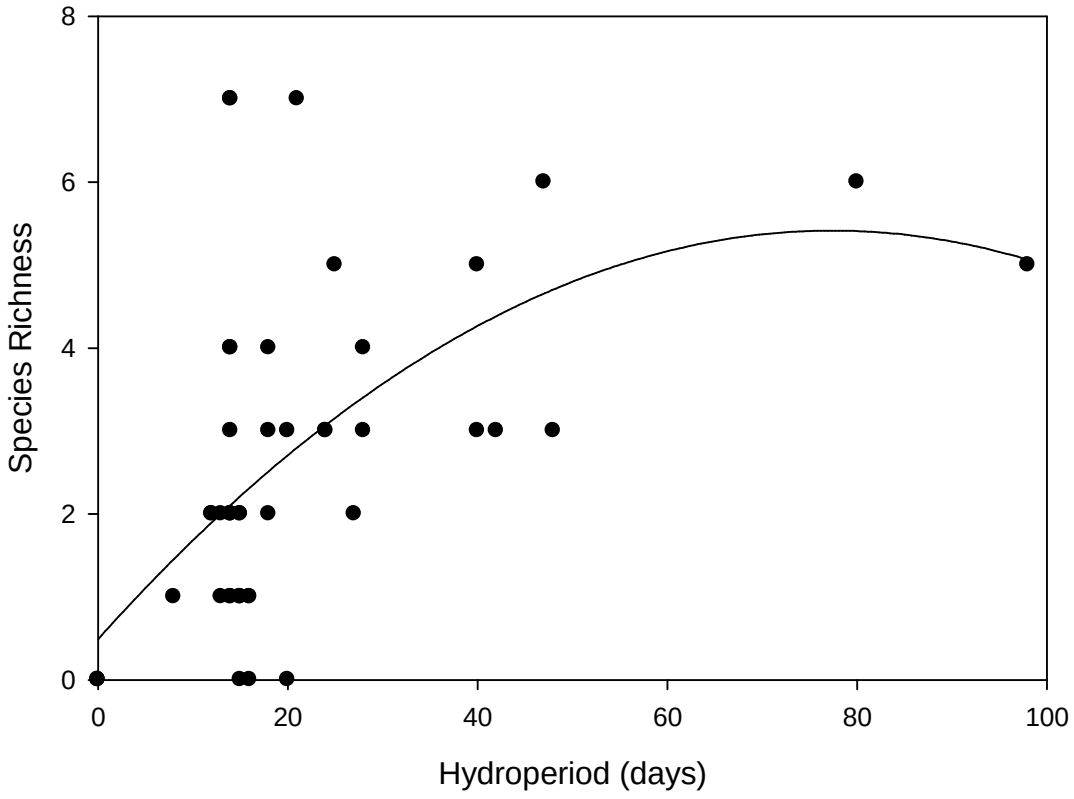


Fig. 4. Correlation between hydroperiod and species richness from playas surveyed in Southern High Plains, TX in 2009. There is no correlation between hydroperiod and species richness in 2009. Species richness was high across all hydroperiod measurements. Black circles illustrate individual playas. Pearson r = 0.150, p = 0.309, R^2 = 0.048.

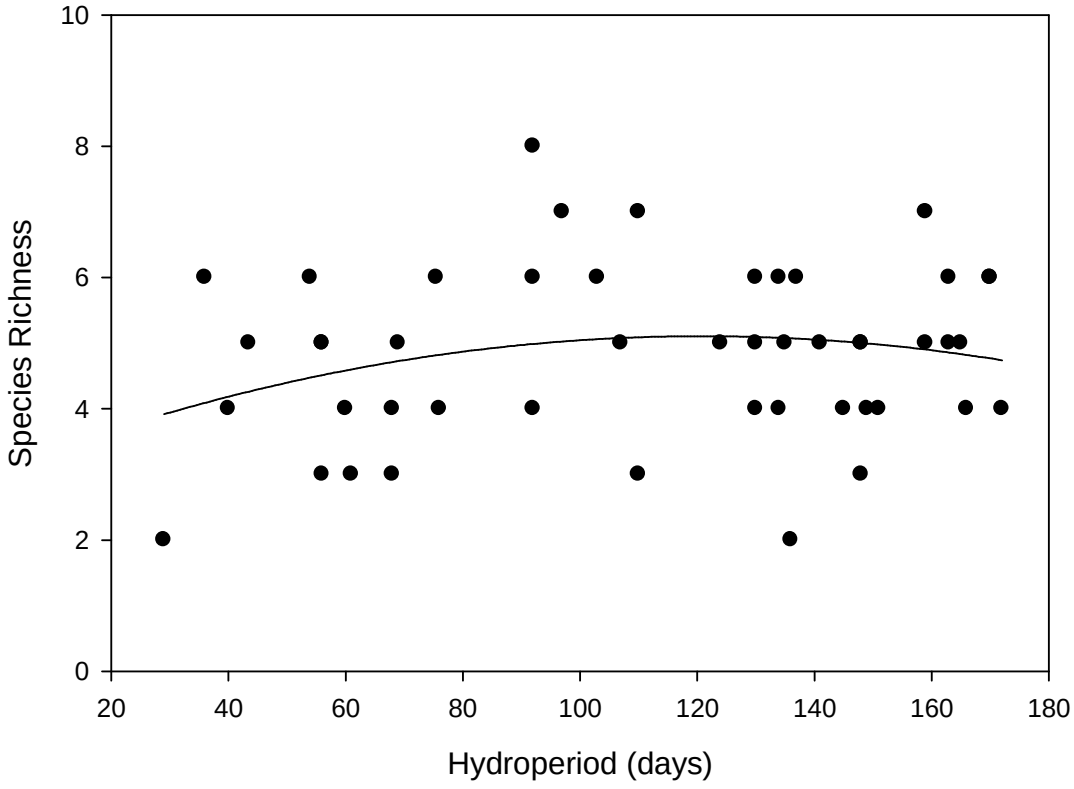


Fig. 5. Generalized linear model fitted for binomial data and modified for predicting the probability of *Ambystoma tigrinum mavortium* occurrence based on hydroperiod for 2008 data. Black dots represent each playa where this species was present or absent. Values of 1 = presence, values of 0 = absent. Hydroperiod (z = 2.22, p = 0.023), Intercept (z = -3.07, p = 0.002), Pearson correlation r = 0.416, p = 0.004.

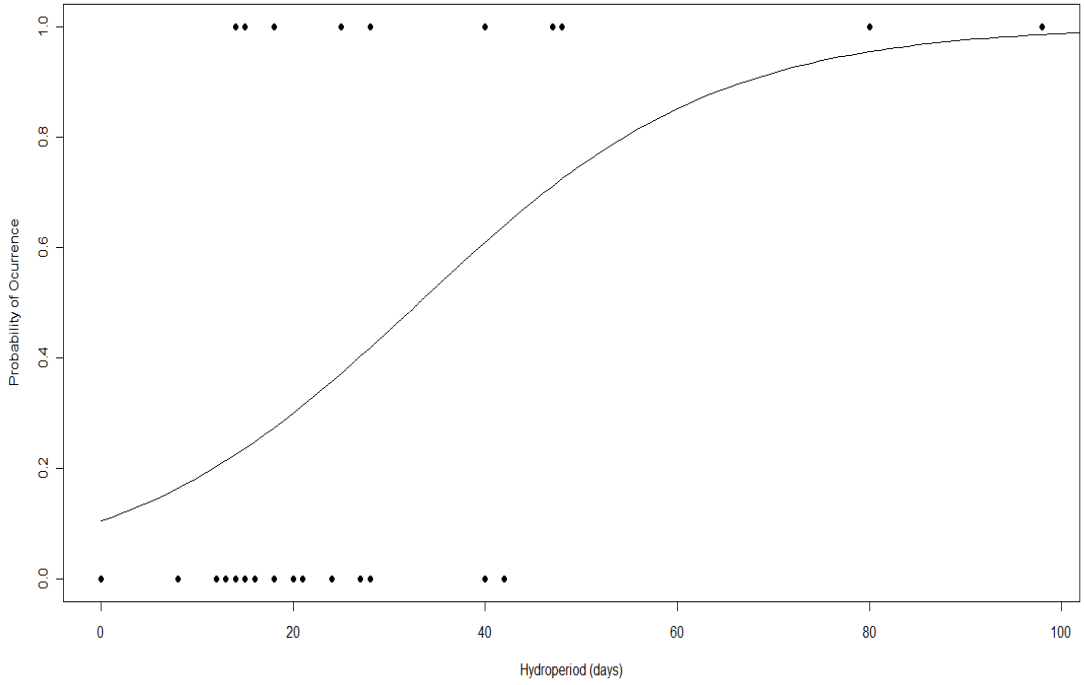


Fig. 6. Generalized linear model fitted for binomial data and modified for predicting the probability of *Bufo cognatus* occurrence based on hydroperiod for 2008 data. Black dots represent each playa where this species was present or absent. Values of 1 = presence, values of 0 = absent. Hydroperiod (z = 1.93, p = 0.053), Intercept (z = -2.21, p = 0.027), Pearson correlation r = 0.337, p = 0.022.

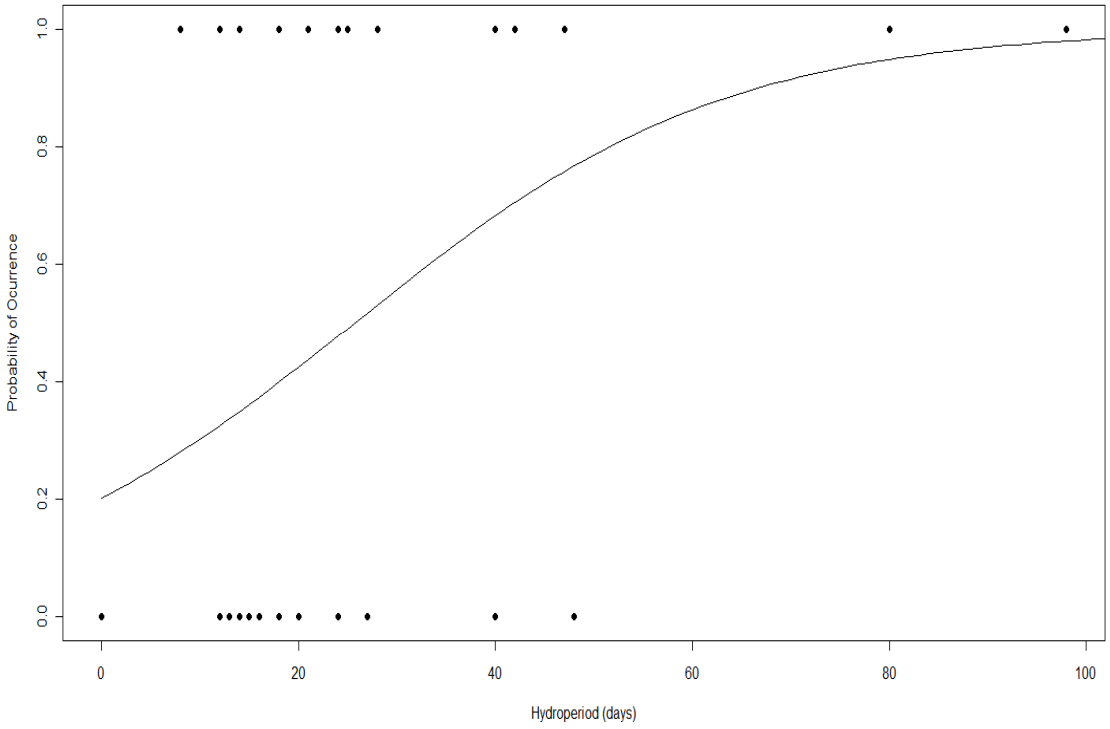


Fig. 7. Generalized linear model fitted for binomial data and modified for predicting the probability of *Bufo woodhousii* occurrence based on sediment depth for 2009 data. Black dots represent each playa where this species was present or absent. Values of 1 = presence, values of 0 = absent. Sediment (z = 2.68, p = 0.007), Intercept (z = -2.56, p = 0.011), Pearson correlation r = 0.441, p = 0.002.

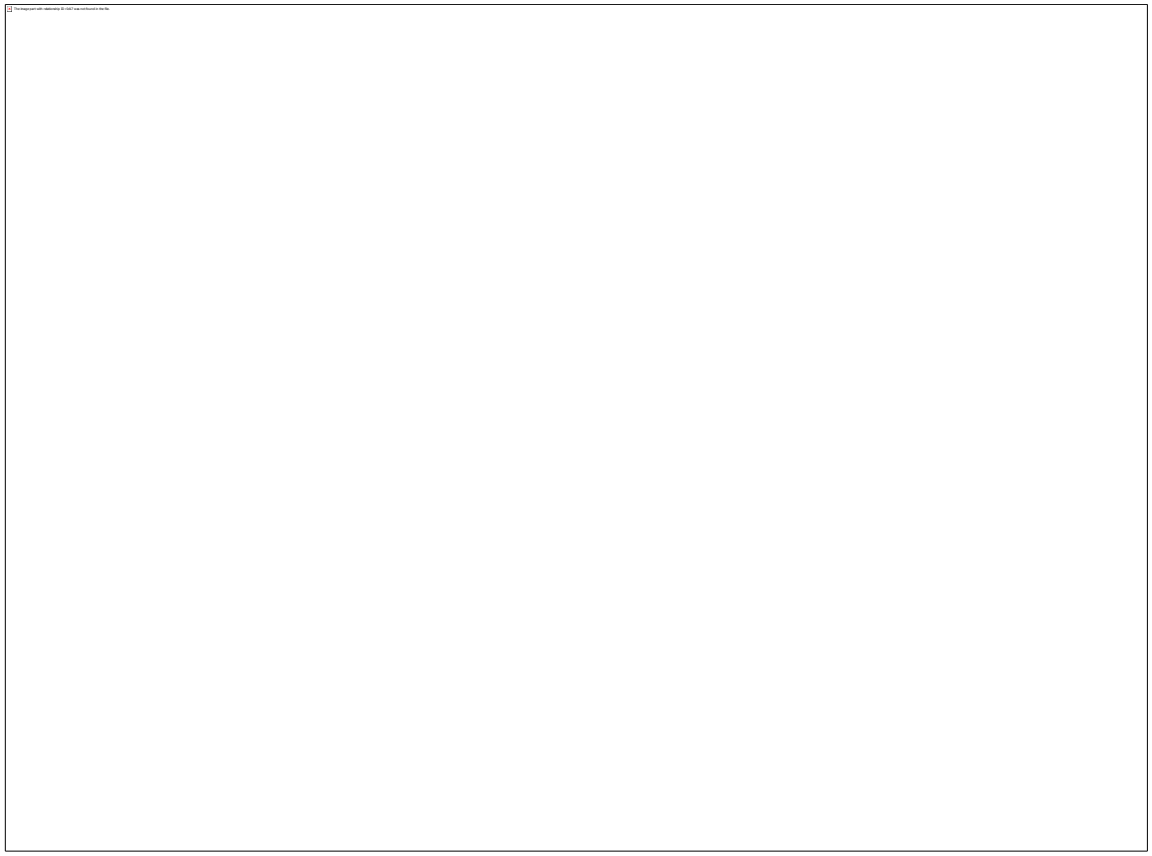


Fig. 8. Canonical correspondence analysis of amphibian species preference of the three landuse categories (crop, CRP, grassland) of playa wetlands in the Southern High Plains, TX in 2008 ($F = 1.50$, $p = 0.075$). CRP explained 52% of the variation in species distribution ($F = 1.95$, $p = 0.043$). Data are shown for the 1st and 2nd axes. Axis 1 explains variation due to CRP and axis 2 is driven by grassland and cropland properties. Both axis 1 and 2 explain 65% of the total variation. Only *Rana blairii* strongly grouped with CRP, all other species tended to group with either grassland or crop playas. Total inertia = 1.116, $\lambda_1 = 0.054$, $\lambda_2 = 0.029$, $\lambda_3 = 0.229$, $\lambda_4 = 0.190$.

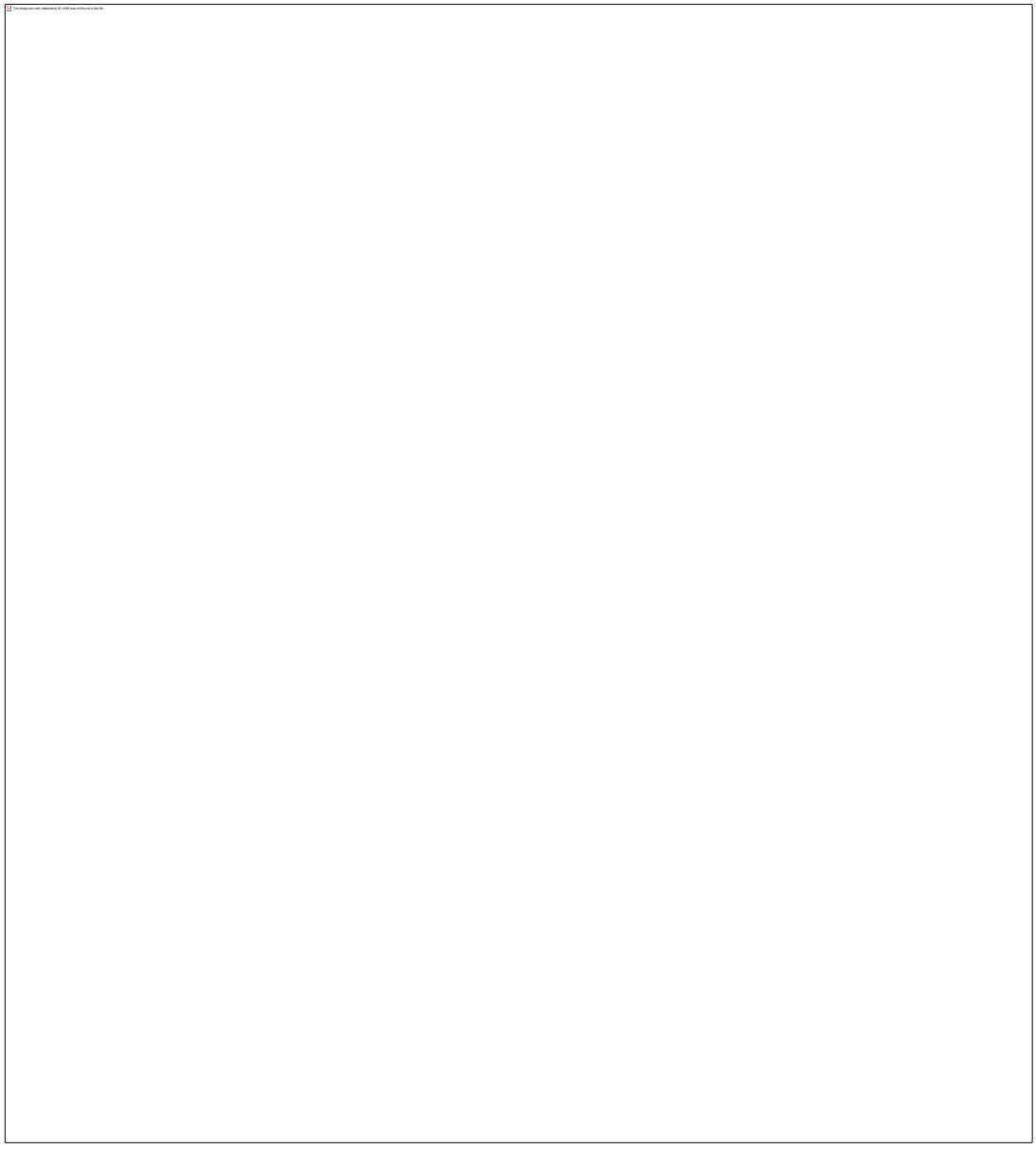


Fig. 9. Canonical correspondence analysis of amphibian species preference of the three landuse categories (crop, CRP, grass) of playa wetlands in the Southern High Plains, TX in 2009. The model is not significant at F = 0.806, p = 0.677. Data are shown for the 1st and 2nd axes.

