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Amphibian Community Composition And Disease Susceptibility In Ridge-Top Wetlands Of The Daniel Boone National Forest

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AMPHIBIAN COMMUNITY COMPOSITION AND DISEASE SUSCEPTIBILITY IN RIDGE-TOP WETLANDS OF THE DANIEL BOONE NATIONAL FOREST

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DEDICATION

I dedicate this thesis to my parents, Jeff and Tari McTaggart, and my sister, Erica McTaggart. Their love, support, and occasional pep talks have motivated me to pursue my passions.

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I would like to thank my advisor and graduate committee chair, Dr. Stephen Richter. His guidance, patience, and enthusiasm for research both motivated and inspired me, and will continue to do so for long after I leave the Richter lab. I would like to thank my committee members, Dr. David Brown and Dr. Kelly Watson, for their instruction and insight, which I am extremely grateful for. I am indebted to Dr. Stacey Lance and Austin Coleman, who were instrumental in the disease portion of my project and, without whom, none of the lab work would have been accomplished. This project would not have been possible without the help of fellow labmates John Bourne, Kristin Hinkson, and Logan Phelps. I thank them for assisting with fieldwork and for their unwavering positivity. I would also like to thank Sandie Kilpatrick and Beth Christensen for facilitating my work in the London District. I truly appreciate the graduate students and faculty of the Department of Biological Sciences who provided assistance and camaraderie throughout my graduate education. Further, I would like to thank the Society of Wetland Scientists and the Department of Biological Sciences for funding for this project. Lastly, I would like to express the deepest gratitude to my family and friends who encouraged and supported me throughout this project.

ABSTRACT

Ephemeral wetlands are a natural feature of the ridge-top ecosystem in the Daniel Boone National Forest (DBNF) in eastern Kentucky, and support a diverse amphibian assemblage characterized by species with short larval periods. However, hundreds of hydrologically permanent ponds have been constructed along the ridge-top system in the last 50 years. The results of previous studies suggest that constructed ponds act as sinks for some historic ridge-top species because they provide habitat for amphibian predators with long larval periods or aquatic adult stages. My objectives were to determine (1) if natural wetlands differ from constructed wetlands in amphibian community composition, (2) the habitat characteristics that predict the presence and abundance of different amphibian species, and (3) if prevalence of either *Batrachochytrium dendrobatidis* (*Bd*) or ranavirus differs between natural and constructed wetlands of the London Ranger District, where construction methods, wetland density, and wetland placement differ from those in previous studies conducted in the Cumberland District. Seven natural wetlands, five wetlands constructed for game use, and five wetlands constructed for bat conservation were surveyed for amphibian larvae and habitat characteristics. Natural wetlands had better wetland condition, indicated by higher Kentucky Wetland Rapid Assessment Method scores, and shallower littoral zones than both constructed wetland types. Natural wetlands also had greater canopy closure than bat wetlands. Using an ADONIS procedure, I found that amphibian communities in natural wetlands differed significantly from those in bat wetlands

($R^2 = 0.22$, $p = 0.017$), and although species richness was similar between natural and game wetlands, the relative species abundances observed between wetland types differed. Ranavirus was detected in large numbers at every wetland; however, there was a higher prevalence in natural wetland types. It is difficult to determine if this was due to the amplifying effect of wood frog larvae or some habitat characteristic present at natural wetland types. *Batrachochytrium dendrobatidis* (*Bd*) was not detected at any of the study wetlands. Overall, results suggest that bat wetlands in the London District are not conducive to recruitment and persistence of historical ridge-top species. Some game wetlands appear to be more favorable to historic species, such as wood frogs (*Lithobates sylvaticus*) and marbled salamanders (*Ambystoma opacum*); these were game wetlands with shallower littoral zones and more complex basin vegetation that mimicked natural wetland characteristics. However, because none of the constructed wetlands were ephemeral, they did not exactly replicate natural wetland habitat function. Lastly, differences between natural and constructed wetland types in the London District were not as pronounced as those in the Cumberland. This was most likely due to the high densities in which permanent wetlands were constructed in the Cumberland, their placement, and also the size and hydroperiod differences observed between natural wetlands in the two areas. For the DBNF, modifying constructed wetlands to replicate natural features such as hydroperiod, littoral zone depth, and vegetation would likely increase the recruitment and persistence of species characteristic of the ridge-top system.

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INTRODUCTION

Freshwater ecosystems account for a disproportionately large amount of species diversity and endemism (Revenga et al 2005). They are relatively small, accounting for only 0.01% of earth's water and roughly 0.8% of earth's surface, yet they support almost 6% of all described species (Dudgeon et al 2006). However, they are rapidly declining. Rivers, lakes, and wetlands have lost a larger proportion of both area and endemic species than any other ecosystem, and losses continue to grow from anthropogenic threats such as pollution, water withdrawals, dams, overharvesting, invasive species introductions, and habitat modification (MEA 2005; Revenga et al 2005).

Kentucky, specifically, has lost an estimated 81% of its historical freshwater wetlands (Dahl 2000), with many being converted for agricultural use. Enacted in 1972, the Clean Water Act was influential in curbing wetland destruction. However, hydrologically isolated wetlands were removed from the jurisdiction of the CWA after a 2001 Supreme Court ruling (Solid Waste Agency of Northern Cook County vs. U.S. Army Corps of Engineers 2001), leaving these habitats unprotected (Zedler 2003). Furthermore, Kentucky continues to rely solely on the section 401 water quality certification program for wetland protection and permitting, sanctioning no additional laws to secure geographically isolated wetlands. This has allowed the continued modification and destruction of key habitat for many wetland species, including amphibians.

The distribution of amphibians in temperate wetlands is influenced by a combination of factors, including natural history, wetland hydroperiod area, canopy closure, amount of forested upland, predation, and competition (Welborn et al. 1996; Van Buskirk 2005; Werner et al. 2007). Hydroperiod is of particular importance (Welborn et al. 1996; Denton and Richter 2013; Calhoun et al. 2014). Permanent wetlands tend to have relatively high amphibian richness, and are characterized by more generalist species and top predators (Babbitt et al. 2003). In contrast, ephemeral pools support a more specialized species assemblage and, therefore, are important for maintaining biological diversity (Snodgrass et al. 2000).

Isolated, ephemeral wetlands are a fundamental feature of the ridge-top wetland ecosystem in the Daniel Boone National Forest (DBNF), Kentucky (Brown and Richter 2012). These wetlands support a comparatively rare and diverse amphibian community and are documented as having high amphibian species richness (Corser 2008). The wetlands are characterized by species with short larval periods, such as wood frogs (*Lithobates sylvaticus*), eastern spadefoots (*Scaphiopus holbrookii*), and marbled salamanders (*Ambystoma opacum*), and the relative absence of top amphibian predators (Denton and Richter 2013; Kross and Richter 2016; Drayer and Richter accepted).

Hundreds of wetlands have been constructed in the DBNF in the last 50 years (Brown and Richter 2012). These wetlands are present in both the Cumberland and London districts of the DBNF, but construction methods differed between the two areas. Wetlands in the Cumberland District are of two general construction types: deep, relatively large ponds, with dammed perimeters that were intended to have permanent hydrology, and smaller, shallower ponds meant to dry and generally replicate natural wetlands. They have also been constructed primarily on the ridge-tops where natural, ephemeral wetlands are found. Wetlands in the London District are also of two general types: game wetlands and bat wetlands. Game wetlands were constructed for deer (*Odocoileus virginianus*) and turkey (*Meleagris gallopavo*) use and resemble the deep constructed wetlands in the Cumberland District, but most were constructed on the sides of ridges, usually by damming an ephemeral stream. Bat wetlands were constructed for Indiana bat (*Myotis sodalis*) use and are long and narrow, have open canopies, and were placed on the ridge-tops. The number of constructed wetlands also differs between the districts, from over 550 in the Cumberland District to fewer than 50 in the London District. Most of these constructed wetlands hold water year-round, and although they provide breeding sites for amphibians, studies in the Cumberland District suggest that they do not support the communities historically found in this ecosystem (Brown and Richter 2012; Denton and Richter 2013; Drayer and Richter accepted).

The addition of permanent water bodies has created suitable habitat for amphibian predators with long larval periods such as bullfrogs (*Lithobates catesbeianus*) and green frogs (*Lithobates clamitans*), or with fully aquatic adult life stages such as eastern newts (*Notophthalmus viridescens*). This has promoted their movement from the lowland basins into the ridge-top systems where they were historically absent or found in low abundance (Drayer and Richter accepted). Furthermore, Kross and Richter (2016) suggested that these permanent, constructed wetlands act as ecological sinks for wood frogs, one of the species historically found in the ridge-top system. In the Cumberland District, wood frog egg clutches were observed in both wetland types, but eastern newts and green frog larvae consumed most eggs in the permanent wetlands (Kross and Richter 2016), and larvae were only detected in one permanent wetland that did not include eastern newts in that district (Drayer and Richter accepted). Thus, permanent wetlands may attract specialized, ephemeral species, but predation by eastern newts and large ranids appears to limit survival rates for some. Furthermore, not only do these lowland species predate those found in the natural, ridge-top wetlands, they are also known reservoirs for disease (Greenspan et al. 2012; Richter et al. 2013).