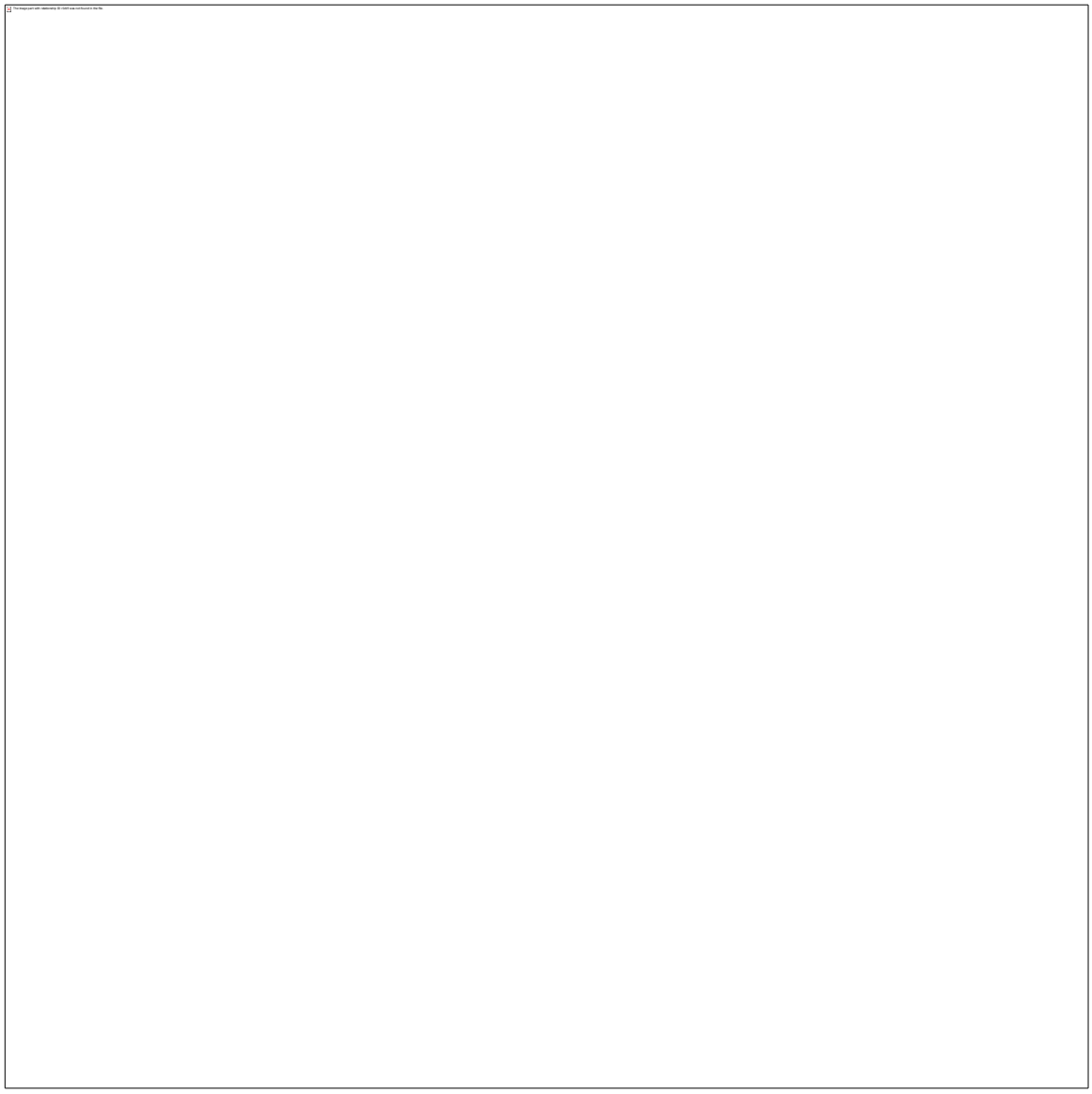
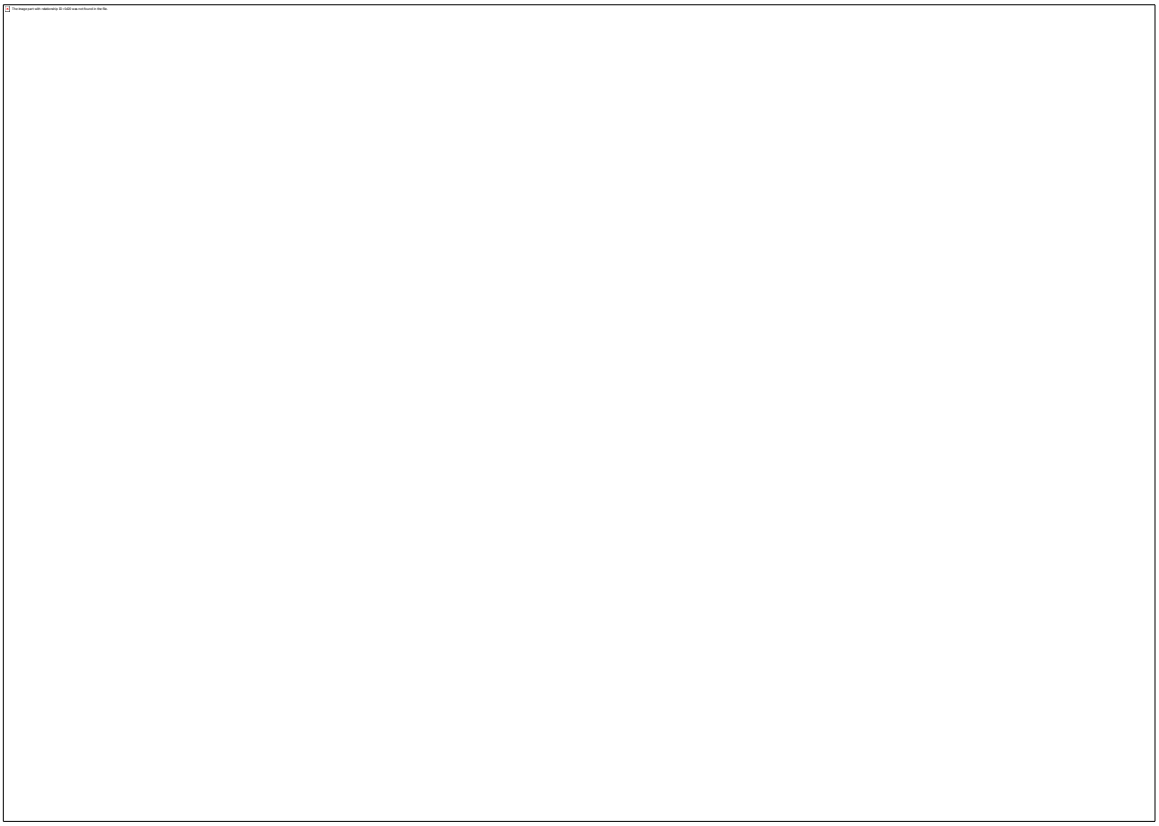


Fig. 10. Canonical correspondence analysis of amphibian species preference of five environmental variables (sediment depth, water loss, hydroperiod, area, and percent grass cover) measured from playa wetlands in the Southern High Plains, TX (F = 1.34, p = 0.10). 2008 and 2009 data were pooled. The length of arrows indicates relative importance to the model. Sediment and water loss are correlated, but sediment is the driving factor for axis 1, whereas hydroperiod and water loss rate are driving axis 2 and 3. **A**. Axis 1 and 2. Area and hydroperiod are correlated. *Rana blairii* is correlated with longer hydroperiods, *Spea* tadpoles are correlated with both hydroperiod and playa area, *Bufo woodhousii* is correlated with sediment depth, and *Spea multiplicata* is correlated with percent grass in the watershed. **B.** Axis 1 and 3. *Rana blairii* is linked with longer hydroperiods, *Bufo woodhousii* is strongly linked with sediment depth, *Spea multiplicata* is linked with percent grass in the watershed, and *Spea* tadpoles are correlated with increasing area and hydroperiod. Area and water loss rate are correlated, as well as hydroperiod and sediment. Total inertia = 1.194, $\lambda_1 = 0.033$, $\lambda_2 = 0.028$, $\lambda_3 = 0.014$, $\lambda_4 = 0.010$.



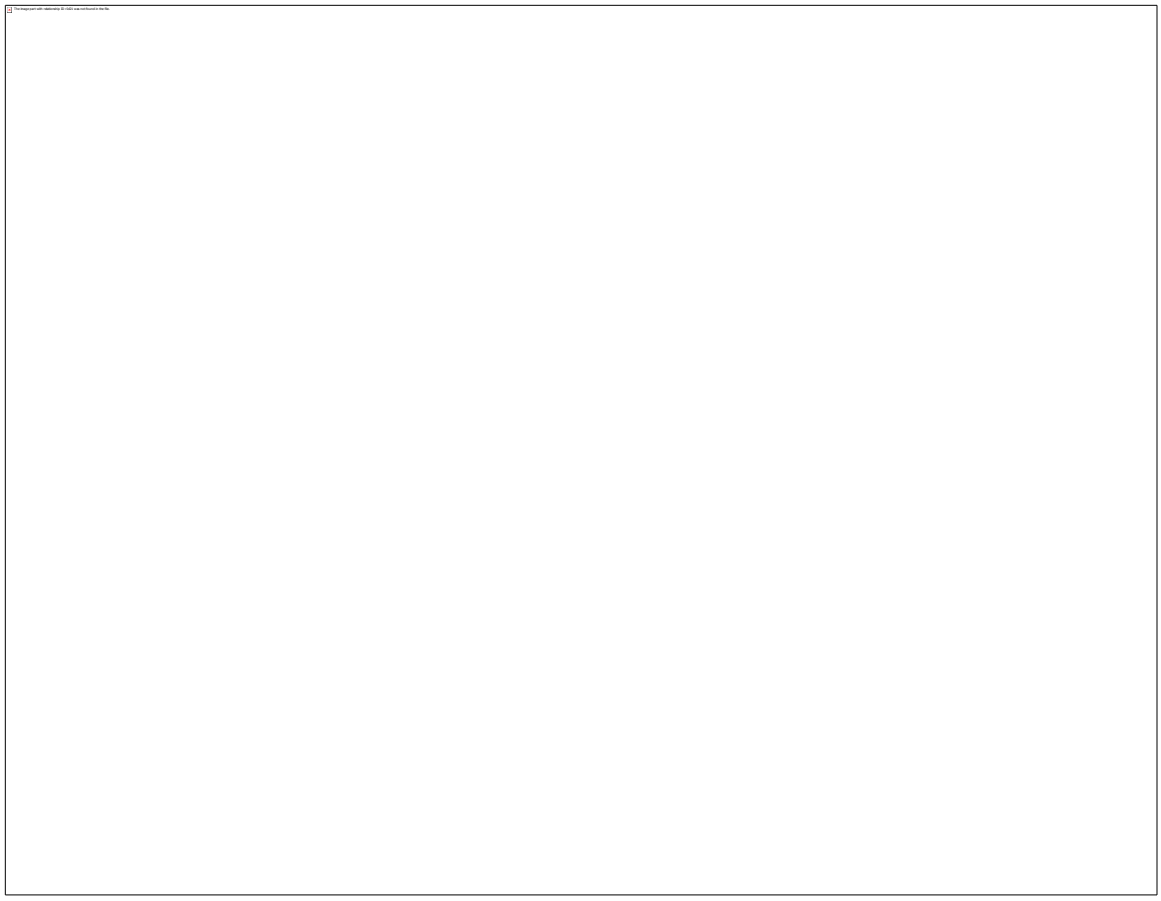


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Fig. 11. Canonical correspondence analysis of amphibian species preference of six environmental variables (sediment depth, water loss, hydroperiod, playa area, and percent grass cover) measured from playa wetlands in the Southern High Plains, TX in 2008. The biplot shows the first and second canonical axes, however, the model was not significant in explaining species distribution ($F = 0.986$, $p = 0.455$).

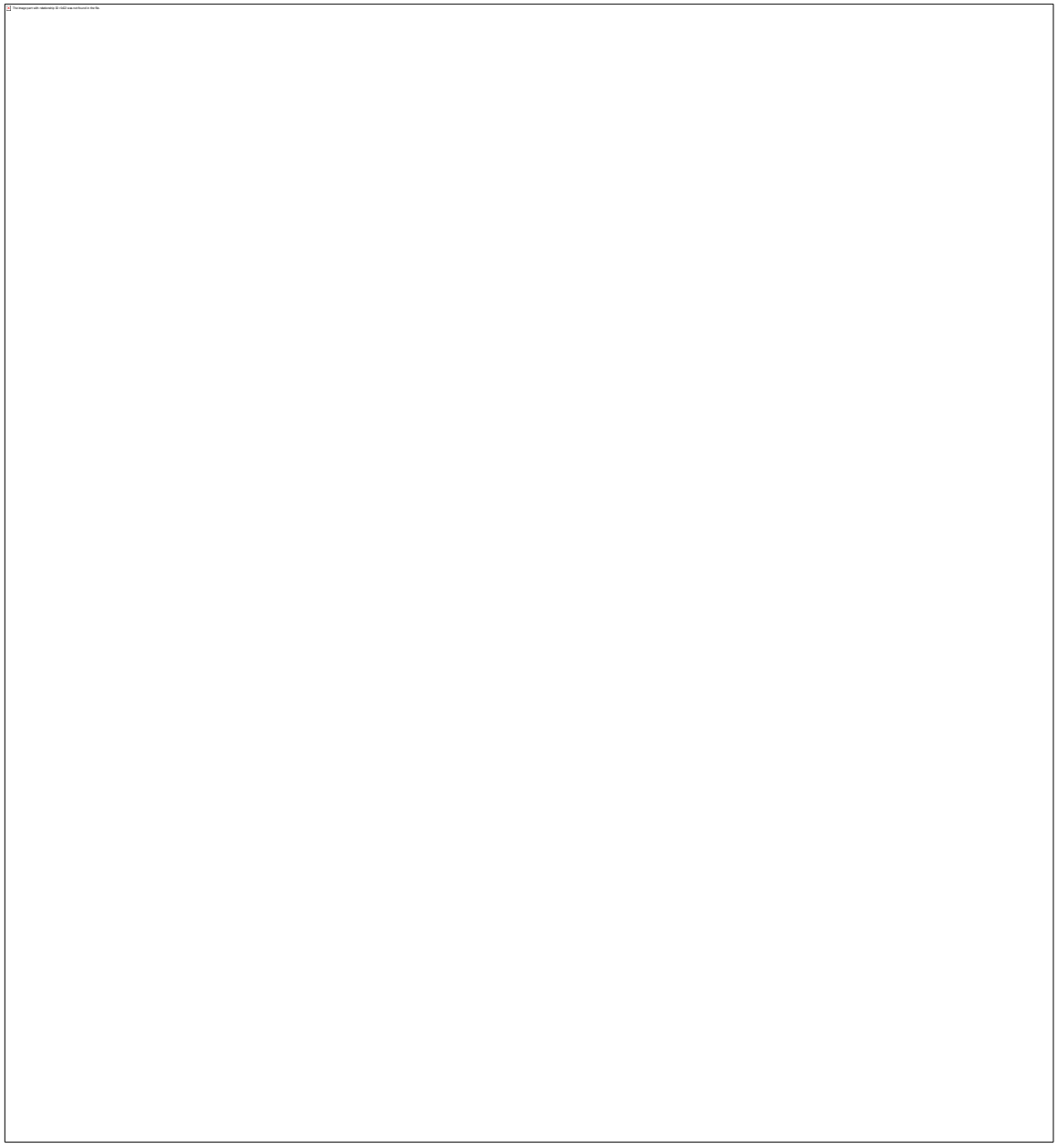
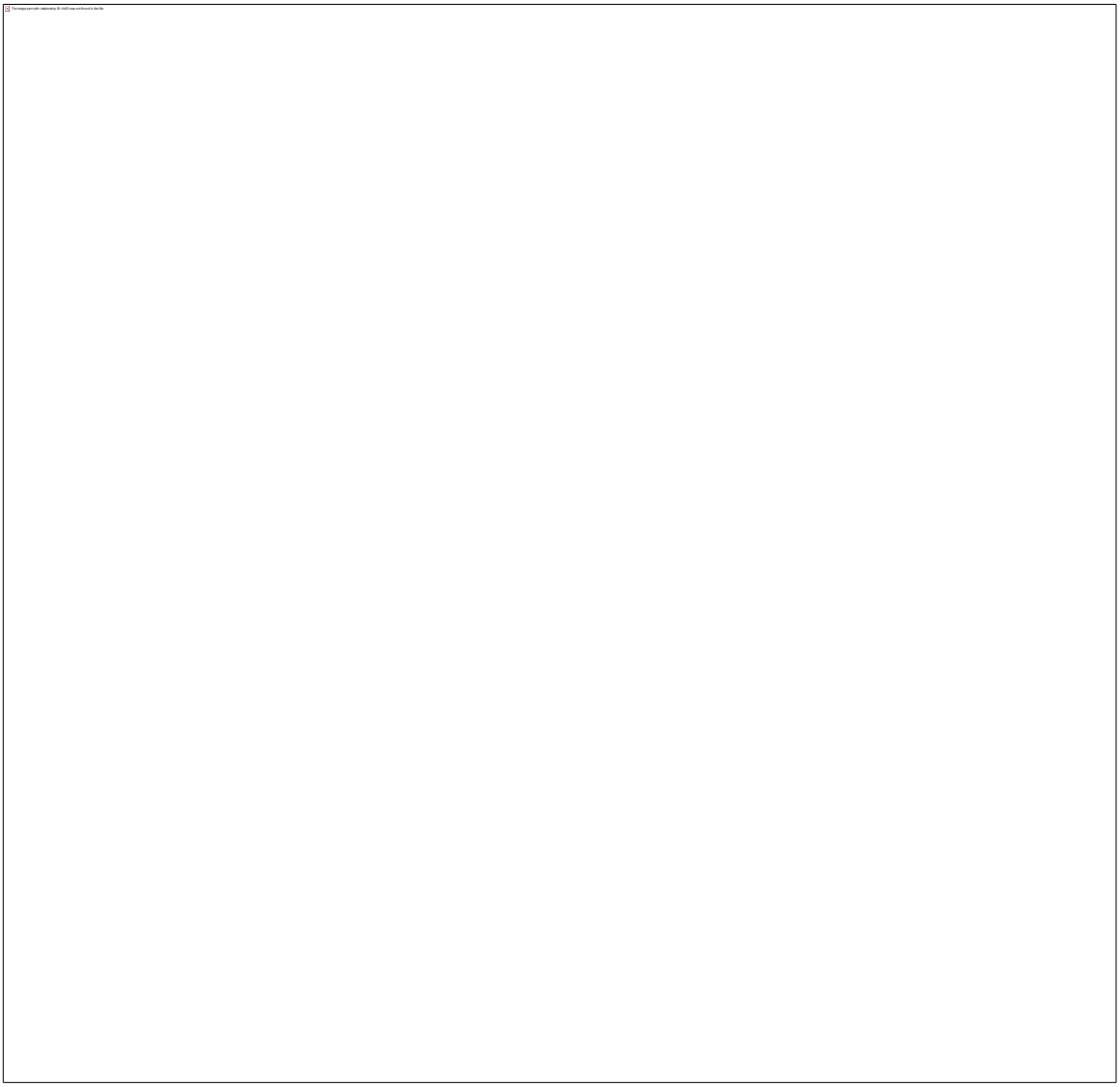


Fig. 12. Canonical correspondence analysis of amphibian species preference of six environmental variables (sediment depth, water loss, hydroperiod, playa area, and percent grass cover) measured from playa wetlands in the Southern High Plains, TX for 2009 data ($F = 1.57$, $p = 0.025$). The biplot shows the first and second canonical axes, explaining 70% of the variation in species distribution. The length of arrows indicates relative importance to the model. Sediment, water loss rate, and playa area are correlated. *Bufo woodhousii* was strongly correlated with sediment and *Rana blairii* was strongly associated with hydroperiod. Overall, hydroperiod explained 37% ($F = 2.87$, $p = 0.01$) of species distribution, sediment depth explained 28.6% ($F = 2.14$, $p = 0.03$), and percent native grass in the watershed loosely explained 24% ($F = 1.80$, $p = 0.11$). Total inertia = 0.442, $\lambda_1 = 0.027$, $\lambda_2 = 0.022$, $\lambda_3 = 0.013$, $\lambda_4 = 0.006$.



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CHAPTER II

EFFECTS OF WATER LOSS ON NEW MEXICO SPADEFOOT TOAD (*SPEA MULTIPLICATA*) DEVELOPMENT, SPLEEN CELLULARITY, AND CORTICOSTERONE LEVELS

Abstract

Amphibian metamorphosis is a complex, energy-demanding event and larval morphology and physiology are completely restructured during this time. Amphibians that live in unpredictable environments are often exposed to environmental stressors that can directly and indirectly alter developmental and affect physiological systems during development, with subsequent consequences later in life. In this study, we investigated the effects of water level reduction on development rate, spleen size and cellularity, and investigated the mechanistic role of corticosterone levels in pre-metamorphic, metamorphic, and post-metamorphic New Mexico spadefoot toads (*Spea multiplicata*). We hypothesized that when exposed to a declining water level, tadpoles would increase developmental rate, but with the trade-off of increasing corticosterone to a level that would subsequently affect spleen size and cellularity, thus prolonging immunological suppression. Declining water levels increased developmental rate by three days, on average; however, there were no direct body size effects due to the water loss treatment.

Corticosterone (CORT) was negatively correlated with total length, snout-vent length, body weight, and spleen weight at metamorphosis. This study supports the hypothesis that environmental stressors that can elevate CORT, can have negative consequences on tadpole development and that, most notably, size at metamorphosis and the immune system may be affected by excessive CORT levels. Our results also strongly support that it may be multiple factors acting as stressors in the field affecting amphibian responses, and simple pathways as tested in this study may not adequately describe field conditions.

Introduction

Metamorphosis is a complex, energy-demanding event that occurs in most amphibian species. Larval amphibians undergo intense morphological and physiological restructuring during development, with changes in the nervous, immune, and endocrine systems at the time of metamorphosis (Burggren and Just, 1992). The majority of larval structures undergo some sort of degeneration during this time (e.g., integumentary cells, lymphatic system, reproductive system, and mouthparts); however, some structures (e.g., lungs, liver, spleen, and thymus) do remain in a functional state (Viertel and Richter, 2000). Energy demands are high due to the restructuring of morphology and rapid growth rates, among others (Denver et al., 2002). Because of these demands, developing amphibians are susceptible to abiotic and biotic stressors such as population density, food availability and intake, temperature, pH, hydroperiod, and chemical contaminants, and these factors can play a role in the decrease of amphibian fecundity and survival (Jørgensen, 1992; Beebee, 1996).

Amphibian metamorphosis is regulated by the neuroendocrine system, and both the thyroid and adrenal glands produce hormones responsible for developmental

regulation (Burggren and Just, 1992; Denver, 1998). Most of our information on the development of larval amphibians stems from research on *Rana catesbeiana* and *Xenopus laevis*. Thyroid hormones induce certain morphological changes in larval amphibians (e.g. limb formation, cranial and intestinal restructuring, tail reabsorption) (Kikuyama et al., 1993; Shi, 2000) and corticosteroids can accelerate metamorphosis (Kikuyama et al., 1993) in response to environmental cues (i.e., photoperiod, conspecific density, temperature, pH, and hydroperiod) (Denver et al., 2002).

A primary system that is affected during metamorphosis is the immune system (reviewed by Du Pasquier et al., 1989). The majority of understanding of the anuran immune system comes from research with the African clawed frog (*Xenopus laevis*). The major organs involved with the immune system are the thymus and spleen (Du Pasquier et al., 1989), being fully formed six days and two weeks after fertilization, respectively. During metamorphosis both the thymus and spleen lose cell numbers, but recover and gradually increase to maximum size and cellularity (Du Pasquier et al., 1989) after metamorphosis.

Thyroid hormones (T_3 and T_4) and corticosteroid hormones (e.g., corticosterone) influence the changes occurring within the thymus and spleen during metamorphosis (Kikuyama et al., 1993; Rollins-Smith, 1998). At metamorphic climax, tadpoles undergo a natural spike in thyroid hormones and corticosterone levels (Rollins-Smith et al., 1997), and the tadpole immune system is reorganized at metamorphosis into the adult-type system (Rollins-Smith, 1998). The increase in corticosterone has been shown to cause apoptosis of lymphocytes in the spleen, thymus, and liver in vitro (Rollins-Smith, 1993). It is thought that the purpose for lymphocyte reduction at metamorphosis is to prevent

interference with the developing adult cells (Rollins-Smith et al., 1992; Barker et al., 1997). However, during this time of decreased lymphocytes, individuals may be more susceptible to environmental pathogens, thereby indirectly increasing sensitivity to the previously mentioned abiotic and biotic factors (population density, food availability and intake, temperature, pH, hydroperiod, and chemical contaminants) (Denver et al., 2002).

The developmental stage at which corticosteroids are produced dictates the outcome of metamorphic size and the timing of metamorphosis (Hayes et al., 1993; Glennemeier and Denver, 2002). Hayes et al. (1993) found that when corticoids were elevated in early larval stages development was inhibited and tadpoles did not reach metamorphosis; however, if corticoids were elevated in later stages, development was accelerated. Inhibition of either thyroid or corticosteroid hormones can be detrimental to the morphological and physiological restructuring that most larval anurans experience because it may reduce or completely halt development (Allen, 1916; Allen, 1938; Jaudet and Hatey, 1984; Kikuyama et al., 1993; Denver, 1998).

Tadpoles are susceptible to environmental stressors at metamorphosis because they are experiencing drastic changes in physiology, possibly altering their feeding and predator avoidance behaviors (Denver et al., 1998; Bridges, 2002). Although corticosterone can aid in expediting amphibian metamorphosis, numerous studies have seen that there is a decrease in metamorphic size with accelerated development (Hayes and Licht, 1993; Wright et al., 1994; Glennemeier and Denver, 2002). This type of phenotypic plasticity is present in many species of amphibians, but can have trade-offs with body size, immunocompetence, and fitness potential (Newman, 1999; Gervasi, 2007). As previously stated, many amphibians are adapted to metamorphosing quickly to

avoid desiccation (Newman, 1988, 1989), and under the pressure of decreasing water levels, tadpoles will begin metamorphosis earlier to leave the water source before drying (Denver, 1997).). Limiting the time spent as larvae can be a distinct advantage for many amphibian species, as tadpoles are generally more vulnerable to mortality than adults when faced with stressors such as reduced hydroperiods, predators, and competition (Duellman and Trueb, 1994).

The goal of this study was to determine the developmental and physiological effects of water loss on spleen size and cellularity and investigate the mechanistic role of corticosterone levels in pre-metamorphic, metamorphic, and post-metamorphic New Mexico spadefoot toads (*Spea multiplicata*). Spleen size and cellularity in *S. bombifrons* and *S. multiplicata* was investigated in a field study looking at the effects of land use surrounding wetland sites (McMurry et al., 2009). They found that *S. bombifrons* tadpoles collected from wetlands where water loss rate was greater had eleven-fold smaller spleen cellularity indices, whereas spleen cellularity indices in *S. multiplicata* tadpoles were approximately two-fold smaller in wetlands with faster water loss (McMurry et al., 2009). We predicted that increased water loss rates would increase corticosterone earlier in development, resulting in premature apoptosis of spleen leukocytes in tadpoles. In post-metamorphic individuals that were reared in a declining environment, we predicted that after thirty days post-metamorphosis, size differences between treatments would remain. Understanding the physiological changes that occur in controlled laboratory experiments are critical to predicting effects in the field, determining the cause of effects in the field, and provide insight to potential threats to amphibian populations.

Methods

Animal Husbandry

Adult male and female *Spea multiplicata* were collected in Gray County, Texas in 2010 and maintained in our animal facility at Oklahoma State University. Adult toads were housed in 38 L aquaria and fed crickets *ad libitum* once a week. Animals were maintained on a 12L:12D photoperiod at a temperature 23-24º C. Breeding was induced via injecting toads with luteinizing hormone releasing hormone (Sigma Aldrich, St Louis, MO) at 2 µg/g toad. Breeding pairs were placed in 76 L aquaria filled with approximately 60 L water and provided 1 mm mesh for egg deposition. Tadpoles were transferred to experimental tanks (38 L) approximately one week after hatching (Gosner Stage 28) (Experiment 1) or 24 hours after hatching (Experiment 2). Staging of tadpoles followed Gosner (1960), with Gosner Stage (GS) 42 being considered metamorphic climax in Experiment 1 and GS 45 in Experiment 2. A single sibship was used for each experiment. Temperature, pH, and dissolved oxygen content were monitored throughout all experiments. All housing and experimental methods were approved by Oklahoma State University IACUC.

Experiment 1

This experiment was designed to assess the effects of water loss on spadefoot tadpole development, corticosterone levels, and spleen leukocyte numbers for three developmental stages (GS 36, GS 42, and 30 days post-metamorphosis). The experiment was carried out in 38 L aquaria using carbon filtered dechlorinated water. Excess food and waste was removed daily and complete water changes occurred every two days to ensure minimum nitrogen build-up due to waste. Two treatments of constant water depth

(10 cm) and declining water level (0.5 or 1.0 cm loss/day) were applied until metamorphosis or 50 days post-hatch. Water level in the decline treatment was reduced over 14 days until depth was 2 cm, and then kept constant for the remainder of the study. To allow for the most non-disruptive removal of water, a siphon with a mesh cover was placed in one corner of the tank. The siphon was also placed into control tanks for approximately the same duration as the declining tanks to account for potential disturbance stress. Tadpoles (GS 28) were randomly assigned to experimental tanks (21 tadpoles/tank) and allowed to acclimate 24 hours prior to starting the experiment. Three developmental stages were assessed: pre-metamorphic (GS 36), metamorphic climax (GS 42), and post-metamorphic (30 days after metamorphosis). Each treatment by developmental stage was replicated four times (24 total tanks). Tadpoles were fed a mixture of Nutrena® Naturewise rabbit chow (Cargill, Minneapolis, MN USA) and Tetra TetraMin® tropical fish flakes (Spectrum Brands Inc., Madison, WI USA) *ad libitum* throughout the experiment. After metamorphosing and reaching GS 45, individuals were placed in 9.5 L aquaria, respective to the same cohorts of the experimental tank, with moist soil and fed crickets once a week for 30 days post-metamorphosis.

This experiment was separated into two distinct ‘groups.’ Tadpoles were hatched and kept in a singular 76 L aquarium, at a density of approximately 650 tadpoles. The experiment started when 25 percent of the tadpoles reached GS 28. These individuals were randomly assigned to half of the experimental tanks, within each treatment. The second half started three days later, when the next group of tadpoles reached GS 28. Throughout the remainder of this paper these groups will be referred to as Group 1 and Group 2.

Experiment 2

Experiment 2 mirrored Experiment 1, investigating effects of water loss on tadpole development, however, in this experiment we assessed effects when the treatment was applied directly after hatching. Two developmental stages were assessed, pre-metamorphic (GS 36) and fully metamorphic (GS 45) individuals. Two treatments of constant water depth (10 cm) and declining water level (0.25 or 0.5 cm loss/day) were applied until metamorphosis or 50 days post-hatch. Similar to Experiment 1, hatching density was approximately 9 tadpoles/L water in 76 L aquaria. To avoid initial density-linked developmental issues experienced in Experiment 1, tadpoles were randomly assigned to experimental tanks (21 tadpoles/tank) one day after hatching (~GS 23) and allowed to acclimate 24 hours prior to starting the experiment. Each treatment by developmental stage was replicated four times (24 total tanks). The decline treatment was carried out for 21 days, until water depth was at 2 cm, and then kept constant for the remainder of the study. All other methods (e.g. water removal, feeding, etc.) were the same as Experiment 1.

Measurements

Animals were euthanized via 0.2% tricaine methanesulfonate (MS-222, Finquel®) (Argent Laboratories, Redmond, WA USA). Directly following euthanization, total length (± 0.01 mm), snout-vent length (SVL) (± 0.01 mm), and wet body weight (± 0.01 g) were measured in all individuals. For each stage, seven individuals were selected for spleen leukocyte counts. These individuals were immediately dissected after being euthanized and wet spleen weight was measured (± 0.01 mg) and the spleen placed in 1 ml amphibian phosphate buffered saline (APBS) in a glass on glass homogenizer, on ice.

To account for a potential bias of body size against spleen weight, a spleen mass index was calculated (spleen weight/svl*100) (McMurry et al. 2009). All carcasses were frozen at -80ºC for future analyses. The date of metamorphosis for each individual was used to determine metamorphic rate for both treatments.

Spleen Leukocyte Counts

Spleens were homogenized and 20 µl of the cell solution mixed with 20 µl of trypan blue in a 13x100 mm culture tube and allowed to sit for five minutes. Duplicate leukocyte counts were performed using a hemocytometer. To account for differences in spleen and body size among individuals, a spleen leukocyte index was used for analysis (number of leukocytes/SVL*100) (McMurry et al, 2009).

Corticosterone (CORT)

Whole body CORT was measured via radioimmunoassay (RIA) (Lovern et al., 2001). Tadpoles were prepared in 1 ml phosphate buffered saline (PBS) using glass on glass homogenizers. A radiolabeled tracer (Corticosterone, [1,2,6,7-3H(N)]-, 1mCi (37MBq), 1000cpm) (NEN Life Science Products) was added to the samples and refrigerated overnight at 4°C for individual recovery determinations. Samples were extracted twice with 5 ml 70:30 diethyl ether:petroleum ether, dried via nitrogen gas, reconstituted in 1 ml 95% ethanol, and frozen overnight at -20°C. The reconstituted samples were centrifuged at 2000 rpm for five minutes at 0°C to separate neutral lipids and proteins. The supernatant was collected in a new tube and extracted with 2 ml hexane. The 95% ethanol was collected and transferred to a new tube and the process repeated by adding 1 ml 95% ethanol to the 2 ml hexane. After extraction, the sample was dried under nitrogen gas and reconstituted in 500 µl 10% ethyl acetate (EA) in

isooctane (2,2,4-trimethylpentane) (I).

Column chromatography was performed to further remove neutral lipids that may interfere with assay performance and to isolate CORT. Columns were made using Fisher 5 ml pipets with a water and glycol phase. Diatomaceous earth (Celpure®, Sigma Aldrich, St. Louis, MO, USA) was combined with either 2 ml ddH_2O (water phase 6:1 m:v) or 3 ml 1:1 propylene glycol:ethylene glycol (glycol phase 3:1 m:v). Samples were forced onto the columns via nitrogen gas and subsequently extracted using increasingly polar solvents (100% I, 10% EA:I, 20% EA:I, 52% EA:I). CORT was collected at 52% EA:I, dried under nitrogen, resuspended in PBS containing gelatin (PBSg), and refrigerated at 4°C overnight.

To determine recovery for all samples, a 50 µl aliquot of each sample was combined with 2 ml scintillation fluid (Ultima Gold™, Perkin Elmer, Waltham, MA, USA) and counted on a scintillation counter (Beckman LS6000SC) set for 2% error or 20 min. For competitive binding RIA, another aliquot of 200 µl each sample was combined with 200 ml PBSg, 100 µl CORT-antibody (Sigma Aldrich), and 100 µl tracer. A standard curve was serially diluted (500-1.95 pg) and run in triplicate, along with total counts, non-specific binding, and maximum binding. 300 µl PBSg was added to total counts and non-specific binding, 200 µl to maximum binding; 100 µl tracer was added to all, and 100 µl antibody added to maximum binding tubes. All samples and standards were refrigerated at 4°C overnight. Dextran-coated charcoal in PBS was added to standards and samples to stop the assay and remove any unbound tracer. All tubes were centrifuged at 2200 rpm for 10 min at 4°C and the supernatant collected. 3.5 ml

scintillation fluid was added to the samples and standards and counted at 2% error or 15 minutes, whichever came first.

Statistics

Experiment 1 data were analyzed via a 2-way analysis of variance (ANOVA) with stage, and treatment as fixed variables. To account for variability between tank and group, tank nested within both treatment and group and group were utilized as random variables (PROC MIXED, SAS/STAT® software, SAS Institute Inc.). Experiment 2 was also analyzed via a 2-way ANOVA with stage and treatment as interaction variables. Again, tank was nested within treatment. We used Restricted Maximum Likelihood (REML) estimation and Kenward-Roger degrees of freedom methods to account for heteroscedasticity and produce unbiased estimates of covariance parameters within the data (Corbeil and Searle, 1976; Kenward and Roger, 1997). Data were square-root transformed when necessary to fit the assumption of normality. Metamorphic timing differences between treatment were analyzed using Student's T-test. Pearson correlation analysis was conducted for comparing total length, snout-vent length, weight, spleen weight, total leukocyte numbers, and leukocytes/µg spleen to corticosterone concentration throughout both experiments. For these comparisons, group and treatment were not separated in analysis to provide a more comprehensive analysis of values. Alpha was set at 0.05 for all analyses.

Results

Stage differences were seen across all metrics in both experiments; however treatment did not have an effect on any variable in either experiment except date to

metamorphosis (Table 1-4). Although group was not analyzed as a fixed effect in Experiment 1, there were noticeable differences that are outlined and discussed below.

Experiment 1

Overall, animals in Group 1 were approximately 10% larger and heavier than those in Group 2 when comparing GS 36 and GS 42, however by thirty days post-metamorphosis there were seemingly no differences between groups (Table 1).

Group 1

When comparing differences among developmental stages in Group 1, SVL at GS 42 18.5% and then increased again to approximately the same size as GS 36 by 30 days post metamorphosis (Table 1 and 2). Weight followed a similar pattern, decreasing at GS 42, however it did not elevate again by thirty days post-metamorphosis. Spleen weight consistently decreased by 35% at GS 42 and then by 55% thirty days after GS 42 (Table 1). The number of leukocytes/µg spleen increased almost 75% at GS 42 and then decreased to a level similar to GS 36 thirty days post-metamorphosis (Table 1). CORT was 86% higher in GS 42 decline animals than GS 36 decline animals, and 76% higher in GS 42 constant than GS 36 constant, for Group 1 (Table 1).

Group 2

Group 2 developmental effects were similar to Group 1 with SVL decreasing at GS 42 and then increasing thirty days post-metamorphosis, weight following the same trend, spleen weight declining throughout development, and leukocytes/µg spleen increasing at GS 42 and then decreasing after thirty days post-metamorphosis. In GS 42, SVL decreased and then increased in post-metamorphs to a size 6% larger than at GS 36

(Table 1). Weight did not increase between GS 42 and thirty days post metamorphosis, but was significantly lower than GS 36 (Table 1 and 2). Spleen weight decreased throughout development but there was no difference between any stage in leukocytes/µg spleen (Table 1 and 2). GS 42 CORT levels were 88 and 83% higher than GS 36 for decline and constant treatments, respectively (Table 1).

By comparing developmental variables to CORT, we were able to determine correlations and potential effects that an environmental stressor may have on tadpole and metamorph physiology. At GS 42, CORT levels were negatively correlated with TL ($r = -0.719$, $p = <0.001$), SVL ($r = -0.480$, $p = <0.001$) (Fig. 1), body weight ($r = -0.623$, $p = <0.001$) (Fig. 2), and spleen weight ($r = -0.540$, $p = <0.001$) (Fig. 3).

Overall, developmental rate was two and a half to three days faster among tadpoles in the decline treatment in Experiment 1 (Table 4) (Fig. 4). The number of individuals that completed metamorphosis was high, with an 87% success rate within both treatments in Group 1 and 76 and 80% success rates in Group 2 decline and constant treatments, respectively (all n = 84). All other tadpoles did not reach metamorphosis before fifty days. When comparing morphing rates between groups, Group 1 individuals from the decline treatment morphed eight and a half days sooner than Group 2 individuals in the constant treatment, as well as morphing six days earlier than tadpoles in Group 2 decline (Table 4).

Experiment 2

In Experiment 2, we exposed tadpoles to the treatments 24 hours after hatching, increasing the number of days of declining water from 14 days (Experiment 1) to 21 days, but at a slower rate. The purpose of doing this was to eliminate the effects of having

two distinct groups of tadpoles and to determine effects when declining water occurs during initial development. No treatment differences ($p > 0.05$) were observed for any metric (Table 3). However, stage differences occurred as smaller size was expressed at the time of metamorphosis (GS 45) compared with GS 36 tadpoles, as well as spleen weight, total leukocytes, leukocytes/µg spleen, and spleen weight index (Table 1 and 3)

Corticosterone (CORT) levels were not affected by treatment (Table 3) but were 25% higher in GS 36 tadpoles reared in the decline treatment than in the constant control. However, this did not occur at GS 45 where both treatments had similar levels of CORT (Table 1). For both treatments, all metrics were significantly larger in GS 36 individuals than metamorphs, except CORT which was over 95% greater in GS 45 for both treatments (Table 1 and 3). The number of leukocytes/spleen µg was 75% greater in GS 36 than in GS 45 in the decline treatment and 70% greater in the constant treatment (Table 1). CORT was negatively correlated with weight in GS 45 individuals ($r = -0.408$, $p = 0.009$) and loosely correlated with SVL in GS 36 individuals ($r = -0.303$, $p = 0.058$). However, CORT was not correlated with spleen mass or leukocytes at either developmental stage. When comparing GS 45 and GS 42 (Experiment 1) CORT levels, there is an 83% increase in GS 45 individuals. Additionally, leukocytes/µg spleen exhibited a 70% decrease from GS 42 to GS 45 (Table 1).

Fifteen percent more individuals metamorphosed in the constant treatment ($n = 147$) than in the declining treatment ($n = 125$). However, morphing rate was high for both treatments with 74% of all decline tadpoles metamorphosing and 87.5% of constant tadpoles. Any mortality occurred early in the experiment (within the first three days) and the remaining tadpoles did not metamorphose by day fifty. Tadpoles in the declining

treatment morphed one and a half days earlier on average than tadpoles in a constant water environment (Table 4) (Fig. 5).

Discussion

Previous studies, when taken together, suggest that a hydrological stress such as declining water level will produce accelerated developmental rate, premature CORT production, greater CORT production, and alterations in the immune system (Newman and Dunham, 1994; Denver, 1997; Denver, 1998; Denver et al., 1998; Lane and Mahony, 2002; Boorse and Denver, 2003; Gervasi and Foufopoulos, 2008; Márquez-Garcia et al., 2009; Márquez-Garcia et al., 2010). Gervasi and Foufopoulos (2008) showed cell responses to desiccation stress in the lab and McMurry et al. (2009) showed similar responses in a field study where amphibians were collected from wetlands with differing water loss rates. Our study attempted to examine the role of CORT in mediating the effect of water loss on spleen cellularity. We hypothesized that when exposed to a declining water level, tadpoles would increase developmental rate, but with the trade-off of increasing corticosterone to a level that would subsequently affect spleen size and cellularity, thus prolonging immunological suppression.

Overall, we did not see a treatment effect in body size, spleen cellularity, or corticosterone levels congruent with previous literature or our hypothesis. However, developmental rate was influenced by hydrology. Similar to previous studies (Newman and Dunham, 1994; Denver, 1997; Denver, 1998; Denver et al., 1998; Gervasi and Foufopoulos, 2008; Márquez-Garcia et al., 2009), development rate accelerated with declining water level in both experiments. Although Group 2 individuals morphed six days later than Group 1, the rate was consistent for both treatments. Phenotypic plasticity

in developmental rate is vital for species living in unpredictable environments, particularly in arid and semi-arid regions where pond duration is variable and mortality rates may be high. Our data show variation within a sibship where some individuals (Group 2) developed slower than others (Group 1) which could be detrimental to slow developers living in natural ponds where six days may have a significant effect on pond duration and tadpole survival (Newman, 1988). Group was not included as a fixed effect in Experiment 1, but variations in development between groups suggest that variable developmental rates within a sibship as a possible bet-hedging strategy (Thumm and Mahony, 2006). This strategy may be advantageous when 1) the pond is long or short-lasting, 2) terrestrial resources are limited, or 3) aquatic predation risk is low or terrestrial predation risk is high. This type of variation can ensure successful metamorphosis for at least some individuals.