Emerging infectious diseases have been increasingly linked to amphibian declines (Collins and Storfer 2003; Daszak et al. 2003; Skerratt et al. 2007). Ubiquitous in North America and implicated in recent amphibian mortality events are *Batrachochytrium dendrobatidis* (*Bd*), a fungal pathogen, and ranaviruses, a DNA-based group of viruses in the family Iridoviridae. *Bd* is the causative agent for chytridiomycosis, a cutaneous fungal infection that inhibits amphibian processes such as respiration and osmoregulation (Berger et al. 1998). This pathogen has been detected in all states throughout the eastern U.S. (www.Bd-maps.net), although highland regions in the southern Appalachians are conspicuously under-sampled (Rollins et al. 2013). *Bd* is typically associated with permanent bodies of water, as zoospores cease to be viable once they are desiccated (Johnson et al. 2003). Zoospores have also been shown to colonize a wide range of amphibian hosts (Gahl et al. 2012). However, susceptibility to the actual disease condition, chytridiomycosis, varies significantly among species and is not always indicative of a mortality event. The ability of certain species to carry sublethal *Bd* infections contributes to the spread of this pathogen. Eastern newts, bullfrogs, and green frogs have been implicated as reservoirs in northeastern amphibian communities due to their ability to harbor *Bd* asymptomatically (Daszak et al. 2003; Raffel et al. 2010; Gahl et al. 2012).

Comparatively little is known about viral emerging infectious diseases when compared to the breadth of knowledge on pathogens such as the *Bd* fungus (Duffus 2006). This lack of knowledge seems counterintuitive as ranaviruses, in particular, are associated with amphibian die-offs in over 20 states (Gray et al. 2009b) and 43% of mortality events in the U.S. from 2001 to 2005 (Muths 2006). Ranavirus monitoring in the southern Appalachians is of particular importance, as studies suggest that wetlands in a high catchment position are at a greater risk of larval mortality events (Gahl and Calhoun 2008), and known die-offs have occurred in such locations in several species (e.g. eastern newts, spotted salamanders, and wood frogs) in 1999 and 2001 (Green et al. 2002). This is important to note, as mortality events are more likely to recur at previously infected sites (Gahl and Calhoun 2008). Susceptibility to ranavirus infection is wide ranging, with individual species and developmental stages differing in disease severity (Gray et al. 2007; Duffus et al. 2008; Schock et al. 2008). Specifically, species that breed in ephemeral to semi-permanent wetlands seem to be more susceptible to this pathogen (Hoverman et al. 2011). Individuals that are infected sublethally act as reservoirs for ranaviruses. In permanent, aquatic environments, larvae that take more than 1 season to develop, such as bullfrogs and green frogs, may host the virus through the duration of the winter and re-infect amphibian populations the following breeding season (Gray et al. 2007). Highly aquatic adults, such as eastern newts, are likely reservoirs as well (Gray et al. 2009a). Furthermore, ranaviral infections have been previously detected in two of five constructed wetlands tested in the Cumberland District of the DBNF (Richter et al. 2013).

The purpose of this research is to further elucidate the impact of constructed wetlands on the ridge-top ecosystem in the southern London District where the natural wetland size, number of constructed wetlands, construction techniques, and wetland placement all differ from the Cumberland District. Specific objectives were to determine (1) if natural wetlands differ from constructed wetlands in amphibian community composition in the London District, (2) what habitat characteristics predict the presence and abundance of different amphibian species, and (3) if prevalence of either *Bd* or ranaviruses differs between natural and constructed wetlands of the DBNF.

METHODS

Study Sites

All wetlands in my study occurred in the ridge-top system of the London District in the Daniel Boone National Forest (DBNF), Kentucky. Three types of wetlands were assessed: natural ephemeral wetlands, wetlands constructed for game use and habitat enhancement, and wetlands constructed specifically for Indiana bat (*Myotis sodalis*) conservation. Both constructed wetland types were intentionally designed to hold water permanently (Dale Lynch, personal communication), but differ in their construction method. Game wetlands were created primarily for use by wild turkey (*Meleagris gallopavo*) and white-tailed deer (*Odocoileus virginianus*). They are round to oval in shape, have raised dams around a portion of the perimeter, and many were made by damming ephemeral streams. Bat wetlands are smaller than game wetlands, and most are long and narrow in shape and were lined to ensure permanence. Also, in most cases, bat wetlands were placed in or near wildlife openings, a management decision reflecting the need for an open canopy to facilitate bat access. I assessed 17 wetlands in my study, including seven natural wetlands, five game wetlands, and five bat wetlands. All wetlands were hydrologically isolated and fishless.

Amphibian Surveys

Amphibian surveys were repeated for three sample periods to encompass peak amphibian breeding in May and June 2015. Each survey included both dipnetting and visual-encounter surveys. Visual surveys were used in occupancy analyses only and began upon arrival at the wetland. All amphibians encountered (adult, juvenile, larval, and egg stages) within 2 m of the wetland edge were recorded. Dipnet sweeps took place every 5 m while walking the perimeter of each wetland. Each sweep consisted of jabbing a D-frame net into the wetland substrate and skimming the bottom of the wetland for approximately 1 m (Denton and Richter 2012). Captured individuals were identified to species. Furthermore, due to the predation of wood frog eggs at constructed wetland sites in the Cumberland District, egg mass surveys were also conducted during the second week of March 2015.

Habitat Characteristics

Habitat characteristics were measured to assess the factors that influence amphibian community composition in each wetland type. Canopy closure was estimated at each of the four cardinal directions and once at the center of the wetland at maximum leaf out using a spherical densiometer and then averaged across the five sample points. Depth of littoral zones and water quality measurements were taken 1 m from shorelines at each of the cardinal directions and averaged. Water quality measurements consisted of conductivity (µmhos), dissolved oxygen (mg/l), and pH, and were taken with a YSI 556 multi-parameter water quality meter (Yellow Springs Instruments; Yellow Springs, OH). Upland coarse woody debris (CWD) was measured using a line-intersect sampling protocol described by Waddell (2002) and modified by Denton and Richter (2013), with 50-m transects established in each cardinal direction perpendicular to the perimeter of the wetland and extending into the surrounding habitat. Coarse Woody Debris intercepted along each transect was recorded if the diameter was >12.5 cm at its narrowest end. Each piece recorded was then measured for total length and diameter at both the narrowest and widest ends, and an estimate of the cubic volume of CWD per hectare was calculated (Husch et al. 1972). Each site was also scored for wetland condition according to the Kentucky Wetland Rapid Assessment Method (KY-WRAM) following the 2013 draft protocol established by the Kentucky Division of Water. This assessment method evaluates six metrics related to the wetland basin and surrounding upland which include: wetland size and distribution, upland buffers, hydrology, habitat alteration, special wetland types, and vegetative complexity.

Disease Surveys

Up to 30 larvae of either green frogs, bullfrogs, or wood frogs were collected via dipnetting at each wetland and sacrificed to test for disease presence. These species do not typically co-occur, usually wood frogs are found in ephemeral wetlands and green frogs and bullfrogs are in found in constructed wetlands, which is why multiple ranid species were used for analysis. Ranid species were targeted because they have been associated with disease outbreaks in eastern North America (Daszak et al. 2003; Raffel et al. 2010; Gahl et al. 2012). Up to 30 larvae of either spotted or Jefferson salamanders were also collected at each wetland and sacrificed to test for disease and were chosen because of the high abundances with which they occur in all wetland types. Difficulty in distinguishing between green frog and bullfrog larvae, and spotted and Jefferson salamander larvae resulted in combining the respective groups. Larvae were handled using sterile methods and a 1% Nolvasan® solution was used to disinfect all field equipment, including boots, to prevent the spread of pathogens between sample sites. Each larva was sacrificed using 5% ethanol and subsequently stored in a 70% ethanol solution (IACUC protocol #07-2015).