Regardless, the first week of development may be crucial to determining timing of metamorphosis. Tadpoles in Group 1 could be stronger competitors and able to initially develop quicker than Group 2, aiding in subsequent development rate. Group 2 individuals took approximately three days longer to reach GS 28 and six days longer, on average, to metamorphose. One of the main assumptions of the Wilbur and Collins (1973) model of amphibian metamorphosis is that there is a size threshold that an individual must reach before metamorphosis can occur, and that some individuals will grow quickly, reach the threshold early, but wait to morph, whereas others will grow more slowly and morph soon after reaching the threshold. Although we did not examine developmental rate prior to starting the experiment, it is possible that initial competition for resources may be related to development and timing of metamorphosis and should be

investigated further.

Spadefoot toads are highly adapted to breeding and living in temporary ponds, however, most studies show a size trade-off for developing faster (Newman, 1988; Denver, 1998; Denver et al., 1998; Morey and Reznick, 2000; Gomez-Mestre and Buchholz, 2006). Although there was no significant treatment effect in our experiment, it is important to note that a size trade-off has not always been consistent across studies. Smaller size at metamorphosis when exposed to an environmental stressor is a widely accepted idea, but as our results indicate it is variable. Gervasi and Foufopolous (2008) also did not see a size trade-off with faster developmental rate. Because previous experiments were conducted with different species, our data may not be congruent because although the species are similar in life history, there are slight differences in the degree of desiccation stressors that these species are exposed to naturally. For example, Couch's spadefoot, *Scaphiopus couchii*, have evolved in more restricted hydroperiods, with the ability to metamorphose in 9.5 days (Newman, 1989). Both *Spea multiplicata* and *Scaphiopus couchii* are subject to different reaction norms which depend on both timing and mass constraints (Rudolf and Rödel, 2007), and therefore this may account for the degree of effect on developmental acceleration. It is also possible that because of low density and ample food resources in our experiment, hydrology effects on size were negligible.

Although we did not see significant differences in size due to treatment, trends across many metrics showed a potentially important biological effect. Mean body weight index of Group 2 GS 42 individuals exposed to declining water levels was 17% of those in the constant treatment, suggesting that size differences may not occur until

metamorphosis, a time when a larger size is more important to survival. Smaller size at metamorphosis has been found to affect both fitness and survival, and Morey and Reznik (2001) found that larger *S. hammondii* juveniles were more likely to survive to reproduction, especially in male anurans which mature faster than females. Semlitsch et al. (1988) found that larger *Ambystoma talpoideum* juveniles remained larger until first reproduction, but survival to reproduction was not dependent on size. In another study, Smith (1987) marked and followed a population of chorus frogs (*Pseudacris triseriata)* to maturity and found that individuals that metamorphosed at a larger size and earlier when faced with pond drying, were more likely to survive to maturity. Because amphibians are both susceptible to predation and are also gape-limited predators, size during development and at metamorphosis is key to survival (Smith and Petranka, 1987; Babbitt and Tanner, 1998; Newman, 1999). It is notable that in our study, any suggestion of differences in size at metamorphosis disappeared by 30 days past metamorphosis. Thus, under favorable conditions (e.g. no predators and ample food) New Mexico spadefoot toads may be able to offset metamorphic size differences during terrestrial growth (Boone, 2005). There have been many studies focusing on size at metamorphosis in spadefoot toads; unfortunately there is essentially no information on terrestrial growth and size at maturity in relation to size at metamorphosis in this group of amphibians.

CORT levels did not statistically differ between treatments, but again, there were relevant trends that occurred at GS 42. CORT along with thyroid hormones (T_3 and T_4) regulate metamorphosis and CORT also mitigates acceleration of metamorphosis when tadpoles are faced with pond drying (Kikuyama et al., 1993; Rollins-Smith et al., 1997; Denver, 1998). Denver (1998) documented elevated CORT levels at GS 42 in tadpoles

when exposed to a water volume reduction; however, our results suggest that there may be unseen factors that can potentially affect CORT levels as we did not see a statistical effect due to water loss alone. CORT levels are exceptionally high at metamorphosis and vary between individuals, contributing to the variability in differences (and similarities) in CORT that we saw at different developmental stages. We expected to see an elevation of CORT at GS 42 from GS 36 and CORT, albeit not statistically significant, had a greater increase in decline animals than in constant animals (Experiment 1) suggesting that water loss may affect CORT (Denver, 1998). However, although we cannot accurately compare GS 42 and GS 45 CORT levels between separate experiments, there is a distinct and dramatic increase (by 83%) between the two developmental stages spanning 2-4 days, leaving metamorphs potentially more susceptible to environmental stressors the first several days after forelimb emergence. The fact that CORT in Experiment 2 GS 45 is almost identical between treatments, may suggest that at the likely peak of CORT (metamorphic climax, GS 45), differences due to water treatment are negated.

As previously stated CORT induces apoptosis of lymphocytes in many organisms and spikes during amphibian metamorphosis (Rollins-Smith et al., 1997; Rollins-Smith, 1998; Verburg-Van Kemenade et al., 1999). Thus, exposure to high CORT levels earlier than normal is predicted to cause premature immunosuppression in developing amphibian larvae (Rollins-Smith and Blair, 1993; Rollins-Smith, 1997). We observed 40 and 50% fewer leukocytes/µg spleen in Experiment 1 GS 36 and post-meta individuals, respectively, reared in a declining versus constant water environment. Gervasi and Foufopolous (2008) found that wood frog metamorphs (*Rana sylvatica*) had fewer

leukocyte numbers when exposed to declining water levels during larval development, as well as having a weaker cell-mediated innate immune response. It is possible that we did not see this cellular suppression consistently throughout our experiment because of differences in species physiology. Spadefoot toads have evolved short development times and thrive in unpredictable environments, whereas *Rana spp.* typically have longer developmental requirements (Martof, 1956; Herreid and Kinney, 1967). Because spadefoot toads are well adapted to hydrological stress, physiological systems (e.g. immune system) may not be as susceptible as with other species with longer developmental times (e.g. *Rana spp.*). McMurry et al. (2009) found that spadefoot tadpoles (*Spea bombifrons* and *S. multiplicata*) living in wetlands with faster water loss rates had fewer spleen leukocytes than those exposed to slower water loss rates. Our data, particularly Experiment 2 in which tadpoles were exposed to the stressor directly after hatching, suggest that declining water alone is not sufficient to cause an effect in spleen cellularity and that the results of McMurry et al. (2009) are more likely due to a combination of stressors (e.g. water loss, contaminants, pH, temperature, *et cetera*) that amphibians are exposed to in natural systems.

Stressful conditions early in development may cause a disproportionate increase in spleen size to occur as we saw in GS 36 tadpoles reared in declining water (Stark et al., 200). For example, we observed this increase possibly due to tadpoles developing under stressful conditions, and the immune system attempting to compensate for the loss of cells early in development. The spleen supplies phagocytic cells and erythrocytes, and mounts both innate and adaptive immune responses (Mebius and Kraal, 2005); therefore if an organism becomes stressed the spleen may become enlarged and can potentially be

resistant to glucocorticoid-induced apoptosis of cells (Stark et al., 2000; Avitsur et al., 2003). Spleen size did vary among developmental stages, if not consistently with treatment, and at metamorphic climax (GS 45) it decreased up to six-fold in size from GS 42, when comparing our two experiments. Similar to differences in CORT, the disparity between GS 42 and GS 45 spleen size is what would be predicted based on previous knowledge of changes in the immune system during development (Flajnik et al., 1987; Du Pasquier et al., 1989). Flajnik et al. (1987) described changes in the immune system of *Xenopus laevis* and noted that the number of lymphocytes increases throughout development through GS 42 and then drastically decreases at GS 45 (corresponding with a prominent spike in CORT), and then increases again after CORT levels drop. This suppression at metamorphosis is attributed to the body eliminating interference from the juvenile immune system and to make way for the new adult-type immune system (Flajnik et al., 1987; Rollins-Smith et al., 1992; Barker et al., 1997). However, the time needed for spleen size recovery may be longer than thirty days, as our results suggest.

Higher CORT at metamorphosis was also negatively correlated with total length, snout-vent length, weight, and spleen weight of GS 42 individuals, suggesting that CORT plays a role in size at metamorphosis; although we did not fully investigate this relationship (Meylan and Clobert, 2005). Meylan and Clobert (2005) found that offspring of female lizards treated with corticosterone had poorer body condition, smaller size, and slower growth than hatchlings from females not exposed to elevated CORT. Therefore, there may not only be a potential suppression of immune function and inhibition of spleen size and cellularity, but a body size effect with accelerated metamorphosis and increased CORT. This relates to what was previously stated about effects of smaller body

size at metamorphosis on potential survival and reproductive success. However, because we did not see body size correlation with CORT throughout both experiments, it is not definitive that this effect occurs.

There have been many studies examining the potential negative effects of increased levels of CORT on amphibians, yielding varied results (Moore, 1983; Moore and Zoeller, 1985; Hayes, 1995; Barker et al., 1997; Hayes et al., 1997; Glennemeier and Denver, 2002; Moore and Jessop, 2003). Most studies agree that increased levels of stress hormones can result in negative effects on fitness, susceptibility to pathogens, *et cetera* of amphibians. This study is another example that higher CORT levels are negatively correlated with developmental and physiological effects and that, most notably, the immune system may be affected by excessive CORT levels. In natural systems animals are subjected to multiple environmental stressors (e.g. desiccation stress, temperature, predation, contaminants, pH) that likely act synergistically on development and body condition. Because our results indicate a high degree of variability within and between experiments in regard to developmental effects due to water loss, it is reasonable to assume that there are other factors that affect tadpole development to a higher degree and CORT is not a singular regulator of lymphocyte numbers in developing tadpoles.

There was a general trend of faster development rates in the declining treatments, throughout both experiments, but size and spleen cellularity data were highly variable. However, we believe that although there may not be significant differences in size and spleen cellularity due to treatment, trends existed that may be biologically relevant. For example, results from Experiment 1 suggest a degree of variability among individuals within a sibship wherein not all members are equally responsive to desiccation stress.

This study supports the hypothesis that environmental stressors, such as desiccation stress, that are experienced in natural systems can have negative consequences on tadpole development. Our results also strongly support that it may be multiple factors acting as stressors in the field affecting amphibian responses, and simple pathways as tested in this study may not adequately describe field conditions. Further studies are needed to elucidate the effects that multiple stressors have on anuran physiology as organisms are exposed to more than one stressor in the field. Understanding the mechanisms driving amphibian development and survival is essential to the protection and conservation of amphibians worldwide. With amphibian declines currently being extensively evaluated, physiological studies are necessary to determine potential underlying causes of decline.

Table 1. Means ± SE of morphometrics, spleen cellularity, and corticosterone for the New Mexico spadefoot toad, *Spea multiplicata*, at three stages of development following exposure to constant and declining water levels in Experiments 1 and 2. Group 1 individuals reached Gosner stage (GS) 28 three days prior to Group 2, and therefore were placed into experimental tanks three days before Group 2. The three developmental stages assessed were pre-metamorphic tadpoles (GS 36), metamorphic tadpoles (GS 42 and 45), and 30 days post metamorphosis (Post-meta).

	Decline			**Constant**		
	GS 36	**GS 42**	**Post-meta**	**GS 36**	**GS 42**	**Post-meta**
Experiment 1 Group 1						
Total length (mm)	47.1 ± 0.70	41.9 ± 0.70	-	47.9 ± 0.49	43.3 ± 0.56	-
Snout vent length (mm)	18.8 ± 0.22	15.3 ± 0.27	19.5 ± 0.57	18.7 ± 0.20	15.7 ± 0.18	19.2 ± 0.42
Body weight index	6.9 ± 0.19	5.6 ± 0.18	4.5 ± 0.23	7.1 ± 0.15	5.9 ± 0.13	4.2 ± 0.17
Spleen weight (mg)	1.01 ± 0.14	0.66 ± 0.04	0.30 ± 0.03	0.70 ± 0.05	0.47 ± 0.03	0.31 ± 0.04
Spleen weight index	5.1 ± 0.68	3.9 ± 0.21	1.4 ± 0.15	3.6 ± 0.24	2.8 ± 0.16	1.5 ± 0.20
Total leukocyte ($x10^3$)	149 ± 32	395 ± 42	44 ± 11	191 ± 21	294 ± 45	71 ± 36
Cells/µg spleen	155 ± 24	593 ± 58	131 ± 26	274 ± 27	646 ± 105	168 ± 50
Corticosterone (ng/g)	0.20 ± 0.01	1.43 ± 0.09	-	0.27 ± 0.03	1.12 ± 0.09	-
Experiment 1 Group 2	**GS 36**	**GS 42**	**Post-meta**	**GS 36**	**GS 42**	**Post-meta**
Total length (mm)	43.8 ± 0.72	38.4 ± 0.63	-	44.1 ± 0.58	39.9 ± 0.79	-
Snout vent length (mm)	16.7 ± 0.22	13.9 ± 0.24	18.4 ± 1.10	16.9 ± 0.19	14.3 ± 0.23	18.1 ± 0.80
Body weight index	5.5 ± 0.22	4.5 ± 0.11	4.2 ± 0.44	5.7 ± 0.17	5.6 ± 0.13	4.0 ± 0.28
Spleen weight (mg)	0.50 ± 0.04	0.36 ± 0.04	0.27 ± 0.04	0.53 ± 0.04	0.39 ± 0.05	0.28 ± 0.04
Spleen weight index	2.9 ± 0.23	2.4 ± 0.27	1.3 ± 0.15	2.9 ± 0.18	2.4 ± 0.33	1.4 ± 0.18
Total leukocyte ($x10^3$)	136 ± 23	160 ± 37	42 ± 22	188 ± 39	137 ± 19	89 ± 31
Cells/µg spleen	260 ± 33	466 ± 106	123 ± 51	336 ± 53	364 ± 40	248 ± 45
Corticosterone (ng/g)	0.21 ± 0.02	1.78 ± 0.17	-	0.20 ± 0.01	1.18 ± 0.12	-

Table 1 Continued

Experiment 2	**GS 36**	**GS 45**	**Post-meta**	**GS 36**	**GS 45**	**Post-meta**
Total length (mm)	41.4 ± 0.44	-	-	42.2 ± 0.40	-	-
Snout vent length (mm)	15.9 ± 0.20	14.5 ± 0.12	-	16.2 ± 0.16	15.0 ± 0.11	-
Body weight index	5.5 ± 0.13	2.6 ± 0.04	-	5.8 ± 0.12	2.8 ± 0.05	-
Spleen weight (mg)	0.33 ± 0.03	0.16 ± 0.02	-	0.30 ± 0.02	0.17 ± 0.01	-
Spleen weight index	2.0 ± 0.14	1.1 ± 0.11	-	1.8 ± 0.12	1.1 ± 0.09	-
Total leukocyte ($x10^3$)	196 ± 23	18 ± 3.0	-	136 ± 20	22 ± 2.0	-
Cells/µg spleen	581 ± 64	131 ± 24	-	469 ± 62	140 ± 14	-
Corticosterone (ng/g)	0.28 ± 0.03	6.40 ± 0.79	-	0.21 ± 0.03	6.48 ± 0.66	-

Body weight index (body weight/svl*100)

Spleen weight index (spleen weight/svl*100)

‘-‘ indicates that no data was collected for this stage/morphometric

Table 2. Results of a 2-way ANOVA investigating effects and interactions among developmental stage and treatment of *Spea multiplicata* tadpoles subjected to two treatments (declining and constant water levels) on various morphometrics in Experiment 1. Two developmental stages were assessed: pre-metamorphic tadpoles (Gosner stage 36) and metamorphic individuals (Gosner stage 42). For the analysis, group, tank within treatment, and tank within group were treated as random variables. Group 1 individuals reached Gosner stage (GS) 28 three days prior to Group 2, and therefore were placed into experimental tanks three days before Group 2. Degrees of freedom were calculated with Kenward-Roger estimation.

	df	F	p
Snout vent length (mm)			
stage	2, 370	138.44	<0.001
treatment	1, 370	0.08	0.776
stage*treatment	2, 370	0.89	0.413
Body Weight Index			
stage	2, 14.3	67.21	<0.001
treatment	1, 14.4	1.42	0.253
stage*treatment	2, 14.2	3.23	0.07
Spleen weight (mg)			
stage	2, 17.1	13.99	<0.001
treatment	1, 17.1	1.35	0.261
stage*treatment	2, 17.1	0.51	0.611
Total leukocyte			
stage	2, 16.9	14.34	<0.001
treatment	1, 16.9	0.07	0.797
stage*treatment	2, 16.9	1.51	0.25
Cells/μg spleen			
stage	2, 17.8	22.67	<0.001
treatment	1, 17.8	1.32	0.266
stage*treatment	2, 17.8	0.7	0.508
Spleen weight index			
stage	2, 17.2	56.03	<0.001
treatment	1, 17.2	0.54	0.472
stage*treatment	2, 17.2	0.7	0.512
Corticosterone (ng/g)			
stage	1, 10.8	78.7	<0.001
treatment	1, 10.8	0.98	0.343
stage*treatment	1, 10.8	2.81	0.122

Body weight index (body weight/svl*100)
Spleen weight index (spleen weight/svl*100)

Table 3. Results of a 2-way ANOVA investigating effects and interactions among developmental stage and treatment of *Spea multiplicata* tadpoles subjected to two treatments (declining and constant water levels) on various morphometrics in Experiment 2. Two developmental stages were assessed: pre-metamorphic tadpoles (Gosner stage 36) and metamorphic climax individuals (Gosner stage 45). Tank was nested within treatment to account for random variation. Degrees of freedom were calculated with Kenward-Roger estimation.

	df	F	p
Snout vent length (mm)			
stage	1, 12	9.84	0.009
treatment	1, 12	1.62	0.227
stage*treatment	1, 12	0.01	0.979
Body Weight Index			
stage	1, 11.9	219.51	<0.001
treatment	1, 11.9	1.69	0.219
stage*treatment	1, 11.9	0.06	0.814
Spleen weight (mg)			
stage	1, 12	18.29	0.001
treatment	1, 12	0.19	0.673
stage*treatment	1, 12	0.22	0.651
Total leukocyte			
stage	1, 12	41.52	<0.001
treatment	1, 12	1.57	0.234
stage*treatment	1, 12	1.95	0.189
Cells/μg spleen			
stage	1, 12	60.33	<0.001
treatment	1, 12	1.06	0.323
stage*treatment	1, 12	1.45	0.252
Spleen weight index			
stage	1, 12	18.33	0.001
treatment	1, 12	0.3	0.593
stage*treatment	1, 12	0.14	0.719
Corticosterone (ng/g)			
stage	1, 12	86.4	<0.001
treatment	1, 12	0.01	0.994
stage*treatment	1, 12	0.01	0.909

Body weight index (body weight/svl*100)
Spleen weight index (spleen weight/svl*100)

Table 4. Mean **(**± SE) days post hatching individuals took to reach GS 42 or 45. New Mexico spadefoot toad (*Spea multiplicata*) tadpoles were subjected to constant or declining water levels in Experiments 1 and 2. In both experiments tadpoles reared in a declining water environment metamorphosed faster. Group 1 individuals reached Gosner stage (GS) 28 three days prior to Group 2, and therefore were placed into experimental tanks three days before Group 2. Not all tadpoles metamorphosed and these values represent data only from tadpoles that successfully reached metamorphosis (Group 1 success rate = 87%, Group 2 = 80%, Experiment 2 = 79%).

	Days to Metamorphosis			
	Decline	**Constant**	**T**	**p**
Experiment 1				
Group 1	24.6 ± 0.44	27.5 ± 0.52	-4.25	< 0.001
Group 2	30.6 ± 0.66	33.1 ± 0.62	-2.84	0.005
Experiment 2	30.2 ± 0.48	31.8 ± 0.42	-2.57	0.011

Fig. 1. Negative correlation between corticosterone (ng/g) and snout-vent length (mm) in New Mexico spadefoot toad (*Spea multiplicata*) GS 42 metamorphs exposed to either declining or constant water level. Each black circle represents a single individual from both treatments (n = 81). r = -0.480, p = <0.0001

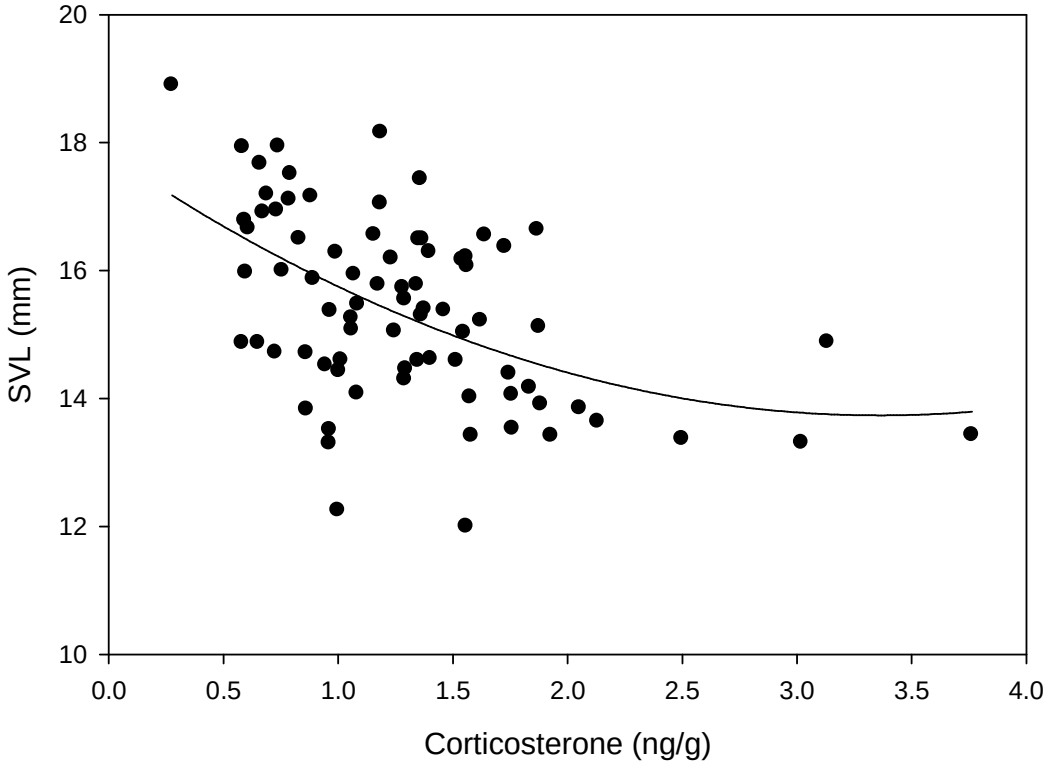


Fig. 2. Negative correlation between corticosterone (ng/g) and wet body weight (g) in New Mexico spadefoot toad (*Spea multiplicata*) GS 42 metamorphs exposed to either declining or constant water level. Each black circle represents a single individual from both treatments (n = 81). r = -0.623, p = <0.0001

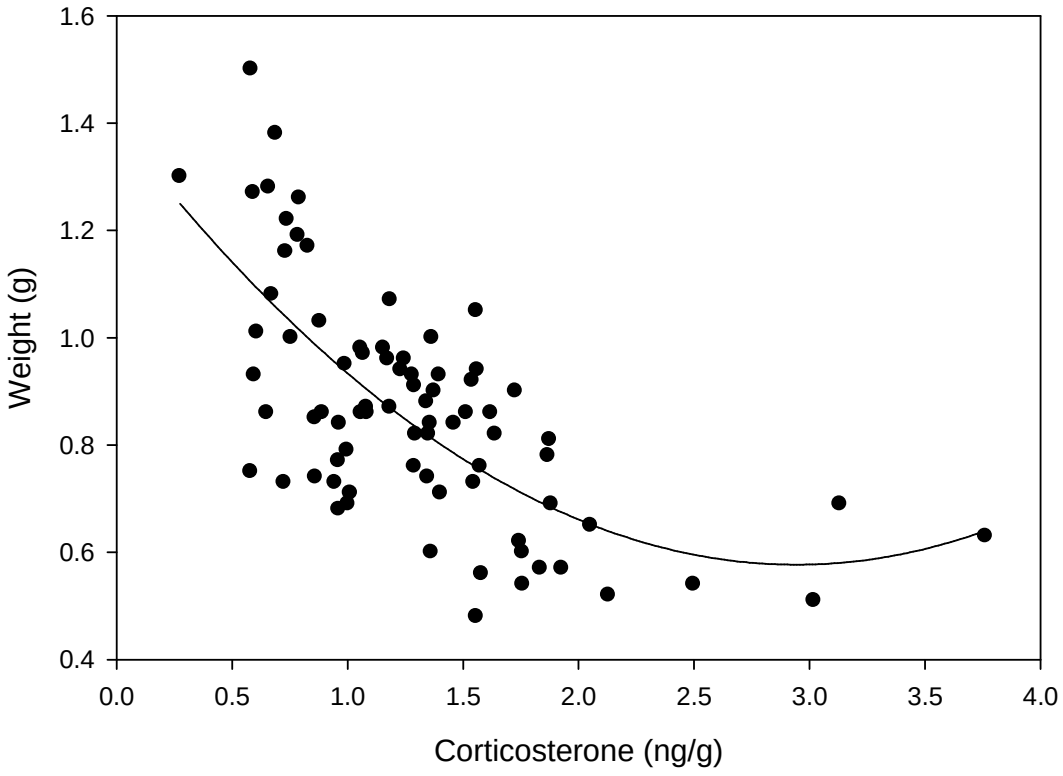


Fig. 3. Negative correlation between corticosterone (ng/g) and spleen weight (mg) in New Mexico spadefoot toad (*Spea multiplicata*) GS 42 metamorphs exposed to either declining or constant water level. Each black circle represents a single individual from both treatments (n = 40). r = -0.540, p = <0.0001

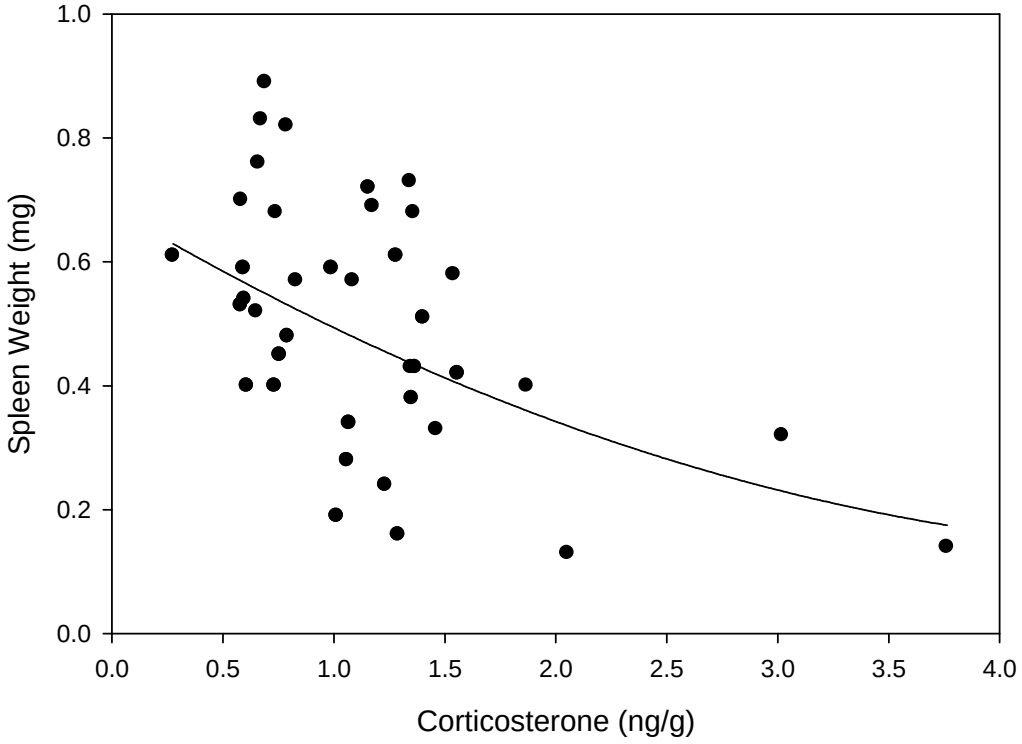


Fig. 4. The figure illustrates the disparity between treatment and group in percentage of morphed individuals of New Mexico spadefoot toads (*Spea multiplicata*) in Experiment 1. Tadpoles were placed into experimental tanks once they reached Gosner stage (GS) 28 and were exposed to declining water depth for 14 days, and then water was held constant at 2 cm. Group 1 individuals reached Gosner stage (GS) 28 three days prior to Group 2, and therefore were placed into experimental tanks three days before Group 2. Data are displayed as the percent of individuals morphed per the number of days since hatching. The values are a percentage of all successfully morphed individuals, not total percent of all tadpoles. Group 1 morphed significantly faster than Group 2 ($F = 25.58$, $p = <0.0001$) and in both groups tadpoles exposed to declining water levels developed faster, Group 1, $T = -4.25$, $p = <0.0001$; Group 2, $T = -2.84$, $p = 0.005$. Decline refers to tadpoles subjected to a declining water level throughout the experiment, whereas Constant refers to tadpoles reared in a constant water level.

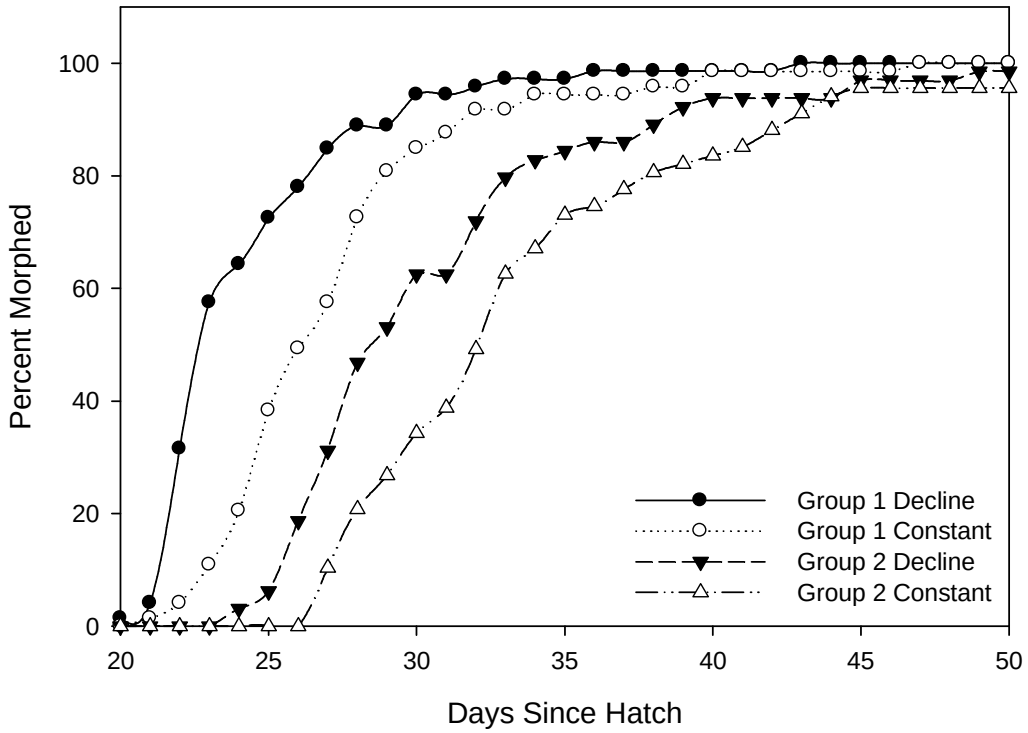
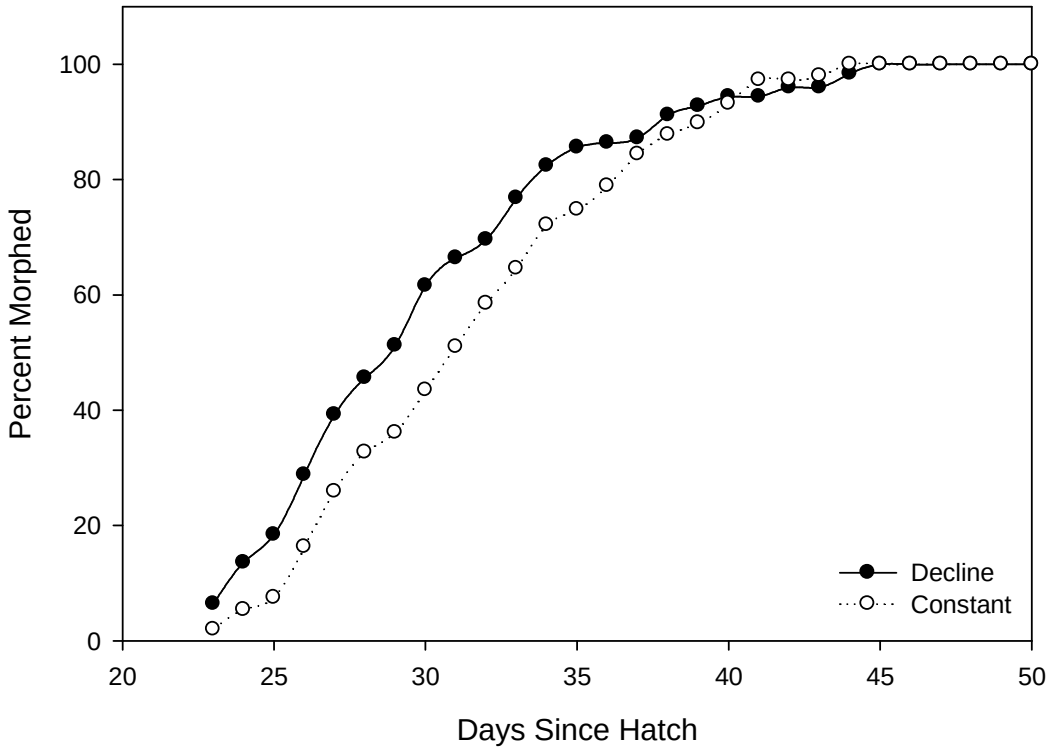


Fig. 5. The figure illustrates the percentage of morphed individuals of New Mexico spadefoot toads (*Spea multiplicata*) in Experiment 2 reared in a declining or constant water level. Tadpoles were placed into experimental tanks 24hrs post-hatching (~GS 23) and exposed to declining water depth for 21 days, and then water was held constant at 2 cm. The data are displayed as the percent of individuals morphed per the number of days since hatching. Data are a percentage of all successfully morphed individuals, not total percent of all tadpoles. Tadpoles reared in a declining water environment morphed 10% faster than tadpoles in the constant treatment. T = -2.57, p = 0.011



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CHAPTER III

DEVELOPMENTAL AND PHYSIOLOGICAL EFFECTS OF TWO ENVIRONMENTAL STRESSORS, ATRAZINE AND WATER LOSS, ON THE NEW MEXICO SPADEFOOT TOAD (*SPEA MULTIPLICATA*)

Abstract

Animals are exposed to a multitude of environmental stressors in natural systems, leading to the possibility of synergistic effects among stressors. Agricultural pesticides can effect amphibian development, growth, and immune function and can have exacerbated effects when coupled with natural stressors. This study investigated the effects of four concentrations of atrazine (0, 0.5, 5.0, 50 μg/L), coupled with either declining or constant water level, on development rate, body size, spleen size and cellularity, and corticosterone levels of pre-metamorphic and metamorphic New Mexico spadefoot toads (*Spea multiplicata*). We hypothesized that increasing atrazine concentrations will result in tadpoles with shorter developmental times, smaller body and spleen size, and fewer spleen leukocytes. We also hypothesized that corticosterone levels will be elevated at higher atrazine concentrations, contributing to cellular suppression. There were significant stage by water regime treatment interactions for snout-vent length,

body weight index, and spleen weight, with all metrics decreasing with declining water and at metamorphosis. At metamorphosis and regardless of atrazine concentration, individuals subjected to constant water levels were 8% larger in snout vent length, had 18% greater body weight indices, and 20% larger spleens, whereas in pre-metamorphic tadpoles there were no, or only slight differences in body size. Corticosterone (CORT) level was twice as high at the highest atrazine concentration (50 µg/L) than at 0, 0.5, or 5 µg/L, and was negatively correlated with snout-vent length, body weight index, spleen weight, and total spleen leukocytes at metamorphosis. Atrazine also influenced metamorphic rate and increased the average number of days to metamorphosis in the 5 µg/L treatment. Our study suggests that atrazine can play an important role in developmental timing, size at metamorphosis, spleen cellularity, and increasing CORT levels when tadpoles are exposed throughout development, but effects do not manifest until metamorphosis when amphibians are already susceptible to environmental stressors.

Introduction

Due to their aquatic dependence, amphibian larvae are susceptible to changes in the water quality and hydrology (Blaustein and Johnson, 2003). Xenobiotics can have both direct and indirect effects on amphibian survival, and even in trace amounts there may still be sub-lethal effects (Hatch and Burton, 1998). Because amphibians are susceptible to agricultural chemicals, they can play an important role in identifying potential threats to other wildlife, as well as humans. Results from numerous studies over the past decade have implied that contaminants may play a key role in many amphibian population declines and will continue to be a problem (Sparling et al, 2001; review Blaunstein and Kiesecker, 2002).

Pesticides have been shown to cause deformities (Bridges, 2000; Hayes et al., 2002; Blaustein and Johnson, 2003), alter corticosterone levels, and cause alterations in the immune system (Gilbertson et al., 2003; Pruett et al., 2003; Christin, et al., 2004; Albert et al., 2007) of various amphibian species. Many of these laboratory studies do not use levels of pesticides similar to those found in the environment, and therefore do not necessarily produce results that mimic the levels of contaminants and/or the effects of multiple stressors on the organisms more commonly found in natural systems. Bridges and Semlitsch (2000) attempted to identify effects of environmentally-relevant carbaryl concentrations among several populations of Southern leopard frogs (*Rana sphenocephala*). Tadpoles were exposed to carbaryl throughout development, with effects on time to death and activity levels, showing that even in short-term exposure to a chemical there could be negative impacts on populations. Bridges (2000) also investigated carbaryl effects on tadpole development and found that although mean age at metamorphosis was not different from controls at lower concentrations, at higher concentrations tadpoles took longer to morph.

Numerous studies conducted in wetland systems have linked immune function (e.g. decreased specific antibody response, increased sensitivity to introduced pathogens, decreased leukocyte counts) to contaminant exposure in amphibians (Gendron et al., 1997; Hopkins et al., 1999; Gilbertson et al., 2003; Hayes et al., 2006; Gibble and Baer, 2011). Lowered immune function potentially increases susceptibility of individuals to disease, parasites, or other pathogens, and many amphibian declines have been attributed to infectious pathogens such as chytrid fungus, *Aeromonas* bacterium, and iridioviruses (Carey et al., 1999). Carey (1993) proposed two hypotheses for increased amphibian

susceptibility to pathogens: 1) environmental contaminant exposure could alter the adaptive and innate immune responses, subsequently causing immunosuppression and 2) changes in the environment (e.g., temperature, pH, UV, xenobiotics) may cause an increase in stress levels, leading to immunosuppression.

Available research suggests that there is a correlation of exposure to agricultural chemicals and decreased resistance to pathogens (Gromysz-Kalkowaska and Szubartowska, 1993; Taylor et al., 1999). Christin et al. (2003) found that when Northern leopard frogs (*Rana* pipiens) were exposed to environmentally relevant mixtures of pesticides, there was a decrease in lymphocytes, and an increase in susceptibility to a parasitic nematode (*Rhabdias ranae).* Christin et al. (2004) studied effects of pesticides on African clawed frogs (*Xenopus laevis*) and Northern leopard frogs (*R. pipiens*) to determine if there were influences on immune response. They found that frogs associated with a mixture of pesticides, similar to that found in the environment, showed a significant reduction in numbers and phagocytic activity of splenocytes (Christin et al., 2004). A decrease in a specific antibody response to an introduced antigen (keyhole limpet hemocyanin), and an increase in delayed-type hypersensitivity to phytohaemagglutinin injection assay (PHA) (increased swelling at the injection site) was also found in *R. pipiens* exposed to environmentally relevant pesticide exposure (Gilbertson et al., 2003).

Along with contaminants, tadpoles are susceptible to other environmental stressors at metamorphosis because they are experiencing drastic changes in physiology (Denver et al., 1998; Bridges, 2002). A hydrological stress such as declining water level will produce accelerated developmental rate, a size trade-off with accelerated

development at metamorphosis, premature CORT production, greater CORT production, and alterations in the immune system (Newman and Dunham, 1994; Denver, 1997; Denver, 1998; Denver et al., 1998; Lane and Mahony, 2002; Boorse and Denver, 2003; Gervasi and Foufopoulos, 2008; Márquez-Garcia et al., 2009; Márquez-Garcia et al., 2010). Limiting the time spent as larvae can be a distinct advantage for many amphibian species, as tadpoles are generally more vulnerable to mortality than adults when faced with stressors such as reduced hydroperiods, predators, and competition (Duellman and Trueb, 1994).

In natural systems animals are exposed to multiple environmental stressors and therefore it is necessary to investigate the possibility of additive effects among these stressors. Previous studies have assessed combined effects of pesticides and predator exposure (Davidson and Knapp, 2007), parasites (Kiesecker, 2002; Koprivnikar, 2010), larval density (Boone and Semlitsch, 2001; Metts et al., 2005; Brodman et al., 2010), resource limitation (Rohr et al., 2004), and pond drying (Boone and Semlitsch, 2002; Rohr et al., 2004). Many species of spadefoot toads, including the New Mexico spadefoot toad (*Spea multiplicata*) breed in temporary ponds and tadpoles are subjected to short hydroperiods, rapid water loss rates, varying pH and temperatures, and agricultural pesticides, including atrazine (Newman, 1988; Buchholz and Hayes, 2002; Smith, 2003). Atrazine is one of the most commonly used pesticides in the United States, being applied to corn, sugarcane, sorghum, and other crops annually (US EPA, 2011a). It is frequently found in ground and surface waters (Thurman et al., 1992; USDA 2002; US EPA 2011b) and is transported aerially and via surface runoff (van Dijk and Guicherit, 1999; Knutson et al., 2004), with an estimated half-life of greater than 100 days and up to three months

in freshwater (Klaassen and Kadoum, 1979; de Noyelles et al., 1989). Because of its widespread use it is also one of the best studied pesticides, with a large amount of information on consequential effects to amphibians and other aquatic organisms (reviewed by Huber, 1993; Solomon et al., 1996, 2008; Giddings et al., 2005; Rohr and McCoy, 2010).