Ranavirus and *Bd* testing was performed at the University of Georgia Savannah River Ecology Laboratory. A DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, California, USA) was used to extract genomic DNA from a 10-50 mg tail clip. DNA extraction was done following the manufacturer's protocol with the exception of eluting in 50µL of buffer. A NanoDrop ND-1000 Spectrophotometer was used to analyze eluted DNA concentrations and DNA was subsequently stored at -20C. Quantitative real-time PCR (qPCR) was performed following Boyle et al. (2004) and Kerby et al. (2013) for *Bd* assays and Allender et al. (2012) for frog virus 3-like ranavirus assays. Individual samples and negative controls were run on a 96 well plate using an iCycler IQ real-time PCR detection system. For *Bd*, single reactions were run using 1x Taqman Universal Master Mix, 1x of Taqman primer/probe, and 3.0 µl of *Bd* DNA template in a total volume of 13µL. Reaction volumes were reduced by 50% from Boyle et al. (2004) based on a successful modification by Kerby et al. (2013). Standard curves for each plate were created using replicates of 100, 10, 1, and 0.1 genome equivalents of *Bd* to quantify sample zoospore loads. For ranavirus, single reactions were run using 1x TaqMan Universal Mastermix, 2x TaqMan primer/probe, and 3.0µL of extracted DNA template in a total volume of 13µL. A serial dilution of positive standard from 10 to 10^6 viral copies/μL was used to produce standard curves and standards were replicated on the plate at least three times with the 10^2 and the 10^1 standards replicated five times. For both *Bd* and ranavirus, the lowest standard (10^1) was considered the threshold Ct (threshold of fluorescence) and a sample had to have a threshold cycle lower than that of the lowest standard to be considered positive.

Data Analyses

Habitat and Amphibian Community Comparisons - Species richness and Shannon-Wiener diversity indices were calculated for each wetland. Dipnetting count data were converted to catch per unit effort (CPUE) and the greatest CPUE value for each species during each sampling period was used for analyses (Shulse et al. 2010; Denton and Richter 2013). Similar to disease-sampling rationale, CPUE for Jefferson salamanders (*Ambystoma jeffersonianum*) and spotted salamanders (*Ambystoma maculatum*) were combined based on their comparable life histories and difficulty in distinguishing them morphologically; CPUE for bullfrogs and green frogs were combined as well.

To examine possible differences in habitat characteristics among wetland types, I used a one-way ANOVA, or a Welch's ANOVA if the data did not meet equal-variance assumptions. A post-hoc Tukey multiple comparison test was then performed using wetland type as the predictor variable in Statistical Package for the Social Sciences (SPSS Inc. Chicago, Illinois). Amphibian CPUE, wetland type, and habitat characteristics were examined using a redundancy analysis (RDA) in R Version 2.12.1 (R Development Core Team, Vienna, Austria). To meet normality assumptions, a Hellinger transformation was performed on species CPUE data. To further measure community similarity between study wetland types, a permutation-based multivariate analysis of variance (ADONIS) was performed in R using the Bray-Curtis Similarity Index in the distance matrix. Individual species associations were also analyzed to determine which habitat variables influence species presence and abundance. This was done using a model selection approach with amphibian CPUE as the response variable and habitat characteristics as predictor covariates. Area was excluded from analyses due to a high degree of correlation with KY-WRAM score. Regression models were evaluated using generalized linear modeling with a compound Tweedie distribution and log-link function. Models were then ranked according to Akaike's Information Criterion values corrected for small sample sizes (AIC_c). If multiple candidate models had $\Delta AIC_c \leq 2.0$, or the top model had an Akaike weight of < 0.9, model averaging was used to determine the relative importance of individual parameters within the top models. Species were evaluated using this approach only if they had a sufficiently large CPUE and they occurred in all wetland types. Values are presented as mean ± SE unless otherwise specified.

Disease Surveys- Amphibian infection prevalence was calculated for both anurans and caudates at each wetland and compared among wetland types using a one-way ANOVA, or a Welch's ANOVA if the data did not meet equal-variance assumptions. A post-hoc Tukey multiple comparison test was then performed using wetland type as the predictor variable in SPSS. A paired T-test was also conducted in SPSS to determine if ranid and ambystomatid groups experienced significantly different infection rates.

RESULTS

Habitat and Amphibian Community Comparisons

All natural wetlands dried during this study and both bat and game wetlands maintained permanent hydroperiods. I found that KY-WRAM score, littoral zone depth, and wetland area differed significantly between natural and constructed wetlands, but did not differ between constructed types. Natural wetlands had shallower littoral zones than both constructed wetland types (game: $p = 0.001$, mean difference = 10.4 ± 2.2 cm; bat: $p = 0.007$, mean difference = 7.9 ± 2.2 cm) and higher KY-WRAM scores (game: $p = 0.009$, mean difference = 10.6 ± 3.02 points; bat: $p = 0.006$, mean difference = 11.3 ± 3.02 points) (Fig. 1). Natural wetlands were also significantly larger than both constructed wetland types (game: $p = 0.002$, mean difference = 586.0 ± 133.5 m^2; bat: $p = 0.001$, mean difference = 650.6 ± 133.5 m^2) (Fig. 1). Canopy closure was significantly higher at natural wetlands than bat wetlands ($p = 0.009$, mean difference = 29.7 ± 8.6%) but did not differ between natural and game wetlands ($p = 0.664$, mean difference = 7.8 ± 8.6%) or game and bat wetlands ($p = 0.081$, mean difference = 22.2 ± 9.5%) (Fig. 1). Water quality measurements and upland coarse woody debris did not differ between types (Fig. 2).

Overall, I captured 5,558 amphibians representing 12 species. Southern two-lined salamanders (*Eurycea cirrigera*) were found in some wetlands constructed from dammed ephemeral streams, but because they are considered to be primarily inhabitants of streams rather than wetlands (Mitchell and Gibbons 2010), they were

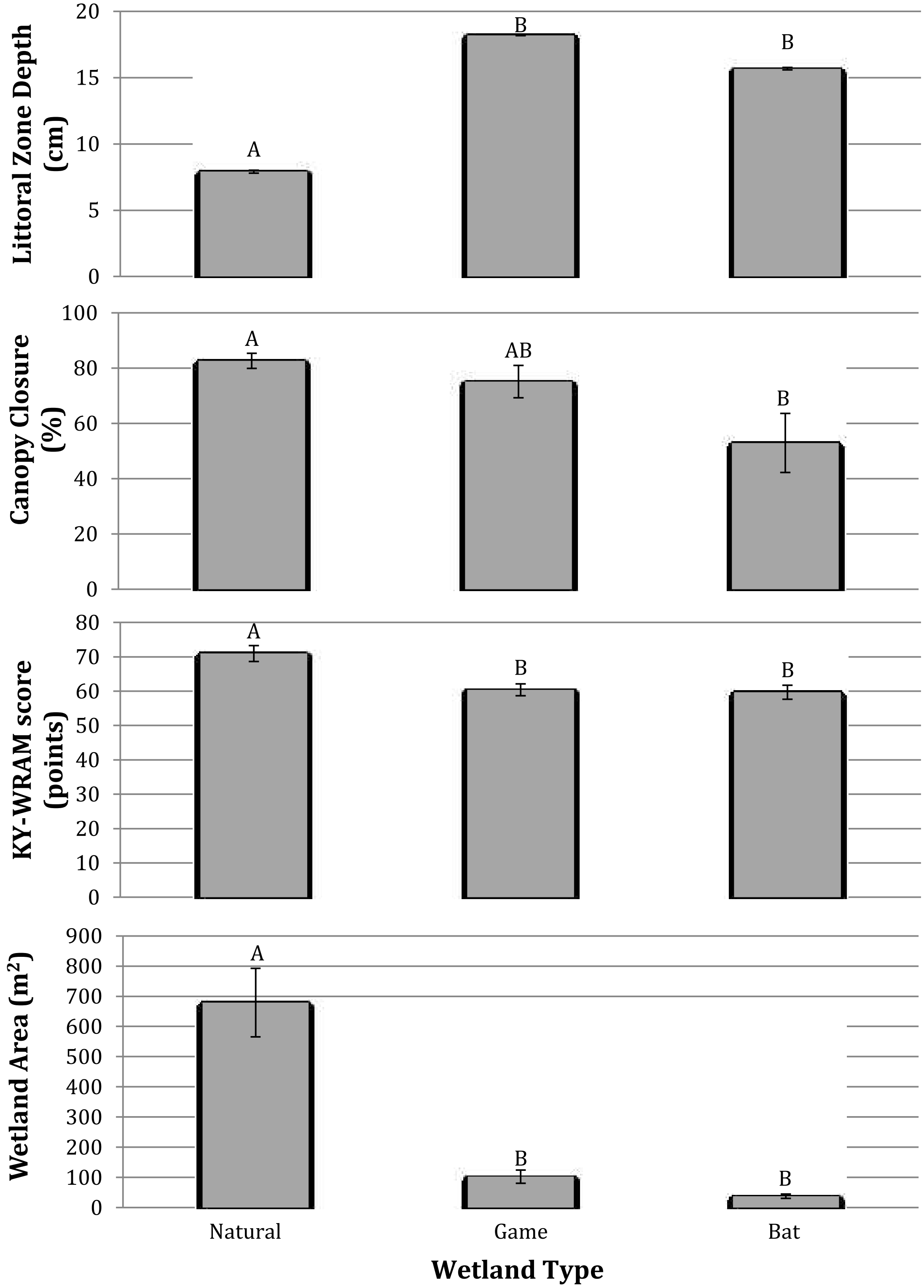

Fig. 1. A comparison of mean littoral zone depth, canopy closure, Kentucky Wetland Rapid Assessment Method score, and wetland area (± SE) between the three wetland types in the Daniel Boone National Forest. The letters above bars indicate post-hoc Tukey comparisons.