The goal of this study was to determine the developmental and physiological effects of atrazine, coupled with water loss, in pre-metamorphic and metamorphic *S. multiplicata*. We wanted to determine if there is an interaction between water loss and atrazine on development rate, body size, spleen size and cellularity, and corticosterone levels. Previous studies show that water loss is a significant factor in determining developmental rate, corticosterone levels, and alterations in the immune system (Newman and Dunham, 1994; Denver, 1997; Denver, 1998; Denver et al., 1998; Lane and Mahony, 2002; Boorse and Denver, 2003; Gervasi and Foufopoulos, 2008; Márquez-Garcia et al., 2009; Márquez-Garcia et al., 2010). Rohr et al. (2004) found that salamanders exposed to pond drying and atrazine had reduced body size and larval survival. We hypothesized that with an increase in atrazine concentration and declining water, tadpoles and metamorphs will have shorter developmental rates, be smaller in body and spleen size, and have fewer spleen leukocytes than tadpoles exposed to no atrazine. We also hypothesized that corticosterone levels will be elevated at higher atrazine concentrations coupled with declining water, contributing to cellular suppression.

Methods

Animal Husbandry

Adult male and female *Spea multiplicata* were collected in Gray County, Texas in

2010 and maintained in our animal facility at Oklahoma State University. Adult toads were housed in 38 L aquaria and fed crickets *ad libitum* once a week. Animals were maintained on a 12L:12D photoperiod at a temperature 23-24º C. Breeding was induced by injecting toads with luteinizing hormone releasing hormone (Sigma Aldrich, St Louis, MO) at 2 µg/g toad. Three breeding pairs were placed in 76 L aquaria filled with approximately 60 L water and provided 1 mm mesh for egg deposition. Tadpoles from all three sibships were equally distributed (21 tadpoles per tank, 7 from each sibship) among experimental tanks 24 hours after hatching and allowed an additional 24 hour acclimation period. Staging of tadpoles followed Gosner (1960), with Gosner Stage (GS) 45 being considered metamorphic climax. Temperature, pH, and dissolved oxygen content were monitored throughout all experiments. All housing and experimental methods were approved by Oklahoma State University IACUC.

Experimental Design

The experiment was set up as a 4x2 factorial design, with four concentrations of atrazine (0, 0.5, 5.0, 50 µg/L) and two water regimes (declining and constant). The experiment was carried out in 38 L aquaria using carbon filtered dechlorinated water. Excess food and waste was removed daily and complete water changes occurred every two days to ensure that atrazine concentrations remained consistent. Two treatments of constant water depth (10 cm) and declining water level (0.25 or 0.5 cm loss/day) were applied until metamorphosis or 45 days post-hatch. The decline treatment was carried out for 21 days to simulate water loss rate of a natural system, until water depth was at 2 cm, and then kept constant for the remainder of the study. To allow for the most non-disruptive removal of water, a siphon with a mesh cover was placed in one corner of the

tank. A siphon was also placed into control tanks for approximately the same duration as the declining tanks to account for potential disturbance stress. Two developmental stages were assessed, pre-metamorphic (GS 36) and metamorphic climax (GS 45), with separate sets of tanks for both stages. Each atrazine concentration (0.5, 5.0, 50 µg/L) by treatment by developmental stage combination was replicated three times (36 tanks), with four replicates for vehicle (acetone = 0 µg/L) controls (2 decline and 2 constant, 8 tanks). For each application, carrier was 100 µL acetone. Tadpoles were fed a mixture of Nutrena® Naturewise rabbit chow (Cargill, Minneapolis, MN USA) and Tetra TetraMin® tropical fish flakes (Spectrum Brands Inc., Madison, WI USA) *ad libitum*.

Atrazine was administered from stock solutions of 0.5, 5, and 50 µg/L in acetone. For each water change, atrazine was added for the proper volume reduction in water and the appropriate volume of acetone was added to the positive control tanks. Water samples were taken at 10 and 30 days into the experiment, before and after water changes, to assess atrazine concentration for quality control purposes. Temperature and dissolved oxygen levels were recorded and averaged.

Measurements

Animals were euthanized via 0.2% tricaine methanesulfonate (MS-222, Finquel®) (Argent Laboratories, Redmond, WA USA) at the appropriate stage for measurement. Directly following euthanization, total length (± 0.1 mm), snout-vent length (SVL) (± 0.1 mm), and wet body weight (± 0.01 g) were measured for all individuals. For each developmental stage, spleens were immediately removed following euthanization and weighed (± 0.01 mg wet weight) from eight tadpoles randomly selected from each tank. Spleens were placed in 1 ml amphibian phosphate buffered saline

(APBS) in a glass on glass homogenizer, stored on ice. To account for a potential bias of body size against spleen weight, a spleen mass index was calculated (spleen weight/svl*100) (McMurry et al. 2009). All carcasses were frozen at -80ºC for corticosterone analysis. The date of metamorphosis (GS 45) for each individual was used to determine metamorphic rate for both treatments.

Spleen Leukocyte Counts

Spleens were homogenized and 20 µl of the cell solution mixed with 20 µl of trypan blue in a 13x100 mm culture tube and allowed to sit for five minutes. Duplicate leukocyte counts were performed using a hemocytometer. To account for differences in spleen and body size among individuals, a spleen leukocyte index was used for analysis (number of leukocytes/SVL*100) (McMurry et al, 2009).

Corticosterone (CORT)

Whole body CORT was measured via radioimmunoassay (RIA) (Lovern et al., 2001). Tadpoles that were randomly selected for spleen cellularity were prepared in 1 ml phosphate buffered saline (PBS) using glass on glass homogenizers. A radiolabeled tracer (Corticosterone, [1,2,6,7-3H(N)]-, 1mCi (37MBq), 1000cpm) (NEN Life Science Products) was added to the samples and refrigerated overnight at 4°C for individual recovery determinations. Samples were extracted twice with 5 ml 70:30 diethyl ether:petroleum ether, dried via nitrogen gas, reconstituted in 1 ml 95% ethanol, and frozen overnight at -20°C. The reconstituted samples were centrifuged at 2000 rpm for five minutes at 0°C to separate neutral lipids and proteins. The supernatant was collected in a new tube and extracted with 2 ml hexane. The 95% ethanol was collected and transferred to a new tube and the process repeated by adding 1 ml 95% ethanol to the 2

ml hexane. After extraction, the sample was dried under nitrogen gas and reconstituted in 500 µl 10% ethyl acetate (EA) in isooctane (2,2,4-trimethylpentane) (I).

Column chromatography was performed to further remove neutral lipids that may interfere with assay performance and to isolate CORT. Columns were made using Fisher 5 ml pipets with a water and glycol phase. Diatomaceous earth (Celpure®, Sigma Aldrich, St. Louis, MO, USA) was combined with either 2 ml ddH_2O (water phase 6:1 m:v) or 3 ml 1:1 propylene glycol:ethylene glycol (glycol phase 3:1 m:v). Samples were forced onto the columns via nitrogen gas and subsequently extracted using increasingly polar solvents (100% I, 10% EA:I, 20% EA:I, 52% EA:I). CORT was collected at 52% EA:I, dried under nitrogen, resuspended in PBS containing gelatin (PBSg), and refrigerated at 4°C overnight.

To determine recovery for all samples, a 50 µl aliquot of each sample was combined with 2 ml scintillation fluid (Ultima Gold™, Perkin Elmer, Waltham, MA, USA) and counted on a scintillation counter (Beckman LS6000SC) set for 2% error or 20 min. For competitive binding RIA, another aliquot of 200 µl each sample was combined with 200 ml PBSg, 100 µl CORT-antibody (Sigma Aldrich), and 100 µl tracer. A standard curve was serially diluted (500-1.95 pg) and run in triplicate, along with total counts, non-specific binding, and maximum binding. 300 µl PBSg was added to total counts and non-specific binding, 200 µl to maximum binding; 100 µl tracer was added to all, and 100 µl antibody added to maximum binding tubes. All samples and standards were refrigerated at 4°C overnight. Dextran-coated charcoal in PBS was added to standards and samples to stop the assay and remove any unbound tracer. All tubes were centrifuged at 2200 rpm for 10 min at 4°C and the supernatant collected. 3.5 ml

scintillation fluid was added to the samples and standards and counted at 2% error or 15 minutes, whichever came first.

Quality Control

Water quality measures were taken twice a week from 10 randomly chosen tanks to account for any differences in water temperature, pH, and dissolved oxygen. Water samples (200 ml) were collected at exposure days 10 and 30, both before and directly after water changes to assess atrazine concentrations. Samples were collected from two, randomly chosen tanks at each atrazine concentration. Samples were extracted via solid phase chromatography and subsequently analyzed via gas chromatography-mass spectrometry. Samples were first spiked with 20 µl deuterated atrazine (atrazine-d_5, Sigma Aldrich, St Louis, MO) as internal standard. Water samples were extracted using silica C_{18} 6 ml solid phase cartridges (SampliQ, Agilent Technologies, Santa Clara, CA, USA) that were previously conditioned with 5 ml methanol followed by 5 ml double-distilled water. After the first extraction onto cartridges, samples were extracted again with 4 ml ethyl ether, followed by 4 ml ethyl acetate, and collected in 16x100 culture tubes. Sample extracts were evaporated to 1 ml under nitrogen and transferred to 2 ml scintillation tubes for analysis. A standard curve was made at 10, 30, 90, 270 ng/ml atrazine (Sigma Aldrich, St. Louis, MO, USA) in ethyl acetate, with 100 µl atrazine-d_5 internal standard. Samples were analyzed on a gas chromatograph-mass spectrometer (Agilent 5975c) and select ion monitoring was utilized: atrazine (200, 220), deisopropylatrazine (DIA, 172), and deethylatrazine (DEA, 173). Both DIA and DEA are common metabolites of atrazine and we wanted to account for any potential break down of atrazine in the experimental tanks. The standard curve was run prior to the samples and

a second 90 ng/ml standard was run after all of the samples for continuing calibration verification. Variation was within 20% error for all concentrations measured. We also performed a four replicate quality assurance study to determine precision and accuracy for atrazine quantification of our standard and stock solutions, with all measures within 10% error.

Statistical Analyses

A 3-way analysis of variance (ANOVA) with stage, water regime, and atrazine concentration as independent variables, was used to test responses of body size, spleen size and cellularity, and CORT levels. Tank was nested within both water regime treatment and atrazine, and treated as random variables (PROC MIXED, SAS/STAT® software, SAS Institute Inc.). For two-way interaction effects (stage*water regime and stage*atrazine), the SLICE option was used to determine pairwise effect differences. We used Restricted Maximum Likelihood (REML) estimation and Kenward-Roger degrees of freedom methods to account for heteroscedasticity and produce unbiased estimates of covariance parameters within the data (Corbeil and Searle, 1976; Kenward and Roger, 1997). Differences in rate of between water regime and atrazine concentration were analyzed using a 2-way ANOVA. A subsequent 1-way ANOVA was used to determine effects of atrazine treatment on the number of days required for at least 50% of individuals to reach GS 45. Pearson correlation analysis was used to compare total length, snout-vent length, weight, spleen weight, total leukocyte numbers, and leukocytes/µg spleen to corticosterone and atrazine concentrations. For these comparisons, water regime and atrazine concentration were not separated in analysis to provide a more comprehensive analysis of values. Alpha was set at 0.05 for all analyses.

Results

Atrazine concentrations remained within 17% error (0.418 ± 0.23SD) throughout the entire study. There was variation at the 0.5 µg/L concentration, as this was at a lower level of our calibration range. At 5.0 and 50 µg/L, concentrations were, on average, 4.2 ± 0.54SD and 49.2 ± 0.72 respectively. Dissolved oxygen averaged 4.6 throughout the experiment and only was lower than 4.0 in one tank on one occasion. Water temperature was also relatively constant across all tanks averaging 24.6 ºC and pH at 7.2.

The majority of effects observed in the study occurred at the time of metamorphic climax (GS 45), with minimal differences due to water regime or atrazine exposure in pre-metamorphic (GS 36) tadpoles (Table 1). Mortality rate was 6%, with the majority occurring within the first four days of the experiment.

Average number of days to GS 45 was not accelerated with declining water but there was a significant influence of atrazine concentration (Table 2)(Fig. 1); at 5 µg/L, tadpoles took almost 2.5 days longer to morph than tadpoles exposed to 50 µg/L atrazine (Fig. 2). However, there were no differences between the other concentrations. Although not statistically different, tadpoles exposed to both 0.5 and 50 µg/L seemingly developed at a faster rate than tadpoles exposed to 0 or 5 µg/L, but this did not occur throughout development. It took approximately three days longer for 50% of all morphed tadpoles to reach metamorphosis in the 0 and 5.0 µg/L treatments than for 0.5 or 50 µg/L ($F = 6.22$, $p = 0.0005$) (Fig. 3). Metamorphic success varied between water regime and atrazine exposure with averages of 67, 83, 70, and 66% for 0, 0.5, 5.0, and 50 µg/L atrazine concentrations, respectively.

There was no interaction among stage, water regime, and atrazine on snout-vent

length, or any interaction between atrazine and either stage or treatment (Table 2). However, there was an interaction between stage and water regime, driven in part by water regime effects on snout-vent length for GS 45 individuals, but not GS 36 tadpoles (Table 2). Metamorphs at GS 45 exposed to constant water levels were 9% longer than those in the decline water regime (Table 1 and 2). For both decline and constant water regime treatments, metamorphs at GS 45 were 6 and 13% longer, respectively, than GS 36 tadpoles (Table 1 and 2).

There was no interaction among stage, water regime, and atrazine on body weight index, or any interaction between atrazine and either stage or treatment (Table 2). However, there was an interaction between stage and water regime, driven in part by water regime effects on body weight index for both GS 36 and GS 45 individuals (Table 2). Tadpoles at GS 36 exposed to constant water level were 4% larger than those in the decline regime, whereas metamorphs at GS 45 exposed to constant water levels were 16% longer than those in the decline water regime (Table 1 and 2). For both decline and constant water regime treatments, tadpoles at GS 36 had 41 and 32% larger body weight indices, respectively, than GS 45 metamorphs (Table 1 and 2).

There was no interaction among stage, water regime, and atrazine on spleen weight, or any interaction between atrazine and either stage or treatment (Table 2). However, there was an interaction between stage and water regime, driven in part by water regime effects on spleen weight for GS 45 individuals, but not GS 36 tadpoles (Table 2). Metamorphs at GS 45 exposed to constant water levels had 15% larger spleens than those in the decline water regime (Table 1 and 2). For both decline and constant water regime treatments, spleens of tadpoles at GS 36 were 52 and 40% larger,

respectively, than GS 45 metamorphs (Table 1 and 2). Spleen weight index only showed variation between developmental stages, but not with water regime or atrazine concentration. GS 45 metamorphs had spleen mass indices 55% smaller than those of GS 36 tadpoles (Table 1 and 2).

Total spleen leukocytes did not differ for any of the main effects or interaction terms (Table 2). However, number of leukocytes/µg spleen differed between developmental stages with GS 36 tadpoles displaying 80% more cells than GS 45 metamorphs (Table 1 and 2).

Across all atrazine concentrations there was a difference in CORT concentration between stages, with GS 45 metamorphs having almost twenty times greater concentrations, on average, than GS 36 tadpoles (Table 1). There was no three-way interaction between atrazine concentration, water regime, and stage, however, there was a stage effect. Within GS 45, there was a difference between atrazine treatments (Table 2), but not at GS 36. There were no differences in CORT at 0, 0.5, or 5.0 µg/L, but at 50 µg/L CORT concentration doubled in both decline and constant GS 45 metamorphs (Table 1).

At GS 36 there were no correlations with CORT in any morphometrics measured. In GS 45 individuals, snout-vent length, body weight, and spleen weight were all negatively correlated with CORT levels (Table 3) (Fig. 4-6). There was also a cellular suppression of total leukocytes with CORT levels, but not to the same degree as body size (Table 3) (Fig. 7). Correlation analyses between atrazine concentration and morphometrics yielded similar results to the regression analyses; CORT was positively correlated with atrazine concentration (Table 4) (Fig. 8), but spleen weight was

negatively correlated with atrazine concentration (Table 4).

Discussion

Previous studies have shown negative and variable effects of atrazine on amphibian metamorphosis, sexual differentiation, growth, development rate, predator avoidance, and immunity (reviewed by Rohr and McCoy, 2010).While studies investigating other natural stressors and atrazine exposure together are limited, they have identified similar consequences in developmental and survival effects as with atrazine alone and that multiple stressors together can exacerbate these effects (Boone and Semlitsch, 2001; Kiesecker, 2002; Boone and Semlitsch, 2002; Rohr et al., 2004; Metts et al., 2005; Davidson and Knapp, 2007; Brodman et al., 2010). This study examined the effects of two stressors, water level and atrazine exposure, and the role of CORT in mediating the effect of these two stressors on development and spleen cellularity in *Spea multiplicata* tadpoles. We hypothesized that when exposed to both water reduction and atrazine, there would be an additive effect causing tadpoles to increase developmental rate, but with the trade-off of increasing corticosterone to a level that would subsequently affect spleen size and cellularity, thus prolonging immunological suppression throughout development.

We observed no interactive or additive effects of water loss and atrazine for any metric, but both stressors did have separate effects on growth, development, and corticosterone levels at the time of metamorphosis. Contrary to previous literature, development rate was not accelerated by declining water volume (Newman and Dunham, 1994; Denver, 1997; Denver, 1998; Denver et al., 1998; Gervasi and Foufopoulos, 2008; Márquez-Garcia et al., 2009), but atrazine exposure did affect average day to

metamorphosis and overall rate, however not in a manner expected. In natural systems, spadefoot toads can metamorphose and leave the pond in as little as 9.5-30 days, depending on the species (Newman, 1988; Denver et al., 1998; Buchholz and Hayes, 2002). We predicted that tadpoles would accelerate development rate when exposed to higher levels of atrazine in a dose-dependent manner, but at 0.5 and 50 µg/L, rate was accelerated, showing that there was no dose-dependent response to atrazine. Acceleration of development when exposed to high levels of contaminants has been previously reported in a number of amphibian species (McDaniel et al., 2004; Rohr et al., 2004; Distel and Boone, 2011; Rohr and McCoy; 2011), but a prolonged larval period is also often reported (Lefcort et al., 1998; Cheek et al., 1999; Boone et al., 2007; Groner and Relyea, 2011; Rohr and McCoy, 2011), and there are some reports of no effect on development (Allran and Karasov, 2009).

Spadefoot toads are commonly associated with depressional wetlands, which in turn are commonly subject to a multitude of contaminants, including atrazine. Spadefoot toads are highly adapted for breeding in temporary ponds, exhibiting short developmental periods that may limit their exposure to aquatic contaminants. However, as our study suggests, the short time that tadpoles are exposed to atrazine is sufficient to cause developmental effects. We did not see any developmental effects of atrazine in pre-metamorphic tadpoles (GS 36), indicating that effects that do occur do not manifest until the time of metamorphosis, when amphibians are already susceptible to numerous stressors due to a suppressed immune system and size (Newman, 1999; Bridges, 2002; Boone, 2005).

In this study, we saw variation in developmental rates in response to atrazine

concentrations therefore it is pertinent to note that although declining water did not show statistical significance in affecting development rate, it may still pose a risk when in combination with contaminant exposure (Rohr et al., 2004). There was no interaction with hydrology treatment and atrazine in average day to metamorphosis, but unseen physiological responses may have manifested due to atrazine exposure and prolonged the number of days to metamorphosis (Boone and James, 2003; Brodeur et al., 2009). It is hard to ignore the copious literature supporting acceleration of spadefoot toad metamorphosis when reared in declining water; therefore our results suggest the possibility that atrazine may somehow negate developmental acceleration due to water loss. Again, there was no dose-response relationship and the results in development were variable, with none the atrazine exposed individuals differing from the control.

Boone and James (2003) found that atrazine increased time to metamorphosis and interacted with hydroperiod in the small mouthed salamander (*Ambystoma texanum*). Salamanders typically have longer larval periods, and because they are not as adapted to unpredictable hydroperiods as spadefoot toads, effects of the combined stress were elevated. Perhaps, in our study, atrazine exposure affected rate at concentrations other than 5 µg/L at an underlying level not strong enough to be detectable via statistical analysis, but to an extent that would negate effects seen by water loss alone. It is also possible that developmental acceleration is variable when tadpoles are exposed to declining water directly after hatching, similar to natural conditions; and because the water loss rate was relatively slow, environmental cues were not perceived prior to meeting size thresholds needed for metamorphosis (Wilbur and Collins, 1973).

Size at metamorphic climax (GS 45) was decreased by hydrology, congruent with

previous literature (Newman and Dunham, 1994; Denver, 1997; Denver, 1998; Denver et al., 1998), but atrazine did not directly affect this relationship as previously reported (review Rohr and McCoy, 2010). Individuals reared in a declining water environment, regardless of atrazine exposure, were on average 17% smaller in body weight index than those in a constant environment and 8% smaller in snout-vent length. However, similar to development rate, atrazine could have an underlying indirect effect on body size at metamorphosis. Atrazine doubled CORT levels in the 50 µg/L treatment and CORT had strong negative correlations with snout-vent length and body weight index at GS 45. In natural systems, atrazine has been found at levels greater than 50 µg/L, where we saw both developmental and physiological effects as low as 5 µg/L (EPA, 2012). Because CORT is directly involved in metamorphosis, anything that affects CORT levels can potentially have a direct impact on metamorphosis. However, although CORT was elevated in this study due to atrazine, there were limited other effects.

Smaller size at metamorphosis has been found to affect both fitness and survival, but there are only a few studies focused on survival and reproductive success of individuals post contaminant exposure (Rohr et al., 2006; Smith et al., 2007; Rohr and Palmer, 2009; Todd et al., 2011). Rohr et al. (2006) found that low level atrazine exposure can affect survival up to 14 months post-exposure in the streamside salamander (*Ambystoma barbouri*) and that density-mediated compensation does occur, but may be negated. Boone (2005) found that small size at metamorphosis after carbaryl exposure was compensated during terrestrial growth, indicating that exposure effects can be ameliorated in both the green frog (*Rana clamitans*) and Woodhouse's toad (*Bufo woodhousii*). Amphibians are both susceptible to and are gape-limited predators, and size

during development and at metamorphosis is key to survival (Smith and Petranka, 1987; Babbitt and Tanner, 1998; Newman, 1999). Morey and Reznik (2001) found that larger western spadefoot toads (*S. hammondii)* juveniles were more likely to survive to reproduction, especially in male anurans which mature faster than females. In another study, Smith (1987) marked and followed a population of chorus frogs (*Pseudacris triseriata*) to maturity and found that individuals that metamorphosed at a larger size and earlier than others, when faced with pond drying, were more likely to survive to maturity. Any stressor that can affect size at metamorphosis may be detrimental to amphibian survival and more studies should attempt to measure post-exposure effects.

CORT is one physiological regulator of amphibian development and can accelerate metamorphosis when tadpoles are faced with pond drying (Rollins-Smith et al., 1997; Denver, 1998), however CORT was not overtly affected by declining water levels in this study, and it is uncertain if water loss did indeed affect CORT. CORT regulates apoptosis of lymphocytes in many organisms and naturally spikes during amphibian metamorphosis (Rollins-Smith et al., 1997; Rollins-Smith, 1998; Verburg-Van Kemenade et al., 1999). However, if tadpoles experience high CORT levels earlier than normal as we predicted, this would cause premature immunosuppression and other physiological responses. We did not see elevated CORT in pre-metamorphic tadpoles (GS 36) at any atrazine concentration, or direct cellular suppression in either stage assessed. However, CORT was negatively correlated with total number of leukocytes and spleen size at metamorphosis, suggesting that elevated levels can cause cellular suppression, but it is not manifested until metamorphosis. Gervasi and Foufopolous (2008) found that wood frog tadpoles (*Rana sylvatica*) had fewer leukocyte numbers

when exposed to declining water levels, as well as having a weaker cell-mediated innate immune response.

Previous studies have also found that atrazine alone causes a decrease in spleen size and cellularity and decreases spleen cellularity and number of circulating white blood cells (Zeeman and Brindley, 1981; Christin et al., 2004; Brodkin et al., 2007) and our study supports this as spleen size was negatively correlated with atrazine concentration. Spleen size and cellularity in *Spea bombifrons* and *S. multiplicata* was investigated in a field study looking at the effects of land use surrounding wetland sites (McMurry et al., 2009). They found that *S. bombifrons* tadpoles collected from wetlands where water loss rate and cultivation around the wetland were greater, tadpoles had 11 fold smaller spleen cellularity indices, whereas spleen cellularity indices in *S. multiplicata* tadpoles were approximately two-fold smaller. Although we did not see a hydrology effect, our results suggest that atrazine exposure can elevate CORT to a concentration capable of potentially causing cellular suppression and increased susceptibility, in concordance with previous laboratory and field studies. This study is another example that higher CORT levels are negatively correlated with developmental and physiological effects and that, most notably, the immune system may be compromised by excessive CORT levels and contaminant exposure.

Atrazine may also have sub-lethal effects on tadpole physiology that can inhibit survival. There are numerous studies investigating LC_{50} values for atrazine, but there is no information on potential neurological and early development effects. In 1% tadpoles in this experiment (3-0.5, 3-5.0, and 2-50 µg/L), we observed behavior that in a natural system would most likely result in mortality from predation. At approximately GS 35 and

throughout the rest of development, these individuals could no longer right themselves (e.g. return to upright), would swim in circles on their back, had tails bent 180°, and in some instances had whole body tremors. Although this only occurred in approximately 1% of all tadpoles in this study, it suggests that atrazine may cause neurological impairment.

Our study suggests that when tadpoles are exposed to atrazine throughout development, there can be effects on developmental timing, size at metamorphosis, and CORT levels. Because amphibians are already susceptible to numerous stressors at the time of metamorphosis, anything that will limit successful survival to reproductive maturity is a direct threat to amphibian populations.

Table 1. Means (± SE) of selected morphometrics, spleen cellularity, corticosterone, and developmental rate for the New Mexico spadefoot toad, *Spea multiplicata*, at two stages of development following exposure to constant and declining water levels and four concentrations of atrazine (0, 0.5, 5.0, 50 µg/L). Developmental stages were pre-metamorphic tadpoles (Gosner stage 36) and metamorphic tadpoles (Gosner stage 45). Sample sizes (n) for Gosner stage 36 total length, snout-vent length, and body weight index was 330, 176 for spleen weight and cellularity, and 110 for corticosterone. Sample sizes (n) for Gosner stage 45 snout-vent length and body weight index were 282, 176 for spleen weight and cellularity, and 110 for corticosterone.

	Decline				**Constant**			
	0	**0.5**	**5**	**50**	**0**	**0.5**	**5**	**50**
Gosner Stage 36								
Total Length (mm)	39.6 (0.58)	39.2 (0.42)	39.2 (0.57)	40.2 (0.56)	41.3 (0.61)	39.9 (0.56)	39.5 (0.57)	40.8 (0.49)
Snout-vent length (mm)	15.1 (0.23)	14.6 (0.14)	14.7 (0.19)	15.1 (0.19)	15.6 (0.24)	14.9 (0.17)	14.89 (0.19)	15.4 (0.16)
Body weight index	5.3 (0.1)	5.2 (0.11)	5.3 (0.12)	5.4 (0.14)	5.9 (0.15)	5.5 (0.16)	5.4 (0.14)	5.5 (0.12)
Spleen weight (mg)	0.31 (0.04)	0.34 (0.03)	0.37 (0.03)	0.37 (0.04)	0.35 (0.05)	0.33 (0.02)	0.33 (0.04)	0.31 (0.03)
Spleen weight index	1.99 (0.22)	2.26 (0.22)	2.5 (0.18)	2.4 (0.23)	2.2 (0.27)	2.2 (0.15)	2.1 (0.25)	2.0 (0.16)
Total leukocytes ($x10^3$)	15.0 (4.0)	25.0 (5.0)	53.0 (13.0)	39.0 (15.0)	64.0 (24.0)	42.0 (7.0)	59.0 (11.0)	50.0 (9.0)
Leukocytes/µg spleen	49.0 (13.0)	72.0 (11.0)	124.0 (25.0)	123 (50.0)	142 (35.0)	125.0 (16.0)	196.0 (38.0)	161.0 (22.0)
Corticosterone (ng/mg)	0.374 (0.05)	0.531 (0.07)	0.543 (0.23)	0.397 (0.09)	0.438 (0.08)	0.315 (0.05)	0.376 (0.05)	0.521 (0.19)
Gosner Stage 45								
Snout-vent length (mm)	15.7 (0.17)	15.8 (0.22)	16.0 (0.21)	15.7 (0.22)	17.6 (0.27)	17.3 (0.21)	17.3 (0.23)	17.1 (0.21)
Body weight index	2.9 (0.13)	3.1 (0.13)	3.2 (0.10)	3.1 (0.11)	3.9 (0.03)	3.8 (0.14)	3.8 (0.15)	3.5 (0.10)
Spleen weight (mg)	0.18 (0.02)	0.17 (0.02)	0.18 (0.02)	0.15 (0.01)	0.22 (0.03)	0.21 (0.03)	0.20 (0.02)	0.19 (0.02)
Spleen weight index	1.1 (0.12)	1.1 (0.11)	1.1 (0.12)	0.9 (0.06)	1.2 (0.15)	1.2 (0.13)	1.1 (0.08)	1.1 (0.12)
Total leukocytes ($x10^3$)	25.0 (6.0)	26.0 (6.0)	35.0 (10.0)	31.0 (5.0)	46.0 (8.0)	327.0 (4.0)	34.0 (7.0)	34.0 (8.0)
Leukocytes/µg spleen	173.0 (50.0)	159.0 (30.0)	224.0 (54)	209 (30.0)	262.0 (47.0)	181.0 (23.0)	150.0 (24.0)	218.0 (50.0)
Corticosterone (ng/mg)	8.14 (2.08)	8.13 (1.46)	6.80 (0.84)	12.07 (1.77)	4.90 (1.02)	8.97 (1.27)	6.54 (1.23)	12.50 (1.98)
Days to metamorphosis	31.9 (1.0)	29.9 (0.80)	32.8 (0.80)	30.9 (0.98)	32.5 (1.0)	32.2 (0.88)	32.8 (0.81)	30.1 (0.78)

Table 2. Results of a 3-way ANOVA (α = 0.05) (PROC MIXED, SAS/STAT® software, SAS Institute Inc.) investigating effects and all interactions among developmental stage, water regime, and atrazine concentration in the New Mexico spadefoot toad (*Spea multiplicata*) tadpoles on selected morphometrics, spleen cellularity, corticosterone, and developmental rate. Tadpoles were subjected to two water regimes (declining water level or constant water level) and exposed to one of four atrazine concentrations (0, 0.5, 5.0, 50 µg/L) throughout development. The two developmental stages assessed were pre-metamorphic tadpoles (GS 36) and metamorphic tadpoles (GS 45). For snout-vent length, body weight index, spleen weight, and corticosterone there was a significant interaction between treatment and developmental stage, therefore results from pairwise comparisons (from the SLICE option) are presented. Degrees of freedom were calculated using Kenward-Roger estimation.

	df	F	p
Snout-vent length			
stage	1, 28.4	123.70	<0.001
water regime	1, 28.4	43.75	<0.001
stage*water regime	1, 28.4	18.84	<0.001
Gosner stage 36	1, 20.1	2.04	0.169
Gosner stage 45	1, 19.8	84.53	<0.001
Decline	1, 20.4	25.48	<0.001
Constant	1, 20.6	131.42	<0.001
atrazine	3, 28.2	1.03	0.396
stage*atrazine	3, 28.2	2.17	0.114
water regime*atrazine	3, 28.2	0.39	0.759
stage*water regime*atrazine	3, 28.2	0.04	0.991
Body weight index			
stage	1, 28.5	758.13	<0.001
water regime	1, 28.5	38.18	<0.001
stage*water regime	1, 28.5	7.23	0.012
Gosner stage 36	1, 19.5	4.44	0.048
Gosner stage 45	1, 19.8	39.49	<0.001
Decline	1, 19.2	456.21	<0.001
Constant	1, 20.7	294.09	<0.001
atrazine	1, 28.2	0.86	0.473
stage*atrazine	1, 28.2	1.32	0.288
water regime*atrazine	1, 28.2	2.12	0.120
stage*water regime*atrazine	1, 28.2	0.19	0.906

Table 2 continued	df	F	p
Spleen weight			
stage	1, 330	118.98	<0.001
water regime	1, 330	0.39	0.531
stage*water regime	1, 330	3.65	0.057
Gosner stage 36	1, 170	0.99	0.321
Gosner stage 45	1, 172	5.74	0.018
Decline	1, 171	91.93	<0.001
Constant	1, 171	42.46	<0.001
atrazine	3, 330	0.27	0.847
stage*atrazine	3, 330	0.37	0.771
water regime*atrazine	3, 330	0.52	0.666
stage*water regime*atrazine	3, 330	0.33	0.805
Spleen weight index			
water regime	1, 330	164.91	<0.001
stage*water regime	1, 330	0.03	0.859
stage*treatment	1, 330	2.32	0.128
atrazine	3, 330	0.28	0.839
stage*atrazine	3, 330	0.61	0.609
water regime*atrazine	3, 330	0.47	0.702
stage*water regime*atrazine	3, 330	0.42	0.738
Total leukocytes			
water regime	1, 28	1.56	0.221
stage*water regime	1, 28	2.84	0.103
stage*treatment	1, 28	0.63	0.433
atrazine	3, 28	0.53	0.665
stage*atrazine	3, 28	0.25	0.858
water regime*atrazine	3, 28	0.71	0.557
stage*water regime*atrazine	3, 28	0.06	0.979
Leukocytes/μg spleen			
water regime	1, 28	7.65	0.010
stage*water regime	1, 28	2.03	0.166
stage*treatment	1, 28	0.88	0.358
atrazine	3, 28	0.61	0.617
stage*atrazine	3, 28	0.49	0.692
water regime*atrazine	3, 28	0.50	0.684
stage*water regime*atrazine	3, 28	0.38	0.769

Table 2 continued	df	F	p
Corticosterone			
water regime	1, 28	127.74	<0.001
stage*water regime	1, 28	0.18	0.645
stage*treatment	1, 28	0.13	0.725
atrazine	3, 28	3.74	0.022
stage*atrazine	3, 28	3.68	0.024
Gosner stage 36	3, 18	0.06	0.982
Gosner stage 45	3, 18	4.46	0.017
0 µg/L	1, 38	26.60	<0.001
0.5 µg/L	1, 58	72.48	<0.001
5.0 µg/L	1, 58	70.21	<0.001
50 µg/L	1, 26	29.13	<0.001
water regime*atrazine	3, 28	0.32	0.808
stage*water regime*atrazine	3, 28	0.38	0.770
Development rate			
water regime	1, 290	0.57	0.450
atrazine	3, 290	3.10	0.027
water regime*atrazine	3, 290	1.20	0.311

Table 3. Pearson correlation coefficients between corticosterone and selected morphometrics and spleen cellularity of New Mexico spadefoot toad tadpoles (*Spea multiplicata*) subjected to two regimes of either declining or constant water level and four concentrations of atrazine (0, 0.5, 5.0, 50 µg/L) ($\alpha = 0.05$). N = 110 for each developmental stage. Individuals from both water regimes and all four atrazine concentrations were combined for this analysis.

	r	p
Gosner stage 36		
Snout-vent length (mm)	-0.103	0.284
Body weight (g)	-0.082	0.392
Spleen weight (mg)	-0.061	0.525
Total leukocytes ($x10^3$)	0.014	0.885
Leukocytes/µg spleen	0.049	0.613
Gosner stage 45		
Snout-vent length (mm)	-0.357	<0.0001
Body weight (g)	-0.468	<0.0001
Spleen weight (mg)	-0.390	<0.0001
Total leukocytes ($x10^3$)	-0.215	0.024
Leukocytes/µg spleen	0.020	0.832

Table 4. Pearson correlation coefficients between atrazine concentration and selected morphometrics, spleen cellularity, and corticosterone concentrations of New Mexico spadefoot toad tadpoles (*Spea multiplicata*) subjected to two regimes of either declining or constant water level and four concentrations of atrazine (0, 0.5, 5.0, 50 µg/L) (α = 0.05). N = 110 for each developmental stage. Individuals from both water regimes and all four atrazine concentrations were combined for this analysis.

	r	p
Gosner stage 36		
Snout-vent length (mm)	0.047	0.626
Body weight (g)	-0.126	0.189
Spleen weight (mg)	0.157	0.101
Total leukocytes ($x10^3$)	0.054	0.575
Leukocytes/µg spleen	0.086	0.370
Corticosterone (ng/mg)	0.030	0.754
Gosner stage 45		
Snout-vent length (mm)	-0.079	0.412
Body weight (g)	-0.111	0.249
Spleen weight (mg)	-0.212	0.026
Total leukocytes ($x10^3$)	0.001	0.995
Leukocytes/µg spleen	0.159	0.097
Corticosterone (ng/mg)	0.364	<0.0001

Fig. 1. Cumulative percentage of morphed (Gosner stage 42) New Mexico spadefoot toads (*Spea multiplicata*) reared in four atrazine concentrations (0, 0.5, 5.0, and 50 µg/L). Tadpoles were placed into experimental tanks 24hrs post-hatching (~GS 23) and exposed to declining (D) or constant (C) water depth and one of the four atrazine concentrations. Data are displayed as the percent of individuals morphed within 42 days post-hatch. Data are a percentage of all successfully morphed individuals, not total percent of all tadpoles. Sample sizes were 151 for constant water individuals and 147 for declining water individuals.

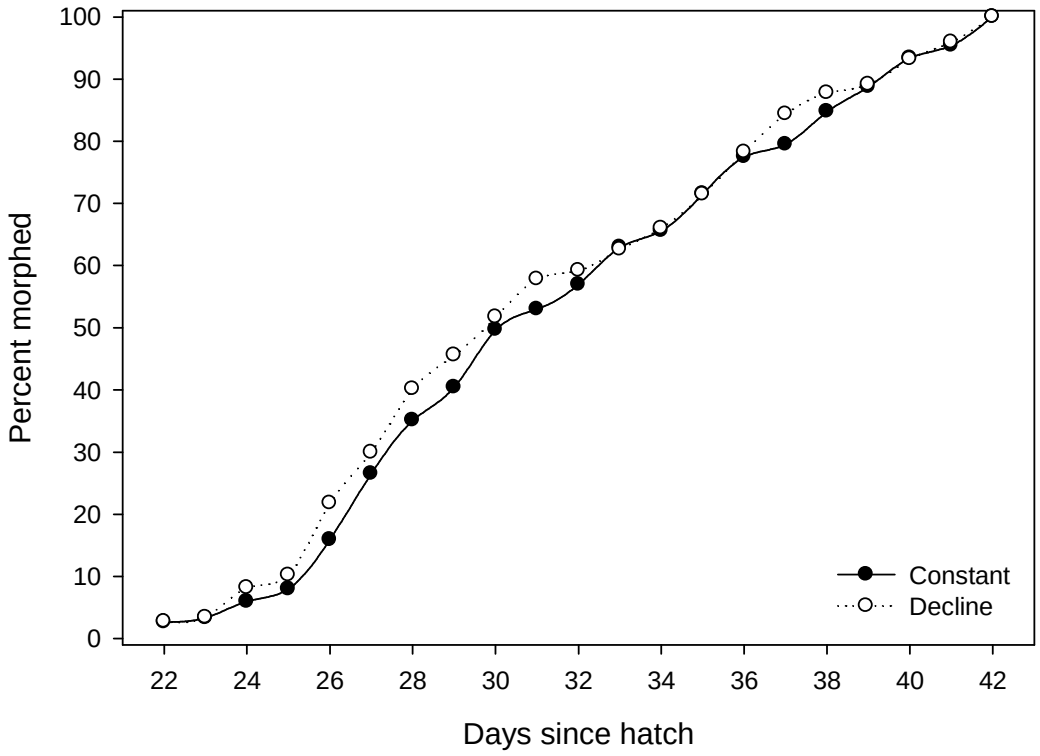


Fig. 2. Mean (± SE) number of days to metamorphosis (Gosner stage 42) for New Mexico spadefoot toads (*Spea multiplicata*) subjected to a decline (D) or constant (C) water level and four atrazine concentrations (0, 0.5, 5.0, 50 µg/L). Sample sizes are listed above error bars.

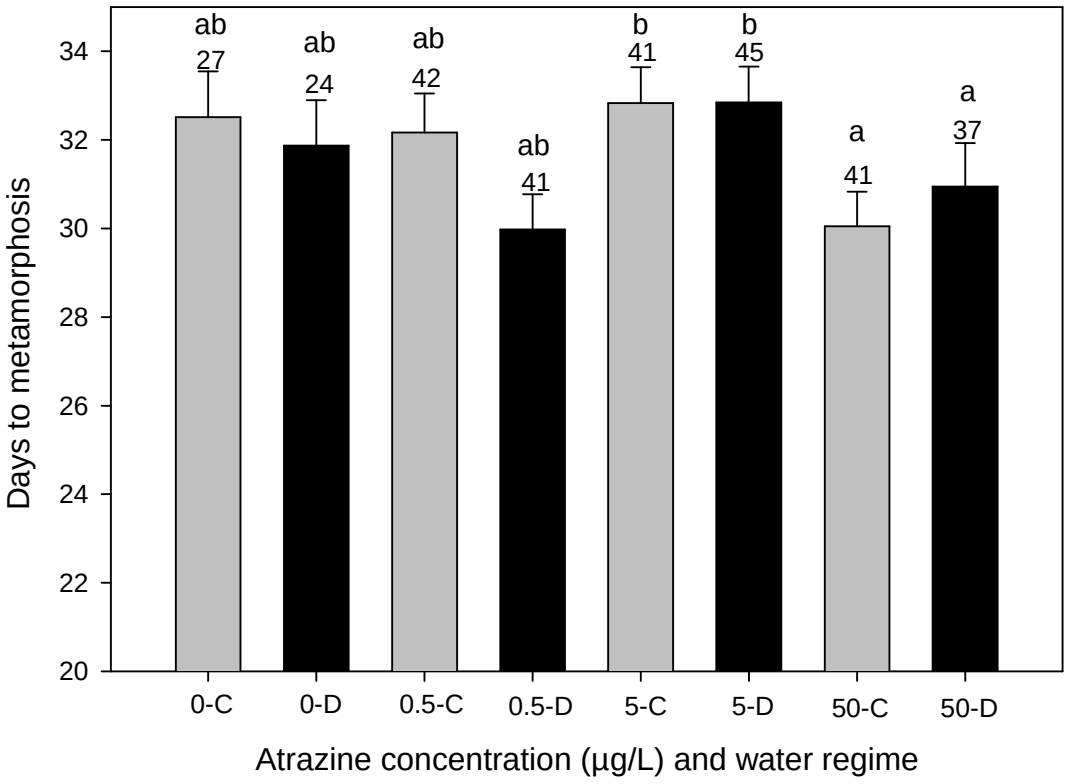


Fig. 3. Cumulative percentage of morphed (Gosner stage 45) New Mexico spadefoot toads (*Spea multiplicata*) reared in four atrazine concentrations (0, 0.5, 5.0, and 50 µg/L). Tadpoles were placed into experimental tanks 24hrs post-hatching (~GS 23) and exposed to declining or constant water depth and one of the four atrazine concentrations. Because there was no water regime effect on developmental rate ($F = 0.57$, $p = 0.450$), data were combined. Data are displayed as the percent of all individuals that successfully morphed within 42 days post-hatch.