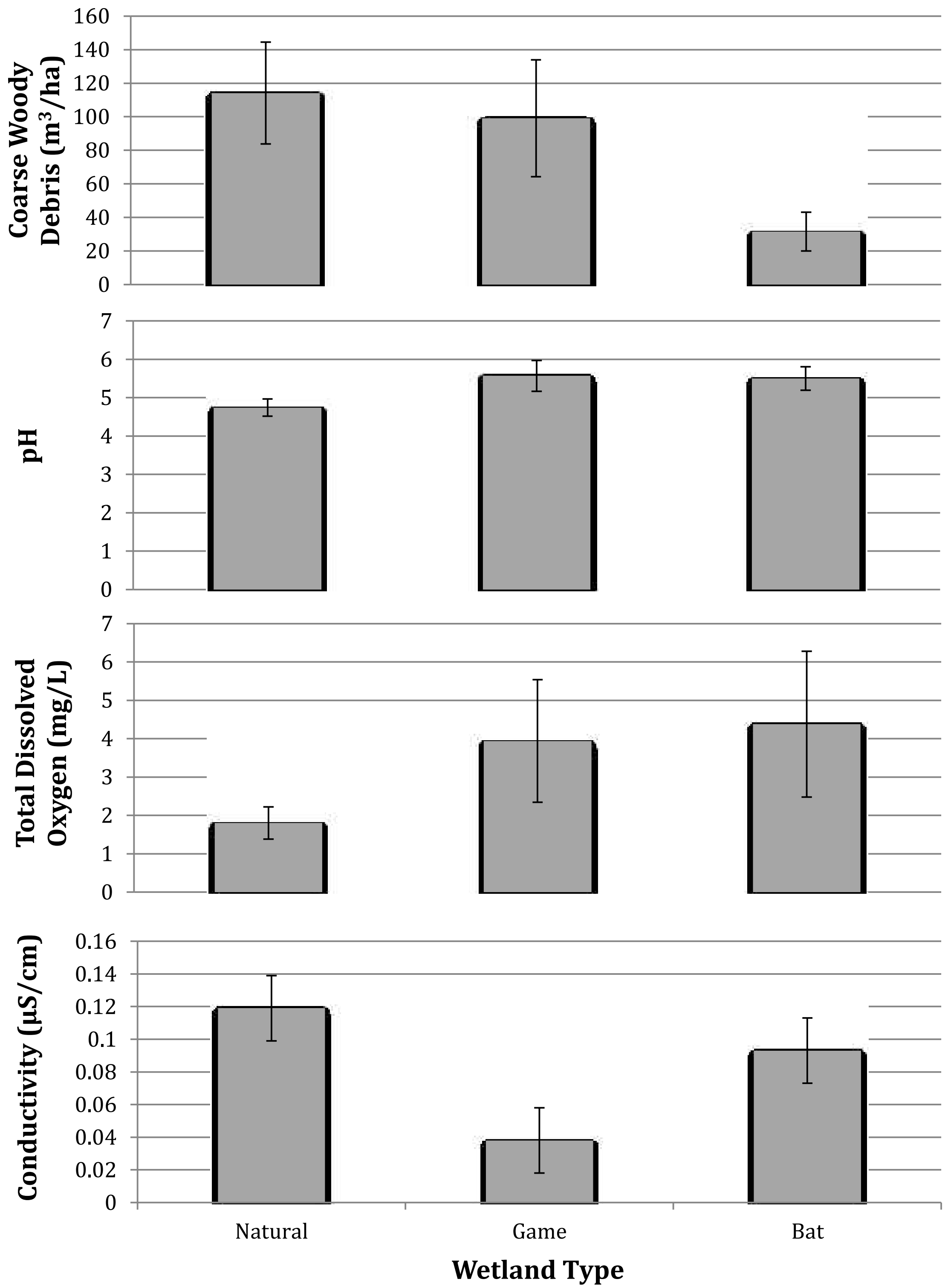

Fig. 2. A comparison of coarse woody debris (CWD), pH, dissolved oxygen (D.O.), and Conductivity (± SE) between the three wetland types in the Daniel Boone National Forest.

excluded from analyses. Natural wetlands had the highest species richness (11) followed by game (10) and bat (9) wetlands. Similarly, abundance was higher in natural wetlands (1,885) than in game (1,428) and bat (789) wetlands. Shannon-Weiner diversity indices were similar for natural (0.98 ± 0.08) and game wetlands (0.90 ± 0.15), and lower for bat wetlands (0.58 ± 0.18), although not significantly ($F_{2,14}$ = 2.58, p = 0.11).

Individual species abundances fell into one of three categories: those that increased from natural to game to bat wetlands, those that decreased from natural to game to bat wetlands, and those that showed no pattern (Table 1). Species that increased in abundance from natural to game and bat wetlands were the green frog-bullfrog group, eastern newts, and the spotted-Jefferson salamander group (Fig. 3). Species that decreased in abundance from natural to game and bat wetlands were wood frogs, marbled salamanders, and spring peepers (*Pseudacris crucifer*) (Fig. 3). Other species showed no discernable pattern across wetland types, presumably due to low overall CPUE and occurrence in five or fewer wetlands. These included four-toed salamanders (*Hemidactylium scutatum*), American toads (*Anaxyrus americanus*), and Cope's gray treefrogs (*Hyla chrysoscelis*).

The RDA accounted for 60% of the total variation in species abundance and habitat data, and canopy closure was the only significant vector term ($F_{1,8}$ = 4.07, p = 0.008) (Fig. 4). Using the ADONIS procedure, community composition of natural and bat wetlands was significantly different (global R_2 = 0.22, p = 0.017); however, community composition did not differ between either natural and game wetlands (global R_2 = 0.16, p = 0.11) or game and bat wetlands (global R_2 = 0.15, p = 0.14).

rt and number of wetlands where a species occurred, per wetland type, in the Daniel
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Species	Natural		Game		Bat	
	mean CPUE ± SE	# of wetlands present	mean CPUE ± SE	# of wetlands present	mean CPUE ± SE	# of wetlands present
Anura						
Anaxyrus americanus (American toad)	1.33	1	-	0	-	0
Hyla chrysoscelis (Cope's gray treefrog)	0.04	1	0.07 ± 0.05	2	0.04	1
Pseudacris crucifer (spring peeper)	5.25 ± 3.86	6	1.90 ± 1.31	5	0.03	1
Lithobates sylvaticus (wood frog)	7.71 ± 3.64	6	3.29 ± 2.43	3	-	0
L. clamitans & *catesbeianus* (green frog & bullfrog)	0.30 ± 0.15	4	0.92 ± 0.27	5	1.80 ± 1.08	4
Caudata						
Notopthalmus viridescens (eastern newt)	0.12 ± 0.09	6	0.84 ± 0.42	4	1.56 ± 0.20	5
Ambystoma opacum (marbled salamander)	1.77 ± 0.88	6	0.33 ± 0.23	2	0.2	1
A. maculatum & *jeffersonianum* (spotted & Jefferson salamanders)	9.11 ± 3.09	7	13.78 ± 2.17	5	18.13 ± 10.44	4
Hemidactylium scutatum (four-toed salamander)	0.13	1	0.19 ± 0.16	2	0.27 ± 0.11	3