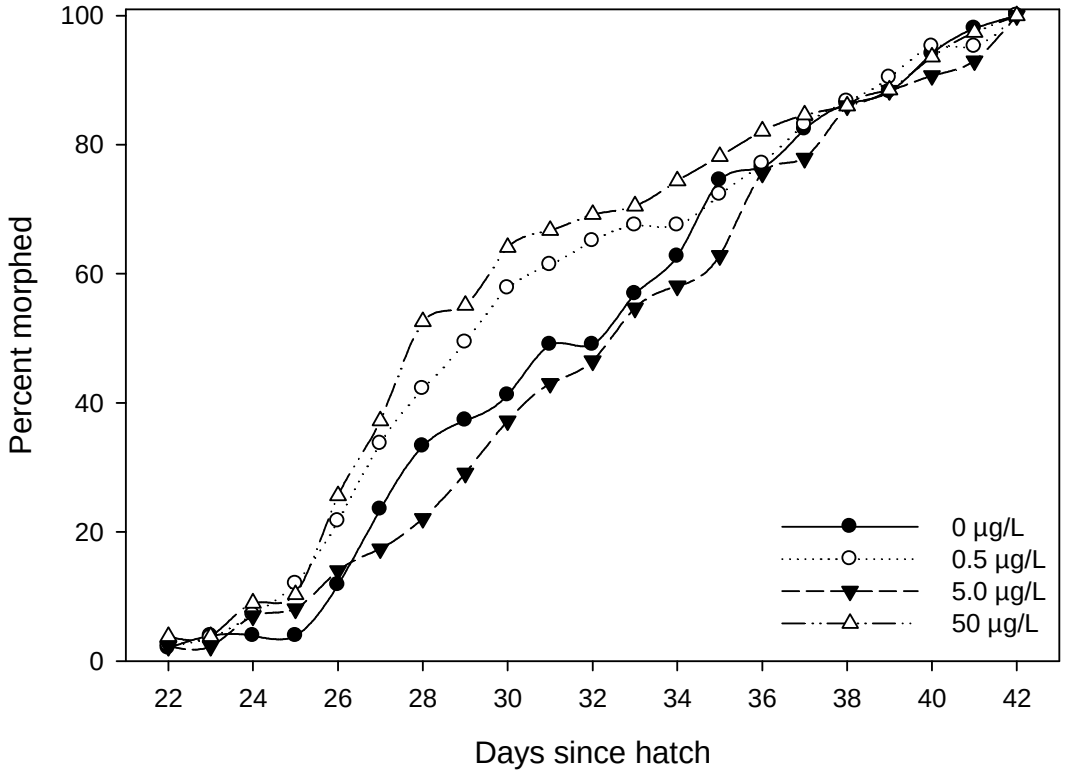


Fig. 4. Scatter plot of corticosterone (pg/mg) versus snout-vent length (SVL) in New Mexico spadefoot toads (*Spea multiplicata*) at Gosner stage 45, exposed to either declining or constant water level and four concentrations of atrazine (0, 0.5, 5.0, 50 µg/L). Each black circle represents a single individual from treatments (n = 110). r = -0.357, p = <0.0001

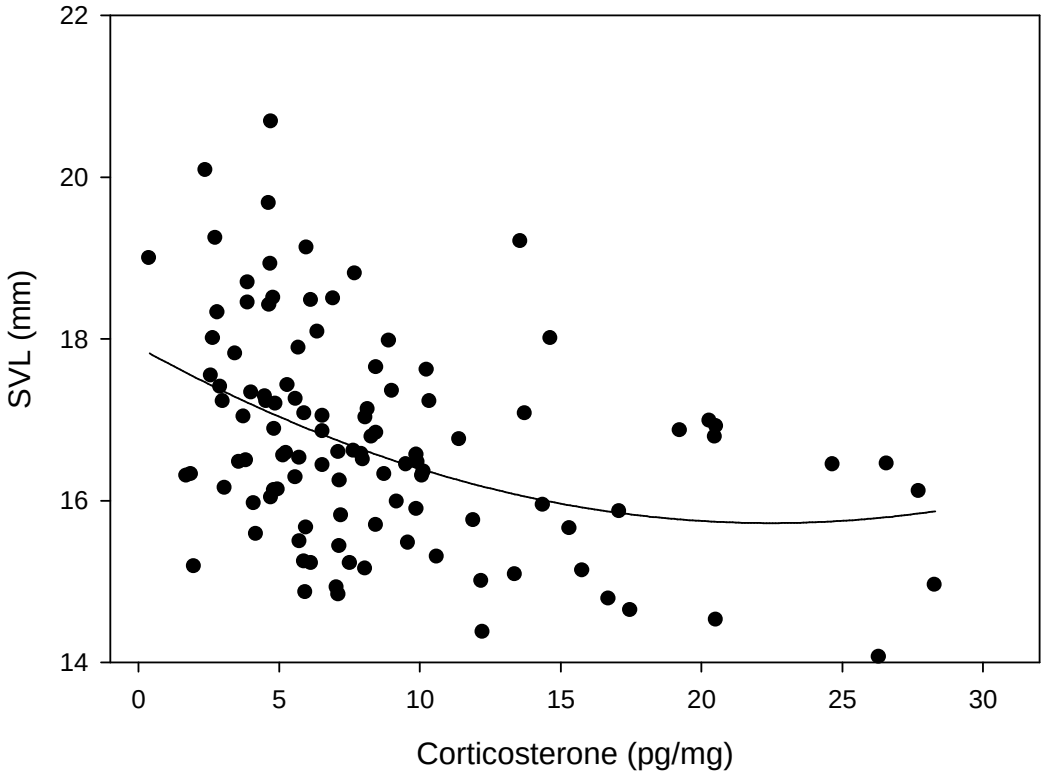


Fig. 5. Scatter plot of corticosterone (pg/mg) versus body weight (g) in New Mexico spadefoot toads (*Spea multiplicata*) at Gosner stage 45, exposed to either declining or constant water level and four concentrations of atrazine (0, 0.5, 5.0, 50 µg/L). Each black circle represents a single individual from treatments (n = 110). r = -0.468, p = <0.0001

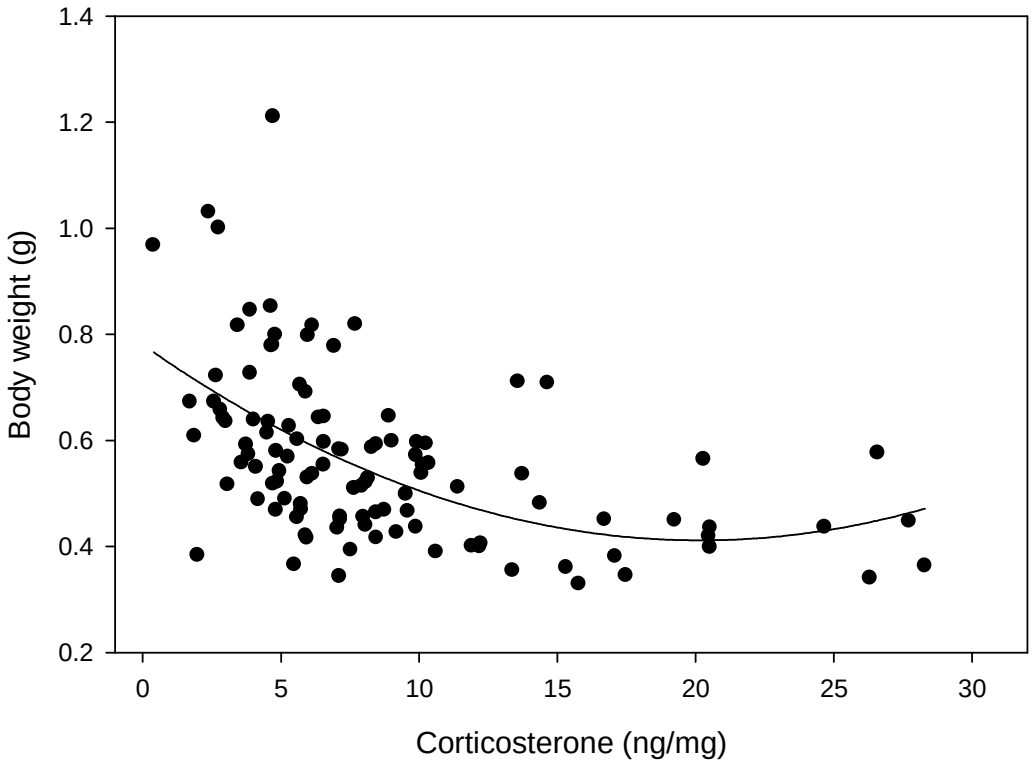


Fig. 6. Scatter plot of corticosterone (pg/mg) versus spleen weight (mg) in New Mexico spadefoot toads (*Spea multiplicata*) at Gosner stage 45, exposed to either declining or constant water level and four concentrations of atrazine (0, 0.5, 5.0, 50 µg/L). Each black circle represents a single individual from treatments (n = 110). r = -0.390, p = <0.0001

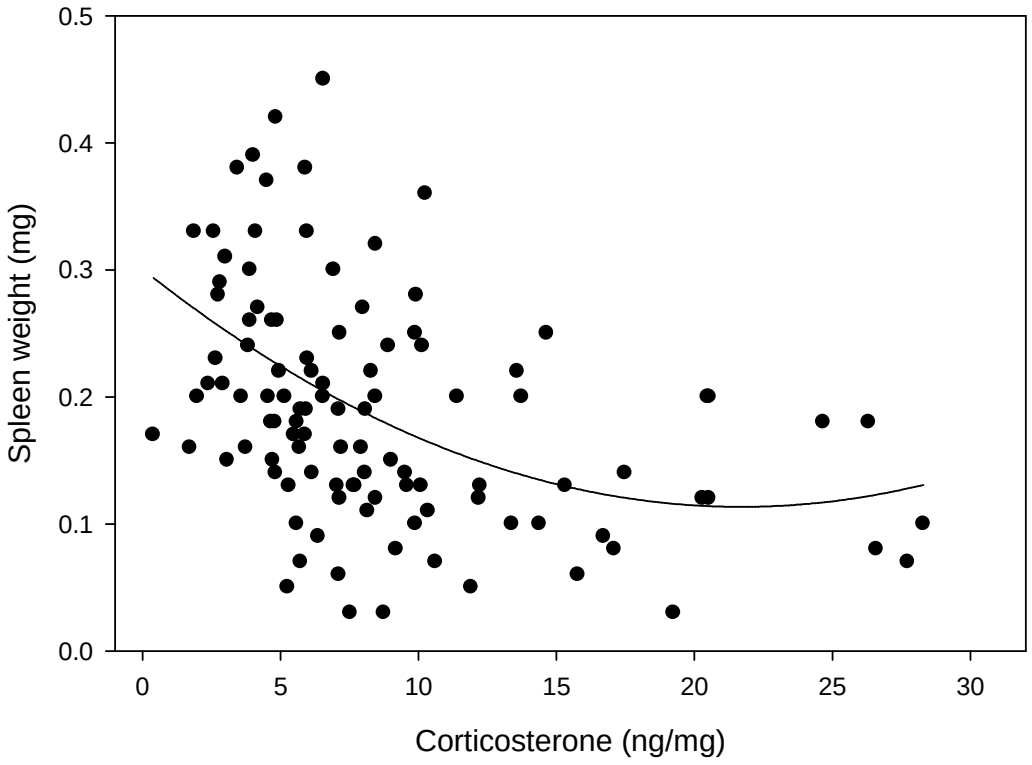


Fig. 7. Scatter plot of corticosterone (pg/mg) versus total spleen leukocytes in New Mexico spadefoot toads (*Spea multiplicata*) at Gosner stage 45, exposed to either declining or constant water level and four concentrations of atrazine (0, 0.5, 5.0, 50 µg/L). Each black circle represents a single individual from treatments (n = 110). r = -0.215, p = 0.024

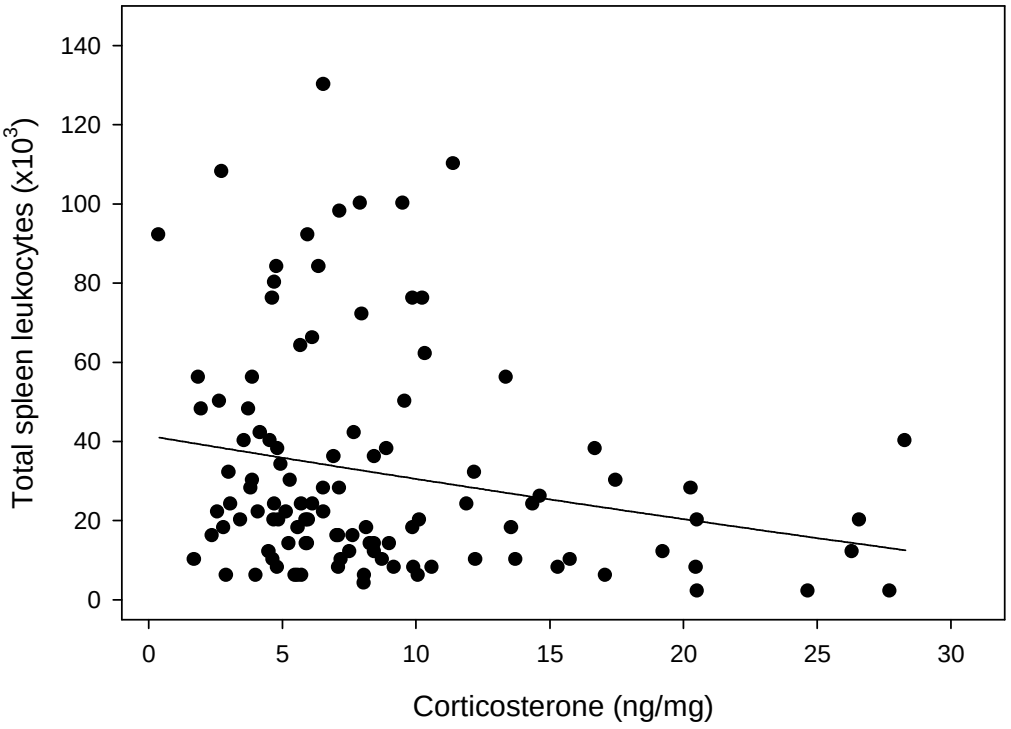
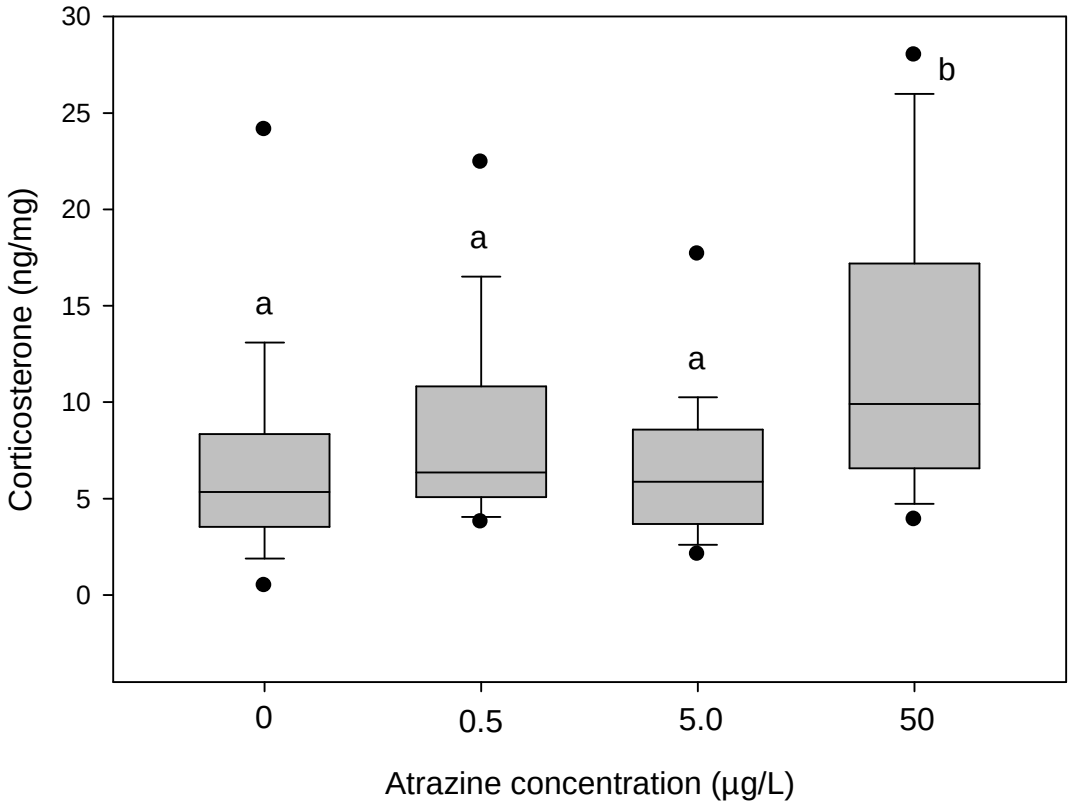


Fig. 8. Box and whisker plot of whole body corticosterone concentration (pg/mg) of New Mexico spadefoot toads (*Spea multiplicata*) at GS 45 exposed to either declining or constant water level at each level of atrazine exposure (0, 0.5, 5.0, 50 µg/L). There are no differences among 0, 0.5, and 5.0 µg/L corticosterone concentrations, but at 50 µg/L, corticosterone levels more than doubled in concentration from 4.90 ± 1.02 at 0 µg/L to 12.50 ± 1.98 ($F = 4.46$, $p = 0.017$). Sample size was 110 for each atrazine concentration. Different letters above bars denote significant difference at $p < 0.05$. Each box denotes the standard deviation from mean and the black line within denotes the median value. The upper whisker denotes the upper quartile (Q_3) while the lower whisker denotes the lower quartile (Q_1). Black circles denote the 5^{th} and 95^{th} percentiles.



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VITA

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Thesis: EVALUATION OF AMPHIBIAN COMMUNITIES OF PLAYA WETLANDS IN THE SOUTHERN HIGH PLAINS, TEXAS AND PHYSIOLOGICAL EFFECTS OF ENVIRONMENTAL STRESSORS ON THE NEW MEXICO SPADEFOOT TOAD, *SPEA MULTIPLICATA*

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Abstract: ***Scope and Methods*** The purpose of this dissertation project was two-fold, 1) to investigate the effects of surrounding land use on playa wetlands and resident amphibian communities and 2) to assess developmental and physiological effects of water loss and agricultural pesticide exposure on the New Mexico spadefoot toad, *Spea multiplicata*. In 2008 and 2009 I collected data on hydroperiod, water loss, sediment depth, and amphibian diversity in playa wetlands that were surrounded by cropland, native grassland, or USDA Conservation Reserve Program (CRP) grasses.
Findings and Conclusions Overall, playas located within CRP watersheds had sediment depths, water loss rates, and starting water depths intermediate to cropland and grassland playas. However, hydroperiod, playa area, and amphibian richness did not differ among landuses. Although species richness did not differ among land use types, distribution of amphibian species among land use types did differ, particularly in the drier 2008 season. This study is the first to definitively investigate CRP effects on playa amphibian communities. Because of environmental stressors observed in the field, I conducted three laboratory experiments investigating effect of water loss and water loss and a pesticide, atrazine, on development, spleen size and cellularity, and corticosterone levels of the New Mexico spadefoot toad, *Spea multiplicata*. Water loss accelerated development in the first two experiments, but did not affect body size or spleen cellularity. Corticosterone was negatively correlated with body size and elevated in tadpoles subjected to declining water levels. In the third experiment, I subjected tadpoles to water loss coupled with four concentrations of atrazine. Overall, there was no interaction between water regime and atrazine exposure. There were significant stage by water regime treatment interactions for snout-vent length, body weight index, and spleen weight. At metamorphosis, constant individuals were 8% larger in snout vent length, had 18% greater body weight indices, and 20% larger spleens. Corticosterone (CORT) level doubled at the highest atrazine concentration (50 µg/L), and was negatively correlated with snout-vent length, body weight index, spleen weight, and total spleen leukocytes at metamorphosis. Atrazine also influenced metamorphic rate and increased the average number of days to metamorphosis. This study suggests that environmental stressors seen in the field can affect development and immune function of amphibians, but effects do not manifest until metamorphosis when animals are already susceptible.

ADVISER'S APPROVAL: Dr. Scott McMurry