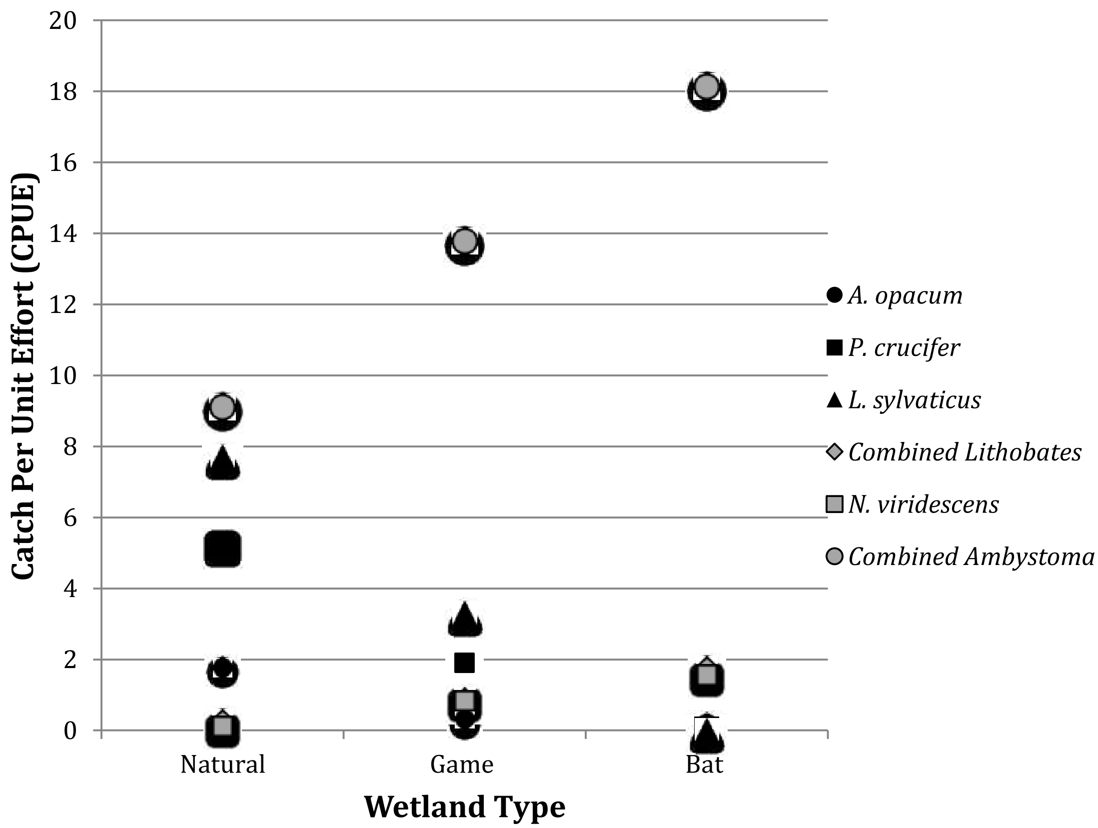

Fig. 3. Mean abundance for species that showed either increasing or decreasing abundances between natural and constructed wetland types in the Daniel Boone National Forest.

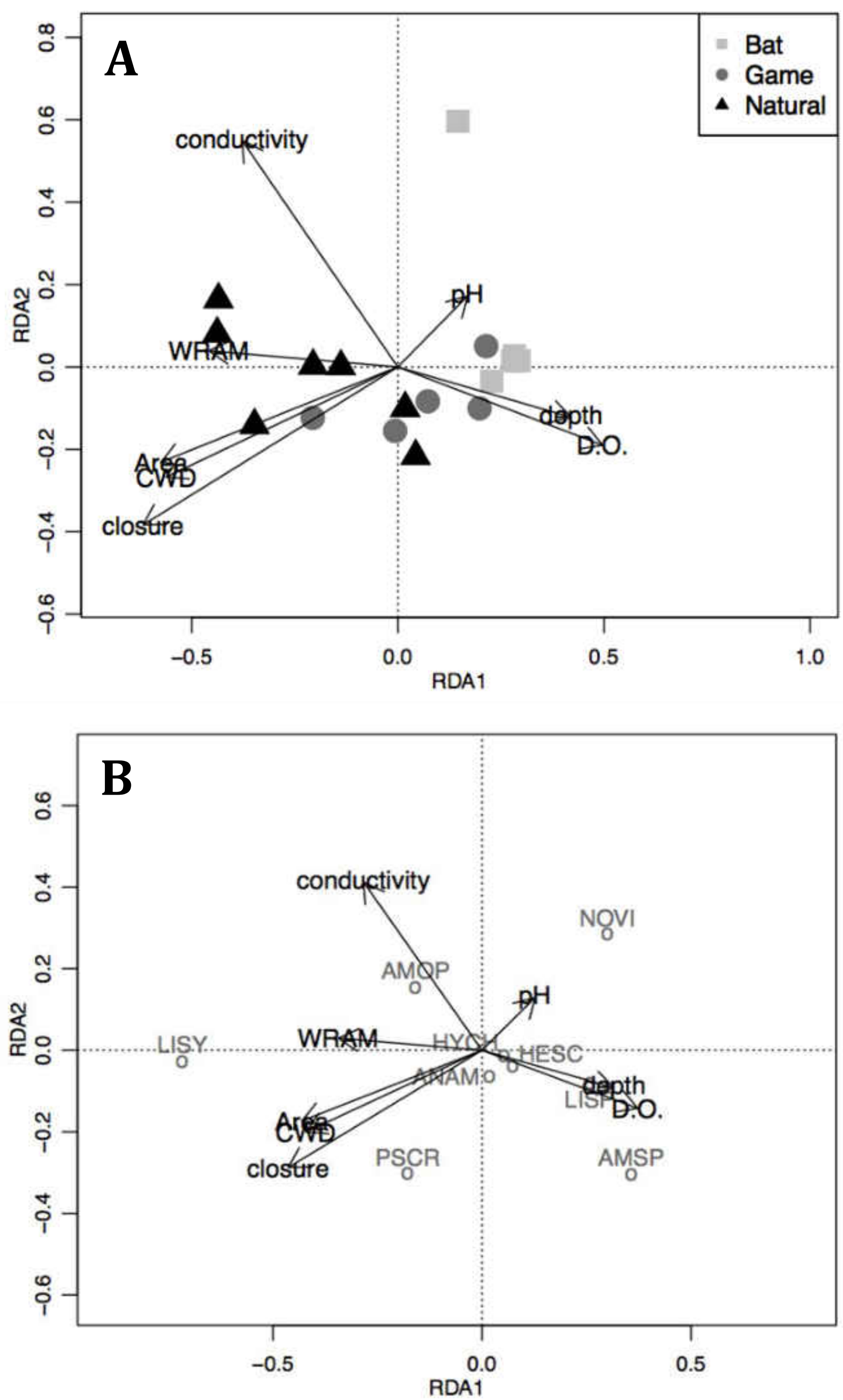

Fig. 4. Redundancy analysis (RDA) triplots for (A) wetlands and (B) species abundance based on catch per unit effort in the Daniel Boone National Forest. The proportion variance in the sample data explained by the RDA was 60% and canopy closure was the only significant vector term ($F_{1,8} = 4.07$, $p = 0.008$).

Individual Species Associations

Tweedie regression models were evaluated for four species (Table A – 2). For each model evaluation, 3–4 models were closely ranked with a $\Delta AIC_c \leq 2.0$ or top model with an Akaike weight of < 0.9 (Table 2), so model averaging was used to produce parameter estimates of factors in the top ranking models for each species (Table 3). Combined green and bullfrog CPUE was best predicted by water conductivity, canopy closure, dissolved oxygen, and KY-WRAM score. These species were negatively associated with water conductivity, canopy closure, and KY-WRAM score, and positively associated with dissolved oxygen. Eastern newt CPUE was negatively associated with natural wetlands, canopy closure, and upland coarse woody debris (CWD). Spring peepers were positively associated with natural wetlands and depth, and negatively associated with canopy closure. Spotted and Jefferson salamander larvae were the most commonly occurring species and were captured in all but one wetland. They were negatively associated with canopy closure.

Regression analyses could not be performed for the remaining five species due to either low capture rates or lack of occurrence in all three wetland types. Wood frogs were captured in high numbers where they were present, but larvae were only detected in natural and game wetlands. Although wood frog egg masses were found in four of the five bat wetlands earlier in the breeding season, no larvae were seen or captured while dipnetting in this wetland type. Marbled salamanders had the highest abundances in natural wetlands, and were detected in all but one of this type. They were also detected in two game wetlands and one bat wetland, but in relatively low numbers. Four-toed salamanders, American toads, and Cope's gray treefrogs occurred in such low abundances that habitat association analyses were not warranted.

Table 2. Tweedie regression models for amphibian species abundance within the ridge-top wetlands of the Daniel Boone National Forest, Kentucky. Displayed models had a difference of ≤ 2.0 in Akaike's Information Criterion corrected for small sample sizes (ΔAIC_c).

Species	**Model**[a]	***K***[b]	**AIC$_c$**	**ΔAIC$_c$**	***w$_i$***[c]
Combined *Lithobates*	WRAM, conductivity	3	45.37	0.00	0.43
	pH, conductivity, D.O.	4	45.47	0.11	0.41
	Closure	2	47.92	2.55	0.12
Pseudacris crucifer	Type	4	78.30	0.00	0.77
	Type, depth, closure, WRAM	7	82.74	4.45	0.08
	WRAM	2	84.29	6.00	0.04
	WRAM, type, depth	6	84.63	6.33	0.03
Notophthalmus. viridescens	Closure, CWD, WRAM	4	46.30	0.00	0.60
	Type	4	47.58	1.28	0.32
Combined *Ambystoma*	Closure	2	128.30	0.00	0.82
	Conductivity	2	133.46	5.16	0.06
	Depth	2	134.31	6.02	0.04

Table 3. Model averaging of the parameters within the AIC$_c$ best models for each amphibian species within the ridge-top wetlands of the Daniel Boone National Forest, Kentucky.

Species	Parameter	Model-averaged estimate (β)	Unconditional SE	85% CI[b]
Combined *Lithobates*	D.O.	0.180	0.05	0.25, 0.11
	Conductivity	-23.815	9.88	-9.587, -38.04
	KY-WRAM	-0.086	0.00	-0.09, -0.09
	Canopy Closure	-0.42	0.01	-0.03, -0.06
Pseudacris crucifer	Wetland Type:			
	Natural	5.680	2.108	8.72, 2.65
	Game use	4.21	1.76	6.74, 1.68
	Bat run	0	0	0.00, 0.00
	Depth	0.201	0.125	0.38, 0.02
	Closure	-0.078	0.041	-0.02, -0.14
Notophthalmus viridescens	Closure	-0.021	0.01	-0.01, -0.03
	Wetland Type:			
	Natural	-2.661	0.64	-1.74, -3.59
	Game use	-0.599	0.35	-0.09, -1.11
	Bat run	0	0	0.00, 0.00
	CWD	-0.011	0.00	-0.01, -0.02
Combined *Ambystoma*	Closure	-0.023	0.008	-0.01, -0.03

Amphibian Disease

All wetlands sampled within the London District were found to harbor ranavirus, but no *Bd* was detected within any of the study sites. Bat wetland 'Buckhorn #3' was excluded from analyses because eastern newts were the only species detected at that site and no ranid or ambystomatid larvae were captured. Ranavirus was detected in ranid tissue samples from every wetland site, and 57% of total individuals surveyed were infected (Table 4). However, ranavirus was detected in ambystomatid tissue samples from only nine of 16 wetland sites, and only 28% of total individuals surveyed were infected. Ranavirus prevalence per site in ranids (mean = 61.2 ± 8.6%) was significantly higher than in ambystomatids (mean = 27.5 ± 9.1%, $t_{15} = 3.115$, $p = 0.007$). Lastly, disease prevalence was compared between wetland types for both ranids and ambystomatids. Only ranid disease prevalence was significantly higher at natural wetlands than at bat wetlands ($p = 0.02$, mean difference = 54.4 ± 17.5%) and no other comparisons were statistically significant (Fig. 5).

ce and species tested per wetland site in the DBNF, Kentucky.

		Lithobates				*Ambystoma*			
Wetland Type	**Site**	**# Tested**	**# Positive**	**Prevalence (%)**	**Species**	**# Tested**	**# Positive**	**Prevalence (%)**	**Species**
Natural	FS 19A	15	14	0.93	Wood frog	10	6	0.60	Jefferson/Spotted salamanders
Natural	High Knob Natural	10	8	0.80	Green/Bullfrogs	10	0	0.00	Jefferson/Spotted salamanders
Natural	Rolling Fork	15	12	0.80	Wood frog	10	2	0.20	Jefferson/Spotted salamanders
Natural	Sandgap	15	14	0.93	Wood frog	10	1	0.10	Jefferson/Spotted salamanders
Natural	Cliff Palace	15	15	1.00	Wood frog	10	9	0.90	Jefferson/Spotted salamanders
Natural	Dale 2	15	15	1.00	Wood frog	10	3	0.30	Jefferson/Spotted salamanders
Natural	Lynch	21	5	0.24	Wood frog	10	9	0.90	Jefferson/Spotted salamanders
Game	FS 19 1	12	5	0.42	Green/Bullfrogs	10	0	0.00	Jefferson/Spotted salamanders
Game	FS 448B	15	10	0.67	Wood frog	10	0	0.00	Jefferson/Spotted salamanders
Game	Good pond	15	15	1.00	Wood frog	10	2	0.20	Jefferson/Spotted salamanders
Game	High Knob 3	20	5	0.25	Green/Bullfrogs	10	0	0.00	Jefferson/Spotted salamanders
Game	Laurel hill	15	10	0.67	Wood frog	10	0	0.00	Jefferson/Spotted salamanders
Bat	Brushy 1	18	1	0.06	Green/Bullfrogs	5	0	0.00	Jefferson/Spotted salamanders
Bat	High Knob 2	19	3	0.16	Green/Bullfrogs	15	3	0.20	Jefferson/Spotted salamanders
Bat	Hurricane 1	20	3	0.15	Green/Bullfrogs	15	0	0.00	Jefferson/Spotted salamanders
Bat	Reece Place	14	10	0.71	Green/Bullfrogs	11	11	1.00	Jefferson/Spotted salamanders
Bat	Buckhorn 3	0	-	-	N/A	0	-	-	N/A
	Total:	**254**	**145**	**0.57**		**166**	**46**	**0.28**	

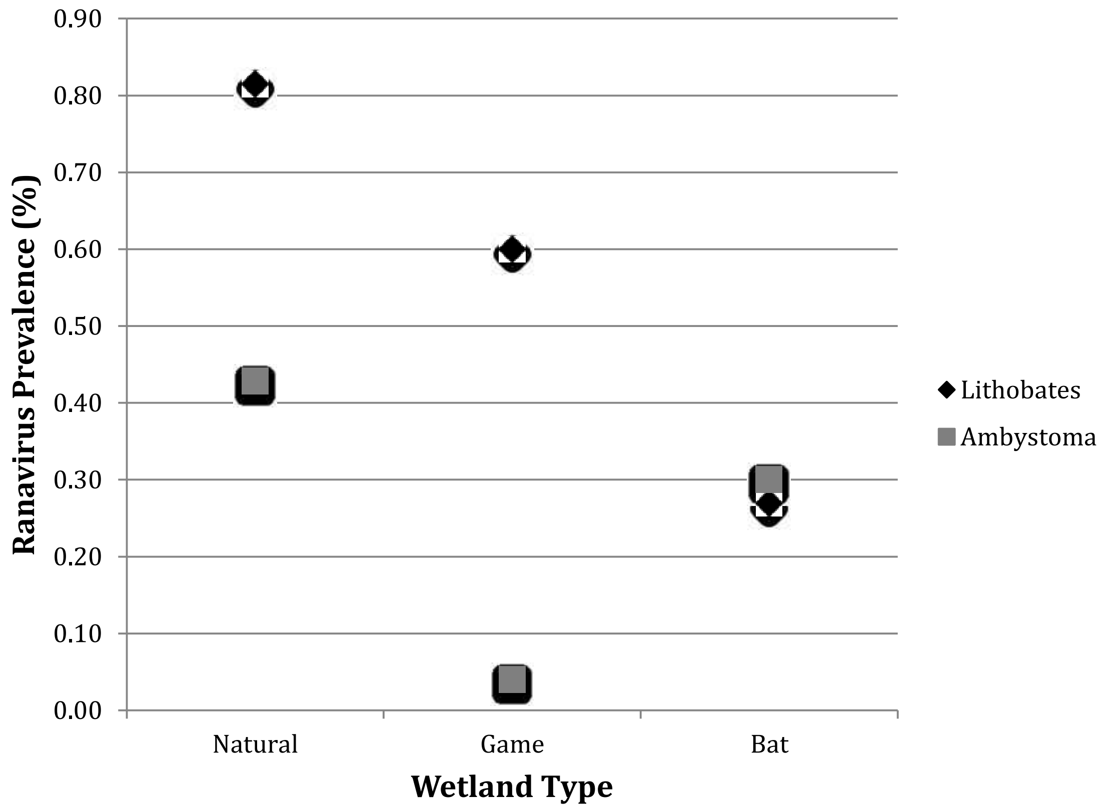

Fig. 5. Ranavirus prevalence for *Lithobates* and *Ambystoma* species per wetland type in the DBNF, Kentucky.

DISCUSSION

Results from this and previous studies in the Daniel Boone National Forest indicate that permanently constructed wetlands are detrimental to many of the amphibian communities of the ridge-top system. Although constructed wetlands have fulfilled their intended purpose by providing year-round water to other ridge-top wildlife such as deer, turkey, and bats, in high densities they encourage colonization by lowland predators like large ranids and newts. This trend is apparent in both the London and Cumberland districts, however the pattern is much more pronounced in the Cumberland. In the London District, game constructed wetland types support relatively sensitive ridge-top amphibians such as wood frogs and marbled salamanders, albeit at lower abundances than natural types. The reason for the disparity between districts likely has to do with constructed wetland density and placement, but also with differences in natural wetland habitat features. Hereafter I will discuss the habitat and community features of both districts and end with some recommendations for making constructed wetlands more conducive to historical amphibian communities.

Habitat and Amphibian Community Comparisons

My results suggest that the characteristics of bat wetlands in the London District are not conducive to recruitment and persistence of historical ridge-top species such as wood frogs and marbled salamanders. Game wetlands had similar species richness and diversity indices as natural wetlands, but supported a relatively high abundance of lowland species and fewer ridge-top amphibians. This may be attributed to the steeper littoral zones and deeper water of both game and bat wetlands which resulted in permanent hydroperiods, contrasting with the ephemeral hydroperiods of naturally occurring ridge-top wetlands. KY-WRAM score was also greater in natural wetlands than both constructed wetland types. Much of the difference in WRAM scores between wetland types can be attributed to differences in Metric 6, the metric that measures vegetative and habitat complexity within a wetland basin. Many amphibian species are positively associated with vegetation quantity and complexity because of its importance for cover and egg deposition (Shulse et al. 2010; Thompson et al. 1980). Lower vegetative complexity in constructed wetland types may be due to their deeper littoral zones (Porej and Hetherington 2005; Calhoun et al. 2014) or to compaction of surrounding soil during wetland construction that may make seed germination more difficult (Alessa et al. 2000). Lastly, differences in canopy closure between natural and bat wetlands likely also influence species richness because most ridge-top amphibian species prefer dense canopies (Dorcas and Gibbons 2008). However, reduced canopy cover was an intentional management decision in the DBNF, as water is easier for bats to access under canopy gaps. These analyses suggest a complex relationship between the gradient of habitat variables and amphibian presence and abundance.

The amphibian community differences detected between natural and bat wetlands is due to certain taxa associating more closely with natural wetlands (marbled salamanders, wood frogs, and spring peepers), and other taxa associating more closely with the hydrologically permanent constructed wetlands (green frogs, bullfrogs, eastern newts, spotted salamanders, and Jefferson salamanders). Most species, with the exception of wood frogs, occurred in all three wetland types. However, relative abundance per species varied among wetland type and was a principal factor affecting the disparity between natural and constructed wetland communities, especially in bat wetlands.

Individual Species Associations

Few green frogs, bullfrogs, and eastern newts occurred in natural wetlands in my study, and newts were negatively associated with this wetland type in regression analyses. Although these species are common in the DBNF, they are typically and historically found in permanent, lowland water bodies such as oxbows, lakes, and marshes (Denton and Richter 2013). Large ranid larvae and aquatic adult newts overwinter in wetlands, a life-history trait that likely promoted their movement from the lowlands to the ridge-tops with the addition of hydrologically permanent wetlands (Sever 2006). This association with larger, more open, lacustrine habitat may also explain the negative relationship of these species with canopy closure in the ridge-top system. Large ranids are fairly tolerant to disturbance and were consistently found in relatively low quality wetland habitat, explaining their negative association with KY-WRAM score. Lastly, eastern newts were negatively associated with upland coarse woody debris. These adults are fully aquatic, and so have little use for upland cover (Mitchell and Gibbons 2010) which would explain this characteristic having no relationship with newt abundance. However, the slight negative relationship indicates that there may be some other habitat characteristic correlated with coarse woody debris that was not measured in this study.

Spotted and Jefferson salamanders were the most common larvae in my study. These species were found in nearly every wetland, and usually in high numbers, but they were most abundant in bat wetlands. Even though spotted and Jefferson salamanders historically used ridge-top wetlands, regression analysis showed them to be negatively correlated with canopy closure, a distinctive feature of the London District natural wetlands. Skelly et al. (2005) described this species as a "canopy generalist" that would utilize breeding habitat regardless of canopy conditions. In the London District, the wetlands with long or permanent hydroperiods tended to also have relatively open canopies. Spotted salamanders have longer developmental periods than other Kentucky ambystomatids (Keen 1975; Nyman 1991), and, albeit not often reported in the literature, larvae can overwinter in the wetland (Whitford and Vinegar 1966; Ireland 1973), which may explain their high abundance in the sparse canopy, long hydroperiod wetlands.

Wood frogs were the most abundant anurans in the study wetlands where they were detected. Larvae occurred in the highest numbers at natural wetlands, and were only found in natural and game wetlands. However, wood frog egg rafts were observed in four of the five bat wetlands earlier in the breeding season. Eastern newts are known to greatly reduce wood frog larvae abundance in this system (Kross and Richter 2016), and eastern newts were observed consuming wood frog eggs at many wetlands in my study (pers. observ.). I postulate that the relatively high newt abundance and predation caused the wood frog larvae absence in bat wetlands as well. Although it is possible that disease caused the wood frog mortality, if this were the case I would have expected to see evidence of a mortality event in the form of deceased larvae while dipnetting at the wetland.

Marbled salamanders were also more abundant in natural wetlands than in constructed wetlands in my study, possibly because of their nesting requirements. In the fall, female marbled salamanders lay eggs terrestrially, under cover objects, in dry wetland beds or the dried margins of reduced ponds. When winter rains and increased water levels flood the nests, the larvae hatch (Mitchell and Gibbons 2010). Although all constructed wetlands in my study held water permanently, water levels did fluctuate throughout the breeding season. The two game wetlands where marbled salamanders were found had relatively shallow littoral zones, which explains their presence at those sites.

Spring peeper abundance was strongly correlated with natural wetlands as well. Early breeding and fairly rapid larval development allows this species to thrive in ephemeral environments (Dorcas and Gibbons 2008). High abundances for this species were also obtained in both natural and ephemerally constructed wetlands in Denton and Richter (2013), making hydroperiod the most likely driver of spring peeper abundance. In the regression models spring peepers were also negatively associated with canopy closure and positively associated with depth, although these models had much lower Akaike weights. This is probably due to the huge abundance of spring peepers at High Knob Natural, a wetland with the least amount of canopy closure and second deepest littoral zone of all the natural wetlands surveyed in this study.

Eastern spadefoots, mountain chorus frogs (*Pseudacris brachyphona*), and pickerel frogs (*Lithobates palustris*) are the only wetland-breeding species known to occur in London County that were not detected during this study. For pickerel and mountain chorus frogs this was expected, since these species tend toward different wetland habitats. Mountain chorus frogs utilize ditches and small puddles (Barbour 1957) while pickerel frogs tend toward more lowland habitat (Cunningham et al. 2007). Eastern spadefoots most likely use London District ephemeral wetlands, and have been previously observed breeding in these wetlands in the Cumberland District (Drayer and Richter accepted). However, breeding effort by this species is known to vary widely between years (Greenberg and Tanner 2005), which may explain their absence in this study.

Amphibian Disease

Ranavirus was ubiquitous throughout the London District wetlands. I had expected ranaviral infection to be present in a higher number of permanent, constructed wetlands because they contain overwintering amphibians that, presumably, would be able to host ranavirus throughout the year and re-infect breeding amphibians and new larvae each spring. Also, although ranavirus can remain viable in dry wetland sediments, its infectivity is greatly reduced over time; Munro et al. (2016) reported a 90% reduction in frog virus 3 (FV3) infectivity in dry pond sediments over just ten days. The detection of ranavirus at all seven ephemeral wetlands in this study suggests that either ranavirus had remained viable in the dried wetland sediments for months, or, more likely, organisms hosting the virus infected the natural wetland populations at the beginning of the breeding season after the wetlands had filled.

Once ranavirus appeared in the natural wetlands, however, it is not surprising that it proliferated. Ranids, in general, are more susceptible than other families to ranaviral infection (Hoverman et al. 2011), specifically the FV3-like strain that was tested for in our study, so the significantly lower infection rates in *Ambystoma* at most wetlands was expected. Additionally, wood frogs, the ranid species most commonly found in London District natural wetlands, are known to be especially susceptible to ranaviral infection. Hoverman et al. (2011) found wood frogs to have the greatest infection prevalence (>90%) of all 19 species tested for ranaviral susceptibility. In many systems they even act as amplifier hosts, providing an avenue for ranavirus virions to multiply rapidly to very high levels (Brenes 2013). For these and other ephemeral wetland species, the high energy cost of rapid larval development may leave less energy available for fighting pathogens (Lochmiller and Deerenberg 2000; Warne et al. 2011). Because wood frogs did not occur in all wetland types, green and bullfrogs were the ranids collected at most constructed wetlands to test for disease. Therefore, it is difficult to parse out whether the high prevalence of ranavirus for both ambystomatids and ranids in London District natural wetlands is due to the high number and amplifying effect of wood frog larvae, or some other habitat variable associated with natural wetlands.

London and Cumberland District Comparisons

Both the Cumberland and London ridge-top systems show similar relationship between habitat structure and amphibian community patterns among natural and constructed wetland types. In this study, as well as previous studies, wood frogs and marbled salamanders were associated with natural wetlands while bullfrogs, green frogs, and eastern newts were more closely associated with constructed wetlands. However, in the Cumberland District these patterns between natural and constructed wetlands are more pronounced. Any community composition similarities between natural and constructed wetlands in the Cumberland District were due to the relative absence of large ranids and newts in certain constructed wetland types, and were not caused by constructed and natural wetlands both supporting historical ridge-top amphibians. In the London District, community similarities were due to game constructed wetlands supporting historical ridge-top amphibians, much like the natural wetlands in the Cumberland District, albeit at lower abundances.

The different amphibian community patterns found between the London and Cumberland districts are most likely due to three factors. First, London natural wetlands are much larger than those in the Cumberland District. While both natural wetlands have ephemeral hydroperiods, London natural wetlands hold water longer, and in especially wet years, might not dry. Therefore, they may naturally support some eastern newts. Second, the high density of constructed wetlands in the Cumberland District has allowed a greater abundance of lowland species to become established in the ridge-top ecosystem, where the traditional species have no natural defense to predation. Lastly, placement of constructed wetlands on ridge-tops near the natural wetlands in the Cumberland District allows for easier dispersal between types when compared to the ridge-side placement of constructed wetlands in the London District.

The ephemeral hydroperiod of the ridge-top wetlands in the DBNF precludes many predatory amphibians from colonization, and, alternatively provides important breeding habitat for those species with weak anti-predator mechanisms (Semlitsch et al. 2015). For many historical ridge-top species, the addition of permanent water bodies has not created extra breeding habitat, but has instead introduced predators that hinder egg and larval survival. This is especially true in the Cumberland District.

Management Recommendations

Comparing results from this and previous studies done in the DBNF ridge-top system, it appears that the Cumberland District would benefit most from reassessing their objectives in terms of constructed wetlands. Constructed wetlands were originally built to maintain a permanent hydroperiod for game and bat use, and in that way they have been a success. However, due to the detrimental effect of these permanent hydroperiods on the historical ridge-top amphibian communities and the high density in which they were constructed, land managers should consider either renovating or removing some of the constructed wetlands. Obviously it is not feasible or advisable to renovate every one of the 500+ constructed wetlands in the Cumberland District, but updating those constructed wetlands that co-occur with natural, ephemeral wetlands on ridge-tops is recommended. Land managers should consider recreating the ephemeral hydroperiods, shallow littoral zones, and high canopy cover and vegetative complexity of the natural wetlands in the area. Created wetlands have the potential to be valuable breeding habitat for the historical amphibians of the ridge-top system if land managers take into account the ecological needs of target species.

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APPENDIX A

le A – 1. Catch per unit effort per species at each ridge-top wetland site in the Daniel Boone National Forest, Kentucky.

Species	Natural							Game					Bat				
	CP	LP	D2	SG	HKN	RF	19A	LH	HK3	GP	448B	191	B1	HK2	B3	H1	RP
Lithobates sylvaticus	8.64	1.16	0.73	27.8	0	9.2	6.5	2.14	0	1.43	12.9	0	0	0	0	0	0
Combined *Lithobates*	0.45	0	0.17	0	1.14	0	0.33	0.43	1.71	0.14	1	1.33	1.5	1	0	6	0.5
Psuedacris crucifer	0.64	1.33	0.53	0	27.9	6.2	0.16	0.71	0.83	0.29	7.14	0.5	0	0.16	0	0	0
Hyla chryscocelis	0	0	0	0	0.29	0	0	0.2	0.17	0	0	0	0	0	0	0.2	0
Anaxyrus americanus	0	0	0	0	9.29	0	0	0	0	0	0	0	0	0	0	0	0
Combined *Ambystoma*	7.45	6	13	5.75	25.7	5	0.33	20.5	8.5	9.71	14.4	15.8	58.8	8.5	0	15.4	8
Ambystoma opacum	0.27	4	1.33	6	0.43	0	0.33	0	1.17	0	0	0.5	1	0	0	0	0
Hemidactylium scutatum	0.91	0	0	0	0	0	0	0.8	0	0.14	0	0	0.5	0.4	0	0	0.43
Notophthalmus viridescens	0.64	0.03	0	0	0.14	0	0	1.14	2.33	0	0.57	0.17	1.5	2	1	2	1.29

Table A – 2. Candidate models for predicting amphibian abundance in ridge-top wetlands, Daniel Boone National Forest, Kentucky.

Model variables [a]	**Model type** [b]
Wetland type, canopy closure, CWD, depth, KY-WRAM, pH, conductivity, D.O.	Global
Type, KY-WRAM, depth, canopy closure	Within-wetland characteristics
CWD, canopy closure, KY-WRAM	Vegetative characteristics
Conductivity, pH, D.O.	Water quality
Type, KY-WRAM, depth	Physical basin characteristics
Conductivity, KY-WRAM	Wetland quality
Type	Wetland type
Canopy closure	Canopy closure
Conductivity	Conductivity
KY-WRAM	KY-WRAM
Depth	Depth

[a] Wetland type = natural, game constructed, or bat constructed; CWD = upland coarse woody debris; KY-WRAM = Kentucky Rapid Wetland Assessment Method score; D.O. = dissolved oxygen.

[b] Variable combinations represent different environmental or wetland construction strategies.

APPENDIX B

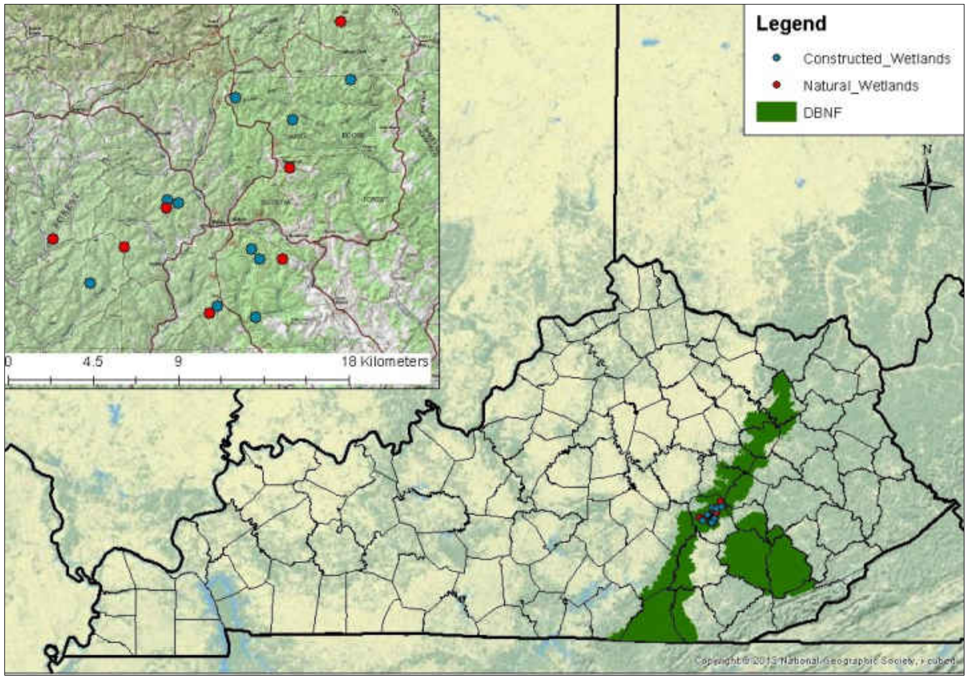

Fig. B – 1. Natural and Constructed ridge-top wetland study sites in the London District, London County, Kentucky.

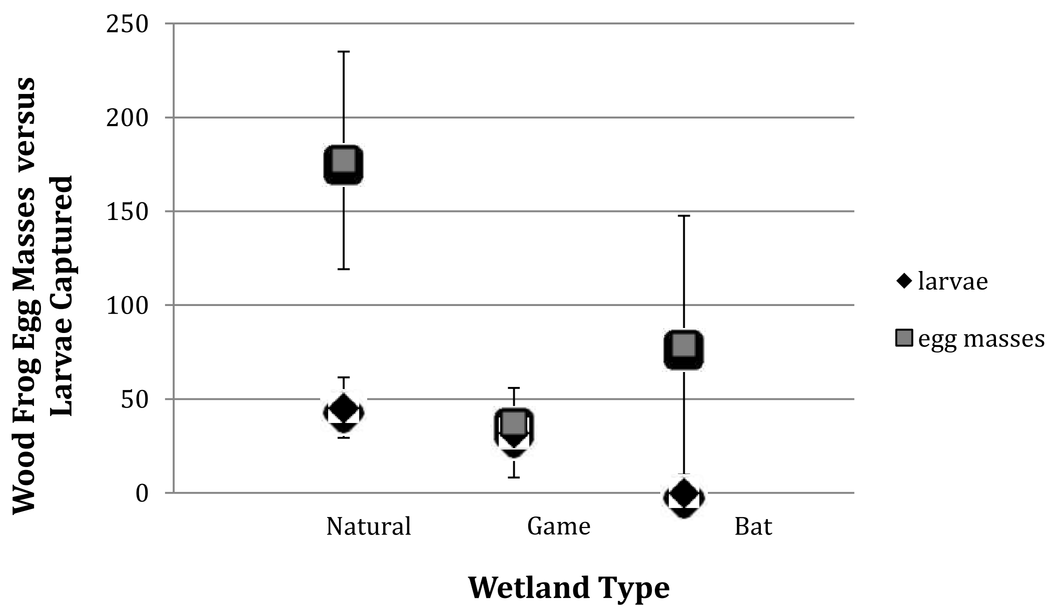

Fig. B – 2. A comparison of wood frog egg masses detected in March versus larvae captured May through June in each ridge-top wetland type.